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# INSTRUMENTATION ISSUES

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# Circular Dichroism Spectroscopy Summer School

*EPSRC summer school in circular dichroism spectroscopy of proteins and nucleic acids*
*16-20 September 2002*

## Instrumentation Issues

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Talking at the end of a course has advantages and drawbacks, the main advantage is that matters have been already well covered and it's a good opportunity to give some suggestions, the drawback is that audience is already well bored for the too many topics already discussed.

We will deal today on a crucial and often neglected point: i.e. how to get reliable measurements.

When Circular Dichroism was a *new* technique instruments around were rare and in most of the cases only *experts* were allowed to put their hands on.
In addition units had many knobs, switches and adjustments ….., *experts* had a real role in playing around.
Today CD units usually have only the main power switch, all the rest is PC assisted and software controlled.
*Experts* made career and are no more involved in practical measurements (even if there are still good cases around) and *medical doctors* (or their students) have often the job to operate them.

We will deal today only with the most popular ECD (electronic circular dichroism) equipments, forgetting about VCD and other more exotic techniques.

First thing to consider is that your expensive CD spectrometer is, after all, a simple single beam UV-VIS spectrophotometer.
There is a light source, a monochromator, a sample, a detector and some electronics to extract the results.
But we deal with circularly polarized light, so between monochromator and detector we do need some components to generate it and our electronics behind must be able to calculate the difference in absorption between left and right circularly polarized light.

In most of the cases the **light source** is a short arc Xenon lamp. You all know that UV-VIS spectrophotometers are equipped with Deuterium and Halogen lamp while short arc Xe sources are more popular with spectrofluorometers.
Why?
Even in CD in the past dual sources were used, however since many years Xe lamps are the standard. Reasons are two:
-higher intensity over most of the wavelength range
-no need to change during a scan
These advantages have drawbacks:
-Xe sources dissipate a lot of heat, so they call for a robust well ventilated housing, this will increase the weight and the cost of your instrument.
-Xe source has a high temperature blackbody emission, so you do need a very good monochromator to limit stray-light (stray light can be defined as the relative portion of the detector signal which arises from light scattered within the monochromator *other than that of the nominal wavelength*[1])

So we do need a good **monochromator** to match the source. Commercial units are typically using double monochromators to keep stray light level at a minimum. This is clearly another source of weight and cost.
Most commercial units are using double prism monochromators.
Why prism? In UV-VIS spectroscopy this was part of the past: original Beckman DU and its British clone (Unicam SP-500) were prism type, but probably most of you had never seen a prism type spectrophotometer apart from your University museum.
Well in CD spectroscopy prisms have (still) a lot of merits.
They do cost more than holographic replica grating, they do need a much more complex scanning drive system (in a grating mount change of wavelength is linearly proportional to rotation …., with prisms you need tailored cam drive to get a linear wavelength scanning). More: a grating monochromator gives practically constant linear dispersion. This means that to operate (as usually) with constant bandpass, the slits are at a fixed width. On the opposite with a prism

[1] Burgess C., Frost T., *Standard and Best Practice in Absorption Spectrometry*, 1999, Blackwell Science, Oxford

monochromator the reciprocal dispersion changes (and a lot) with wavelengths, so you need a servo system to control actual slitwidth to scan any spectra at constant bandpass.

So why prisms, here too a few good reasons:
-most of the CD measurements you have seen this week are in the UV or far UV range. Here prisms show better reciprocal dispersion than gratings, so you can operate for same bandpass with wider slits putting more *light* in your system
-prisms are not producing second and multiple order spectra (in a grating monochromator at 600nm also radiation at 300nm will pass through ….). While in a grating spectrophotometer spurious orders are simply eliminated inserting automatically long pass filters, this typically distort slightly the baseline, particularly using polarized light ...
-prisms monochromators have no Wood's anomalies, present in any grating system. These are spurious efficiency bands you can easily detect scanning a single beam spectra, they are difficult to compensate even in conventional absorption spectrophotometers, in CD case they are very unpleasant since strictly related to the light polarization.
-finally grating monochromators are *by definition* a sort of linear polarizer. Their output is partially polarized and polarization effect is not achromatic. These problems are not present in prism designs.
That's all, no there is another reason: you can use the prisms inside the monochromator to generate not only monochromatic radiation, but also well linearly polarized, saving an external **polarizer**.

This introduces the next argument: how to generate circularly polarized light.
There are many ways to do it[2], but all modern units are based on the method presented and patented in France in the sixties[3].

*monochromatic light is linearly polarized, than it pass through an electro optical modulator where the polarization is modulated at the modulator frequency alternating between left and right circular polarization. If sample is CD active the detector output signal will contain an alternating component $V_{ac}$ and a continuous component $V_{dc}$. Ratio between $V_{ac}$ and $V_{dc}$ is the CD effect we want to measure. A non CD active sample will have $V_{ac}$= 0, while the phase of the AC component will indicate the polarity of the phenomena.*

Today the early Pockel cells used originally has electro optical modulators have been replaced by photoelastic modulators (**PEM**)[4], in which linear birefringence is induced on a quartz plate by mechanical stress.
These crystals are forced to oscillate at the natural frequency of their base (typically 50KHz) and if proper stress is applied they operate as quarter wave retarder. Actually the stress to give ¼ delay is related to the actual wavelength, with a nearly linear relationship.
So we get on our **sample** light cyclically changing from left to right circular polarization and a suitable light detector will measure it.
The detector is typically a **photomultiplier** tube (a device, similar to the valves of old radio-sets) able to convert photons into current. Photomultiplier tubes are also widely used in UV-VIS spectrophotometers, in CD apparatus however the tendency is to use end-on models (i.e. pieces with flat surface) that despite the higher cost (once again) have the sensitive window inducing less birefringence.

As we said above the CD effect we want to measure is $V_{ac}/V_{dc}$.
In most commercial units only $V_{ac}$ is measured since $V_{dc}$ is kept constant by dynode feedback.
Dynode feedback means that the high voltage applied to the photomultiplier tube is changed automatically in order to give a $V_{dc}$ constant level, so only $V_{ac}$ may be measured.

To measure $V_{ac}$ the **electronics** of our instrument is typically using a lock-in amplifier, which is tuned to the PEM oscillating frequency,
the signal generated will have an amplitude and a phase ….., so our CD spectra will show its zero on the center of the scale.
The extracted CD signal is shown against wavelength (CD spectra) or against time (CD Kinetics) as in any normal spectrophotometer. So nothing new in this respect.
In the past display was on strip-chart recorders and now you use your PC to see/plot and manipulate the data!

[2] Abu-Shumays A., Duffield J.J., *Anal. Chem.*, 38, 1966, 29A
[3] Grosjean M., Legrand M., *Compt. rend.*, 2651, 1960, 2150
[4] Kemp J.C., *J. Opt. Sci. Amer.* , 59, 1969, 950

We have seen so far how our instrument measures CD spectra, so we can go a bit further with practical comments/answer to questions in a rather casual order:

**Nitrogen purge**
You do need to flush your optics with dry nitrogen, one obvious reason is related to wavelength range. In low UV (below 195nm) air (or better oxygen, is strongly absorbing, so you need to flush optics with $N_2$ gas to get proper far UV penetration.
But you do need to purge always, all over the wavelength range, in order to protect your optics surface from photochemical damages from the high amount of ozone generate otherwise by the strongly UV emitting Xe source.
While nitrogen flow may be adjusted, depending from the actual low wavelength limit desired, purge is s a real must.
Furthermore nitrogen optics protection may be invalidated if the gas used is not dry and not of the best purity.
A few minutes of pre-flushing are always necessary before switching on the lamp.

**Warming-up**
Modern electronics require little warming-up. Furthermore the most delicate component (the PEM) is usually kept at a stable temperature even if main-power is switched off.
Nevertheless due to the sizeable heat transferred by the source it's a good practice to warm-up your unit before starting high sensitivity measurements, moreover measurement is basically single beam …., despite the dynode feedback.
The necessary time may be dependent from instrument to instrument and may be different even comparing same models.

**Why do we collect CD and HT spectra?**
A good point. In our systems there is the dynode feedback, so high voltage applied to the photomultiplier tube is automatically changed to keep $V_{dc}$ constant. In practical terms in the region of the spectra where your system is less efficient or its source is emitting less the high voltage will increase*.
Typically an HT of 700V is the real maximum you can afford to get realistic data.
Only ways to keep it down are:
-larger slits, but this is not so effective in low UV and in any case you are limited by natural band-shapes, so 2nm seems for example the ideal for protein analysis in far UV
-smaller sample concentration, but this will correspondingly decrease also signal
-shorter path cells, increasing eventually concentration
-change of buffer with more transparent one
In any case HT simultaneous recording is a very effective and simple way to check data integrity. The collection may not be necessary when the method is fully known.
Last the HT spectra can be easily converted into single beam absorption spectra ……
** changing the high voltage applied on a PM tube you change its gain (current emitted for a given number of photons received. You get nothing free of charge: increasing the H.T. applied also noise goes up!*

**What's expected source lifetime?**
Xe arc lamps get old since electrodes are eroded by use; this will decrease the brightness. You can easily verify the phenomena monitoring the HT plot …., as time is passing higher values will be obtained.
So replacement is due when values are no more satisfactory: about 800 hours in average.

**Are mirrors too getting old?**
Yes, and this is part of running cost you should consider in advance. Despite $N_2$ flushing mirrors are slowly, but continuously deteriorated by photochemical effects.
Here too the monitoring way is by HT plotting.
Typical lifetime is around 3000 hours.

**What's the best sample concentration?**
On paper easy to answer, the one which gives an absorbance of $0.5 \approx 1$. But mainly when working in the low UV the buffer own absorption will play a relevant role. So for spectra acquisition the idea is to use cells as short as possible with a high concentration. Dealing with melting experiments (to monitor changes of structure versus temperature) it's often better to compromise ….. using longer path cells to enable a more accurate temperature distribution (via stirring for example) in the cuvette.

**What's best buffer?**
The question relates to the previous one. Buffer should be as transparent as possible in the wavelength range you are measuring, otherwise shorter path cells must be used.
1≅20mM of potassium phosphate is the ideal choice, EDTA must be <1mM, detergents are not allowed ….
In any case run spectra of your blank and check the HT!

**Why do we measure CD in millidegrees?**
Another good question, CD is an absorption technique, but we measure it as ellipticity not in ΔA.
Reason is purely historical since Americans approached CD from ORD …., indeed you can go to ΔA simply:
$\Delta A = \Theta/32980$

where:
ΔA in absorbance units
Θ is ellipticity in mdeg
But literature data are usually reported in molar ellipticity [Θ]:
$[\Theta] = \Theta/(10xCxl)$ or $[\Theta] = (\Theta xM)/(cx\ lx10000)$
where:
Θ is ellipticity in mdeg
C is the molar concentration (mole/l)
l is the cell path in cm
M is molecular weight
c is concentration in g/ml
since obviously
$C = (1000xc)/M$
[Θ] is expressed in deg x $cm^2$ x $decimole^{-1}$
For macromolecules such as proteins the mean residue molar ellipticity is used $[\Theta]_{MRW}$:
$[\Theta]_{MRW} = \Theta/(10xC_rxl)$
Formula is still the same, but $C_r$ is the mean residue molar concentration
$C_r = (nx1000xc_g)/M_r$
where:
n is the number of peptide bonds (residue)
$c_g$ is the macromolecule concentration (g/ml)
$M_r$ is the molecular weight of the species

**Experimental errors**
It's good to refer to an old paper from a real expert: W.Curtis Johnson[5].
He pointed out seven types of errors that may interfere with proper analysis of protein spectra.

***Operational errors (1-3)***
*1 wavelength error*
The work shows how a relative minor shift (reported as + or – 2nm) may significantly distort secondary structure estimation.
Fortunately mainly prism type CD monochromators are very reliable in this respect and they tend to keep calibration forever (in the VIS and NIR range situation is much worst).
An accurate check of the calibration is however rather problematic, since no liquid or solid standard is available with proper sharp peaks in the 180-260 nm region. So when really necessary an Hg source may help, but data should be complemented by measurement of absorption spectra of gases such as ammonia and benzene.

*2 scan rate errors*
These errors originate when the scanning speed is too fast for the integration time used*. Since in CD it's a common practice to use long integration time to reduce the noise, the risk to fall in this error is present.
Usually best criteria to select the maximum scanning speed is as follows:

$SS \leq SBW/IT \cong SBW/5xATC$

Where: SS = scanning speed (nm/sec)
SBW = selected bandpass of the monochromator (nm)
IT = integration time of the photometer (sec)
ATC = time constant of the photometer (sec) ….. this was widely used in old apparatus

** you'll avoid this potential error using step-scan scanning mode, but not everybody would like this way of collection, which drastically limits the number of data points ….*

[5] Hennessey J-P., Curtis Johnson W. Jr., *Anal. Biochem.*, 125, 1982, 177

*Spectral bandwidth error*
Selection of monochromator bandpass (SBW) should be based on the natural halfwidth (NBW) of the bands to be detected.
The old rules indicate that SBW/NBW ≤ 0.1, but in practical terms 0.15 may be a reasonable compromise, this means a SBW of 2nm for the low UV range of protein/peptide spectra (aromatic region in near-UV may call for smaller SBW). If SBW is too large spectra are flattened and proper intensities are lost.

***Experimental errors (4-6)***
*Intensity errors*
Caused by:
- improper CD calibration, this is easy to verify and correct using ACS standard (0.06% aqueous solution of ammonium-d-camphorsulfonate in 1 mm cell), you can check the ratio between negative peak at 192.5 and the positive one at 290.5, ratio should be 2:1*. This method is also suggested by European Pharmacopoeia[6].
  * *calibration at a single wavelength as typically suggested by manufacturers is not enough, since the PEM chromatic performance depends on the correct driving program*
- improper cell path, this is a potential trouble using mainly short path or demountable cells
- incorrect sample concentration. This is probably the most important and sometime neglected step. Measuring absorbance at 280nm of the aromatic sidechains may not be enough.

*Baseline error A*
It's showing up when baseline is not really at zero, but shifted somehow. This shift will cause errors in the zero crossing. Use of same cell (and in same orientation) for sample and buffer is a clear must. Do not use *automatic* baseline software features …., you'll end-up with correction done by somebody else! So collect baseline as normal spectra with same parameters and subtract it later on. Repeat the run if you doubt about instrumental shifts.

*Skewed baseline error B*
You get this effect when baseline shifts in time, particularly in the far UV region. Since 1982 instruments are more stable, but trying to extract information at the limits may be difficult. Spectra averaging is a good way to improve S/N in your spectra, but baseline drifts can distort sensitive measurements. The use of accessories such as sample alternator which allows to measure sample→air→sample→air etc and average only air-baseline subtracted spectra was a way well used in the past, but taking twice the time.

***Analytical Constraints error (7)***
This is last source of error reported by Curtis Johnson, it relates to the problem of secondary structure estimation used at that time (but also today!) that total of secondary structures may be (even significantly) different from 100%. Very often you can get also negative numbers, and correction algorithms are used. With this argument we go far outside the purpose of this talk, since it was treated previously by much more qualified people.

***Other sources of errors***
At least two other sources should be mentioned:

*Linear anisotropies of the sample*
Particularly when dealing with films, liquid crystals or when sampling with small cuvettes. Sometime difficult to detect and to compensate. An LD accessory is a recommended warning device.

*Light scattering*
CIDS (circular intensity differential scattering) may significantly distort the spectral shape. Solid sampling, membrane proteins etc are good examples. The trial of alternative sampling devices is a way to sort out these artifacts.

To close with, I hope you have a more clear pictures on the instrumental aspects which may limit the quality of your measurement. Your results will be in any case limited by your strategic experimental approach and by the condition of your instruments.
To assist newcomers I started ands I'm still continuing with a very modest web ( http://digilander.libero.it/ecssrl) which is periodically updated with Technical Reports in .pdf format dealing mainly with different instrumental aspects behind CD technique.

[6] European Pharmacopoeia (Addendum 2001) 2.2.41