Circular Dichroism Spectral Similarity Plots to Extend Validation and Correction to All Measured Wavelengths

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Abstract

Interlaboratory comparisons of circular dichroism (CD) spectra are useful for developing confidence in the measurements associated with biopharmaceuticals. These measurements help define such drugs' higher-order (secondary and tertiary) structure. Unfortunately, the extent of the validity of these measurements has been unclear. In this work, a method is described to extend CD validation over the entire observed wavelength range using what will be called spectral similarity plots. The method involves plotting, wavelength by wavelength, all measured spectral intensities of a sample at one concentration against the intensity values of the same material at a different concentration or pathlength. These spectral similarity plots validate the instrument in terms of spectral shape and whether the shape is shifted in intensity and/or in wavelength. This comparison tests the linearity of instrument's signal, the balance of its left and right polarizations, its wavelengths, and its spectral intensity scales. When the process is applied to materials with accepted and archived intensity values, the method can be linked to older single-wavelength and double-wavelength calibration techniques. Further, spectral similarity testing of CD spectra from samples with different concentrations run in different labs suggests that improved inter-laboratory validation of CD data is possible. Since a database of archival-CD measurements is available online, spectral similarity comparisons could possibly provide the ability to compare linearity, polarization balance, wavelength and spectral intensity between all current CD instruments. If the preliminary results published here prove robust and transferable, then comparisons of full-wavelength-range spectra to archived data using spectral similarity plots should become part of the standard process to validate and calibrate the performance of CD instruments.

Introduction

Circular dichroism (CD) is one of the original methods identified by the International Conferences for Harmonization for the quantitative assay of higher-order protein structure.[1, 2] In turn, the importance of CD as an assay of higher-order structure has created an impetus to understand and characterize interlaboratory variations in CD measurements for biopharmaceutical materials.[3, 4, 5] However, no consensus method exists for robust calibration over the full-wavelength range.

CD is usually run in the UV-visible range, and the magnitude of a typical CD signal is on the order of one part in 10^3 of the total absorbance. Circular dichroism intensities are usually calibrated using the spectral maxima and/or minima of small chiral molecules such as ammonium *d*-10-camphorsulfonate (ACS), camphorsulfonic acid (CSA), pantolactone, and cobalt (III) tris-ethylenediamine; the choice depends on the wavelength range of interest, but a paucity of spectral features limits such calibrations to one or two wavelengths.[5, 6, 7, 8, 9]

The uncertainties at a single wavelength in UV-visible absorbance spectroscopy for measurements of standards (like NIST Standard Reference Material (SRM) 935 and SRM 2034) are on the order of ± 0.5%.[10, 11] CD uncertainty for a series of measurements on a single instrument, is also ± 0.5%,[5] even with the small magnitudes of the CD signals. However, interlaboratory comparison studies report uncertainties of ± 8% for the ammonium salt of the highly chiral (1S)-(+)-10-camphor sulfonic acid and uncertainties as poor as ± 50% for measurements on weakly chiral proteins.[5, 12, 13] The multivariate statistical analysis in the latest inter-laboratory study[13] suggests that, despite efforts to standardize the measurement processes, these uncertainties arise primarily from uncontrolled differences in the instrument settings and practices of the labs. Subsequently, an uncertainty analysis of CD spectroscopy ascertained the most crucial parts of the uncertainty budget to improve interlaboratory measurement comparisons.[14] More recent work suggests that non-stochastic effects also contribute.[15]

The interlaboratory uncertainty of molar CD ($\Delta\varepsilon$ with units of $mol^{-1}dm^3cm^{-1}$) has contributions from both pathlength and sample concentration uncertainty.[14] Since nearly all modern instruments now record both absorbance and CD simultaneously, [16, 17] it is suggested that absorbance and CD spectra should always be published together.[18] Since sample absorptivity is more likely to be known, always having a sample's absorbance spectra often allows sample concentration to be determined and $\Delta\varepsilon$ calculated if not already known. In addition, the same group also recommends that measurements of samples at more than one concentration should be used to ascertain if the CD instrument response is linear.[18]

DiNitto and Kenney discuss the noise/error of CD instruments and postulate that shot noise is the largest source of uncertainty.[19] They characterize instrument performance for a range of slit widths, bandwidths, scan speeds, dark currents, and stray light by plotting the ratios of the standard deviations of two spectra run with different numbers of scans to support their statement. Beyond spectral noise, Sutherland describes two sources of dichrometer error: the use of mathematical approximations more suited to smaller relative signals and varying modulator phasing at different wavelengths.[20] Sutherland has also described how to maintain a consistent modulator phase over the full-wavelength range.[21]

As mentioned earlier, the differences between CD measurements made on more than one instrument arise from both wavelength and spectral intensity uncertainties.[13] Wavelength calibrations usually are based on the spectral lines emitted by a lamp or a sample containing sharp peaks such as a solution of holmium perchlorate and/or a glass filter doped with holmium oxide or didymium.[22] The spectral intensities in CD measurements are calibrated against one, two, or as many as five published values for small molecule calibrants.[22] The spectral lines of the wavelength standards are only present at particular wavelengths, and the small molecule calibrants have only a small number of peaks and/or valleys with published intensity values. Therefore, neither of these calibration procedures are easily

extended to address the valibration of every point across the measured spectra. All-wavelength calibrations, based on matching CD measurements from artificial devices with known optical properties and calculations of the expected CD spectra, have been implemented.[23] This method has yet to be widely adopted because precisely duplicating the optical devices and/or measuring the necessary optical parameters is difficult.

The biggest advantage of full spectral shape comparisons is that it tests whether two instruments agree on both the wavelength scale and the intensity scale at the same time. In contrast, typical calibrations are completed first for wavelength and then for intensity, leaving the possibility that intensity calibration can incorrectly remap the wavelength calibrations. While instrument linearity tests can be done as functions of either cell pathlength or sample concentration, these experiments aren't done frequently because they are time consuming.[5, 6, 7, 24] In contrast, the spectral similarity based linearity tests presented here, as functions of wavelength, only require one extra measurement.

Once spectral similarity and instrument linearity have been tested by varying sample concentrations or pathlengths for a single instrument, the next desirable step is to compare with other CD instruments. A convenient resource is the Protein Circular Dichroism Data Bank (PCDDB), [25, 26] an online public archive that freely distributes CD data. The authors of the data bank state that all entries undergo validation and curation procedures to ensure completeness, consistency, and quality of the data included. The data and metadata are accessed through a web-based interface that provides both graphical displays and downloadable text files. By applying our multi-wavelength spectral similarity comparison between archived and new data, a CD instrument's wavelength and intensity can be validated and calibrated. This process focuses on analyzing nonlinearities in both spectral intensity and wavelength and uses the results to correct errors that distort the true CD spectrum in the measured results. As noted

below, by matching the full CD spectrum to data archived in the PCDDB, we learn that the robustness of CD instrument response has improved since the international comparison studies mentioned earlier.

Materials and Methods[++]

(1S)-(+)-10-camphor sulfonic acid (CSA), human serum albumin (A3762), and lysozyme (L-7651) were purchased from Sigma-Aldrich. The 10-mm pathlength cuvettes were purchased from Starna, Inc. (Atascadero, CA). The cuvette spacers were purchased from the Firefly Cuvette Shop (Staten Island, NY). A cuvette spacer is a quartz optical plug that occupies the center of a 10-mm cuvette to decrease the pathlength of the cell to values appropriate for CD measurements. CD spectra were obtained using an Applied Photophysics Chirascan V100 spectrometer (Leatherhead, Surrey, UK). The data for this report was collected within 10 weeks of the instrument being calibrated by an Applied Photophysics technician using their standard protocols. Simultaneous UV-vis measurements of our CSA/water, human serum albumin/water, and lysozyme/water solutions were used to determine their concentrations.[24, 27, 28, 29, 30, 31] The data conversions and analysis were performed using the Chirascan v.4.5 software, Excel (Microsoft), and OriginPro 2021 (OriginLab Corporation, Northhampton, MA). The two files from the PCDDB used for comparison were the human serum albumin file CD0000038000,[32] and the lysozyme file CD0003675000.[33] The CSA data is not listed as part of a separate publication.

The most obvious place to start to describe how a spectral similarity plot tests instrument response and linearity begins with the Beer-Lambert Law's formula:

$$A(\lambda) = \varepsilon(\lambda)\, bC \qquad (1)$$

where $A(\lambda)$ is the sample absorbance in absorbance units at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient at λ, b is the pathlength in cm, and C is the concentration in mol/L. A similar equation is also true for circular dichroism measurements:

$$(A_L\text{-}A_R)(\lambda) = \Delta\varepsilon(\lambda)\, bC \qquad (2)$$

where $(A_L\text{-}A_R)(\lambda)$ is the differential circularly polarized absorbance measurement in absorbance units at wavelength λ, and $\Delta\varepsilon(\lambda)$ is the molar CD at λ. For CD comparisons between samples with differences in sample pathlength and/or concentration, the idea is to solve for $\Delta\varepsilon(\lambda)$ at each λ for two samples with known pathlengths and sample concentrations. The two lists of numerical spectral points from the two trials then become the x and y values to plot on a graph. As will be shown next, by plotting the pairs of $\Delta\varepsilon(\lambda)$ values from the two digitized spectra this way, the linearity of the instrument response can be obtained. The precision of the match is described by fitting a straight line to the spectral similarity plot. If the measurements are identical, the fit results in a line with a slope of 1.000, an x-value of 0.000 for the y = 0 plot, and an adjusted R^2 value of 1.000. Demonstrations of a number of different types of comparisons and corrections using this general scheme follow.

Results and Discussion

Comparing two instruments

Figure 1A shows two CD spectra of the same lysozyme sample obtained using two different instruments. Instrument #1 (black line) had recently undergone routine maintenance and had its wavelength scale calibrated. Instrument #2 (gray line) was due for maintenance and recalibration. The most obvious difference appears to be the peaks and valleys of Instrument #2 are shifted to longer wavelengths. A difference in intensities is also observed although the magnitudes of both instruments' peak-to-peak signals are close. A wavelength calibration and a one-point calibration for intensity would

not allow correcting Instrument #2 to match Instrument #1. The spectra don't differ in magnitude, as much as their intensities are shifted. Even a two-point calibration for maximum and minimum values would not address the shift downward of the spectrum from Instrument #2.

Figure 1A CD measurements of the same lysozyme sample from two different instruments don't match

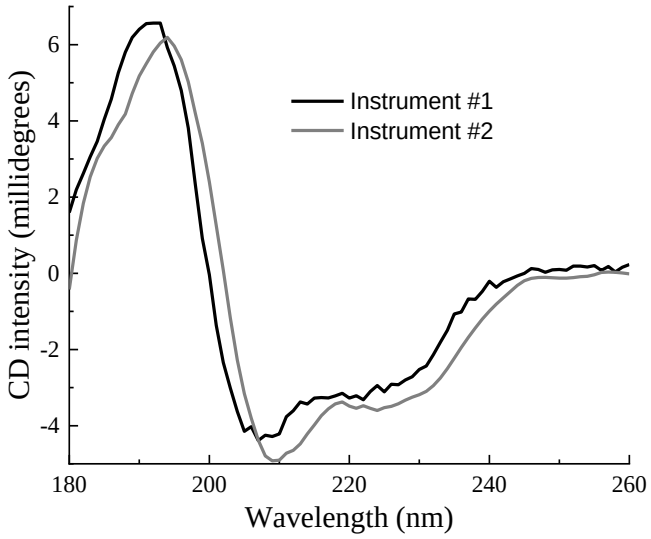


Figure 1B The spectral similarity plot of the CD spectrum of lysozyme from two different instruments isn't ideal

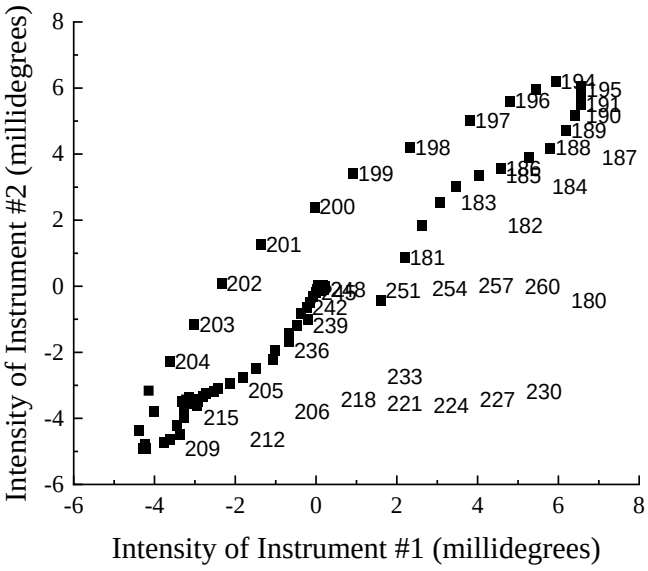


Figure 1C Reassigning the wavelengths of one instrument improves the spectral similarity plot

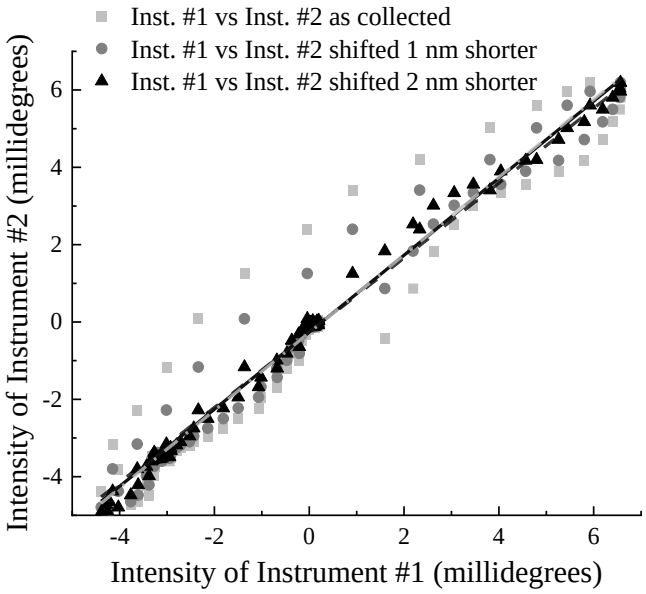


Figure 1B shows a spectral similarity plot of the data from Figure 1A. Here the intensities observed by Instrument #1 and Instrument #2 at each wavelength are plotted against each other. This process is illustrated by labeling many of the data points with the wavelengths they represent. The plot of the data describes a tilted, near-oval shape. Most of the plotted points approximate a line passing near the (0, 0) point but some - those labeled from 194 to 204 - extend out away from the line. The improperly matched sides of the peaks and valleys of the two spectra systematically create curved shapes. If the two spectral shapes measured by the two instruments were identical, then the plots would describe a straight line with a correlation coefficient of one, a slope of one, and pass through the (0, 0) point. The deviations of the data points from their ideal matching values characterize and quantitate the differences between the two instruments that account for differences in intensity and wavelength calibrations, sample composition and concentration, cuvette pathlength, material and optical quality.

So which of these possible factors are most likely to create a plot with a tilted oval shape? If the peak and valley positions of the two curves are different, then issues with sample composition might be expected. If the instruments were randomly observing intensity differences, say from intermittent instrumental artifacts or very low light throughput, then the shape and spread of the data plots would be random, but it is not. Figure 1B shows that the set of data points from about wavelengths 195 to 204 consistently vary from the others, and the manner of the shift is informative. The relationship of the 195 to 204 points to the others is systematic because the spectrum of Instrument #2, in that range, is higher than the spectrum of Instrument #1 in Figure 1A. For the rest of the points, before and after 195 to 204, the data from Instrument #2 is lower than the data from Instrument #1. The alternating nature of these three wavelength sections of Figure 1A suggests that the data from Instrument #2 is shifted to lower wavelengths (to the right in Figure 1A and Figure 1B) than the spectrum from Instrument #1. Comparisons of the band positions of lysozyme CD spectra from the PCDDB confirm the shift.[25, 26] It makes sense that it is the data from Instrument #2 that is shifted because it is not well calibrated for wavelength. Next, Figure 1C and Table 1 demonstrate how adjustments in the assigned wavelengths correct the spectral similarity plot closer to its ideal form.

Table 1 Spectral similarity parameters from data in Figure 1C

Plot	Slope	Graph value at y = 0	Adjusted R^2
Inst #1 v Inst #2	0.97 ± 0.03	-0.28 ± 0.10	0.918
Inst #2 shifted 1 nm vs Inst #1	0.996 ± 0.018	-0.265 ± 0.059	0.973
Inst #2 shifted 2 nm vs Inst #1	1.007 ± 0.009	-0.275 ± 0.029	0.994

In Figure 1C, the light gray squares repeat the plot of Figure 1B and the spectral similarity parameters of slope, intercept, and adjusted R^2 are listed in the first row of Table 1. The medium gray

circular points result from shifting Instrument #2 data to 1 nm shorter wavelengths (spectral similarity parameters: second row of Table 1) and the black triangular points specify a spectrum shift to 2 nm shorter wavelengths (spectral similarity parameters: third row of Table 1). These tabulated fitting parameters show that, by shifting intensity-paired points with one another by shifting the wavelength values of Instrument #2 by one or two nanometers, the relationships between the two spectra trend toward an ideal match. The spectral similarity plot provides a clear visual and statistically straightforward relationship. These relationships can be used to match both intensity and wavelength scales simultaneously.

Comparing the spectral similarity approach to a two-point calibration

How does the spectral similarity approach compare to more typical one-point and two-point calibrations? Figure 2A shows CD spectra of 2.675 mM and 4.013 mM (1S)-(+)-10-camphor sulfonic acid (CSA) using ΔA rather than $\Delta\varepsilon$ to allow a quantitative comparison between the spectral similarity approach and a two-point calibration using the known concentration values. The plot also shows (grey squares and circles) the two-point calibration values calculated from the known molar ellipticity values of CSA at 192.5 nm (-15,600 deg cm^2 $dmol^{-1}$)[27, 28] and 290.5 nm (7800 deg cm^2 $dmol^{-1}$)[27, 28] for these two sample concentrations and for the pathlength used (0.0509 cm ± 0.00016 cm). Three of the calculated calibration values are close to the observed spectral values and the fourth (for the 192.5 nm band of the 4.013 mM sample) appears slightly low. In a two-point calibration, the ratio of the 192.5 nm band intensity to the 290.5 nm band intensity should be 2.0. However, in Figure 2A the ratio is 1.95 for the 4.013 mM sample but is greater at 2.02 for the 2.675 mM sample. If the 1.95 ratio of the 4.013 mM sample is scaled up to 2.0 and the 2.02 ratio for the 2.675 mM sample is scaled down to 2.0, then the spectra intersect with the calibration points. (plot not shown)

Figure 2A CD spectra of 2.675 mM and 4.013 mM (1S)-(+)-10-camphor sulfonic acid with expected calibration intensities at the maxima and minima of each curve

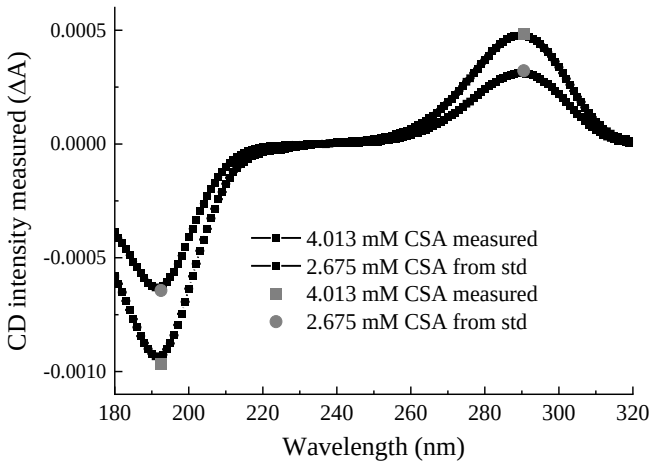
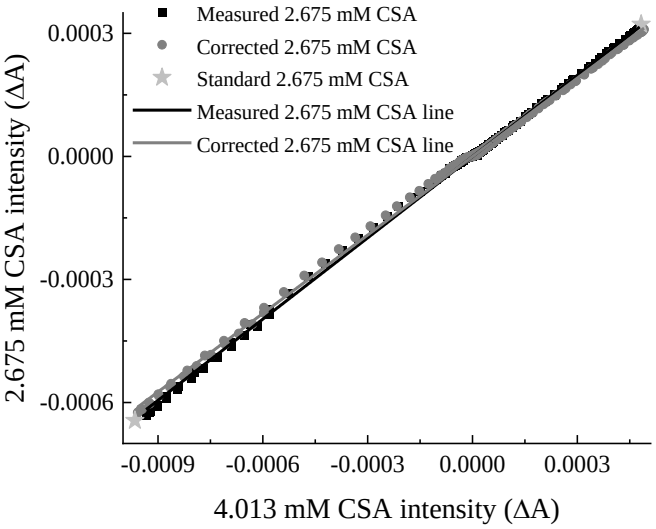


Figure 2B Spectral similarity plots of CD spectra of 2.675 mM and 4.013 mM (1S)-(+)-10-camphor sulfonic acid: measured and corrected



A spectral similarity plot of the data from Figure 2A, as measured and additionally after calibration, clarifies the comparison in Figure 2B. The parameters of the fitted lines are listed in Table 2. On the y-axis values of the CD intensities of the 2.675 mM sample are plotted and on the x-axis values of the CD intensities of the 4.013 mM sample are plotted. Here, the similarity plot compares the

measured intensities at each wavelength in millidegrees to illustrate the similarity of the spectral shapes with concentration change both before (black squares) and after (dark grey circles) correction. The calculated two-point calibration intensities (light grey stars) are included to indicate how the line of a spectral similarity plot intersects the calculated two-point calibration values.

Table 2 Spectral similarity parameters from data in Figure 2B

Plot	Slope	Graph value at y = 0	Adjusted R^2
Measured 2.675 mM CSA	0.6605 ±- 0.0016	-0.0175 ± 0.020	0.999
Corrected 2.675 mM CSA	0.6376 ± 0.0016	-0.173 ± 0.020	0.999

Since the cell pathlength of the two samples was identical, the ratio of the known sample concentrations (calculated from UV-vis absorbance spectrum values as 0.6666 ± 0.0067) should define the slope of the spectral similarity line and allow the spectra before and after a two-point correction to be compared to a known value. As measured, a slope of 0.6605 (top row of Table 2) indicates a value that is 0.9% low. After a two-point correction to make the maxima and minima of the CD spectra match the literature values, the slope of the spectral similarity line changes to 0.6376 (second row of Table 2) and indicates a value that is worse: at 4.4% low. This shows that the spectral similarity description of the as-measured sample concentration ratio is more accurate than the concentration ratio obtained after a two-point CD correction.

Comparing protein CD spectra using the spectral similarity

Figure 3A shows ΔA CD spectra of human serum albumin (HSA) at three different concentrations: 2.72 μM (thick black), 2.23 μM (medium dark grey) and 1.59 μM (thin grey). The same cell (pathlength of 0.0140 cm ± 0.00012 cm) was used for all three and the measurements were run on a

newly calibrated Chirascan 100 instrument. Figure 3B shows the data from Figure 3A, after being converted to Δε to combine the instrument uncertainty of the ΔA spectrum with the uncertainties of pathlength and sample concentration into spectral similarity plots that comprehensively test the measurements. The square points plot the 1.59 μM HSA vs the 2.23 μM HSA and the circular points compare 2.72 μM HSA to 2.23 μM HSA. The parameters describing the lines fitted in Figure 3B are compiled in rows two and three of Table 3. In contrast to the slope being related to the ratio of the sample concentrations as shown in Figure 2B and Table 2, by working in Δε, here the spectral similarity plots compare not only the spectral shape but also the protein concentrations because these measurements use the same cuvette. Given how well these values approximate an ideal spectral similarity graph, the instrument is clearly well calibrated.

Figure 3A Comparison of three different CD spectra of human serum albumin concentrations with identical pathlengths

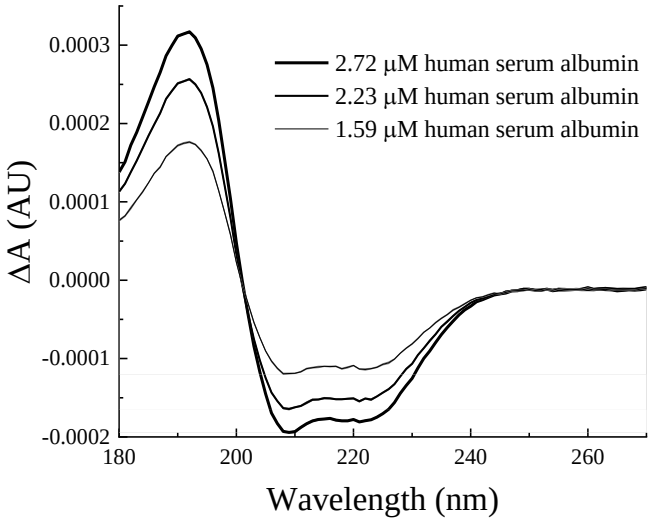


Figure 3B Comparisons of measured Δε spectra of HSA after the ΔA spectra have been normalized for concentration and pathlength

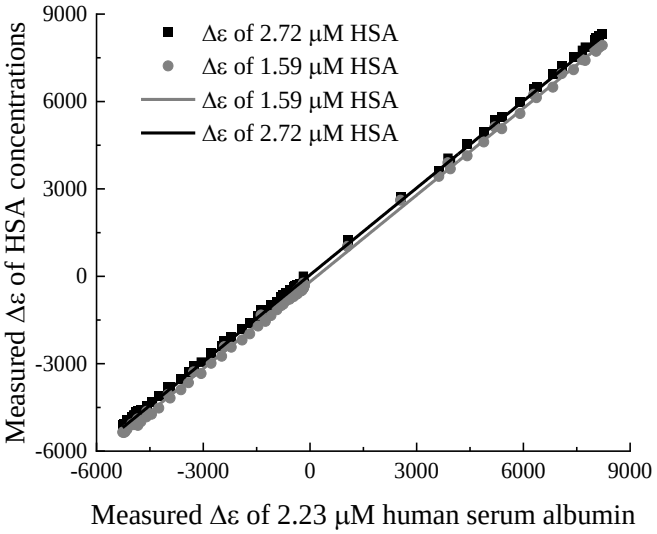
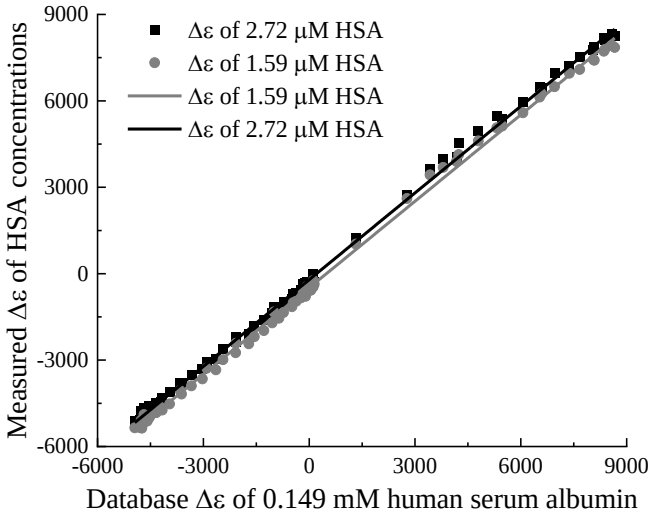


Figure 3C Comparisons of Δε spectra of HSA from Figure 3B Δε spectra from the PDCCB after normalization for concentration and pathlength



Interlaboratory comparisons

Figure 3C shows a spectral similarity plot of HSA data from both our laboratory (y-axis) and from another laboratory's contribution to the PCDDB (x-axis).[32] The square points plot Δε of 2.72 μM HSA vs Δε of 0.149 mM HSA, and the circular points compare the Δεs of 1.59 μM HSA and 0.149 mM HSA. The parameters describing the linear fits of the two spectral similarity plots are compiled in the third and fifth rows of Table 3. The fourth row of Table 3 shows the parameters describing the spectral

similarity plot of 2.23 μM HSA and 0.149 mM HSA. (data not shown) The data was collected using two different cuvettes on two different instruments in different years. Here the average 0.43% deviation of the slope from the ideal value of 1.000 is smaller than the 0.9% error of our CSA concentration ratio measurement of Figure 2. Given how well these values approximate a perfect spectral similarity line, this suggests that both instruments are well calibrated for making comparisons between laboratories despite using different sample sources, cuvettes, and sample concentrations. This is a significant improvement from the International Comparison Studies of CD Measurements where the protein CD intensity differences were as large as ± 50%.[13] While some of the improvement can be ascribed to improvements in instrument capabilities over time, much of the improvement is likely due to the comparison studies' finding that their uncertainties arose primarily from uncontrolled differences in the operator practices of the labs such as instrument settings, cell cleaning, and care applied in measuring sample concentrations and pathlengths.

Table 3 Compiled comparisons of Δε HSA from different concentrations and sources

Plot	Adjusted R^2	Graph value at y = 0	Intercept
2.23 μM v 1.59 μM	0.999	0.993	-190
2.23 μM v 2.72 μM	0.999	0.995	47
0.15 mM v 1.59 μM	0.999	1.001	-488
0.15 mM v 2.23 μM	0.999	1.008	-301
0.15 mM v 2.72 μM	0.999	1.004	-207

Spectral similarity comparisons with imperfect slope matches

Figure 4 repeats the processes of Figure 3 using lysozyme instead of HSA. Figure 4A shows ΔA spectra from lysozyme at three different concentrations (0.1040 mM, 0.0603 mM and 0.346 mM) collected using the same cell with a pathlength of 0.0140 cm ± 0.00012 cm using the newly calibrated Chirascan 100 instrument. Figure 4B shows two spectral similarity plots of Δε data from Figure 4A.

The square points compare 0.1040 mM lysozyme to 0.0603 mM lysozyme, and the circular points compare 0.0346 mM lysozyme to 0.0603 mM lysozyme. The parameters describing the best-fit lines appear in the second and third rows of Table 4. Both lines described by the plots are good approximations of our ideal spectral similarity plots and have adjusted R^2 values of 1.000 and 0.999 respectively. However, in these cases, the slopes of the fitted lines deviate from the ideal by between 3 and 4%. Given the recent instrument maintenance and the accuracy of the HSA samples across all parameters of the spectral similarity lines collected on the same day, the plots in Figure 4B suggest that the error in the measured slopes could be due to imperfect sample dilution or concentration determination rather than issues with the performance of the CD instrument.

Figure 4A Comparison of three different CD spectra of lysozyme concentrations with identical pathlengths

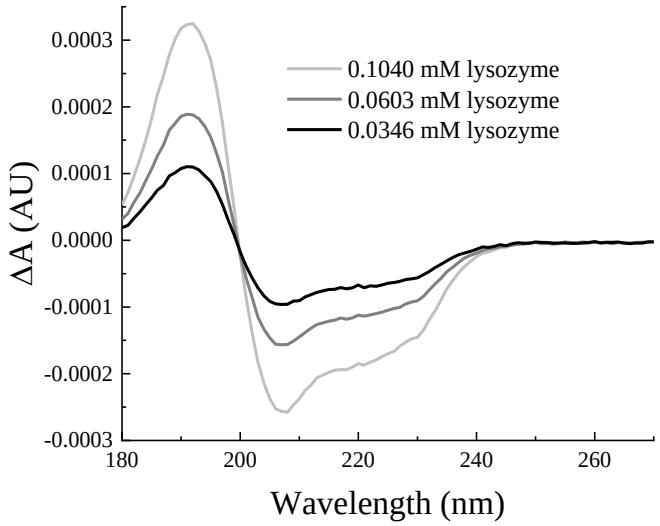


Figure 4B Comparisons of measured Δε spectra of lysozyme after the ΔA spectra have been normalized for concentration and pathlength

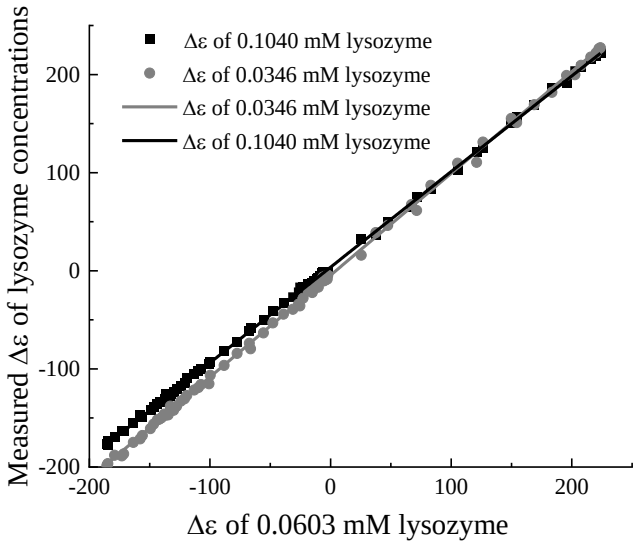
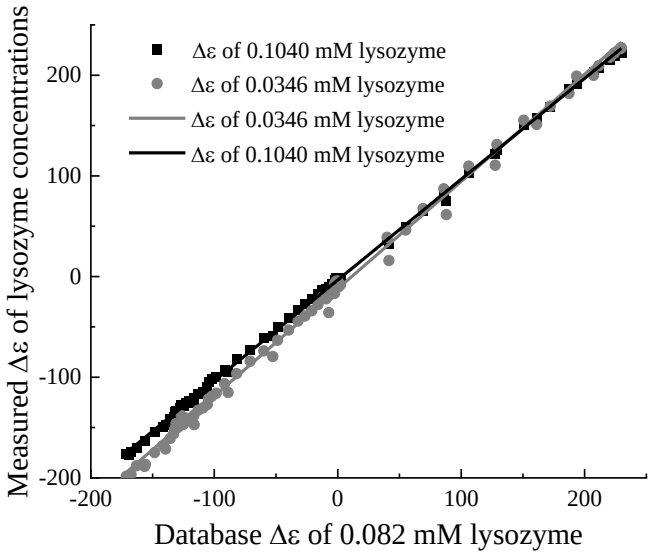


Figure 4C Comparisons of Δε spectra of lysozyme from Figure 4B Δε spectra from the PDCCB after normalization for concentration and pathlength



Interlaboratory comparisons with imperfect slope matches

Figure 4C shows lysozyme data from both our laboratory (y-axis) and from another laboratory's contribution to the PCDDB (x-axis)[33] after adjusting to normalize cuvette pathlength and protein concentration by comparing Δε using a spectral similarity plot. The square points compare 0.035 mM lysozyme to 0.082 mM lysozyme, and the circular points compare 0.104 mM lysozyme to 0.082 mM lysozyme. The parameters describing the linear fits of the spectral similarity plots are compiled in the

last three rows of Table 4. To avoid clutter in the figure and to keep the same format as Figure 4B, the spectral similarity plot of 0.082 mM lysozyme vs 0.060 mM lysozyme is not shown. Again the data was collected using different cuvettes on different instruments in different years. Once again the adjusted R-square values of 0.999 and 0.997 show good approximations of our ideal line. Like Figure 4B, the deviation from a slope of 1.000 suggests that our concentration measurements are the origins of the observed slope error. However, this time there is a third comparison between the 0.82 mM sample from the PCDDB and the 0.104 mM sample that does not have significant slope error. This suggests that only the concentrations of the 0.035 mM and 0.060 mM samples, which were created by dilution from the 0.104 mM, are likely to be the sources of the observed slope error.

Table 4 Compiled comparisons of Δε lysozyme from different concentrations and sources

Plot	Adjusted R^2	Graph value at y = 0	Intercept
0.060 mM v 0.035 mM	0.999	1.037	-4.5
0.060 mM v 0.104 mM	1.000	0.974	3.7
0.082 mM v 0.035 mM	0.997	1.065	-12.1
0.082 mM v 0.060 mM	0.999	1.028	-7.4
0.082 mM v 0.104 mM	0.999	1.001	-3.5

Using spectral similarity corrections to improve congruence of CD spectra

The Δε data in Figures 5A and 5B was collected using calibrated CD instruments and samples with known concentrations and pathlengths. Nevertheless, these spectra do not overlap. Small errors in single-wavelength measurements of either UV-vis absorbance and/or CD intensity contribute to small errors in concentration, pathlength, and calibration, which produce imperfect congruence of the spectra. The spectral similarities of these spectra are described by the slopes, graph values at y = 0, and adjusted R^2 in Table 3 and Table 4.

Figure 5A Comparison of Δε HSA with different concentrations and from different sources

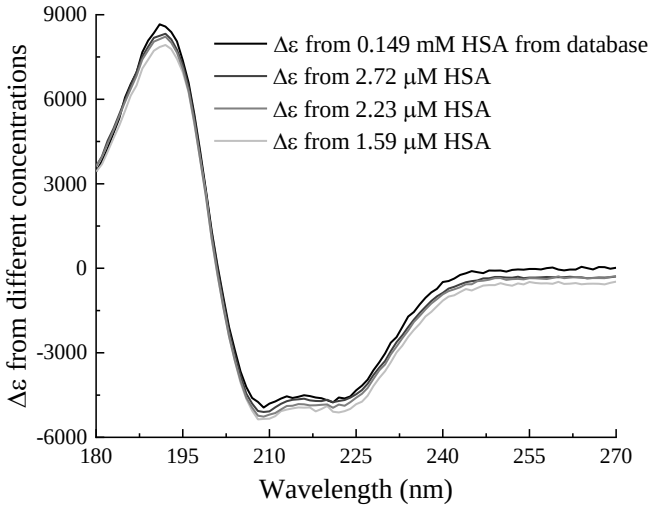
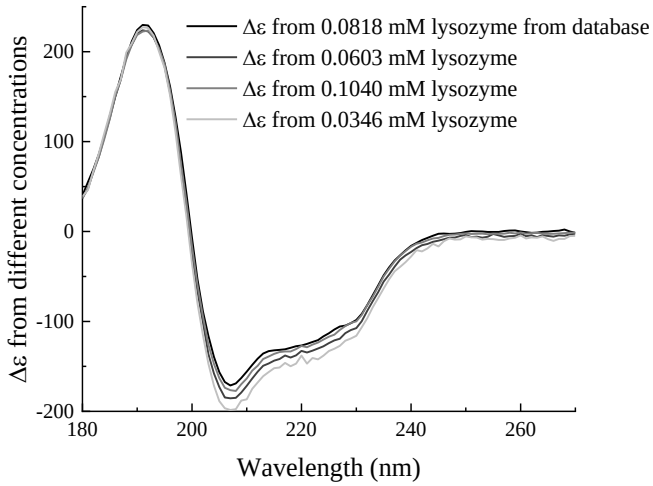


Figure 5B Comparison of Δε lysozyme with different concentrations and from different sources



The graphs in Figure 5A and Figure 5B show similar full-wavelength range uncertainties for the CD spectra of both HSA and lysozyme. In the different examples, the slope data in Table 3 shows only small deviations from ideal for HSA, yet larger deviations from the ideal are seen in the slope data of Table 4 for lysozyme. If slope uncertainty were only a matter of sample concentration and/or pathlength uncertainty, as we considered in the section concerning Figure 4C, the spectra in Figure 5A, with its smaller slope deviations from ideal, would lie on top of each other. Then, the spectra in Figure 5B, with

larger slope deviations, would not. Since neither group of Δε spectra are congruent based on single-wavelength based properties, it makes sense to try full-wavelength range based corrections.

To do this, each spectrum for a given material can be adjusted to become more congruent with any other in two steps. 1) Add the x graph value at y=0 from the spectral similarity plot to the intensity values for each wavelength of the spectrum. 2) Divide each wavelength result from 1) by the slope of the best-fit line of the spectral similarity plot to produce the corrected CD spectrum.

This process has been carried out for each of the pairs of spectra in Figure 5. The three in-house spectra were adjusted to become more congruent with the PCDDB spectrum for each sample. After the correction, the resulting HSA and lysozyme Δε spectra plotted in Figures 6A and 6B can be seen to overlap better than they do in Figure 5.

Figure 6A Comparison of Δε spectra of HSA from different concentrations and sources after many wavelength slope and y-intercept corrections

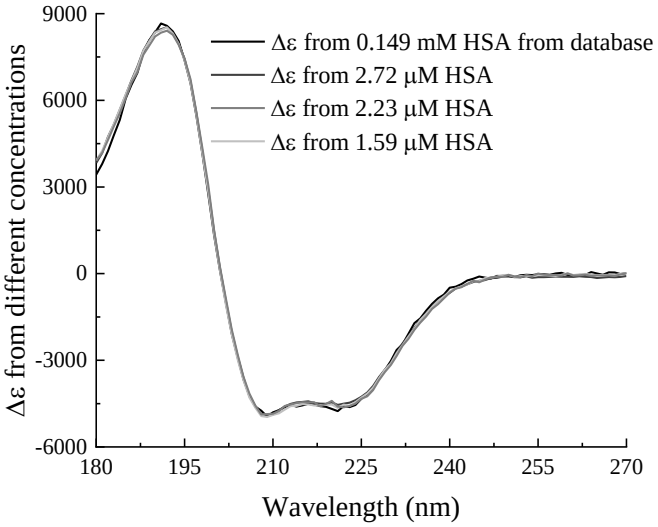
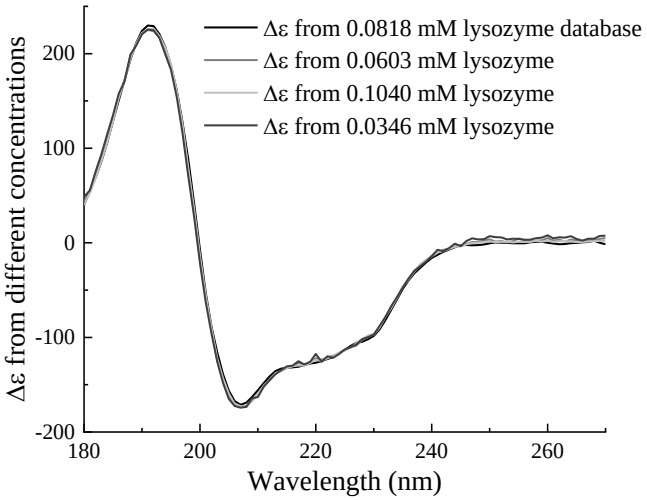


Figure 6B Comparison of Δε spectra of lysozyme from different concentrations and sources after many wavelength slope and y-intercept corrections



The reasons the spectra in Figure 6A and Figure 6B are more congruent than those in Figure 5A and Figure 5B are straightforward. The slopes and x graph values at y = 0 of the spectral similarity plots are not greatly influenced by any deviations from a single-wavelength intensity. Like integrated band intensities, they are more accurate measures of sample concentration or pathlength than a single wavelength measurement. In addition, imbalances between the left and right circular polarizations (the small vertical shift in Figure 1A) and improper wavelength values (the significant wavelength shift in Figure 1B and Figure 1C) are all address in a systematic and encompassing manner.

Methods to determine and improve how well the spectra in Figure 5A or in Figure 5B match each other are not straightforward. Simple single-factor comparisons like weighted spectral differences[34] or single- or double-wavelength calibrations are insufficient because spectral shapes and positions are influenced by both the independent individual and the correlated collective values of the data points in both contributing spectra. To address these issues, our spectral similarity plots break this difficult situation up into two orthogonal, multiwavelength, and correctable factors: the fitted slope, the graph value of x at y=0, and the adjusted R^2 value reporting the quality of the wavelength and intensity correlation. The success of this method is shown in the near congruence of the CD spectra in Figure 6.

Conclusion

Successful interlaboratory comparisons of CD spectra require more than separate wavelength and one or two-point intensity calibrations. Interplay between wavelength and intensity values is never ending. Low-quality wavelength calibration can be hidden during intensity calibration and improper intensity calibration can be necessary if wavelength calibration is poor or infrequent. By simultaneously comparing all intensities at all wavelengths for a CD spectrum of a calibrant in a spectral similarity plot, these issues are addressed. Spectral similarity plots are based in terms of spectral shape and how that shape is placed vertically and horizontally on it's graph. The resulting all-wavelength tests of instrument linearity and offset utilize the properties of spectral similarity to also detect errors in wavelength calibration. These ideas are first tested between CD spectra from samples with different concentrations utilizing measurements acquired in a single lab and then extended to compare CD spectra from different labs. These experiments show that spectral similarity methods for comparing CD spectra at all wavelengths can be run between multiple cuvettes, using more than on instrument, in different labs with several operators around the world. This suggests that virtual inter-laboratory comparisons of CD data are possible and much closer than they seemed with the publication of the International Comparison Studies of CD data.[13] This success should encourage others to test the spectral similarity and offset errors in their CD instruments and to compare their data with data archived in the PCDDB.

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The Author declares that there are no conflicts of interest.

References

[++]Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

1. “International Conference on Harmonisation; Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products”. Federal Register. 1999. 64(159): 44928-44935.

2. A. B. Harris, R. D. Kamien, T. C. Lubensky. ”Molecular chirality and chiral parameters”. Rev. Mod. Phys. 1999. 71: 1745-1757.

3. J. P. Gabrielson, W. F. Weiss IV. “Technical decision-making with higher order structure data:starting a new dialogue”. J. Pharm. Sci. 2015. 104: 1240-1245.

4 J. Wen, D. Batabyal, N. Knutson, H. Lord, M. Wikström. “A comparison between emerging and current biophysical methods for the assessment of higher-order structure of biopharmaceuticals”. J. Pharm. Sci. 2020. 109: 247-253.

5. D. Schiffmann, D. Butterfield, R. Yardley, A. Knight, S. Windsor, C. Jones. “Val-CD Appendix A: CD spectroscopy: an inter-laboratory study”. 2004. National Physical Laboratory, Teddington, Middlesex, U. K., Crown Copyright 2004 (ISSN 1744-0602).

6. C. Jones, D. Schiffmann, A. Knight, S. Windsor. “Val-CD best practice guide: CD spectroscopyfor the quality control of biopharmaceuticals”. NPL Report DQL-AS 008. 2004. National Physics Laboratory.

7. S. M. Kelly, T. J. Jess, N. C. Price. “How to study proteins by circular dichroism”. Biochim. Biophys. Acta. 2005. 1751: 119-139.

8. A. J. Miles, F. Wien, J. G. Lees, B. A. Wallace. “Calibration and standardisation of synchrotron radiation and conventional circular dichroism spectrometers. Part 2: Factors affecting magnitude and wavelength”. Spectroscopy. 2005. 19: 43-51.

9. A. Damianoglou, E. J. Crust, M. R. Hicks, S. E. Howson, A. E. Knight, J. Ravi, P. Scott, A. Rodger. “A new reference material for UV–visible circular dichroism spectroscopy”. Chirality. 2008. 20: 1029-1038.

10. R. W. Burke, R. Mavrodineanu. NIST Special Publication 260-54. 1977. Copies may be obtained from the Standard Reference Materials Program, National Institute of Standards and Technology, Gaithersburg, MD 20899 or online at www.nist.gov.

11. R. W. Burke, R. Mavrodineanu. NIST Special Publication 260-81. 1983. Copies may be obtained from the Standard Reference Materials Program, National Institute of Standards and Technology, Gaithersburg, MD 20899 or online at www.nist.gov.

12. A. J. Miles, F. Wien, J. G. Lees, A. Rodger, R. W. Janes, B. A. Wallance. “Calibration and Standardisation of Synchrotron Radiation Circular Dichroism and Conventional Circular Dichroism Spectrophotometers”. Spectroscopy. 2003. 17: 653-661.

13. J. Ravi, D. Schiffmann, R. Tantra, S. Cox, J. Eady, C. W. Meuse, M. J. A. Bailey, A. E. Knight. “International comparability in spectroscopic measurements of protein structure by circular dichroism: CCQM-P59.1“. Metrologia. 2010. 47: 631-641.

14. M. G. Cox, J. Ravi, P. D. Rakowska, A. E. Knight. “Uncertainty in measurement of protein circular dichroism spectra”. Metrologia. 2014. 51: 67-79.

15. C. W. Meuse, J. J. Filiben, K. A. Rubinson. “Submultiple Data Collection to Explore Spectroscopic Instrument Instabilities Shows that Much of the “Noise” is not Stochastic”. Anal. Chem. 2018. 90: 5066-5074.

16. A. A. Bahab, D. El-Hag, A. F. Drake. “Simultaneous determination of photometric accuracy during circular dichroism measurements”. Anal. Methods. 2010. 2: 929-935.

17. G. V. Barnett, G. Balakrishnan, N. Chennamsetty, B. Meengs, J. Meyer, J. Bongers, R. Ludwig, L. Tao, T. K. Das, A. Leone, S. R. Kar. “Enhanced precision of circular dichroism spectral measurements permits detection of subtle higher order structural changes in therapeutic proteins”. J. Pharm. Sci. 2018. 107: 2559-2569.

18. E. Castiglioni, P. Albertini, S. Abbate. “Evaluation of instrumental errors built in circular dichroism spectrometers”. Chirality. 2010. 22: E142-E148.

19. J. M. DiNitto, J. M. Kenney. “Noise characterization in circular dichroism spectroscopy”. Appl. Spec. 2012. 66: 180-187.

20. J. C. Sutherland. “Dichrometer errors resulting from large signals or improper modulator phasing”. Chirality. 2012. 24: 706-717.

21. J. C. Sutherland. “Calibration of photoelastic modulator based dichrometers maintaining constant phase across the spectrum”. JINST. 2016. 11: P07016.

22. W. C. Johnson. “Circular Dichroism Instrumentation”. In: G. Fasman, editor. Circular Dichroism and the Conformational Analysis of Biomolecules’s. New York: Plenum Press, 1996. 635-652.

23. N. T. H. White. “Calibration device and uses thereof”. US Patent 10,352,846 B2. 2017. 2019.

24. N. J. Greenfield. “Using circular dichroism spectra to estimate protein secondary structure”. Nature Protocols. 2006. 1: 2876-2890.

25. L. Whitmore, B. Woollett, A. J. Miles, D. P. Klose, R. W. Janes, B. A. Wallace. “PCDDB: the protein circular dichroism data bank, a repository for circular dichroism spectral and metadata”. Nucleic Acids Res. 2011. 39(suppl 1): D480-D486.

26. L. Whitmore, A. J. Miles, L. Mavridis, R. W. Janes, B. A. Wallace. “PCDDB: new developments at the Protein Circular Dichroism Data Bank”. Nucleic Acids Res. 2017. 45 (D1): D303-D307.

27. G. C. Chen, J. T. Yang. “Two-Point Calibration of Circular Dichrometer with d-10-Camphorsulfonic Acid”. Anal. Let. 1977. 10: 1195-1207.

28. P. H. Schippers, H. P. J. M. Dekkers. “Direct determination of absolute circular dichroism data and calibration of commercial instruments”. Anal. Chem. 1981. 53: 778-782.

29. A.J. Miles F.W. Wien B.A. Wallace. “Redetermination of the extinction coefficient of camphor-10-sulfonic acid, a calibration standard for circular dichroism”. Anal. Biochem. 2004. 335: 338-339.

30. R. C. Davies, A. Neuberger, B. M. Wilson. “The dependence of lysozyme activity on pH and ionic strength”. Biochim. Biophys. Acta. 1969. 178: 294-305.

31. S. C. Gill, P. H. von Hippel. “Calculation of protein extinction coefficients from amino acid sequence data”. Anal. Biochem. 1989. 182: 319–326.

32. J. G. Lees, F. Wien, A. J. Miles, B. A. Wallace. “A reference database for circular dichroism spectroscopy covering fold and secondary structure space”. Bioinformatics. 2006. 22: 1955-1962.

33. F. Meersman, C. Atilgan, A. J. Miles, R. Bader, W. Shang, A. Matagne, B.A. Wallace, M. H. J. Koch. “Consistent picture of the reversible thermal unfolding of hen egg-white lysozyme from experiment and molecular dynamics”. Biophys. J. 2010. 99: 2255-2263.

34. N. N. Dinh, B. C. Winn, K. K. Arthur, J. P. Gabrielson. “Quantitative spectral comparison by weighted spectral difference for protein higher order structure confirmation”. Anal. Biochem. 2014. 464: 60-62.