# A SIMPLE TECHNIQUE FOR CALIBRATING THE VOLUME OF HPLC INJECTION VALVE LOOPS.

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## *Introduction*

The six-port sampling valve is the most commonly used injection device in HPLC instrumentation. Several designs and implementations exist, depending on the manufacturer, but the basic operating principle is the same. They can be used for partially filled loop injections or completely filled (Fixed) loop injections. In addition, six-port sampling valves can be used not only for manual injections, but they can be incorporated into automatic injection devices as well.

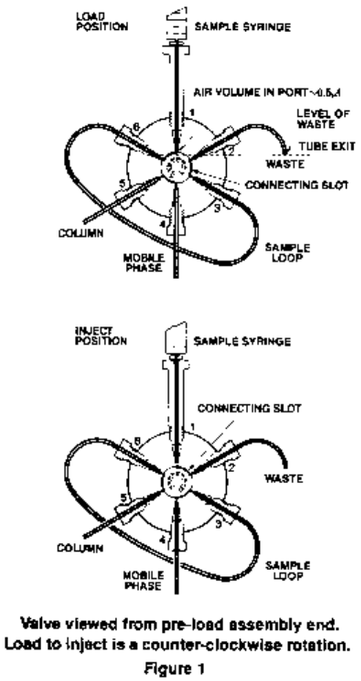


**Figure 1**

Traditionally, the requirement to know the exact volume of the six-port valve's sample loop was not necessary as long as all standards and unknowns were injected using the same technique with the same loop. However, due to new regulations for the validation of instrument performance for GLP, some laboratories require that the loop volume be accurately known. Loops are typically constructed from tubing of a known internal diameter, cut to the required length to provide the desired volume. Many laboratories fabricate their own sample loops in this fashion. However, the internal diameter of the

tubing may vary as much as 20% depending on the manufacturer of the tubing and the tubing batch. In addition, the total injectable volume of the HPLC injection valve loop is a combination of the physical volume of the loop itself, plus the flow path volume within the valve. Additionally, any "Dead Volume" that results from tubing with ends that are not cut squarely or deburred will also increase the injection system volume. These flow paths are illustrated in Figure 1. Note that the labeled volume of the loops sold by most valve manufacturers for use with their product includes the additional injectable flow path volume of their valve. Furthermore, some valve manufacturers offer calibrated loops for their valves at an additional expense. Regardless of the source of the loop, proper loop volume calibration requires that both the loop and valve combination be calibrated together. The technique described here provides a simple volumetric procedure for calibrating the total injectable volume of an HPLC injection valve and loop.

## *Calibration Procedure*

The HPLC system used for the loop calibration consisted of a Model 765 HPLC Pump, (Alcott Chromatography, Inc., Norcross, GA), a Linear Model UVIS 200 Variable Wavelength HPLC Detector and a Spectra Physic Model 4400 Integrator (both from ThermoSeparations, Riviera Beach, FL). The valve and loops calibrated in this study were built into the Alcott Model 708 Autosampler(Alcott Chromatography, Inc., Norcross, GA). The valve in the autosampler is a Valco Model C6W (Valco Valve, Houston, TX). The loops evaluated were either homemade in the laboratory or purchased (also from Valco). The chromatography was conducted using a 150 mm $\times$ 4.5 mm ID column packed with Spherisorb ODS II, 5 μm dp (Phenomenex, Torrance, CA) using a 70/30 Methanol/Water Mobile Phase (HPLC Grade, Fisher Scientific, Norcross, GA). The 70/30 Methanol/Water solution was used for all

sample and standard dilutions as well as the chromatographic Mobile Phase. Standard stock solutions were pipetted using a Hamilton Model 1710, 100 μL syringe. Note that although HPLC was used to assay the calibration solutions and unknowns, other techniques such as UV/Vis spectrophotometry could have been used for this calibration procedure.

The standard, stock solution was prepared by weighing 0.150 g of Propyl Paraben (n-Propyl *p*-Hydroxy Benzoate, Sigma Chemical, St. Louis, MO) into a 10 mL volumetric flask. The flask was then filled to the mark with the 70/30 Methanol/Water Mobile Phase. The four calibration solutions were prepared by pipetting 5 μL, 10 μL, 20 μL, and 50 μL of this standard stock solution into individual 10 mL volumetric flasks and then filling the flasks to the mark with the 70/30 Methanol/Water Mobile Phase.

The Autosampler was connected to the HPLC pump and the loop installed. Approximately 10 mL of Mobile Phase was pumped through the Autosampler's valve first in the LOAD position and then in the INJECT position. After rinsing, the valve was placed in the LOAD position, and the loop was overfilled with 500 μL of the Propyl Paraben standard stock solution. The valve was switched to the INJECT position, and the valve and loop contents were flushed into a 10 mL volumetric flask with 1 mL of Mobile phase. After flushing the loop contents, the pump flow was discontinued, and the flask was removed and filled to the mark with Mobile Phase. This procedure was repeated for two additional loop sizes and replicate measurements were made for each loop size.

Once all calibration curve and loop solutions were prepared, the resulting Propyl Paraben concentrations were measured chromatographically. The Autosampler was fitted with a 10 μL loop, and a column and detector were attached. The prepared solutions were placed into autosampler vials and injected into the HPLC.

## *Results and Discussion*

Figure 2 and Table 1 are the results for the calibration solutions. The calibration curve exhibited good linearity (r = 0.9999) over injection volume range chosen. The results for the individual loops are listed in Table 2. The 10 µL loop was purchased from Valco while the 20 µL and 40 µL loops were homemade in the laboratory. Note that the percent difference between the loop's labeled volume and its actual volume is much less for the factory made loop indicating the close tolerances the manufacturer observes in fabricating its loops to match its valves. The homemade loops exhibited much larger volumes than intended. The Valco C6W valve used in the autosampler has an internal volume of approximately 0.5 µL and the measured volumes for the homemade loops show a larger discrepancy than this auxiliary volume would add. This additional volume is more than likely a result of either the variability of the tubing's internal diameter, the craftsmanship of the loop fabricator, or a combination of both of these factors. The overall precision of the procedure was better than ± 0.30% indicating the high reliability of the

***Table 1***

Calibration Standards' Statistics

| | Peak Areas | | | |
|---|---|---|---|---|
| *Volume* | *50 uL* | *20 uL* | *10 uL* | *5 uL* |
| | 6805181 | 2719871 | 1368808 | 654243 |
| | 6793120 | 2713367 | 1366785 | 651122 |
| | 6804431 | 2717653 | 1367704 | 652883 |
| | 6817383 | 2725062 | 1375293 | 651915 |
| | 6799493 | 2719932 | 1369631 | 653133 |
| | 6786845 | 2721604 | 1370138 | 654138 |
| *Average* | 6801075.50 | 2719581.50 | 1369726.50 | 652905.67 |
| *Stand. Dev.* | 10605.19 | 3915.92 | 2991.69 | 1226.56 |
| *RSD (%)* | 0.16 | 0.14 | 0.22 | 0.19 |

*Regression Output:*

| | | |
|---|---|---|
| *Constant* | | 0 |
| *Std Err of Y Est* | | 16633.52 |
| *R Squared* | | 0.99996334 |
| *No. of Observations* | | 4 |
| *Degrees of Freedom* | | 3 |
| *X Coefficient(s)* | 136002.38 | |
| *Std Err of Coef.* | 302.43 | |

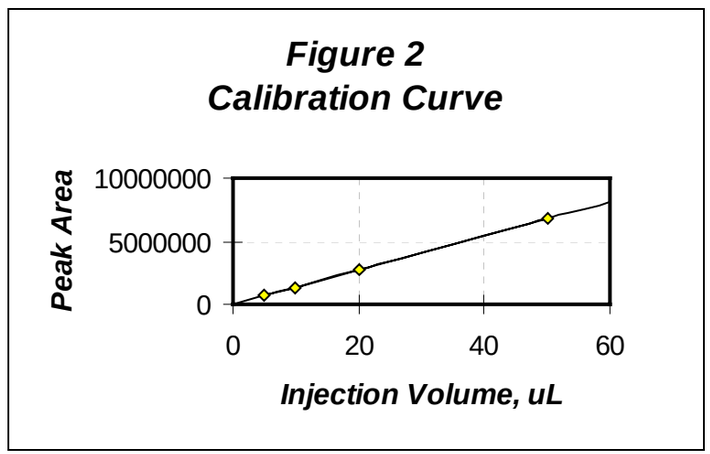


***Table 2***

Loop Volume Determination Data

| | *10uL Loop* | | *Peak Areas 20uL Loop* | | *40uL Loop* | |
|---|---|---|---|---|---|---|
| | 1389.67 | 1387.73 | 3156.02 | 3143.41 | 5994.64 | 6014.89 |
| | 1378.40 | 1380.57 | 3157.43 | 3146.72 | 5967.40 | 5991.15 |
| | 1385.10 | 1383.52 | 3146.80 | 3149.85 | 5975.89 | 5996.34 |
| | 1385.24 | 1388.26 | 3145.46 | 3140.12 | 6006.34 | 6011.32 |
| | 1384.92 | 1382.95 | 3163.44 | 3140.19 | 5980.88 | 5993.41 |
| | 1389.70 | 1383.94 | 3150.92 | 3136.98 | 5970.26 | 5988.04 |
| *Average* | 1386383.83 | 1384572.83 | 3153757.83 | 3142771.17 | 5982476.83 | 5999430.83 |
| *Stand. Dev.* | 417.665 | 305.562 | 664.009 | 468.853 | 1509.766 | 1098.001 |
| *RSD* | 0.30 | 0.22 | 0.21 | 0.15 | 0.25 | 0.18 |
| *Loop Volume, uL* | 10.2 | 10.2 | 23.2 | 23.1 | 44.0 | 44.1 |
| *%Error* | 1.9 | 1.8 | 15.9 | 15.5 | 10.0 | 10.3 |

measurement.

## *Conclusion*

The valve and loop calibration procedure presented here provides the chromatographer with a simple solution for accurately determining the total volume of the injection system when this parameter is required. Since the loop volume is determined with the loop installed in the valve, all additional volumes such as the valve's internal flow path volume and all "Dead Volume" associated with poorly cut tubing and improperly swaged fittings are included in the measurement.