

Addressing Complex Sample Matrices with a Dual Cell Changer Configuration

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Key Words

- Complex Matrices
- Extinction Coefficient
- Linear Range
- Proteins and Peptides
- UV Spectroscopy
- Vaccines and Injectable Pharmaceuticals

Introduction

Complex matrices, which may include additives like surfactants and preservatives, are found in many industrial and research samples, including:

- Vaccines and Injectable Pharmaceuticals
- Nutritional Supplements
- Food and Beverages
- Cosmetics
- Specialty Chemicals
- Biological Materials

One of the difficulties in analyzing these matrices is that they often exhibit a significant absorption in the same wavelength range as the analyte of interest. Compensating for these matrix effects can be a challenging and tedious process for the analyst.

The Thermo Scientific Evolution 300 UV-Visible spectrophotometer offers a unique dual cell changer configuration to overcome the challenges posed by strongly absorbing matrices. The extinction coefficient assay to measure protein concentration is a good example of the advantage of this configuration.

Extinction Coefficients of Proteins or Peptides

The use of proteins and peptides as therapeutic agents for the treatment of diseases such as cancer and neurological anomalies has risen dramatically. A similar trend can be observed in the pharmaceutical industry as more companies are exploring large molecule therapeutics. The analysis of these large, biological molecules often involves an extinction coefficient assay. Extinction coefficient assays can be used to identify both proteins and peptides, and to determine their concentration in a sample based on Beer's Law.

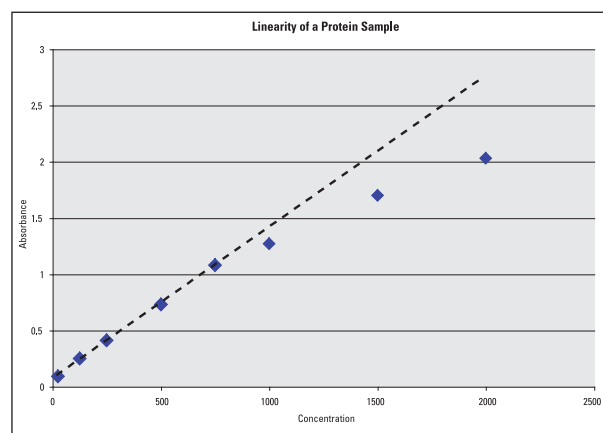


Figure 1: Protein absorption as a function of concentration is linear within a limited working range. Assay linearity is limited by the sample, not the instrument.



The Thermo Scientific Evolution 300 spectrophotometer is shown with a dual rotary 7-cell changer configuration

According to Beer's Law, the absorption (A_λ) of a given substance dissolved in solution at a particular wavelength (λ) is constant and proportional to its concentration (c) at a fixed pathlength (L), in this case 1 cm. This relationship gives rise to the constant ϵ , called the extinction coefficient, which is an intrinsic property of the substance and is illustrated in the following equation.¹

$$A_\lambda = \epsilon c L, \text{ where } L = 1 \text{ cm}$$

When the concentration (c) of the analyte is known, the extinction coefficient (ϵ) can be determined using UV-visible spectroscopy. The absorbance (A_λ) of the analyte is measured at the maximum of the absorbance peak, and its value is substituted for A_λ in the equation. A simple rearrangement of the equation then returns a measured value for the extinction coefficient (ϵ).

The extinction coefficient is constant only over a limited working range (See Figure 1); therefore, measurements made outside the linear working range of the assay produce erroneous results. Variations in the measured values of extinction coefficients at high concentrations negatively affect the precision and repeatability of these measurements. For this reason, analyzing the extinction coefficient across the linear range of the assay, as opposed to a single point, is preferred. In addition, once the extinction coefficient has been determined over a linear working range, accurate determination of analyte concentration is possible using a simple UV-Visible absorption assay.

The extinction coefficient of a protein or peptide is based primarily on the absorption of the amino acids tryptophan, tyrosine, phenylalanine and, to a small degree, cysteine. Consequently, proteins and peptides exhibit a peak maximum at or near 280 nm and are typically analyzed at this wavelength. Unfortunately, many sample matrices in the pharmaceutical industry include additives like Polysorbate, which also have a substantial absorbance in this wavelength range. In order to compensate for this, a reference sample must be prepared to minimize the impact of the sample matrix's contribution to the absorption measurement.

Ideally, the reference sample should be identical to the analytical sample but should not contain the analyte of interest. In other words, a reference sample should be prepared containing all of the components of the analytical sample matrix at the same concentration. The analyte of interest in the analytical sample, the protein in this case, is not added to the reference sample.

For analyses containing various dilutions of the analytical sample, such as a linearity curve for determining concentration or for validating an assay, compensating for matrix interference becomes increasingly difficult. This is because the level of contribution of the additive is also dependent on its concentration in the analytical sample. *Therefore, in order to compensate for the interfering absorbance of an additive in the sample matrix over the entire concentration range, each analytical sample must be analyzed against a unique reference sample at each concentration, as seen in Table 1.*

Analytical Sample API Concentration	Analytical Sample Components		Reference Sample
	BSA	Poly 80	Poly 80
Nominal	1.0 mg/mL	0.10%	0.10%
80%	0.8 mg/mL	0.08%	0.08%
60%	0.6 mg/mL	0.06%	0.06%
40%	0.4 mg/mL	0.04%	0.04%
20%	0.2 mg/mL	0.02%	0.02%
0%	0.0 mg/mL	0.00%	0.00%

Table 1: Spectral scan of Polysorbate 80 in saline at working concentrations of 0.1, 0.5 and 1.0% (w/v)

The dual cell changer configuration of the Evolution™ 300 automates the correction for complex matrices, thereby reducing the amount of time required to compensate for their contributing absorption values. The importance of this correction is demonstrated further in the example analysis in the following section.

Additive Absorption and Analysis of a Protein Product

A common scenario in the biopharmaceutical industry, particularly for products comprised of vaccines and other injectables, is to have the protein or peptide active pharmaceutical ingredient (API) suspended in a saline solution. To obtain the final product, one or more additives must be incorporated into the saline solution matrix before it can be released for therapeutic use. These additives, comprised of materials like preservatives and surfactants, frequently contribute a meaningful level of absorption in the region of measurement for proteins and peptides. In this simulation, Bovine Serum Albumin (BSA), a well characterized and commonly used protein standard, will represent the protein API. Polysorbate will represent the additive necessary to convert the API into the final product.

Polysorbate, also known as Tween®, is a common additive in the pharmaceutical industry. The most common forms of Polysorbate are Polysorbate 20, 40 and 80. Polysorbate 80, known chemically as polyoxyethylene-sorbitan monooleate, is a nonionic polymeric surfactant that can increase the solubility of hydrophobic compounds or act as a stabilizer for emulsions. The level of absorbance of Polysorbate 80 varies with concentration (Figure 2). Accordingly, the contribution of Polysorbate 80 to the absorbance of a solution across a linearity curve will also vary, illustrating the potential significance of the contribution of matrix additives in absorption analyses. It is important to note that the UV spectra for Polysorbate 80 may exhibit significant variation between lots and manufacturers.²

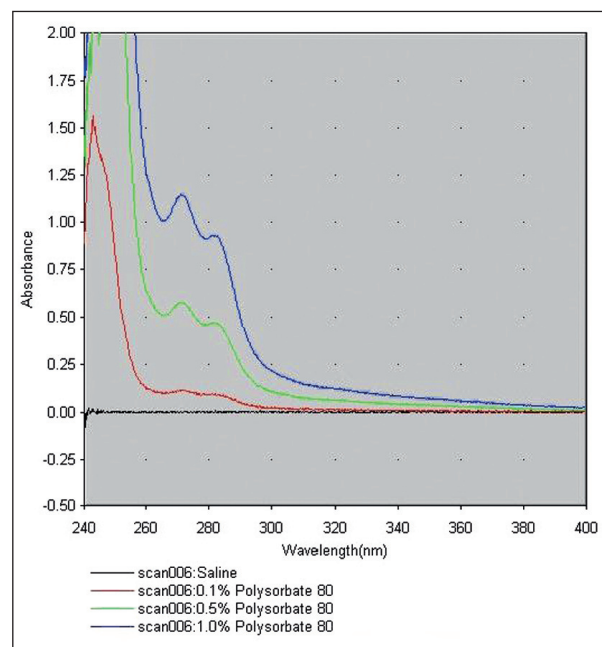


Figure 2: Spectral scan of Polysorbate 80 in saline at working concentrations of 0.1, 0.5 and 1.0% (w/v)

A solution of 1.0 mg/mL Bovine Serum Albumin (BSA) Standard +0.1% Polysorbate 80⁴ in saline was prepared as a representative analytical sample.³ A reference sample identical to the analytical sample matrix was also prepared. Four subsequent dilutions of each were made at 80%, 60%, 40% and 20% resulting in the solutions described earlier in Table 1.

The analytical samples were analyzed with and without correction against their corresponding reference samples. For simplicity, the absorbance values were plotted against the API concentration of each analytical sample assuming a molecular weight of 66,400 Da for BSA.² When plotted in this manner, the slope of a linear fit of the data is the molar extinction coefficient of the protein, where $\epsilon = A_{280}/c$.

In Figure 3, it is observed that the molar extinction coefficient for BSA without the correction for Polysorbate 80 is 51,801 M⁻¹cm⁻¹ and with correction is 45,920 M⁻¹cm⁻¹. This is a difference of more than 11% and is significant enough to negatively affect the accuracy and precision of any results calculated without correction for the contribution of Polysorbate 80 to the absorbance of the analytical sample.

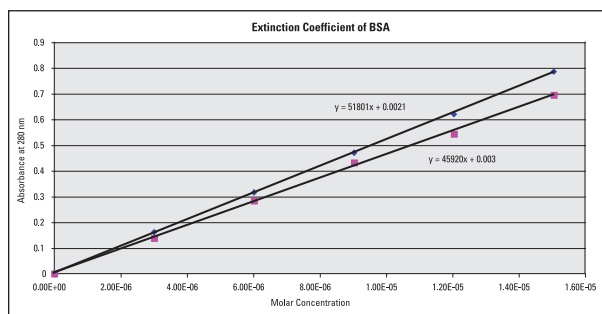


Figure 3: Graph of Absorbance at 280 nm vs. Molar Concentration of BSA for Analytical samples with and without correction for Polysorbate 80

Advantages of the Dual Cell Changer Configuration

The large sample compartment and separation between reference and sample beams offered by the Evolution 300 UV-Visible spectrophotometer allows two, seven-cell changers to be placed into the compartment in tandem. This allows the analyst to compare each sample to a unique reference automatically by placing one cell changer in the sample beam and a second cell changer in the reference beam. The spectrophotometer can then be programmed to compare each analytical sample to its corresponding reference sample using Thermo Scientific VISION software.

Summary

When performing spectrophotometric assays, it is important to correct sample absorption measurements for matrix ingredients that absorb in the same range as the analyte of interest. The Evolution 300 UV-Visible spectrophotometer equipped with dual cell changers simplifies this process through automation. This feature is useful not only for analyzing matrices with additives, but also for many other applications that require comparison of multiple analytical samples to a set of unique reference samples. This is particularly true in applications involving highly variable samples, such as allergy testing and human and plant tissue analyses. Automating your complicated methods reduces hands-on-time, minimizes the potential for human error, and increases productivity.

References

1. See Thermo Scientific Tech Tip #6, Extinction Coefficients, at www.thermo.com/pierce for more information on this topic.
2. W. Peter Wuelfing, Kathryn Kosuda, Allen C. Templeton, Amy Harman, Mark D. Mowery, Robert A. Reed, *Journal of Pharmaceutical and Biomedical Analysis*, 41 (2006) 774-782.
3. Thermo Scientific Pierce, BSA Standard
4. Fluka, Ph.EUR. grade

Ordering Information

Description	Part Number
Evolution 300 UV-Visible Spectrophotometer with PC Control	10300501
VISION ^{pro} [™]	10040101
VISION ^{security} [™]	10040301
Smart Rotary 7-cell Changer	10010101
4 mm Semi-micro Quartz Cuvettes	268-800000

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