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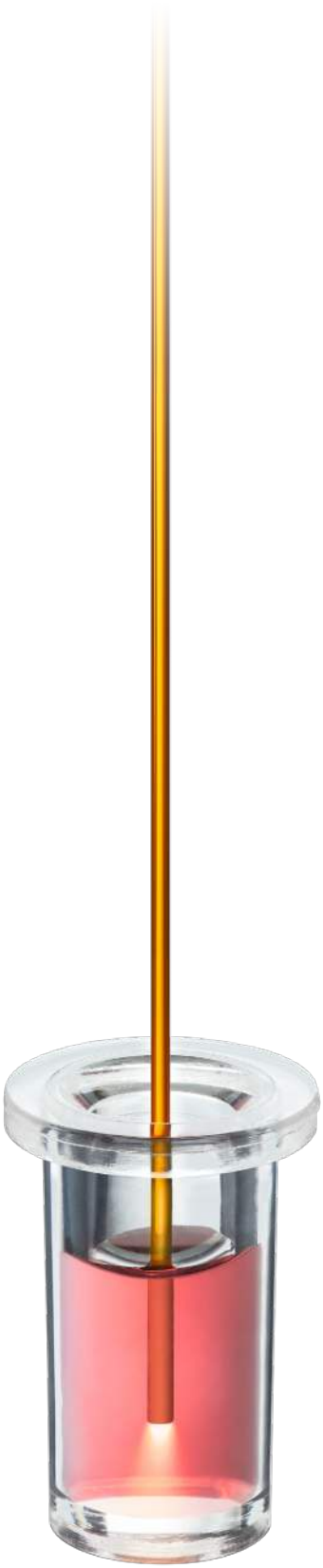
Validation of ADC Platform for Protein Concentration and the Drug-Antibody Ratio (DAR) using Variable Pathlength Technology



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Antibody Drug Conjugates (ADCs) are biopharmaceutical drugs which are designed to treat cancer by targeting and killing the cancerous cells while leaving the healthy tissue intact. ADCs are comprised of an antibody connected to a cytotoxic drug by a linker.

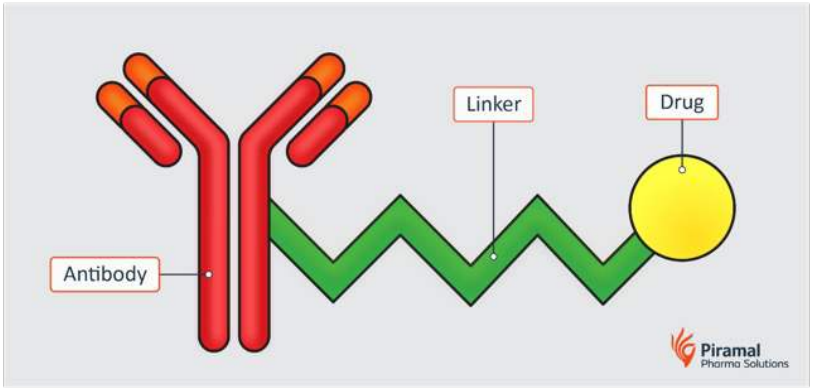

Figure 1.
Schematic showing the components of an ADC.

The antibody targets a specific antigen which is present in cancerous cells but not healthy cells. The ADC binds to the cancer cell and becomes internalized where the cytotoxic drug is released and kills the cancer cell.

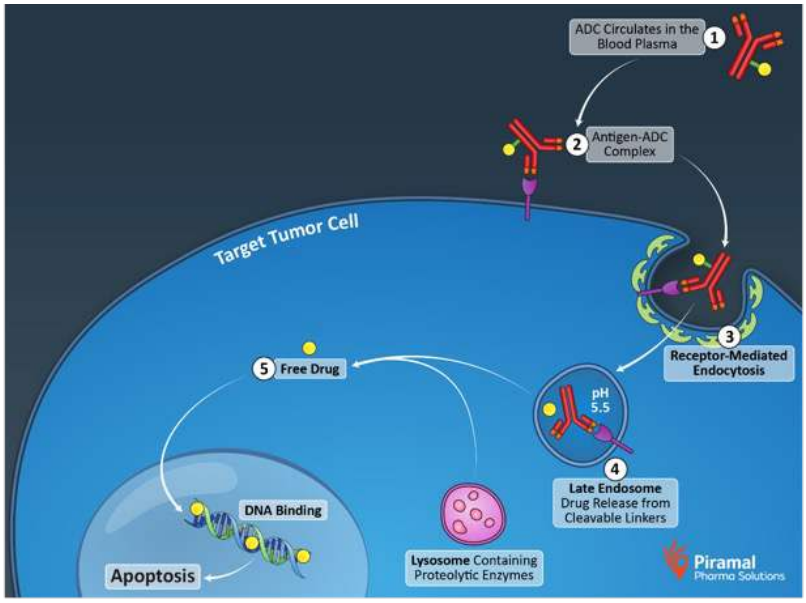

Figure 2.
Figure showing the mechanism of action of an ADC.

KEY TERMS:
ADC, bioconjugation, Mass Spectroscopy, hydrophobic interaction chromatography, Drug-Antibody Ratio, reverse phase chromatography

Two important pieces of information required about the ADC are the protein concentration and the Drug-Antibody Ratio (DAR). The DAR is the number of drug molecules attached to the antibody. It is important during the manufacturing steps to ensure that the process is proceeding as expected and that there is not more or less drug present than required.

When choosing a technique to measure the DAR there are several to choose from: DAR by HIC (hydrophobic interaction chromatography), DAR by RP (reverse phase chromatography), DAR by MS (mass spectroscopy) and DAR by UV/Vis (ultra-violet/visible spectroscopy). Each technique comes with its own advantages and disadvantages, and use varying amounts of consumables, reagents and time (Table 1).

Technique	Advantages	Disadvantages
DAR by MS	• Gives extra information about the samples. • Can be used for cysteine and lysine linked ADCs.	• Requires large amount of data processing. • Complex technique.
DAR by RP HPLC	• Better peak resolution compared to HIC. • Easy DAR calculation (Area%).	• Only works for cysteine linked ADCs. • Can require complicated sample preparation.
DAR by HIC	•Robust method due to easy DAR calculation (Area%). • Maintains the structure of the ADC.	• Only works for cysteine linked ADCs. • Poor peak resolution.
DAR by UV/Vis	• Can be used for both cysteine and lysine linked ADCs. • Simple technique.	• Can be affected by buffer and pH. • Calculation complicated by extinction coefficient similarities. • Poor data information.

Table 1.
Comparison of some advantages and disadvantages for the four DAR techniques.

The information quality and ease of use of the technique can be a deciding factor when choosing a method. Mass Spectroscopy (MS) can give extra information about the samples being tested when used in conjunction with HIC and RP-HPLC including structural or compositional characteristics. However, it is a complex method and requires a large amount of time spent to process the data.

DAR by HIC, RP-HPLC and MS provide precise results on most time-consuming limited depending on the linker used on the ADC. They also involve time consuming sample preparation steps and data processing. All require mobile phases and reagents to be prepared which can be costly, especially for the HIC method which requires large amounts of buffer salts for mobile phases.

UV/Vis Spectroscopy is a simple, easy to use method and the DAR can be quickly calculated from the absorbance, though it can require long periods of time spent performing dilutions. It has been found that performing the dilutions gravimetrically rather than volumetrically removes some of the error during preparation. When performing gravimetric dilutions the weight of the sample and the weight of the dilution buffer are recorded and used to calculate the dilution factor.[2]

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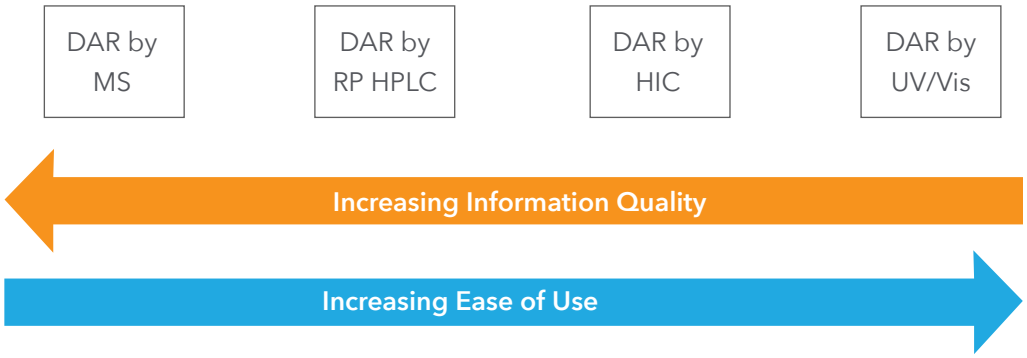
Figure 3.
Diagram comparing information quality and ease of use of each technique.



When taking into account restrictions within the quality control (QC) department, DAR by UV/Vis is the most frequently chosen technique. Figure 3 shows a simplified schematic of information quality and ease of use of each of the four techniques.
Protein concentration can be calculated by rearranging the Beer-Lambert law from

$$A = \varepsilon * c * l \quad \text{(Equation 1)}$$

To

$$c = \frac{A}{(\varepsilon * l)} \quad \text{(Equation 2)}$$

Where A = absorbance, ε = molar extinction coefficient, c = concentration and l = path length. When taking the dilution factor (DF) into account the equation becomes

$$c = \frac{A * DF}{\varepsilon * l} \quad \text{(Equation 3)}$$

To calculate DAR, first the molar concentration of the drug [Drug] (M) and the molar concentration of the protein [Protein] (M) must be calculated by simultaneous equations:

$$c_{mAb} = (A_{280}\varepsilon^{\lambda(drug)}_{drug} - A_{\lambda(drug)}\varepsilon^{280}_{drug})/[(\varepsilon^{280}_{mAb}\varepsilon^{\lambda(drug)}_{drug} - \varepsilon^{\lambda(drug)}_{drug}\varepsilon^{280}_{drug})l] \quad \text{(Equation 4)}$$

And

$$c_{drug} = (A_{280}\varepsilon^{\lambda(drug)}_{mAb} - A_{\lambda(drug)}\varepsilon^{280}_{mAb})/\varepsilon^{280}_{drug}\varepsilon^{\lambda(drug)}_{mAb} - \varepsilon^{\lambda(drug)}_{drug}\varepsilon^{280}_{mAb})l] \quad \text{(Equation 5)}$$

Where λ(drug) is the wavelength of the drug linker.

Dividing c_{drug} by c_{mAb} will give the final DAR result.

The CTech™ SoloVPE is an innovative instrument for UV-Vis spectroscopy. The SoloVPE uses Variable Pathlength Technology (VPT) to provide analysts with easy access to another dimension of measurement using the Beer-Lambert law, namely fine pathlength control.

The SoloVPE changes the pathlength by altering the depth of the Fibrette® in the sample. Figure 4 shows a sample vessel with a Fibrette inserted; the Fibrette will move up and down in the sample as the pathlength is changed. The system finds the pathlength where the absorbance of the sample is 1 Au (absorbance unit) and then selects 10 successively shorter pathlengths and measures the corresponding absorbances to plot a graph.

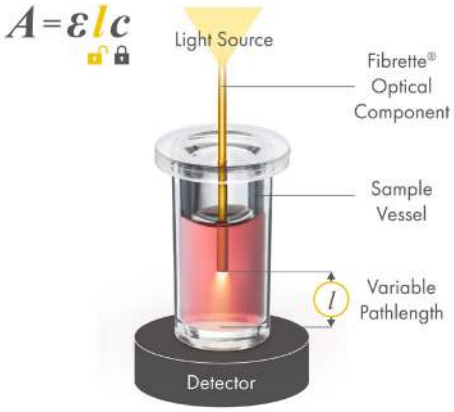


Figure 4.
Diagram showing the basic set up of the SoloVPE during the analysis of a sample.

Unlike traditional UV-Vis methods that rely on a single absolute absorbance value, the Slope Spectroscopy® method uses absorbance vs. pathlength data to determine a slope value for the sample concentration. The Slope Spectroscopy equation is derived from the Beer-Lambert law (Equation 1) by moving the pathlength term l to the left-hand side of the equation, so it becomes:

$$A / l = \varepsilon \times c \quad \text{(Equation 6)}$$

The linear equation from the regression of absorbance vs. pathlength data can be written as:

$$A = m \times l + b \quad \text{(Equation 7)}$$

Where **m** is the slope of the regression line, and **b** is the y-intercept of the linear equation. The units of the slope **m** in Equation 7 are absorbance/ pathlength, in this case, Abs/mm. This dimensional equality allows direct replacement of the left-hand side of Equation 6 (A/l) with the slope term (m) from Equation 7.

This substitution results in the Slope Spectroscopy equation:

$$m = \varepsilon \times c \quad \text{(Equation 8)}$$

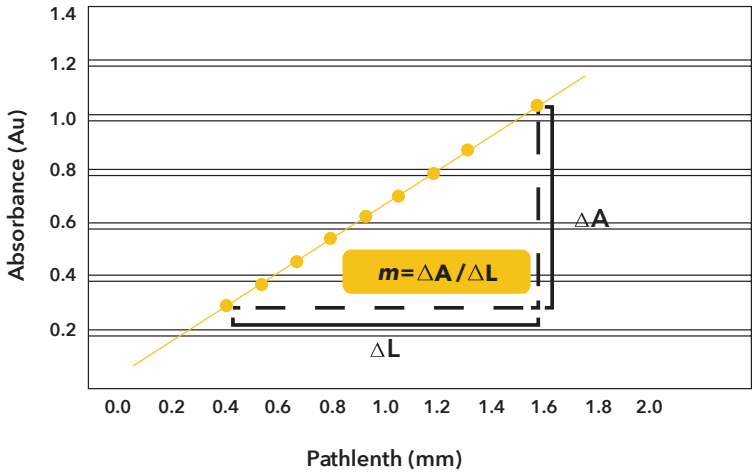


Figure 5.
Example graph plotted by the SoloVPE.

Figure 5 shows 10 points measured and plotted by the SoloVPE System. The slope of the line is then used to calculate the concentration of the sample using equation 9.

$$c = m / \varepsilon \quad \text{(Equation 9)}$$

The SoloVPE analysis tool provides two key parameters: The slope and the coefficient of determination (R^2). The coefficient of determination indicates how well the regression line matches the measured data, ensuring the reliability of the measurement.

The Slope Spectroscopy method allows samples to be measured without any dilution or baseline correction and with reliable and reproducible results. It has been found that the use of the SoloVPE minimises the time spent performing sample dilutions and removes the error involved.

As part of a comparison between the traditional UV and the SoloVPE, the cost of reagents, consumables and FTE (full time equivalents, a measure of operator time) were taken into consideration. The data has been tabulated in Table 2.

Table 2.
Comparing costs of SoloVPE analysis vs Traditional UV analysis. Based on an assay of 5 samples in triplicate. Prices are correct at the time of publication.

	Cost	
Reagents/Consumables	**Traditional UV (£)**	**SoloVPE (£)**
Formulation Buffer Components	0.31	N/A
pH Calibration Solution	0.60	N/A
Filters	12.07	N/A
SST STD	8.98	2.36
Fibrettes	N/A	121.72
Cuvettes	N/A	36.80
FTE	1200.00	600.00
Total	**1221.96**	**760.88**

As can be seen from Table 2, the SoloVPE System saves approximately one-third the cost compared to traditional UV spectroscopy due to reduced sample preparation and analysis time. SoloVPE allows analysis of 5 samples in triplicate to be completed in half the time it would take using a traditional UV instrument.

Before SoloVPE could be used in project work, experiments were carried out to confirm that the results would be comparable to the results generated by the traditional UV. The project required protein concentration and DAR to be calculated for the final BDS (bulk drug substance). Often this analysis is required at various points during the manufacturing process. An example process is shown in Figure 6. Typically protein concentration and DAR are monitored at the pH Adjust 2, conjugation, pre-formulation and Final Formulation steps.

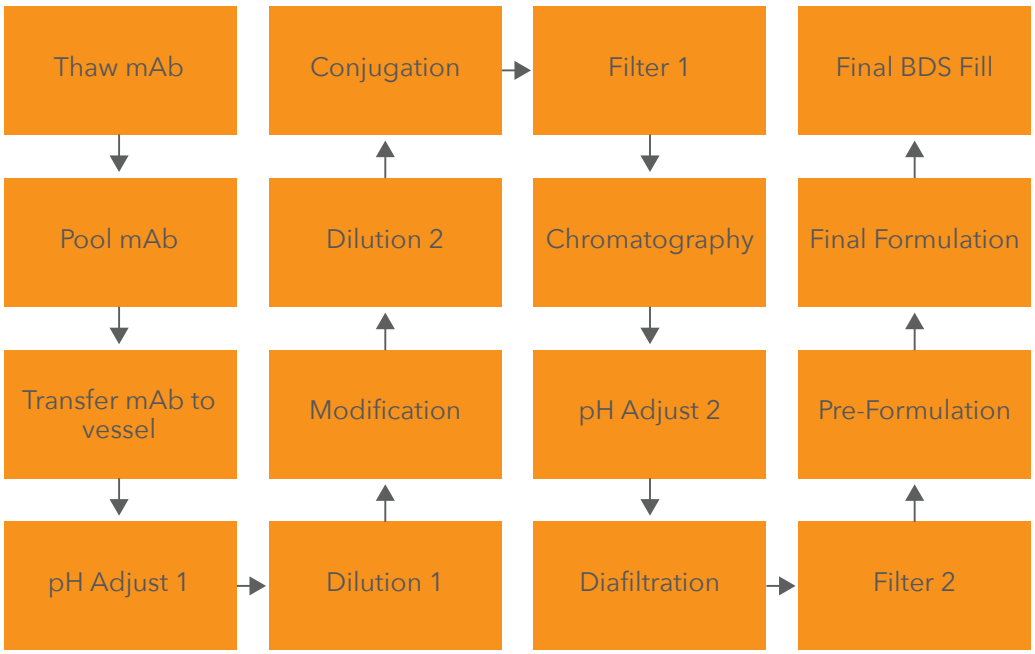


Figure 6.
Process flow diagram showing the steps performed during the manufacture of an ADC.

The traditional UV method for the BDS was converted to a SoloVPE method with slopes collected at 248nm for the drug linker and 280nm for the protein. The software was used to calculate the protein concentration of the sample in mg/mL. The molar concentrations of the drug linker and the mAb along with the DAR were calculated using Microsoft Excel. The results obtained were then compared to the results generated using a Shimadzu 1800 spectrophotometer.

	Shimadzu 1800		**SoloVPE**		**Difference between Shimadzu and SoloVPE**	
Sample	**Mean (P) (mg/mL)**	**Mean DAR**	**Mean (P) (mg/mL)**	**Mean DAR**	**ΔMean (P) (mg/mL)**	**ΔMean DAR**
ADC Test Sample	5.0	4.0	5.3	3.9	0.3	0.1

Table 3.
Summary table of mean protein concentration and DAR results for both Shimadzu 1800 and SoloVPE and the calculated difference between the results.

The data displayed in Table 3 showed that the results from the SoloVPE were within 0.3mg/mL for the protein concentration and 0.1 for the DAR. It was concluded that the results for both the protein concentration and DAR obtained using the SoloVPE were comparable to the results obtained using the Shimadzu 1800.

Qualification experiments were carried out to assess specificity, linearity, repeatability and intermediate precision for the BDS sample. In traditional UV analysis, samples can be diluted to cover a range of approximately 80% to 120% of the nominal concentration; however, as the SoloVPE requires no sample dilution, pre-concentration of the qualification material was required to allow for dilutions to be performed to cover the range required.

A specificity experiment was carried out by performing a full UV scan of both the antibody and the ADC samples at varying pathlengths. Refer to figures 7 and 8. Differences in absorbance were assessed at 248nm and 280nm. The ADC has a higher absorbance at 248nm compared to the antibody due to contribution of the drug linker.

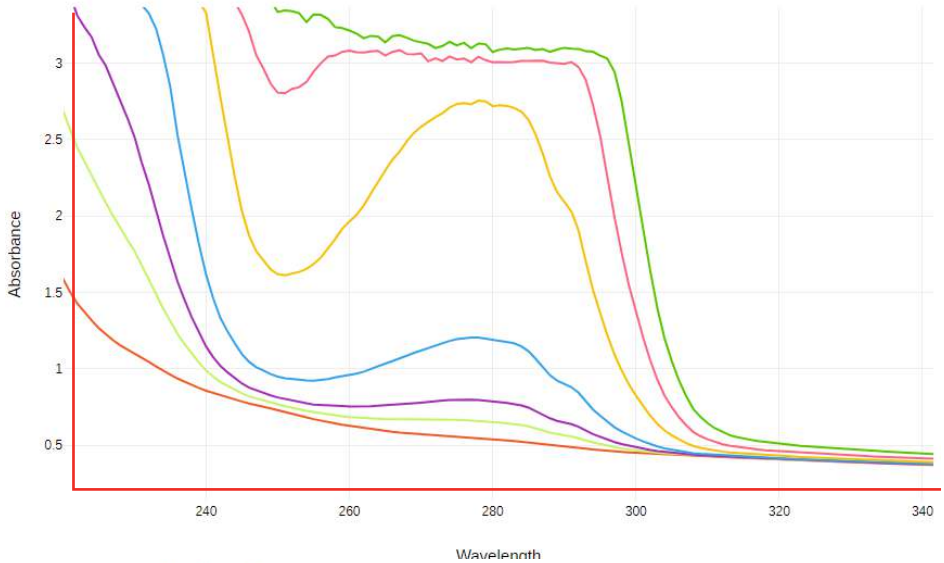


Figure 7.
Antibody Test Sample scan at multiple search pathlengths. Red trace is 0.005mm; light green trace is 0.05mm; purple trace is 0.1mm; blue trace is 0.25mm; yellow trace is 0.5mm; pink trace is 1.0mm; dark green is 2.5mm and turquoise trace is 5.0mm.

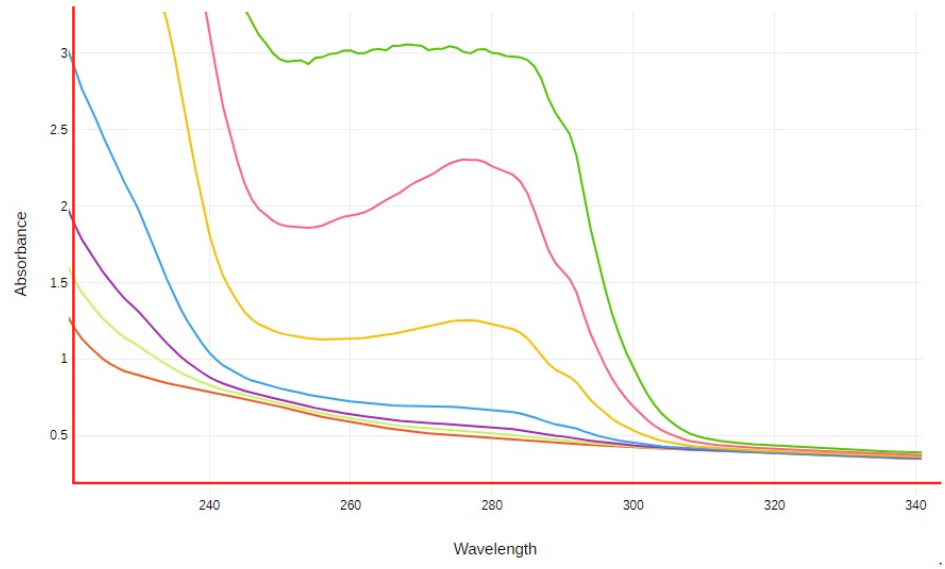


Figure 8.
ADC Test Sample scan at multiple search pathlengths. Red trace is 0.005mm; light green trace is 0.05mm; purple trace is 0.1mm; blue trace is 0.25mm; yellow trace is 0.5mm; pink trace is 1.0mm; dark green is 2.5mm and turquoise trace is 5.0mm.

Table 4 shows the nominal concentration of the sample, the target range for the linearity and the target concentration of the concentrated sample.

Sample	Nominal Concentration (mg/mL)	Linearity Target Range (mg/mL)	Target Concentration of Concentrated Sample (mg/mL)
ADC Test Sample	5	2.5 - 15	20

Table 4.
Linearity study plan including target values.

Table 5 shows the actual concentration of the concentrated sample, which was determined by using the existing UV method and averaging the results of six dilution replicates over two assays performed by two analysts (3 replicates each) using different spectrophotometers.

Sample	Concentration (mg/mL)	DAR
ADC Test Sample	24.65	4.1

Table 5.
Determination of concentration of concentrated linearity sample.

A dilution scheme was prepared for the linearity using the values in Table 5. The calculated theoretical concentrations can be seen in Table 6 below and covers the target range specified in Table 4.

Level	Theoretical Concentration (mg/mL)
1	2.6
2	3.9
3	5.2
4	10.5
5	15.8

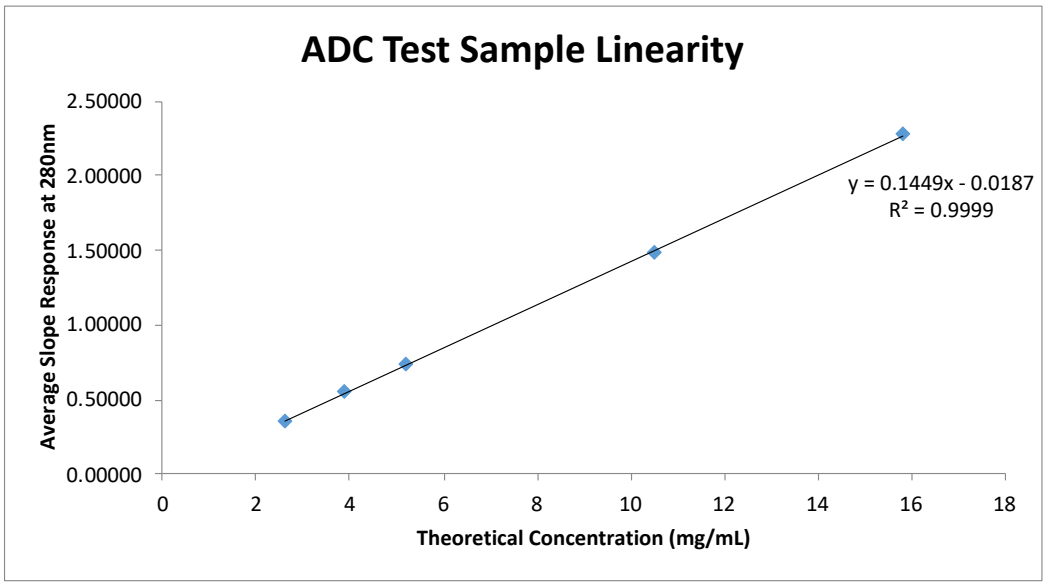
Table 6.
Theoretical concentration of each sample at each linearity level.

Level	Average Measured Concentration (mg/mL)	%RSD
1	2.5	0.6
2	3.8	0.7
3	5.1	0.2
4	10.3	0.2
5	15.8	0.4

Table 7.
Measured concentration of each sample at each linearity level.

All measured concentrations are within 0.2mg/mL of the theoretical concentration with all %RSD ≤0.7% showing that the SoloVPE is an accurate method. %Recovery was calculated for each level and was found to be between 96% and 100%, meeting the acceptance criteria of 90% - 110%.

Figure 9.
Line fit plot for ADC Test Sample linearity.



Regression analysis was carried out on the linearity results and line fit plots were generated for each sample as can be seen from Figure 9. All the results met the required acceptance criteria for the linearity. As can be seen from Figure 9, the protein concentration is linear with an $R^2 = 0.9999$. This met the acceptance criteria of ≥0.99.

Table 8.
Measured DAR at each linearity level.

Level	Average Measured DAR	%RSD
1	4.1	0.6
2	4.1	0.9
3	4.1	0.2
4	4.1	0.3
5	4.0	0.1

The expected DAR of the sample is 4. As can be seen from Table 8, the results achieved are within ±0.1 of the expected value.

Sample	Protein Concentration (mg/mL)	DAR
1	5.17	4.06
2	5.14	3.95
3	5.17	4.07
4	5.18	4.07
5	5.17	4.08
6	5.18	4.07
7	5.22	4.10
8	5.23	4.03
9	5.20	4.11
10	5.20	4.08
11	5.19	3.86
12	5.19	3.95
Average	5.2	4.0
%RSD	0.5	1.8

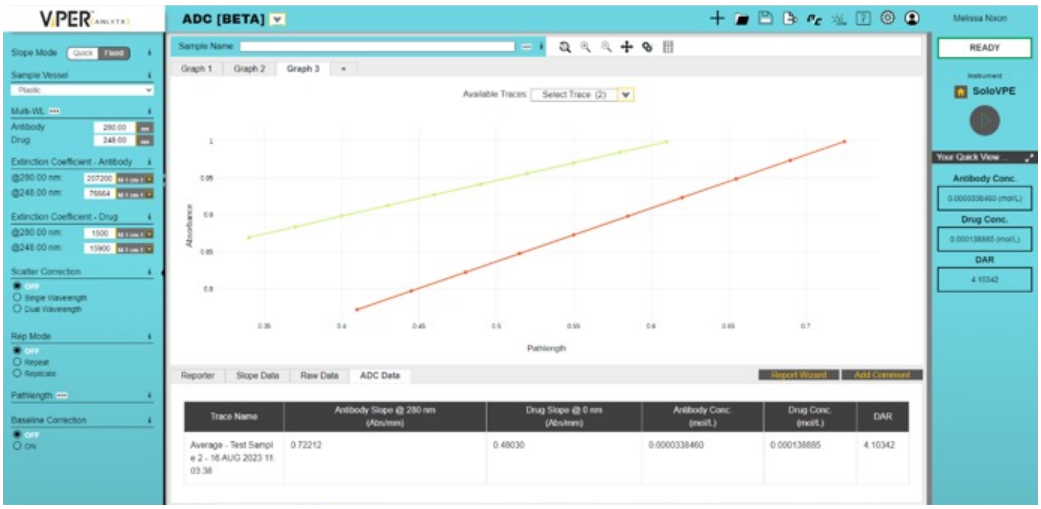
Table 9.
Intermediate precision results for protein concentration and DAR.

Repeatability and intermediate precision were also performed as part of the qualification experiments. From Table 9, the %RSD of protein concentration and DAR can be seen to be less than 2% which is well within the acceptance limit of 5% specified for the qualification. The intermediate precision was performed by a second analyst analysing six preparations of the sample to give a total of 12 results. The %RSD of all 12 results met the specified qualification criteria of ≤5%.

All method qualification acceptance criteria were met and comparable results obtained between the SoloVPE and traditional UV experiments. Therefore, the SoloVPE and Slope Spectroscopy method were deemed qualified and suitable for use.

The ViPER software for controlling the SoloVPE has recently updated to add an application specifically to perform and calculate DAR. This app requires the analyst to input the wavelength of the drug linker (248nm for this drug linker) and the extinction coefficients of both the antibody and the drug linker at 280nm and the wavelength of the drug linker. The software measures the absorbance at 10 different pathlengths and plots the results. The slope value is then used to calculate the molar concentrations of the antibody and the drug linker by replacing the absorbance values in equations 4 and 5. The DAR is calculated by dividing the molar drug linker concentration by the molar antibody concentration.

Figure 10.
Screenshot of ADC App result view.



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A DAR of 4.1 was generated using the ADC app for the sample. This matched the DAR result generated using the quick slope app for linearity, repeatability and intermediate precision experiments. This indicates that the ADC app is accurate and capable of calculating the DAR of a sample.

As can be seen from the evidence above, Slope Spectroscopy method using SoloVPE is a precise and accurate technique with the ability to be used for a variety of sample points during the ADC manufacturing process to calculate protein concentration and DAR. It has many advantages over traditional techniques used in the past including cost savings as mobile phase and sample buffers don't have to be prepared; analyst time is minimal due to a decrease in time spent preparing samples (5 minutes per sample instead of 30) and processing the data; and method error is lower as there is no requirement to dilute samples. As the SoloVPE is an extension to a UV Spectrophotometer and the ViPER software runs using a browser, there are many opportunities as technology advances to update the software and improve parameters and applications to further simplify the SoloVPE technique in the future. The user-friendly interface and minimal sample preparation attributed to the SoloVPE is proving to be a success, as evidenced by the ever-growing demand, as 9 out of 10 new projects coming into the company have requested the use of this system.

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