Simplified Quantitation of Proteins at A280 using a Low Cost, One Millimeter Matched Pathlength Plastic Cuvette

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Routine measurement of protein concentration in a production environment is typically done using a 1 cm pathlength quartz cuvette. The protein to be measured requires dilution to be within the dynamic range of the spectrometer. Dilution involves an additional step in the concentration measurement process, and often leads to random or systematic errors that skew the measurement and either under or over estimate the concentration. These errors in concentration can increase manufacturing variability for diagnostic tests that require a known quantity of protein to be incorporated into a test device. To avoid experimental error due to dilution, 1 mm pathlength quartz cuvettes have been used, however the extra steps required for cleaning can also introduce errors into the measurement. The technician might not clean the contents completely, or be unable to clean it due to non-reversible adsorption of proteins on the quartz surface. To avoid these issues, a single-use 1 mm pathlength cuvette has been developed and shown to permit quantitation of protein concentrations with accuracy and precision similar to that of a 1 mm quartz cuvette.

Introduction

SpecVettes are simple, single-use microfluidic cuvettes for use in standard spectrometers. Each device accommodates two samples, introduced using a pipette. They are a polymer laminate construction comprising two layers of a UV transparent window film, Zeonor, bonded to the body of the cuvette using a pressure sensitive adhesive. The body of the device, made from close tolerance cast acrylic, determines the pathlength of the cuvette. The cuvette slides into a holder to position it in a standard 1 cm^2 sample holder. Two different holders are available for z-heights ranging from 8.5 to 15 mm and 15 to 20 mm.

The absorbance of proteins at A280 is often used as a routine method for determining protein concentration. The amino acids tyrosine, tryptophan and phenylalanine contain aromatic ring moieties with strong absorbance at 280 nm. By using Beer's law and measuring the absorbance of a known concentration of protein in a known pathlength, one can calculate the absorbance constant of the protein of interest. The absorbance constant can then be used to find the concentration of an unknown sample of the same protein when the pathlength is known.

Materials and Methods:

Creating Sets of matched Pathlength SpecVettes Matched pathlength cuvettes were made by sorting a batch of SpecVettes™ according to measured pathlength and proximity on

the sheet. Each batch is fabricated in a 12" x 24" sheet and produces over 200 cuvettes. Each SpecVette is labeled with a letter and number which maps its location on the sheet. Three to five SpecVettes are taken from each column on the sheet and the pathlength is measured using an optical comparator. The comparator focuses on the edge of the part at 4.5 x and the thickness of the cell is measured and recorded on each end of the SpecVette. The pathlength of the SpecVette is then reported as the average of each measurement minus the thickness of the window material, 50 microns, +/- 5 microns. The measured pathlengths are then plotted on a graph to permit grouping by location on the sheet. Error bars that represent + and - .5% error are also plotted for each pathlength. This defines the maximum and minimum pathlength with which another SpecVette can be grouped. In a typical fabrication run, about 80 to 90% of the batch can be grouped reasonably into matched sets of 25 cuvettes. Once grouped, the average of the combined measured pathlengths contained in the set is averaged. If the % std deviation from the average is less than 2%, the box is labeled with the average pathlength. If not, a few more SpecVettes are measured based on their location on the sheet, and the cuvettes regrouped to keep the % standard deviation from the average less than 1%.

Preparation of Protein Solution Used for All Concentration Measurements. Bovine serum albumin (BSA) from Proliant Biologicals (30% solution) was diluted (PBS buffer pH 7.2) to approximately 10.5 mg/ml for an absorbance of about 0.7 AU (extinction coefficient = 0.67 cm^{-1} ml/mg). This solution was used for all concentration measurements.

Protein Concentration Measurements Using Quartz Cuvettes. Ten individual 1 mm pathlength quartz cuvettes purchased from Varian, Inc. were used for reference. Measurements involved filling a cuvette with PBS, blanking the instrument, then rinsing the cuvette at least twice with the BSA solution before filling with BSA a third time and taking the absorbance measurements at 280 nm and 320 nm. The absorbance at 320 nm was subtracted from the measurement at 280 nm to correct for light scattering. A total of forty-two individual concentration measurements were made using these cuvettes and the BSA solution described above.

Protein Concentration Measurements Using SpecVettes. Eleven separate matched SpecVette sets from across four lots were analyzed. Seven sets were analyzed using a Cary 50 spectrophotometer, two using a Schimidzu, one using a Beckman, and one using a Cary 300. Each set was analyzed by one of three different technicians. Each SpecVette BSA concentration measurement involved blanking with PBS using one SpecVette chamber, then filling the other chamber with BSA and measuring the absorbance at 280 nm and 320 nm. The absorbance at 320 nm was subtracted from that at 280 nm to correct for light scattering. Each SpecVette set was analyzed side by side with the Varian quartz cuvettes, except for the set analyzed on the Beckman which resulted in beam clipping for the quartz cuvettes as the Z-dimension for the Beckman is lower (8.5 mm) than that for

the other spectrophotometers (15 – 20 mm). The concentration of the BSA solution was measured using eighty-eight individual SpecVettes.

Data Analysis:

Data were analyzed using JMP software (version 8.0.1, SAS Institute Inc.).

Results:

Protein Concentration Measurements Using Quartz Cuvettes. All protein concentration measurements generated using Varian 1 mm pathlength quartz cuvettes were used to calculate a grand mean protein concentration of 10.41 mg/ml (N = 42), with an overall CV of 1.2%.

Protein Concentration Measurements Using SpecVettes. All protein concentration measurements from SpecVettes were used to prepare the histogram shown in Figure X. A grand mean protein concentration of 10.44 mg/ml was obtained (N = 88), with an overall CV of 2.4%. The % difference between the overall SpecVette grand mean and the overall 1 mm pathlength Varian quartz grand mean is 0.29%.

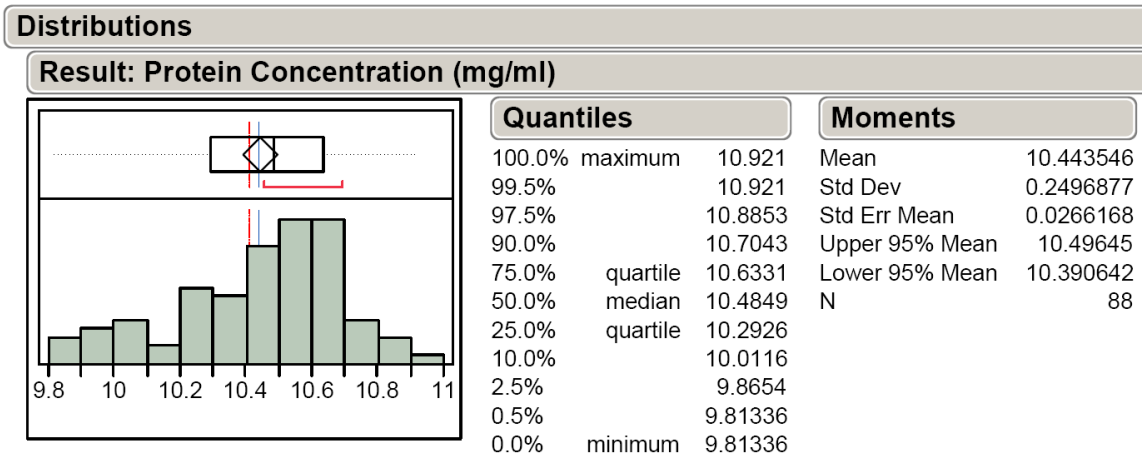

Figure X: Histogram of SpecVette Data with 1 mm Pathlength Quartz Grand Mean (10.41 mg/ml, Red Line) and SpecVette Grand Mean (10.44 mg/ml, Blue Line) Indicated.

Individual variance components for SpecVette measurements were determined. While 47% of the total variation came from set to set differences, this variation can be decreased by taking two repetitions with each SpecVette from a different matched set. 12% of the variation came from lot to lot differences, 13% from instrument to instrument variation, 6% from operator to operator variation, and 22% from measurement to measurement variation within a set. The average of two repetitions with both SpecVettes coming from different matched sets would provide a CV of 2.00% with corresponding 95% confidence limits of +/- 3.92% (Table X).

Table X: Summary Table for Expected Variation in Means of Two SpecVette Measurements.

Conditions	CV	CV X 1.96 for 95% confidence interval
Average of 2 Specvette measurements from the same matched set	2.32%	4.55%
Average of 2 Specvette measurements from different matched sets	2.00%	3.92%

Discussion:

SpecVettes are disposable 1 mm pathlength cuvettes that can be used for measuring protein concentrations without the need for dilution. Error coming from pipettes used for dilution as well as dilution technique, dilution calculations, and insufficient cleaning of cuvettes is thus eliminated.

A total of eighty-eight individual SpecVettes were analyzed side by side with ten 1 mm Pathlength Varian quartz cuvettes. The SpecVettes had an overall %CV of 2.4%, and the grand mean protein concentration was 0.29% different from the grand mean for the 1 mm pathlength quartz cuvettes. Data indicate that when two SpecVette measurements of the same solution are averaged, the concentration will be within < 5% of the mean quartz value with 95% confidence.