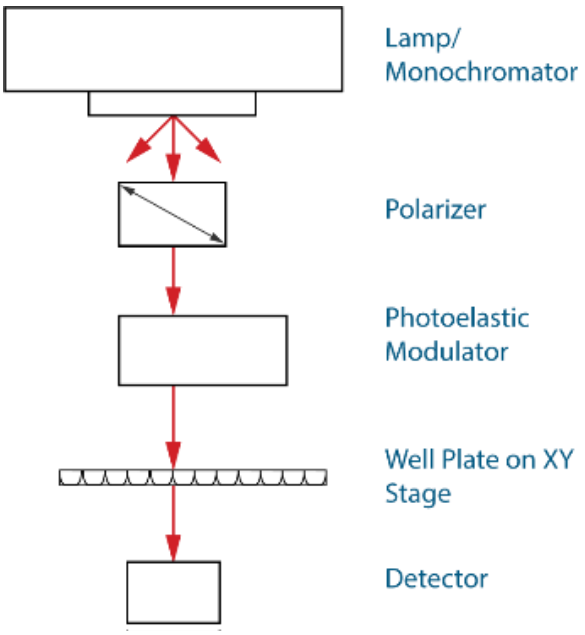
EKKO™ CD Microplate Reader

Solvent evaporation effects on measurement stability

I – INTRODUCTION

Circular dichroism (CD), the commonly used technique for chiral analysis, refers to the differential absorption between left and right circularly polarized light. It is often used for assigning the secondary structures of proteins and determining enantiomeric purities in asymmetric syntheses, both of which require the ability to do the measurements in a high-throughput fashion [1,2].

The primary advantage of the EKKO™ CD Microplate Reader is its speed resulting from the use of well plates allowing for the highest throughput possible. It accomplishes this by turning the light path from the horizontal to vertical, allowing for the use of a computer-controlled XY stage so that CD signals are read directly from a well plate.



Block Diagram of the EKKO™ CD Microplate Reader

This removes the time-consuming steps of 1) transferring the contents from each well of a well plate into a cuvette, and 2) cleaning the cuvette between measurements. As a result, it takes only two minutes to measure the CD signal in all 96 wells of a standard well plate at any given single wavelength and needs less than 90 minutes to measure all 96 CD spectra over 50 wavelengths in a standard well plate. This results in a significant increase in productivity, as much as 100-fold with respect to conventional CD systems coupled to liquid handling robotics[1,2,3].

Under normal operation, a loaded well plate is not exposed to the environment for more than 90 minutes. The evaporation of solvent in an open well over these periods of time is not likely. Yet measurements often take longer to acquire if cleaner or more detailed spectroscopic information is required. In these cases, solvent evaporation is a concern given that the effective pathlength in a well plate is dependent on the volume within the well.

In this paper, we address the effects of solvent evaporation in well plate applications using the EKKO™ CD Microplate Reader with CD measurements of (+)-camphorsulfonic acid (CSA), (-)-pantolactone (PL), and bovine serum albumin (BSA) with purposely extended exposure to and protection from the environment to ensure the presence and absence of the evaporative loss of solvent.

II – RESULTS & DISCUSSION

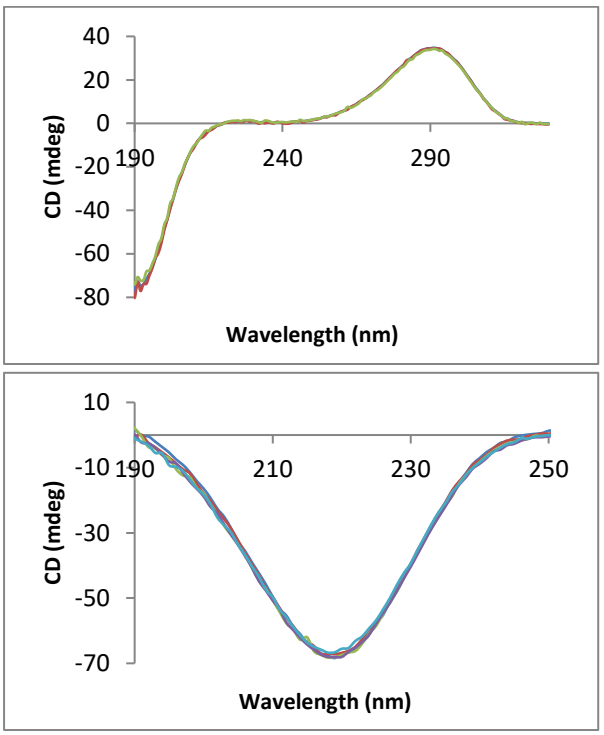


Fig. 1. Raw CD spectra of CSA (top) and PL (bottom) with larger effective path lengths as a function of time. 200 µl of CSA (Sigma) at 0.2 mg/ml in well #F10 and 200 µl of PL (Sigma) at 0.1 mg/ml in well #C9 of a solid fused silica 96 well plate (Hellma). Raw spectra (blanking not performed) were collected after 0, 2, 4, 6 and 23 hours of constant exposure to the environment of the sample chamber at a room temperature of 25°C. No efforts to minimize evaporation (samples were not protected with an optically clear cover) or reduce the noise (data were collected with the shortest integration times possible) were taken.

Figure 1 illustrates that regardless of the evaporative loss of solvent which surely must have occurred over the course of 23 hours, there was no meaningful differences between the between the initial and final CD spectra of either CSA or PL with average deviations for the time points of 1.6% and 2.4% from the initial spectra across the peaks (CSA, 290 ± 20 nm; PL, 220 ± 20 nm), respectively. The simplest interpretation of this observation is that when the solvent is evaporated, the decrease in the effective pathlength in the well is compensated for by the proportional increase in the concentration of solute in accord with the Beer-Lambert and Mass Balance laws.

Since the wells of the well plate used are cylindrical, the volume in the well converts linearly to the path length assuming a constant meniscus effect. This interpretation of the observed results was easily tested because it implies that increasing the path length while reducing solute concentration will not alter the CD signature of the sample.

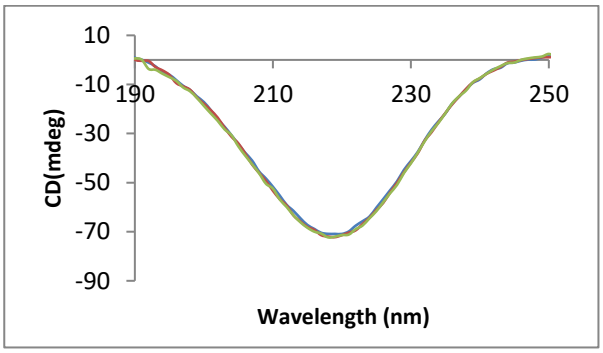


Fig. 2. Raw CD spectra of systematically diluted PL with larger effective path lengths. The CD spectrum of 200 µl of PL (Sigma) at 0.1 mg/ml in well #F9 of a solid fused silica 96 well plate (Hellma) was recorded. The CD spectrum was then retaken after dilution with an additional 20 µl of solvent added twice sequentially. Raw spectra were collected with no efforts to minimize the noise and were taken at a room temperature of 25°C.

The nearly identical spectra of PL, average deviation of 1.2% from the initial spectra across the peak, 220 ± 20 nm, even though it has been systematically diluted in figure 2 illustrates that the increase in the effective pathlength compensates for the decrease in the concentration of the solute; affirming that Mass Balance accounted for the results observed in figure 1.

While evaporation has very little to no effect on CD signals when measurements begin with relatively high starting volumes (e.g., ≥ 200 µl – ~ 6 mm pathlength), what is the case when

relatively low starting volumes are desired either because of the need to minimize the effective pathlength because of solvent absorption or the cost of the precious sample?

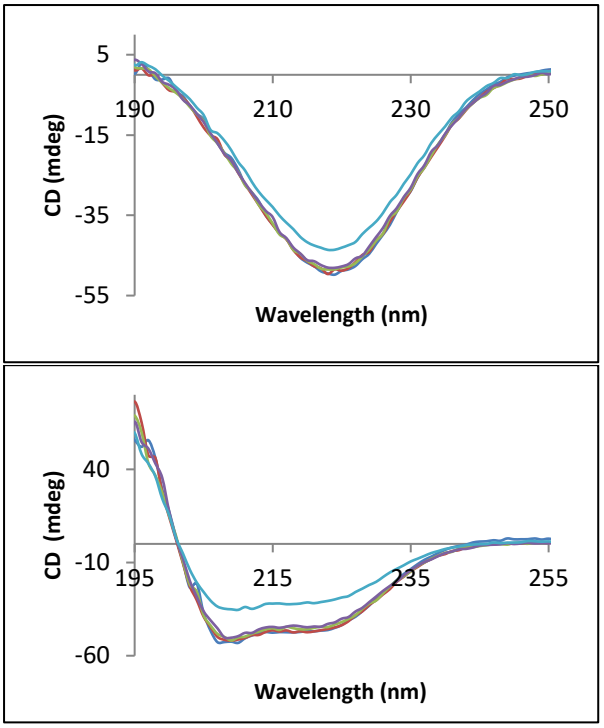


Fig. 3. Raw CD spectra of PL (top) and BSA (bottom) with moderate effective pathlengths as a function of time. 150 µl of PL (Sigma) at 0.1 mg/ml in well #D9 and 140 µl of BSA (Sigma) at 0.1 mg/ml in well #C10 of a solid fused silica 96 well plate (Hellma). Raw spectra were collected after 0, 2, 4, 6 and 23 hours of constant exposure to the environment of the sample chamber. No efforts to minimize evaporation at a room temperature of 25°C or noise were taken.

Figure 3 demonstrates the effects of evaporative loss of solvent when a moderate starting volume (140 or 150 µl with an effective path length of ~ 4 mm) was used. In this case, noteworthy alterations in the spectra were not observed until the well containing PL had been exposed to the environment for at least 6 hours (purple trace). With only a 2.8% deviation from the initial spectra across the peak 220 ± 20 nm, it is arguable that this is not significant. After 23 hours, the deviation was 12.2% across the peak (light blue trace). Similar indications were obtained using 140 µls of BSA, it did not show significant deviation from the initial trace until the 6-hour time point (purple trace) with a deviation of 6.8% for the wavelengths between 200 and 240 nm, the deviation at 4 hours was only 2.1%. This deviation was 29.9% at 23 hours. Given that under normal operating conditions, it takes less than 90 minutes to measure all 96 CD spectra over 50 nm, evaporative loss is not an issue for experiments beginning with moderate well volumes that are ≥ 140 µls.

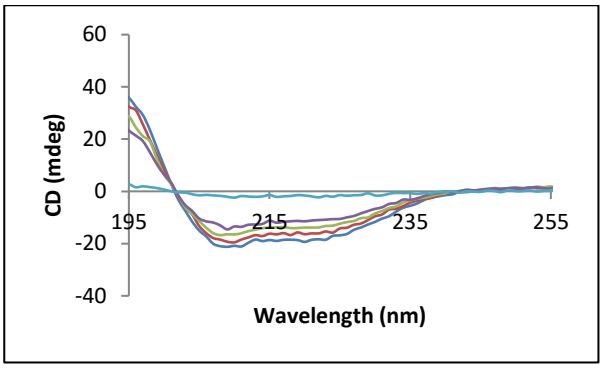


Fig. 4. Raw CD spectra of BSA with shorter effective pathlengths as a function of time. 70 µl of BSA (Sigma) at 0.05 mg/ml in well #F8 of a solid fused silica 96 well plate (Hellma). Raw spectra were collected after 0, 2, 4, 6 and 23 hours of constant exposure to the environment of the sample chamber. No efforts to minimize evaporation or noise were taken. Room temperature was 25°C.

Figure 4 illustrates the effects of solvent evaporation when the starting volume is 70 µl, or a starting effective pathlength of ~ 2 mm. Even at two hours (red trace), the evaporative loss of solvent had a significant effect on the spectral characteristics of BSA, causing a 12.1% deviation from the initial spectra. The deviation became progressively greater as a function of time, by 23 hours it was at a deviation of 88.9%. The deviations for PL were 8.1% and 87.7% at the 2 and 23-hour time-points, respectively (data not shown).

The noteworthy changes observed in figure 3 at the longer time points and dramatic alterations in figure 4 were surprising after the observations with larger volumes and the concomitantly larger effective pathlengths.

The earlier results appeared to obey Beer-Lambert and Mass Balance laws precisely. This deviation at lower volumes suggests this is the result of not having a consistent meniscus effect and warrants further investigation. Regardless; however, there is a simple solution to this problem to ensure stability of the measurements.

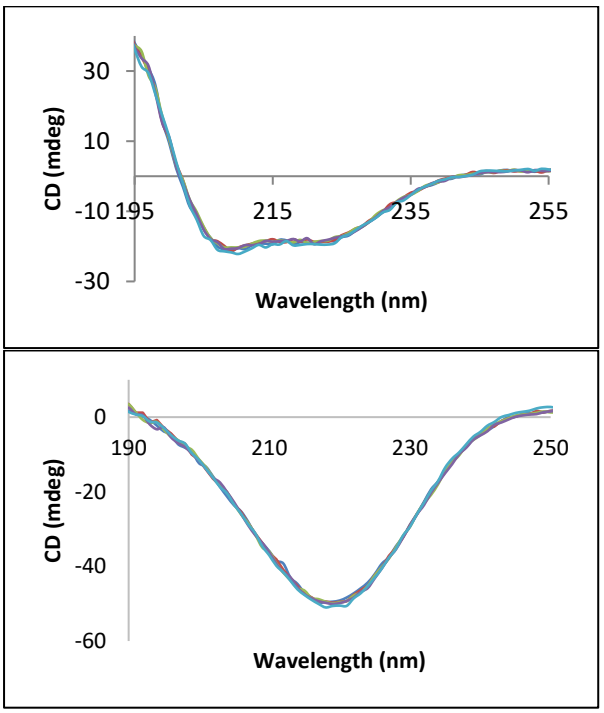


Fig. 5. Raw CD spectra of BSA (top) and PL (bottom) with environmentally protected short effective pathlengths as a function of time. 70 µl of BSA (Sigma) at 0.05 mg/ml in well #F4 and 150 µl of PL (Sigma) at 0.1mg/ml in well #D3 of a covered solid fused silica 96 well plate (Hellma). Raw spectra were collected after 0, 2, 4, 6 and 23 hours. No efforts to minimize noise were taken. The room temperature was 25°C.

Figure 5 illustrates the effect of protecting the sample from evaporative loss. In this case, the plate was covered with a 0.5 mm thick fused silica sheet to reduce the evaporation related effects. Even after 23 hours, the CD spectra of 70 µl of BSA demonstrated only a 2.1% average deviation with respect to the initial spectra while the CD spectra of PL demonstrated only a 1.7% average deviation. Thus, protecting the sample with a cover mitigates evaporative loss effects.

III – SUMMARY & RECOMMENDATIONS

1. Most experiments using the EKKO™ CD Microplate Reader and a 96 well plate will be completed in less than two hours. Evaporation will have negligible effect on CD results when the wells are not covered due the effects of Mass Balance. This holds for experiments that begin with moderate or higher starting volumes (≥140 µl for 96 well plates with a well diameter of 6.6 mm), simply due to the longer effective sample pathlength.

2. When a low starting volume is ideal empirically, a protective cover made of optical glass or fused silica, depending on wavelengths needed, should be used to minimize the effects of evaporation on CD measurements made with the EKKO™ CD Microplate Reader.

3. There may be cases where it will take longer than 2 hours to complete an experiment using the EKKO™ CD Microplate Reader and a 96 well plate. If an increase in the solvent pathlength is not expected to generate strong adverse effects for the measurement, it is recommended that the starting volume of the wells be relatively high (≥ 200 µl in wells with a 6.6 mm diameter) eliminating the need for a protective cover. For a 96 quartz well plate made by Hellma, the maximum recommended well volume is 300 µl with the minimum well volume is 60 µl.

4. For long term tests and experiments involving highly evaporative solvents, a protective cover made of optical glass or fused silica should be used to minimize the effects of evaporation on the CD measurements made with the EKKO™ CD Microplate Reader.

REFERENCES

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2) Jo, H.H., Cao, X. You, L., Anslyn, E.V., and Krische, M.J., Application of high-throughput enantiomeric excess optical assay involving a dynamic covalent assembly: parallel asymmetric allylation and ee sensing of homoallylic alcohols. *Chem. Science.* 6, 6747-6753 (2015).
3) Fielder, S., Cole, L., and Keller, S., Automated Circular Dichroism spectroscopy for medium throughput analysis of protein conformation. *Anal. Chem.* 85, 1868-1872 (2013).