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A New Reference Material for UV–visible Circular Dichroism Spectroscopy

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ABSTRACT To obtain accurate and consistent measurements from circular dichroism (CD) instruments over time and from different laboratories, it is important that they are properly calibrated. The characteristics of the available reference materials are not ideal to ensure proper calibration as they typically only give peaks in one or two spectral regions, and often have issues concerning purity and stability. Currently either camphor sulfonic acid or ammonium camphor sulfonate are used. The latter can be an unstable, slightly hygroscopic secondary standard compound with only one characterized CD band. The former is the very hygroscopic primary standard for which only one enantiomer is readily available. We have synthesized a new reference material for CD, Na[Co(EDDS)]·H_2O (EDDS = *N*,*N*-ethylenediaminedisuccinic acid) which addresses these problems. It is extremely stable and available in both enantiomeric forms. The CD spectrum of Na[Co(EDDS)]·H_2O has nine distinct peaks between 180 and 599 nm. It thus fulfils the principal requirements for CD calibration chemical standards and has the potential to be used to ensure good practice in the measurement of CD data, providing two spectra of equal magnitude and opposite sign for a given concentration and path length. We have carried out an interlaboratory comparison using this material and show how it can be used to improve CD comparability between laboratories. A fitting algorithm has been developed to assess CD spectropolarimeter performance between 750 and 178 nm. This could be the basis of a formal quality control process once criteria for performance have been decided. *Chirality 20:1029–1038, 2008.*

KEY WORDS: circular dichroism; calibration; enantiomers; standard

INTRODUCTION

Circular dichroism (CD) is a powerful spectroscopic technique with many applications in organic chemistry and biochemistry. For example, one can qualitatively and quantitatively assess the purity of two enantiomers of a chiral molecule; one can sensitively detect changes in the structure of protein molecules; and one can predict the secondary structure of proteins. It is important, prior to the analysis, to establish quality assurance for factors affecting the reliability of the data. This is especially true when spectra are crucial evidence of the structure and stability of a protein component of a pharmaceutical product, but it is also important when spectra are to be reported for almost any purpose.

To achieve comparability of CD spectra between instruments, it is important that the instruments are well-maintained and used correctly.[1,2] However, even where this is done, differences between instruments will mean that data are typically not comparable.[3,4] One route to the comparability of data is to characterize CD instruments in terms of both CD intensity calibration and wavelength calibration across the spectral range of interest. However, currently available "standard" materials typically only provide a single CD peak, and are often poorly characterized, as is discussed below. The lack of confidence that data can meaningfully be compared when they are measured in different laboratories, or at different times, undermines the usefulness of CD as a technique. This is particularly true in heavily regulated areas such as biopharmaceutical quality control.

In the past decades, a series of optically active substances have been identified and used extensively for the calibration of CD spectropolarimeters. D-10-camphorsulfonic acid (CSA) in water at 290.5 nm has been regarded as the primary standard and has been the one used extensively throughout the world. However, differences in the magni-

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tude of its spectra due to its hygroscopic nature have repeatedly been reported in the literature.[5] Other substances such as androsterone and isoandrosterone in dioxane have also been considered[5,6] as well as glucurono-γ-lactone and D-pantolactone in other organic solvents.[7] Glucurono-γ-lactone was found to be less suitable than the D-pantolactone that became commercially available as optically pure crystals having relatively high optical rotation with weak UV absorption and less water uptake than CSA.[7] A study was also conducted by Chen and Yang[6] comparing CSA in water at 290.5 nm, D-pantolactone in methanol at 222 nm and (+)-*tris*-(ethylenediamine) Co(III) iodide monohydrate in water at 490 nm. The study revealed deviations in molar ellipticity of up to 30% for D-pantolactone and (+)-*tris*-(ethylenediamine) Co(III) iodide monohydrate[8] between measurements carried by different spectropolarimeters. That study may suggest that [Co(ethylenediamine)$_3$]$^{3+}$ would make a good standard. It is certainly chemically and enantiomerically stable. However, it is challenging to make up solutions accurately for UV measurements (literature extinction coefficient data is all for the visible region and the UV intensity is orders of magnitude larger). In addition, a single solution cannot be used from the visible region to the UV region. The final nail in its coffin is that it is not commercially available and it extremely difficult to produce 100% enantiomerically pure as it is resolved by repeated co-crystallization with chiral anions.[9]

Ammonium D-10-camphorsulfonate (ACS) (see Fig. 1), which is less hygroscopic, but much more expensive, than CSA, has been widely adopted as a secondary standard. ACS was found to be essentially non-hygroscopic, easily handled and to have the same spectrometric behavior as CSA because of the fact that both compounds form the same ion in solution.[2,5] Calibration with both CSA and ACS routinely involves using only the 290.5 nm peak. Thus, there is no calibration of the visible region and none at lower wavelength. CSA does have a CD band at 191.5 nm, however, there is still some debate as to what its magnitude is (the consensus is that the 192.5 nm:290.5 nm ratio should be about 2.0).[3] A further concern with ACS is that its stability is variable. Jones et al.[10] showed its intensity can change within 2 wk even when refrigerated. Thus fresh standards need to be made regularly and accurately to a standardized protocol.[1,2] The material used also needs to be of a known chemical purity and enantiomeric purity. In a recent interlaboratory comparison study coordinated by the National Physical Laboratory (NPL),[11] it became apparent that the current single point calibration even with an externally provided standard did not ensure instrument comparability or even provide enough data to indicate whether instruments recorded comparable data.[4] For all types of measurement, the ideal is that standards should be traceable to the International System of Units (the SI)[12] with a known uncertainty.[13,14] This means that all measurements that are made in this way can be compared within a known uncertainty; moreover this is recognized as an efficient means of achieving comparability. Traceability is typically established through a chain of measurements, starting from a primary, absolute measurement (often made by a National Measurement Institute, such as NPL) and disseminated through primary and secondary standards. No such chain exists for CD, and therefore the absolute values of the CD of the standard compounds are not known with any confidence. However, where such a chain cannot be established, it may be sufficient for practical purposes to establish the comparability of measurements by other means, and this is what we have set out to achieve.

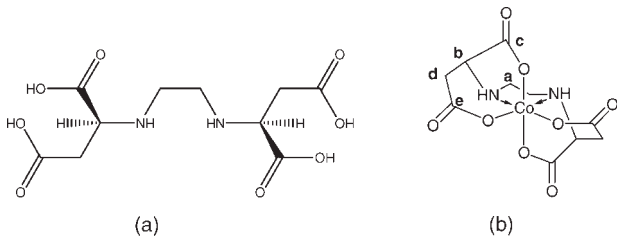

Fig. 1. (**a**) *S,S-N,N*-ethylenediaminedisuccinic acid (EDDS); (**b**) Co-EDDS with proton labeling indicated.

What impacts do uncertainties in the measurement of CD have? A good example is the use of CD spectra to predict the secondary structure of proteins. With some algorithms, errors in the intensity and wavelength scales of CD spectrometers have a significant effect on the predicted structures.[15] Similar problems arise when CD is used to measure enantiomeric purity since CD intensity is proportional to enantiomeric excess. In empirical applications such as comparing the spectra of biopharmaceutical formulations, the comparisons between data measured on different occasions and/or in different laboratories are only valid if the instruments can be relied on to produce consistent data. Thus it is essential that, even in the absence of an absolute standard for CD, all instruments used would produce essentially the same data for the same sample. Furthermore we need a measure of what we mean by 'the same'.

There has been a long running project at Warwick University to find an alternative CD secondary standard with the following prerequisites: chemical stability in solution; enantiomeric stability in solution; availability of bands across the full UV–visible region (including below 200 nm) that can be measured on the same concentration solution; and availability of both enantiomers. One of the issues with low wavelength instrument performance is that in most instruments stray light becomes a significant problem. Instrument problems, in this or any other region of the spectrum, apart from a simple magnitude scaling, are likely to result in enantiomers not having mirror image spectra. The availability of both enantiomers and a requirement for equal magnitude and opposite signed CD signals at all wavelengths were therefore among the requirements of this work. We have screened many compounds over the years and have finally selected and tested *R,R*- and *S,S*-Na[Co(EDDS)]·H_2O ((EDDS = *N,N*-ethylenediaminedisuccinic acid), henceforth referred to as *R,R*-CoEDDS and *S,S*-CoEDDS, respectively). CoEDDS is a transition metal complex with d→d transitions which was first synthesized by Neal and Rose in 1968.[16] Its visible region CD spectrum

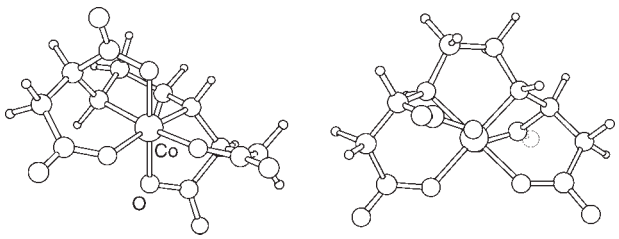

Fig. 2. Stereogenic arrangements of the five- and six-membered rings in the EDDS complex $Li(H_2O)_3[Co^{III}(S,S\text{-EDDS})]$.[18]

has been previously published[16,17] though no UV CD data were available prior to this study. With modern instruments, it is now possible to collect data for this compound from the visible to well into the far UV region with the same sample in the cuvette as shown later. In fact by using both bench top and synchrotron radiation instruments we have acquired data from the visible down to 165 nm. Together with its stability (see later) and the availability of both enantiomers this makes it an attractive calibration standard.

For both enantiomers of $Na[Co(EDDS)]\cdot H_2O$ we have considered the effects of temperature and concentration on the CD spectra line shapes and also tested the chemical and enantiomeric stability of the compounds over a period of 12 mo. In addition we undertook a small interlaboratory comparison (including Warwick University, National Physical Laboratory, the National Institute for Biological Standards and Control, and Chiralabs). Data were also collected at the synchrotron source at Aarhus University to determine low wavelength spectral shape. The aim of the comparison study was to investigate the utility of the new material in achieving comparability between laboratories and to confirm the spectral characterization performed at Warwick. We also investigated the use of the material in instrument performance verification testing.

MATERIALS AND METHODS

Optically pure *S,S*-EDDS (see Fig. 2) was donated by Innospec. Other materials were obtained from Sigma-Aldrich and used without further purification. 18.2 MΩ water was used throughout.

R,R-*EDDS*[16]

A mixture of D-aspartic acid (50.73 g, 0.38 mol, ee = 98%), NaOH (30 ml, 50% aqueous solution), $Ca(OH)_2$ (13.94 g, 0.19 mol), and deionized water (70 ml) was placed in a 1-liter three-necked flask fitted with a reflux condenser and 50 ml pressure-equalized dropping funnel and large magnetic follower. 1,2-dibromoethane (28 ml, 61.0 g, 0.33 mol) was added carefully with stirring via the third side-arm and the mixture was heated to gentle reflux. A further portion of NaOH (24 ml, 50% aqueous) was added dropwise over about 6 hr, maintaining the reflux. Water (100 ml) was added and the solution was heated at reflux for 1 hr. The reaction mixture was left to cool with stirring for 1 hr. The mixture was acidified with concentrated HCl until pH 3 by which time a copious white precipitate had formed. The filtrate was collected and added to water (225 ml) followed by NaOH (50% aqueous) to pH 11. The solution was carefully acidified with HCl to pH 3.5 and the subsequent precipitate was collected, washed with dilute HCl and dried in air at 65°C in vacuo. Yield 17.9 g (32%). Anal. Found (Calcd. for $C_{10}H_{16}N_2O_8$) %C 41.02 (41.10), H 5.50 (5.52), N 9.31 (9.59). ^{1}H NMR (400 MHz, 293 K, NaOH/D_2O ref. 4.79) δ ca. 2.35 (dd, 2H, CH_2CO_2), 2.51 (dd, 2H, CH_2CO_2) 2.64 (m, 4H, CH_2CH_2), 3.39 (dd, 2H, CH) (NH and CO_2H not observed at this pH). ^{13}Cpp NMR δ 41.5 (CH_2), 46.8 (CH), 61.5 (CH_2—CH_2), 179.9 (C_q), 181.6 (C_q). MS (FAB) *m/z* 292 (M^+).

$Na_3[Co(CO_3)_3]\cdot 3H_2O$[16]

$Co(NO_3)_2.6H_2O$ (29.1 g, 0.1 mol) in water (50 ml) and H_2O_2 (10 ml, 30%) was added dropwise to a cold slurry of $NaH(CO_3)$ (42.0 g, 0.5 mol) in water (50 ml). The mixture was allowed to stand for 1 hr at 0°C with continuous stirring. The resultant green product was filtered, washed with cold water (thrice in 10 ml), ethanol, and diethyl ether, and then dried in vacuo. Yield 29 g, 80%.

*Na[Co(*S,S*-EDDS)]H$_2$O (*R,R*-Isomer Prepared Similarly)*[16]

S,S-EDDS (3.10 g, 10 mmol) was added with stirring to an ice cold slurry of $Na_3[Co(CO_3)_3]\cdot 3H_20$ (3.63 g, 10 mmol) and activated charcoal (3 g) in water (75 ml). The reaction mixture was allowed to warm to ambient temperature and was stirred until CO_2 evolution ceased. The mixture was then heated to 80°C and stirred for 20 min, followed by slow addition of small amounts of acetic acid (5% aqueous, total 10 ml) to complete evolution of CO_2. The solution was heated again to 80°C for 5 min and allowed to cool. Acetic acid solution was added to pH 4.25. The solution was filtered through Celite to remove the charcoal and the volume of the solution was reduced to about 30 ml. Slow addition of ethanol (ca. 100 ml) resulted in precipitation of a purple solid which was isolated by filtration. The solid was suspended in ethanol (ca. 100 ml) and sufficient water was added to dissolve the solid. The solution was filtered and ethanol was slowly added with stirring until a solid began to precipitate. The mixture was then heated to redissolve the complex and was left to cool. The fine crystals of $Na[Co(EDDS)]\cdot H_2O$ (see Fig. 2) obtained were isolated by filtration and left to dry in air overnight (yield 1.7 g, 44%). Intriguingly drying the crystals in a vacuum oven removed the water of crystallization. However, on exposure to the atmosphere (most obviously on a balance), one molecule of water per Co was reincorporated. *S,S*-isomer: Anal. found (Calcd. for $C_{10}H_{14}O_9CoN_2Na$) % C 30.17 (30.94), H 3.75 (3.64), N 7.00 (7.22). ^{1}H NMR (400 MHz, 298 K, D_2O) δ 3.51 (2H, dd, $^3J_{HH}$ = 5.5 Hz, $^3J_{HH}$ = 1.5 Hz, H_c), 3.16 (2H, d, $^2J_{HH}$ = 10.5 Hz, H_a/H_b), 3.09 (2H, dd, $^2J_{HH}$ = 24 Hz, $^3J_{HH}$ = 5.5 Hz, H_e), 2.84 (2H, dd, $^2J_{HH}$ = 24 Hz, 1.5 Hz, H_d), 2.71 (2H, d, $^2J_{HH}$ = 10.5 Hz, H_a/H_b). ^{13}C {} NMR (100 MHz, 298 K, D_2O) δ_C 183.1 (C_c/C_e), 183.0 (C_c/C_e), 66.6 (C_b), 52.6 (C_a) 40.0 (C_d, observed at 353 K). MS (ESI negative) m/z 346.9 ($[M]^-$), 302.9 ($[M]^-$, CO_2), 259.0 ($[M]^-$, 2 × CO_2). IR ν cm^{-1}:

1563 s (COO asymmetric stretch), 1383 s (COO symmetric stretch), 1209 m, 1134 w, 1040 m, 925 w, 879 m.

Circular Dichroism Analysis

The spectral characterization and stability study spectra of the CoEDDS compounds were all obtained using the Jasco J-715 spectropolarimeter at Warwick (calibrated using 0.060% ACS for intensity and a neodymium filter for wavelength) or the Jasco J-810 at NPL (calibrated using 0.060% ACS for intensity and a holmium filter for wavelength). As a rule of thumb, with the Jasco's photomultiplier tubes we choose sample concentrations to keep the high tension voltage below 600 V. The standard parameters used were: bandwidth 1 nm; response time 1 sec; wavelength scan range 190–750 nm; data pitch 0.5 nm; and scanning speed 100 nm/min. These parameters gave spectra that overlaid with those collected at slower scan speeds and narrower bandwidth and so were deemed satisfactory. If one wishes only to calibrate in the visible region then a concentration of ~2 mM is appropriate (higher concentrations can be used but with Jasco instruments require the low sensitivity setting to be selected, otherwise the signal goes off scale); in the UV region a smaller concentration (0.05 mM) is required to ensure the sample's absorbance is not too high.

All cuvettes used in this study were washed three times with water (18.2 MΩ) and three times with ethanol and dried with compressed air. Before establishing the stability study, the instrument-only (i.e. air only in the sample compartment) baseline and the water baseline of each cuvette used were collected. Instrument-only baselines were then collected at each time point. Thus at intermediate time points, *t*, the baseline that was subtracted from the sample spectrum was the water baseline of the cuvette at time 0 plus the difference between the instrument-only baseline at time *t* and the instrument-only baseline at time 0. Samples of each of the *R,R* and *S,S*-[Co(EDDS)] at a concentration of 0.05 mM in water were stored at 4°C, at room temperature in the dark, and at room temperature in the light for 12 mo.

Interlaboratory Comparison

The interlaboratory comparison followed a "star" design where samples were distributed from the coordinating laboratory to the participants. The study participants are listed in Table 1. The samples that were distributed included both the *R,R*- and the *S,S*-CoEDDS enantiomers of the standard (of concentrations 0.067 mM) and a racemic mixture of the two; an ACS solution for comparison, and a water blank. The participants were provided with a common protocol for measurements. All spectra were acquired in 10 mm path length cuvettes provided by the participant laboratory; CoEDDS spectra were collected from 180 to 800 nm and ACS spectra from 200 to 360 nm. The spectra were collected with a 1 nm bandwidth, 0.1 nm data pitch, and 6 accumulations. The reference spectra collected at NPL were collected at a scan speed of 10 nm/min with a 4 sec response time. Participants were left to choose the scan speed they felt most appropriate, but 50 nm/min with a 1 sec response was suggested. Baseline and sample spectra were then returned to the organizing laboratory for baseline subtraction and further analysis. To investigate the use of the standards for instrument performance verification, one participant (designated 4) intentionally used an instrument that was known to be in need of calibration and with a poorly performing lamp.

TABLE 1. Participants in the interlaboratory comparison

Institution	Participants
NPL (Coordinator)	Alex Knight; Jascindra Ravi
University of Warwick	Alison Rodger; Angeliki Damianoglou
National Institute for Biological Standards and Control	Christopher Jones
Chiralabs	George Tranter; Delphine LePevelen; Ann Talbert

Curve Fitting

Manipulation and analysis of the interlaboratory comparison data was performed in MATLAB (The MathWorks, Natick, MA). Curve fitting of the spectra was performed using the MATLAB Curve Fitting Toolbox. The model chosen for the interlaboratory comparison data was a sum of 9 Gaussians (reflecting the 9 maxima in the observed spectra), with constraints applied to peak position, height, and width to ensure an accurate fit. The equation used was of the form:

$$a_1 e^{-((x-b_1)/c_1)^2} + a_2 e^{-((x-b_2)/c_2)^2} + \cdots + a_9 e^{-((x-b_9)/c_9)^2}$$

where for the *n*th Gaussian peak a_n corresponds to the peak height, b_n to the peak (or center) wavelength, and c_n is related to the peak width (with the full width at half maximum given by $2\sqrt{2\ln(2)}c$). To achieve accurate fitting of the spectra it was necessary to include constraints in the fitting method, and these are listed in Table 2. The peak heights (*a* parameters) were constrained to be either positive (0 to $+\infty$) or negative ($-\infty$ to 0); the peak wavelengths (*b* parameters) were constrained to a 10 nm or 15 nm window. In addition, the *c* (width) parameters were constrained to a minimum of 10 nm for all peaks except 1, which was constrained to a minimum of 5 nm. These constraints were developed empirically by iteratively adding terms to the model until the residuals approached a flat line. Additionally, spectra were truncated at short wavelengths to exclude noisy data.

RESULTS

Structure of the Complexes

EDDS (EDDS = *N,N*-ethylenediaminedisuccinic acid) is a hexadentate chelating agent, isomeric with EDTA, and forms an octahedral complex with two nitrogen and four oxygen donors; the latter are typically deprotonated under basic conditions.[16] A key structural difference between H_4EDDS and H_4EDTA is that the former contains two

TABLE 2. Constraints used in Gaussian fitting of CoEDDS and fit coefficients for NPL data

	Fit constraints				Fit parameters					
	Wavelength (nm)		Sign		Wavelength (nm)		Height (mdeg)		Width (nm)	
Peak	Min	Max	*S,S*	*R,R*	*S,S*	*R,R*	*S,S*	*R,R*	*S,S*	*R,R*
1	175	185	+	−	179.2	181.4	25.494	−19.650	8.770	7.804
2	205	215	+	−	211.0	211.0	61.189	−62.025	12.120	12.117
3	225	240	−	+	234.9	233.6	−11.691	12.165	26.215	26.479
4	260	275	+	−	267.6	268.6	9.985	−9.577	28.034	27.307
5	367	377	+	−	368.0	370.3	1.740	−1.919	23.656	25.343
6	399	409	+	−	406.8	409.0	2.129	−2.016	29.775	27.371
7	478	488	+	−	482.6	484.2	1.719	−1.791	32.972	36.096
8	540	550	−	+	544.9	545.4	−5.947	6.170	31.572	33.284
9	595	610	+	−	598.8	595.0	1.188	−1.382	30.218	34.488

Confidence limits for these data are available in Supplementary Information. Additionally, minimum width constraints were applied to the peaks to ensure an accurate fit. Note that *S,S*- and *R,R*- data are in most cases consistent within 95% confidence limits (see Supplementary Information). Where peaks are not well constrained by the data they may show differences but these peaks would not be used for calibration purposes.

stereogenic carbon atoms which retain their stereochemistry upon chelation. However, unlike with EDTA where the four carboxyl arms all form five-membered rings upon coordination, EDDS forms two five-membered rings and two six-membered rings (see Fig. 2). When the two stereogenic carbon atoms have the same absolute configuration (i.e. both *S,S*- or both *R,R*-), only one arrangement, that with the five-membered rings in the axial positions and the six-membered rings in the equatorial positions has been observed.[16,17,19,20] This is a result of excessive strain energy associated with having the five-membered rings in equatorial positions.[16,18,20] As a result, all homochiral EDDS complexes are diastereomerically pure; the chirality of the ligand is expressed perfectly in the absolute configuration of the metal atom such that e.g. *S,S*-EDDS gives only the complexes Δ-[M(*S,S*-EDDS)]$^{n-}$. It is this stereogenic arrangement of the ligand around the metal atom that leads to the observed CD spectrum.

Characterization and Purity by NMR

The cobalt complexes produced by the methods described in this article were chemically, diasteriomerically, and enantiomerically pure within limits of detection. After the reaction there is at most 0.01% of any *R,R*-EDDS in the *S,S*- product due to the high enantiomeric purity of the starting materials. (The aspartic acid is >98% ee (er = 99:1), but the product diastereomer is homochiral. Any heterochiral (*R,S*) is crystallized out, and is absent according to NMR. Chances of any homochiral product of wrong handedness is thus 1% of 1%, i.e. 0.01%.) Recrystallization will have further reduced this. The ^{1}H-NMR spectra of Na[Co(*S,S*-EDDS)]·H_2O and Na[Co(*R,R*-EDDS)]·H_2O in D_2O (see Fig. 3) were superposable on one another. They showed no detectable impurities; small apparent fluctuations in the baselines in Figure 3 (e.g. about 3.1 and 3.4 ppm) are due to ^{13}C satellites. The spectra showed the expected five sets of multiplets, the NH groups having been deuteriated by the solvent. The assignments given in the experimental section were made via ^{1}H—^{1}H and ^{13}C—^{1}H correlation spectra and other standard methods. The H atoms on the ethylene backbone, H_a and H_b, gave two second-order doublets of doublets centered at 2.81 and 3.28 ppm. H_d and H_e gave two sets of mutually coupled doublets centered at 2.98 ppm and 3.22 ppm with the smaller and inequivalent couplings to H_c (3.59 ppm) allowing their assignment via the Karplus equations. In the ^{13}C spectrum the resonance for the C atom bonded to $H_{d/e}$ was observed only at high temperature or via ^{1}H observed heteronuclear correlation, presumably as a result of an exchange between conformers of this 6-membered ring. We note that the corresponding ^{1}H resonances are also relatively broad. Unfortunately, the freezing point of

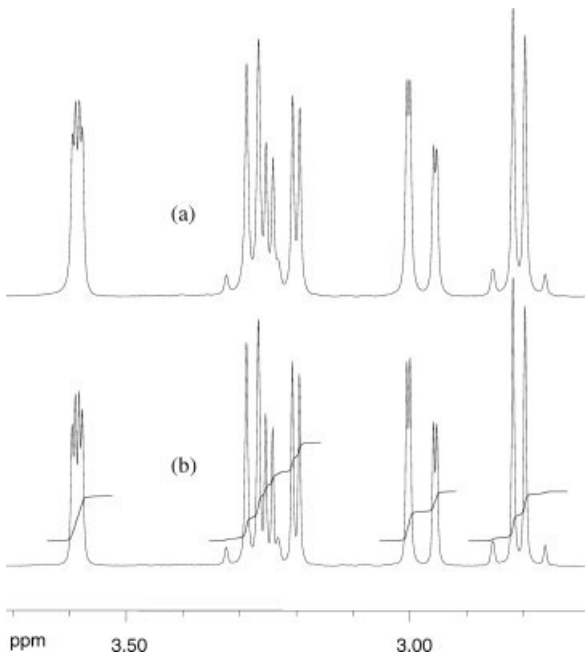

Fig. 3. ^{1}H NMR spectra of **(a)** Na[Co(*R,R*-EDDS)]·H_2O and **(b)** Na[Co(*S,S*-EDDS)]·H_2O.

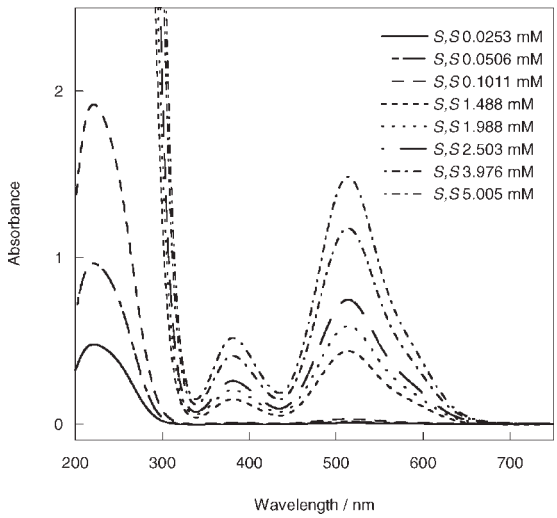

Fig. 4. Absorbance spectra (1 cm path length) as a function of increasing sample concentration (indicated in figure) for *S,S*-CoEDDS.

the solvent precluded measurement of low temperature spectra.

Extinction Coefficient Determination

Most laboratories with CD machines have UV/visible absorption instruments but not all have high quality balances. Thus, accurate values for the extinction coefficients of CoEDDS are required in all application wavelength ranges. Absorbance data were collected over a series of separately prepared samples of each enantiomer at a range of concentrations (determined by weighing four independent samples and making to volume in a volumetric flask, then diluting these samples) (see Fig. 4). Data at the wavelength maxima were extracted and plotted to determine extinction coefficients as summarized in Table 3.

CD Spectra of R,R- *and* S,S-*CoEDDS*

A full wavelength spectrum of *R,R*-CoEDDS is given in Figure 5a. It is a concatenation of data collected at Warwick, NPL, and the synchrotron source at Aarhus (calibrated using 0.060% ACS for intensity and benzene vapor for wavelength) to give the best signal-to-noise at each wavelength and to ensure significant overlap of the different instruments (spectra in common wavelength regions were identical within the noise envelope of the data). The spectra for the two enantiomers are equal and opposite in sign at each wavelength, as illustrated in Figure 5b where the spectra of the *R,R* and $-S,S$ (minus 1 times the *S,S* spectrum) spectra overlay within instrument noise. $\Delta\varepsilon$ values are given in Table 3. The low wavelength spectra collected with a 1 cm cell overlaid those collected with 100 μm and 18 μm pathlengths, when scaled to account for the pathlengths and concentrations (data not shown).

Stability Studies by CD

The CD spectral shapes of the two enantiomers show that the samples are stable for at least 1 yr even at room temperature exposed to light. All storage conditions retained the same spectral shapes over the 12 mo period (most data not shown) as illustrated in Figure 6. Despite sealing the cuvettes with Teflon tape, a slight degree of evaporation occurred for one sample, which has been corrected for in Figure 6 by a scaling factor determined from the change in the absorbance.

Curve Fitting

Comparison of spectra by eye is sufficient for many purposes. However, if we wish to use CD to, for example, Good Laboratory Practice (GLP) standard, a more rigorous and quantitative evaluation of instrument performance is required. To this end, we first showed that the CoEDDS CD spectra could be fitted using a sum of 9 Gaussian functions (see methods). To illustrate the fitting results, fits and residuals for the NPL data from the round robin study are shown in Figure 7. The constraints and fit parameters are given in Table 2. It is clear that the model fits the data accurately from 185 to 800 nm. The residuals are small and randomly distributed, although greater noise in the spectra is evident at the wavelength extrema. The R^2 for the *S,S* enantiomer was 0.9998 and for the *R,R* enantiomer, 0.9997. Once a data set has been reduced to a sum of Gaussians, comparison with a similarly represented standard spectral data set is straightforward.

Interlaboratory Comparison

The interlaboratory comparison (see Supplementary Information for details) clearly illustrated the value of the fitting methodology. It was possible to show pictorially with spectra (Supplementary Information Fig. S3) and quantitatively with residual plots (Supplementary Information Fig.

TABLE 3. Extinction coefficient and delta epsilon values for both enantiomers, fitted using Kaleidagraph

Wavelength (nm)	$\varepsilon_{S,S}$ (mol^{-1} dm^3 cm^{-1})	$\varepsilon_{R,R}$ (mol^{-1} dm^3 cm^{-1})	$\Delta\varepsilon_{S,S}$ (mol^{-1} dm^3 cm^{-1})	$\Delta\varepsilon_{R,R}$ (mol^{-1} dm^3 cm^{-1})
545	209 ± 0.4	211+0.9	**−2.40 ± 0.05**	**+2.33 ± 0.05**
515	**296 ± 0.4**	**299 ± 0.3**		
480			**+0.68 ± 0.015**	**−0.67 ± 0.015**
382	**103 ± 0.5**	**104 ± 0.6**	**−0.95 ± 0.025**	**+0.93 ± 0.025**
274	6142 ± 20	6131 ± 33	**+3.35 ± 0.10**	**−3.30 ± 0.10**
237			**+3 ± 0.3**	**+4 ± 0.3**
221.5	**18,997 ± 24**	**18,985 ± 32**	**+0.665 ± 0.10**	**−0.675 ± 0.10**
210	17,490 ± 35	17,500 ± 60	**+24.0 ± 0.3**	**−22.5 ± 0.3**

Error quoted is standard deviation. Data and its analysis are given in Supplementary information. Data in bold are peak maxima.

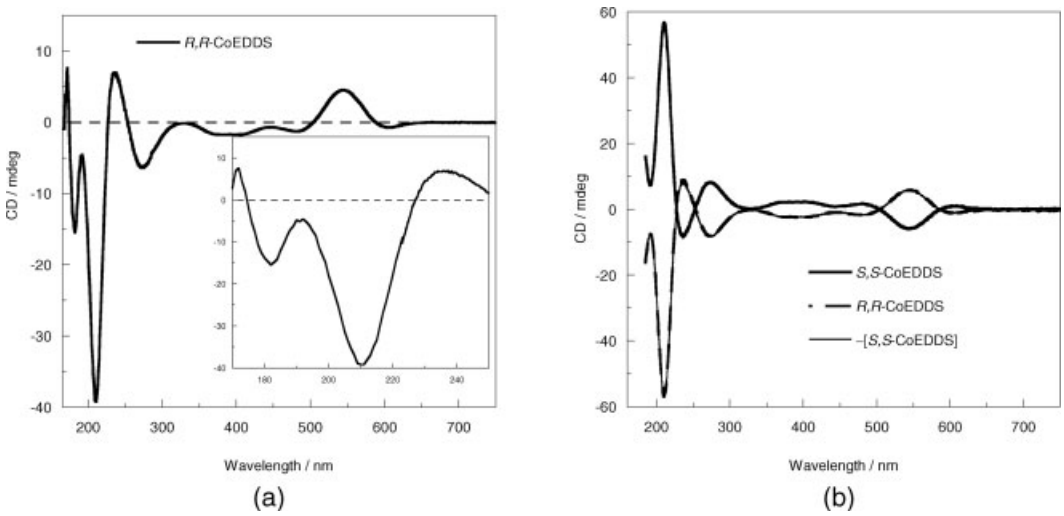

Fig. 5. (**a**) CD spectrum in millidegrees of *R,R*-CoEDDS in water (concentration of 0.056 mM, 1 cm path length and 0.56 mM, 100 μM pathlength). *S,S*-CoEDDS is equal in magnitude and opppsite in sign at each wavelength but is not shown for clarity. The insert shows the low wavelength part of the spectrum. (**b**) Overlaid CD spectra of *R,R*-, *S,S*-CoEDDS and minus *S,S*-CoEDDS (0.072 mM, 1 cm path length) illustrating the equal magnitude and opposite sign of the two enantiomers.

S4) that 3 of the 4 laboratories were giving consistent data. As expected Laboratory 4 clearly had problems, giving R^2 values with respect to the standard of 0.44 (*S,S*) and 0.79 (*R,R*). This implies that "rogue" spectra can be automatically rejected by software based on fit quality statistics such as the R^2 value. The 211 nm peak is particularly useful for instrument validation as it typically gives the narrowest confidence intervals, shown by the error bars in the plots in Supplementary Information Figure S4.

A more subtle application of the standards and the fitting method to analyze instrument performance is provided by the spectra of Figure 8. These *R,R*- and *S,S*- spectra should match the ones in Figures 5 and 6 as the samples were the same but the data collected with a 2 nm bandwidth. Our expectation with a 2 nm bandwidth was that most of the spectra should be of reasonable quality (and usually better than with 1 nm bandwidth, as more light passes through the sample with wider slits) but that the sharp peak at 210 nm might show some flattening. In practice, however, on the Warwick J-715 instrument when these spectra were collected, it is apparent from the overlay of the two enantiomers and more clearly from the *R,R*- plus *S,S*- spectrum (which should be a flat line of zero magnitude), that below 300 nm the instrument performance with 2 nm bandwidth would not be satisfactory for many applications.

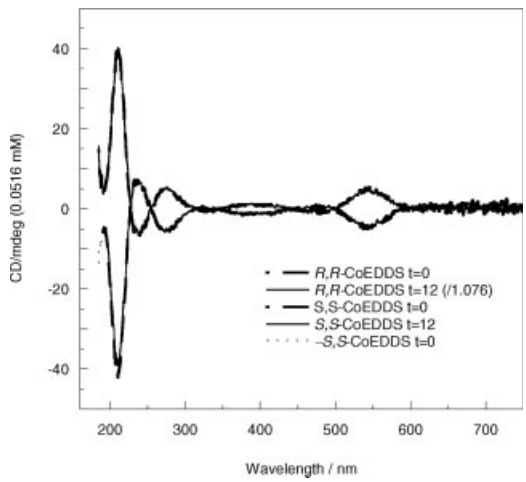

Fig. 6. CD spectra of *R,R*- and *S,S*-Co EDDS (0.56 mM in water, 1 cm pathlength) obtained at time = 0 and time = 12 mo (labeled $t = 0$ and $t = 12$, respectively) and stored on the bench at room temperature in the light between measurements which were using the Jasco J-715 instrument. A water baseline measured on the same day as the sample was subtracted from the initial and final spectra. *R,R*-CoEDDS at $t = 12$ mo has been scaled to account for a slight increase in concentration (as determined from absorbance measurements) of the sample due to evaporation.

CONCLUSIONS

To meet the pressing requirement for better consistency and comparability of circular dichroism measurements, we have set out, in the program reported in this article, to synthesize and characterize a suitable reference material. The synthesis of the two enantiomers of the metal complex $Na[Co(EDDS)]\cdot H_2O$ (EDDS = *N,N*-ethylenediaminedisuccinic acid) has been accomplished and its properties assessed. The stereochemistry of EDDS ensures that the metal complexes adopt only one diastereomeric form, thus the enantiomeric purity of the ligand determines the enantiomeric purity of the final complex. ε and $\Delta\varepsilon$ values have been determined for a number of wavelengths. We have shown that both enantiomers remain stable in solution at room temperature on the bench for at least 12 mo (in contrast to ACS which can often be surprisingly unstable[10]). Greater care does need to be taken to prevent evaporation and we recommend sealing the cuvettes to be used. We have also demonstrated that consistent results can be obtained with these standards (when used according to good practice recommenda-

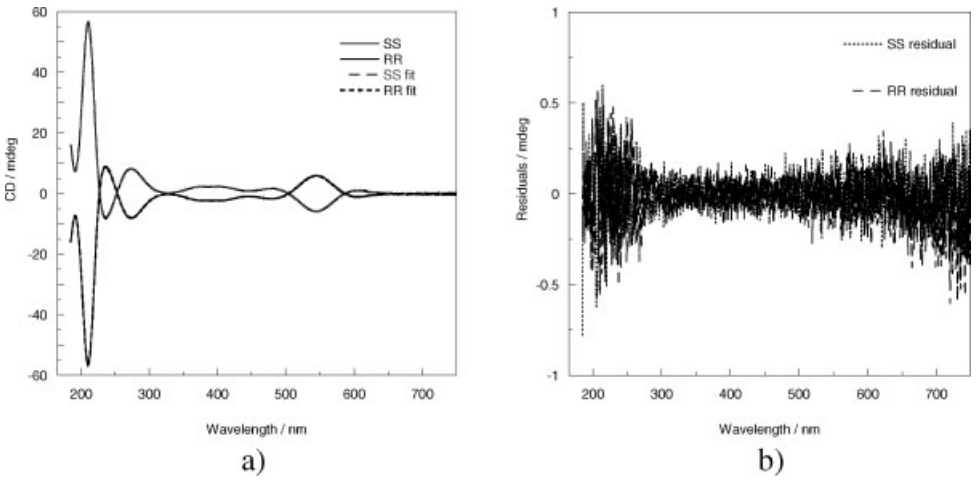

Fig. 7. (**a**) Overlay of NPL experimental CD data for *R,R*- and *S,S*-CoEDDS (0.067 mM) with its fit to a sum of 9 Gaussian curves; (**b**) the fit residuals.

tions)[1,2] between laboratories, particularly between conventional instruments and the synchrotron CD instrument at Aarhus in Denmark. By combining data from different instruments we have produced a standard spectrum from a single solution of concentration ~0.05 mM which can be used as an instrument performance verification tool and to compare the calibration status of CD instruments from 750 to 168 nm. For more critical work, however, higher concentrations could be used for measurements of the longer wavelength peaks.

The broad spectral range of this standard means that it will be useful for a wide range of applications. For example, if we consider only proteins, in conventional CD, the far UV range (typically 180 to 260 nm) is primarily used for protein secondary structure measurements. The main peak of ACS or CSA is at 290.5 nm, which lies outside this range. However, CoEDDS has intense peaks at 210 and 237 nm; it also has a peak at around 179 nm, which is readily accessible on synchrotron CD instruments. The near UV region (typically 240 to 320 nm) provides information on the aromatic side-chains and disulfide bridges in proteins, and can be calibrated using the CoEDDS peak at 270 nm. Further, peaks are found at 370, 410, 480, 545, and 599 nm, extending well into the visible region. In protein work this spectral region is principally used for studies of prosthetic groups such as haems. This wide spectral range should prove to be of similar benefit in other applications.

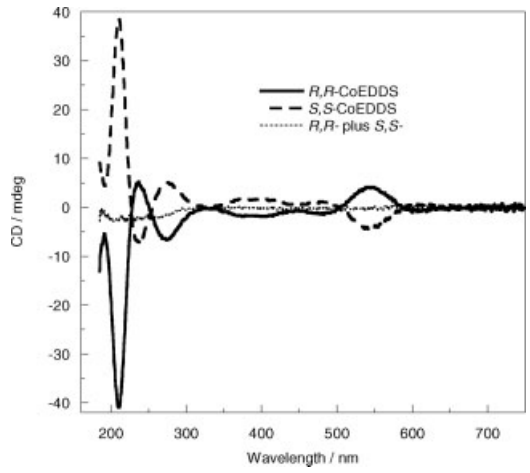

Fig. 8. CD spectra of *R,R*-CoEDDS and *S,S*-CoEDDS (0.056 mM and 1 cm path length) collected with 2 nm bandwidth. Also show is the sum of the two enantiomeric spectra.

The CD spectrum of CoEDDS thus has a number of peaks across the spectral range from the far UV to the visible region, all of which can be measured with a single solution. Furthermore, we noted that the spectrum can be accurately modeled as a summation of nine Gaussian functions. This means that the characteristics of the instrument across the spectral range where virtually all electronic CD measurements are made can be reduced down to a set of a few numbers, which greatly facilitates the comparison across instruments. Indeed, such a set of parameters could be used to "correct" for differences between instrument sensitivity profiles, as previously described by Miles et al.[3] but with the benefit that only one standard is required rather than three. We have therefore developed a protocol for CD wavelength and intensity calibrations using a method for 'automatic' instrument validation. The data from participant 4 and also the Warwick data collected with a 2 nm bandwidth provided a test of the standard material in instrument performance verification. Automated pass/fail testing based on this standard could readily be implemented in software used as part of a quality control regime, for example in industrial applications. Criteria could include a threshold for fit quality, and acceptable ranges for the wavelength and intensity of each peak.

We would suggest the following procedure for the use of the standard:

1. Certified samples of both enantiomers of CoEDDS are distributed to users in sealed cuvettes, together with

appropriate documentation, a protocol for their use, software for analysis of the resulting spectra, and the cuvette baseline. The certification would indicate the cell path length, peak parameters (intensity and wavelength), and recalibration intervals.

2. A spectrum is acquired from each of the two enantiomers of CoEDDS using appropriate parameters, together with baseline spectra (determined from the local instrument baseline and the provided cuvette baseline). If desired, the spectra can be truncated to the range of wavelengths of interest.
3. The spectra are imported into the analysis software, baselines are subtracted, and the curve fitting method described earlier is applied to the spectra.
4. The parameters of the fit are then compared to reference values. If the values fall outside a predefined range, the instrument will be considered to have failed the test. These parameters would include:
   (a) The R^2 (coefficient of determination) of the fit should exceed 0.99. If the value is less than this, it indicates poor quality spectra, for example due to severe wavelength errors, noise, or other distortions. This also serves as a check that the correct spectrum is being used.
   (b) The peak wavelength parameters should fit the reference values to within a defined range, for example ± 1 nm. (This should only be applied to peaks within the measured range. This would also take into account the confidence limits of the fit, and should only be applied to peaks which have positions well constrained by the data.)
   (c) The peak heights should match the reference values to within a predefined limit. For example, for critical work we recommend the value of the 211 nm peak should be correct to within, say 0.5% (in which case the above quoted 2 nm bandwidth spectrum of Figure 8 would 'fail') or less critically to within 5% (in which case it would pass). Again, this criterion would only apply to these peaks within the measured spectral range and that are sufficiently well estimated by the fitting algorithm.
   (d) Implicit within the above criteria is that the two enantiomers will exhibit equal and opposite spectra, confirming that the instrument is performing correctly.
5. The software would then generate a report, including the measured and reference spectra, the results of the curve fitting, and a parameter-by-parameter pass/fail assessment of the spectrum. Finally, the software would generate an overall pass/fail decision and a recommendation for the next action in the event of a failure:
   (a) In the event that the spectra pass on the fit quality and wavelength checks, but fail on the peak intensities, the user will be first recommended to remeasure the spectra, checking all instrument parameters. If this situation still holds, the software will offer the option of generating a calibration correction curve. This curve can be applied to data to make them comparable to data measured on the reference instrument. This option serves to accommodate the scenario where a simple single-wavelength intensity calibration adjustment (as available on most instruments) is insufficient to achieve comparability; for example, where instruments exhibit wavelength-dependent variation in sensitivity.
   (b) If the spectra fail on fit quality or wavelength checks, or if the intensity correction option is not appropriate, the user will be recommended to perform further calibration checks or adjustments, or call in a service engineer.

We believe that this new standard, when used as described above, will greatly improve the reproducibility and comparability of circular dichroism spectroscopy. Although we anticipate most users will want calibration in the UV region, CoEDDS has already been used by physicists at Durham who were building a visible region CD machine and could not find a commercially available standard.[21] The usefulness of this material could be greater still if its absolute, traceable spectral characteristics were determined. It could then be used to disseminate traceable CD to end-user laboratories. However, this will require the development of CD reference instruments by a National Measurement Institute.

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