

GOOD LABORATORY PRACTICE

Agilent Cary 8454 UV-Visible Spectroscopy System



Introduction

The objective of good laboratory practice (GLP) is to obtain accurate and precise results, using a measurement system that is functioning properly and to the specifications required by global regulatory bodies such as the United States Pharmacopeia (USP) and European Pharmacopoeia (EP). The Agilent Cary 8454 UV-Visible spectroscopy system includes a UV-Visible instrument based on leading LC photodiode array detector technology, which has been designed and manufactured to meet the strictest standards and regulatory requirements. Agilent offers a comprehensive portfolio of service plans and regulatory compliance services to help achieve validation of the 8454 UV-Visible system and its components to ensure the highest levels of reliability and performance. This note looks at specific components that require validation; the instrument, software, and validated test methods, and describes the features of the Agilent Cary 8454 UV-Visible system that is designed to meet the requirements of both initial and recurrent validation.

Spectrophotometer

The UV-Visible system is based on the Agilent Cary 8454 UV-Visible spectrophotometer. Unlike conventional scanning spectrophotometers, diode-array spectrophotometers such as the Cary 8454 UV-Visible have no optically-active moving parts and have proven to be extremely reproducible and stable both in the short (hours or days) and long term (months or years) from over 30 years in production and operation. With no moving parts, there are no mechanical components to adjust or recalibrate, thus verification is primarily required to:

- Check that the instrument is within specifications
- Check for significant changes in performance

Changes can affect the accuracy of quantitative analyses and can indicate potential problems. In most cases, changes in performance are due to the intensity of the deuterium lamp decreasing over its lifecycle.



Verification

The Agilent Cary 8454 UV-Visible system has a built-in self verification feature in the software that makes frequent, full verification unnecessary. In principle, the full verification procedure is necessary only after a repair, or when the lamp is changed. The lamp should be changed when the noise reaches an unacceptable level.

However, based on common practices with conventional spectrophotometers, we suggest full verification at six-monthly intervals. There is a significant difference between the USP and EP regarding performance verification of UV-Visible spectrophotometers. The USP states: "The instrument performance should be verified as prescribed by the manufacturer". In contrast, the EP gives specific tests and performance requirements for wavelength accuracy, photometric accuracy, stray light, and resolution. The Agilent Cary 8454 UV-Visible system meets both sets of requirements by providing a range of tests, including all EP required tests, and by allowing the user to select those tests which should be performed to provide full verification according to company or national regulatory needs. The specifications for the components of spectrophotometer performance verification are listed below.

Performance verification specifications

Wavelength Accuracy

Within ± 0.5 nm for 14 validated transmission peak minima of a certified NIST-traceable holmium oxide in perchlorate solution^{1,2}. The instrument is blanked on air or a blank solution and the transmittance spectrum of the standard is measured. The wavelengths of the peak minima found are then compared to the specified wavelengths.

Photometric Accuracy

- Within ± 0.005 AU at 440.0, 465.0, 546.1, 590.0, and 635.0 nm, at approximately 1 AU using the NIST-traceable 930e, 10% transmittance, absorbing glass standard. The instrument is blanked on air and the absorbance values at the specified wavelengths are measured. These values are then compared to the specified value for the standard.
- ± 0.01 AU 235, 257, 313, 350 nm using a 6 % w/v potassium dichromate NIST-traceable 935a solution in 0.01 N sulfuric acid (also available in 0.001M perchloric acid). The instrument is blanked and the absorbance values at the specified wavelengths measured. These values are then compared to the specified value for the standard.

Stray Light

- Less than 0.03% at 340 nm measured with 50 g/l NaNO_2 solution.
- Less than 0.05% at 220 nm measured with 10 g/l NaI solution.
- Less than 1% at 198 nm measured with 1.2% KCl solution.

In transmittance mode the instrument is blanked on air. The transmittance value at the appropriate wavelength is measured with the appropriate solution in place.

Resolution

The ratio of the absorbances at 269 and 266 nm of a 0.02 % v/v solution toluene in hexane is greater than 1.5. The instrument is blanked on hexane and the absorbance values at the specified wavelengths measured. The ratio is calculated and compared to the specified value.

Noise

Less than 0.0002 AU rms (root mean square) at 500 nm and 0 AU. No filters or standards are required. With the sample area empty, 60 consecutive absorbance measurements of 0.5-second integration time are made at the user specified wavelength(s). Noise is calculated from the measured data using the equation:

$$\text{Noise (rms)} = \sqrt{(\sum(X-x)^2)/n}$$

X is a 11-point moving average, x is the measured value and n is the number of points.

Baseline Flatness

Less than 0.001 AU rms at 0 AU. No filters or standards are required. With nothing in the sample area a blank is measured, then a sample absorbance spectrum is measured using 0.5 second integration time. The baseline rms value is calculated using the same equation as above but with a normal average.

Stability

Less than 0.001 AU at 340 nm at 0 AU, measured over one hour. No filters or standards are required.

The Agilent Cary 8454 UV-Visible verification procedure allows the user to select those tests that are appropriate to national or international guidelines, as shown in Figure 1. The system's verification procedure prompts the operator to insert standards and make the appropriate measurements. It performs calculations and generates a validation report. In addition, the results are archived for reporting or reviewing.

Agilent 8453/8454 Instrument Performance Verification Setup

Instrument serial number: CN22808688

1. Wavelength Accuracy and Reproducibility

- Holmium oxide
- Deuterium lamp emission lines

2. Photometric Accuracy

- Neutral density filter (NIST 930E)
- Potassium dichromate
- Potassium dichromate (430 nm)

3. Stray Light

- KCl solution at 198nm
- NaI solution at 220nm
- NaNO2 solution at 340nm

4. Resolution

- Toluene in hexane

5. Noise

- No filter required

6. Baseline Flatness

- No filter required

7. Absorbance Stability (1 hour test)

- No filter required

OK Cancel Help

Figure 1. Verification tests selectable by the user

Self Test

Full performance verification as described above takes time and is not practicable on a daily basis. It is common practice to do full performance verification on a monthly to six monthly basis. However, if it is not done frequently, there should be some way to monitor performance between verifications. The Agilent Cary 8454 UV-Visible spectrophotometer has a built-in self-test procedure that the user can initiate at any time. This self-test comprises electronic tests (that are also performed automatically when the spectrophotometer is switched on), and optical performance tests, which are summarized in below. The self-test detects any changes in performance between full performance verifications. The results of the self-test are stored in an electronic logbook and can be printed and reviewed as required. The self-test should be performed at weekly or even daily intervals.

Key components of the spectrophotometer self test:

Electronic Tests: Performed to ensure proper operation of microprocessors, RAM, communications interface, analog-to-digital converter and shutter.

Optics: Dark current, intensity profile, wavelength accuracy and resolution at the 486.1 and 656.3 nm emission lines of the deuterium lamp are checked.

Computer and software

The operating software, ChemStation UV-Vis, undergoes extensive validation during development, as documented by the Declaration of Validation shipped with each instrument. The equipment itself also contains additional software routines for revalidation of particular parts of the electronic data processing. Data acquisition is implicitly tested by the automated validation routine for the Agilent Cary 8454 spectrophotometer as described above.

Data analysis and the reporting components of the software can be verified using the software validation kit

shipped with every copy of the ChemStation software. These validation files can be used under non-21 CFR Part 11 as well as under 21 CFR Part 11 software configurations.

This kit includes:

- Spectra for single component calibration and evaluation,
- Spectra for multi component calibration and analysis,
- Methods designed to exercise spectral processing and quantification,
- An automation file that performs the validation process automatically, and
- Documentation of expected results.

A test is performed by loading and running the appropriate automation file. The software automatically steps through a series of method loading, data loading, data analysis and reporting processes to produce printed reports that are then compared with the expected results. The user can adapt the validation process to meet specific needs. Detailed descriptions of the transformations used by the Agilent Cary 8454 system are given in the Understanding Your ChemStation manual. This enables the user, if required by an auditor, to reproduce manually all the calculations performed by the software.

Method validation

Method validation is the process of establishing that the performance characteristics of the analytical method are suitable for the intended application. The United States Pharmacopeia (USP) position on method validation is well documented.

The analytical variables considered in the validation of methods should typically include precision, accuracy, limit of detection, limit of quantification, selectivity, linearity and ruggedness.

Advanced software for Agilent Cary 8454 UV-Visible systems includes tools that simplify the validation of an analytical method. To evaluate variables in an analytical method requires proper statistical tools. The advanced software includes full statistical evaluation of all results. The statistics include the ability to enter the standard deviation for the preparation of standards and the statistics obtained by the Agilent Cary 8454 UV-Visible spectrophotometer on the measurement of each sample. Such measurement statistics are only available with a diode-array spectrophotometer. When maximum likelihood is selected, these measurement statistics are used in the calibration routine to improve the fit.

- *Accuracy is the closeness of an individual test result to the true value*
The Optimize Wavelength function of the advanced software measures the accuracy of a method. A typical sample is measured and quantified at all wavelengths over the measured wavelength range to determine the wavelength giving the most accurate results.
- *Precision is the degree of agreement among individual test results obtained by applying the analytical method to multiple samplings of a homogeneous sample*
The advanced software includes a test method that calculates the standard deviation of the results of a series of sample analyses. This is a direct measure of the precision of the method.
- *Sensitivity is a measure of the response obtained for a given amount of analyte. Sensitivity is denoted by two analytical factors called the limit of detection and the limit of quantification.*
Limit of detection is the lowest concentration of analyte that is detectable at the most sensitive instrument settings. The detection limit is considered to be reached when the signal from the analyte is equal to three times the noise in the measurement.

The advanced ChemStation software gives quantitative results with standard deviations that are based on the noise in the measurement. The detection limit is approximately three times the standard deviation.

- *Limit of quantification is the lowest concentration of analyte that can be determined with acceptable precision and accuracy*
The acceptable limits of precision and accuracy depend on the objectives for the analysis. With these defined, the tools described above can be used to determine the acceptable limits.
- *Linearity is the ability of the method to produce test results that are proportional, either directly or by a mathematical transformation, to the concentration of analyte in samples within a given range*
For UV-Visible measurements, the usual linear relationship is Beer's law. The advanced ChemStation software gives both graphical and statistical evaluation of the linearity of the calibration curve (Figure 2). The graphical display shows the calibration curve with 95% confidence intervals. Statistical values include the percent error of each standard from the calculated relationship, and the correlation coefficient and standard error of regression of the fit.

The Evaluate Standards function of the advanced software can be used to optimize linearity. This feature performs calibrations using the specified calibration parameters at all wavelengths available using all standards. The quality of the calibration curve at each wavelength is indicated by the correlation coefficient, standard deviation of regression or uncertainty which are plotted against wavelength. The best calibration is the one at the wavelengths where the chosen statistic gives the best value (Figure 3).

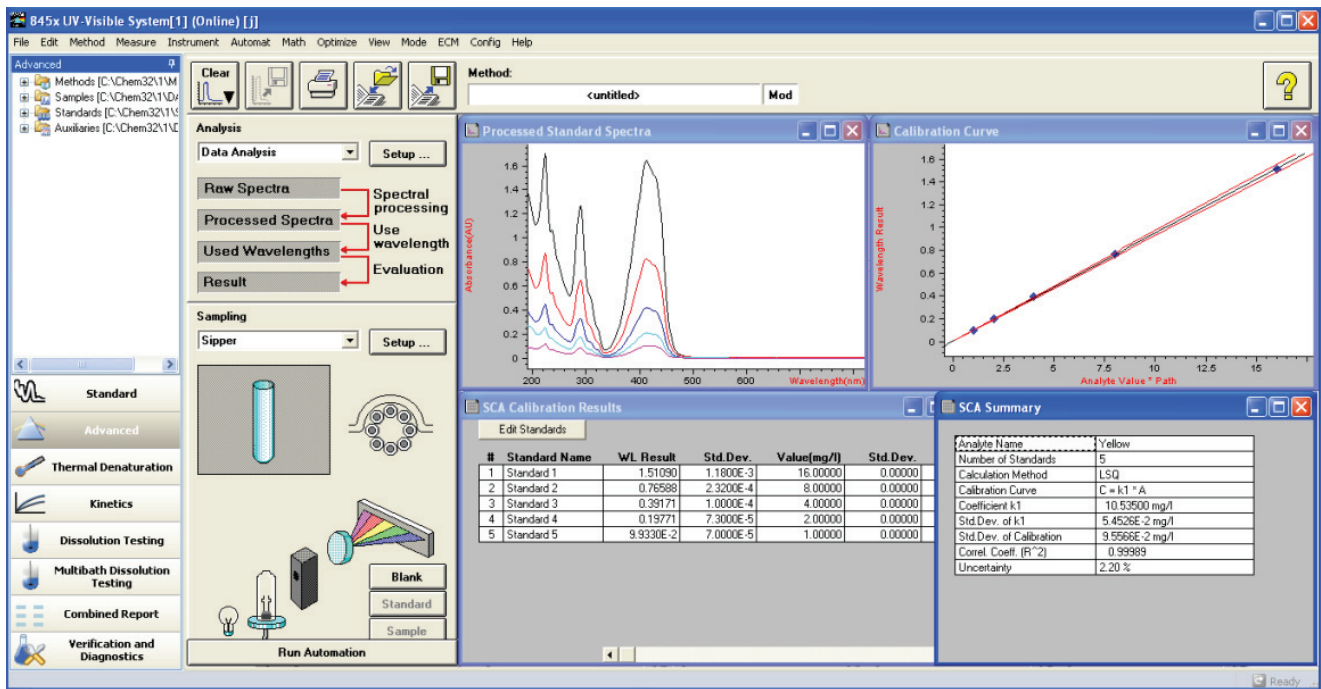


Figure 2. Calibration results.

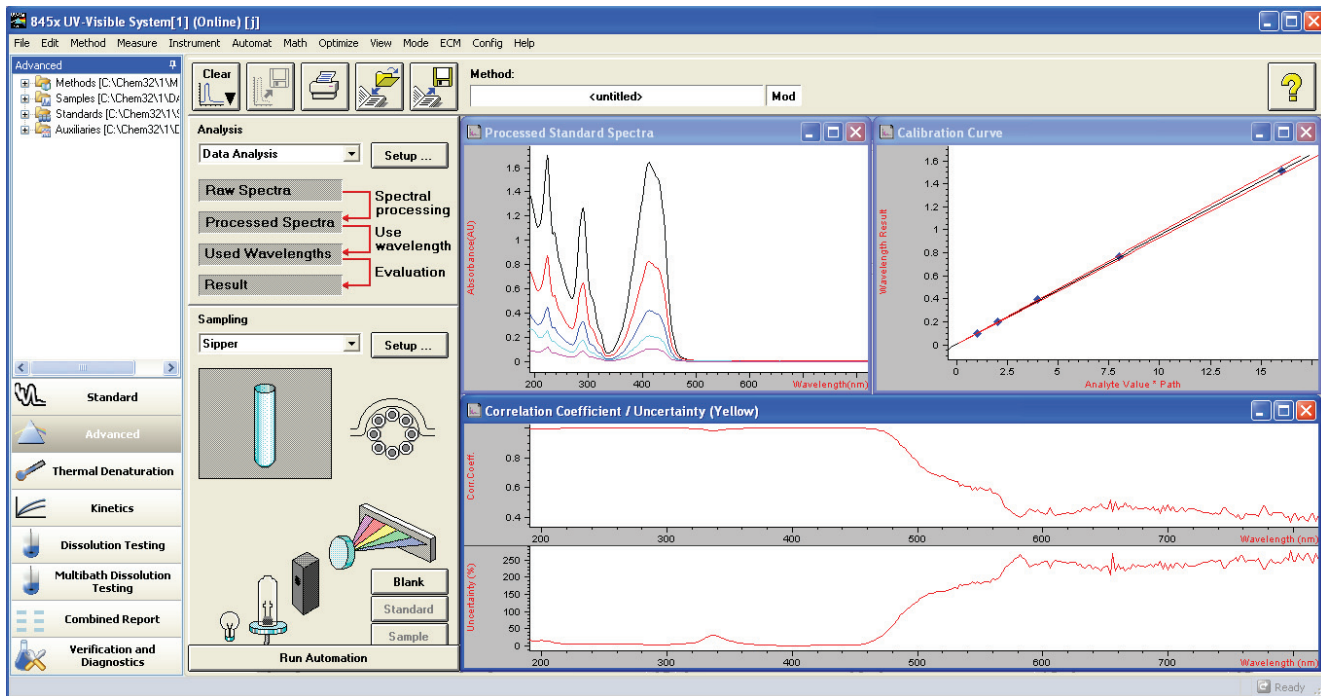


Figure 3. Results of the Evaluate Standards function of the advanced software.

- Range is the interval between the upper and lower levels of analyte that have been determined with the required precision, accuracy and linearity.*
The range may be determined by analyzing samples containing varying concentrations of the analyte and using the tools described above to test whether the required levels of precision, accuracy and linearity are achieved.
- Selectivity is the ability of a method to quantify, accurately and specifically, the analytes in the presence of other compounds.*
Other compounds could be synthesis precursors, known impurities, excipients, and degradation products, that may be expected to be present in the sample matrix and that also have a UV-Visible absorbance. The advanced ChemStation software includes an Optimize Wavelength feature that is used to find the wavelengths giving the best sensitivity for the target analyte.

To use this feature, the calibration standards are first measured and the proposed analytical parameters entered. Then the spectrum of a typical sample is measured and the Optimize Wavelength function of the advanced software quantifies the sample using calculated calibration coefficients at all available wavelengths and plots the results against wavelength (Figure 4).

When the concentration of the analyte in the sample has been determined by an alternative technique, the wavelength giving the best selectivity is the one with the result closest to the known value. When the concentration of the analyte is unknown, the wavelength giving the lowest concentration is usually the one that gives best selectivity (impurities always add absorbance causing erroneously high results).

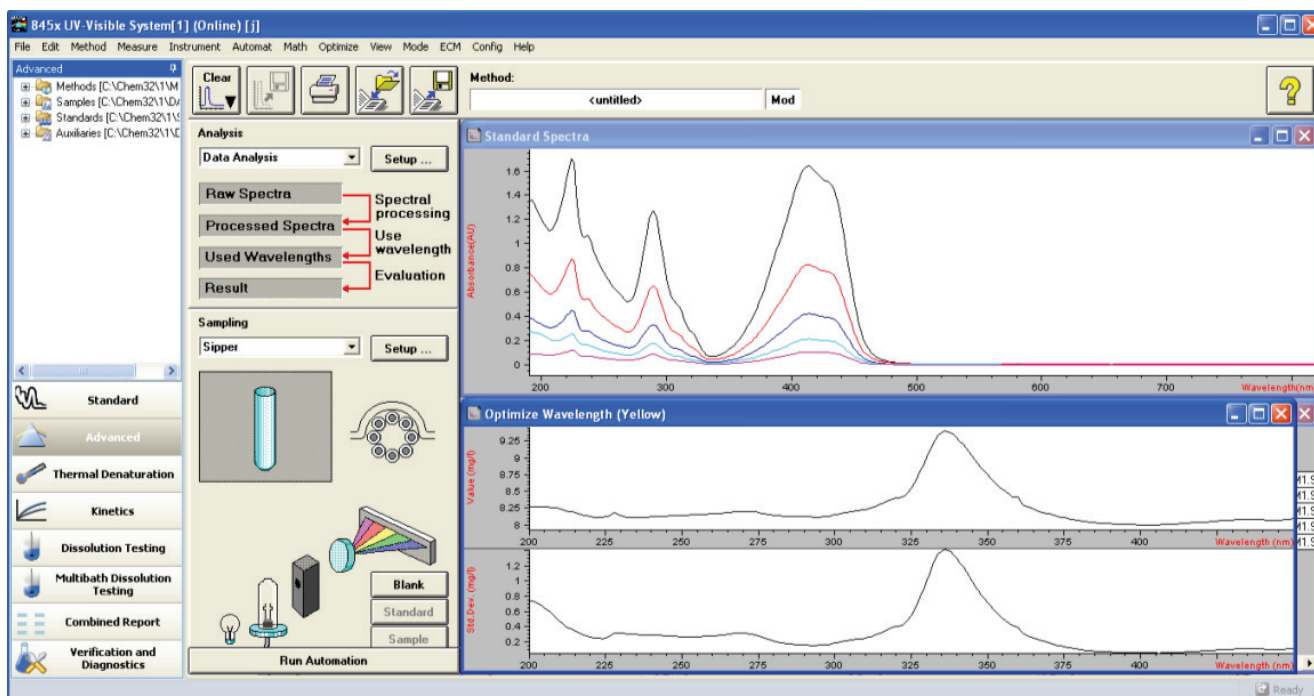


Figure 4. Results of the optimize wavelength function of the advanced software.

- Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions.*

The method should not be prone to variations due to time or location. The reproducibility of the method should be established under varied conditions, for example, different reagent batches or different assay temperatures. Testing for ruggedness involves inter-instrument and inter-laboratory measurements that are beyond the scope of a single instrument.

Documentation

A vital part of any validation process is proper documentation. Figure 5 shows an example of the method documentation that is generated by the system. The report gives a complete overview of all parameters, data acquisition, data analysis and report for a specific method. Additional documentation is available if the method uses a calibration (see below).

System suitability

System suitability should not be confused with method validation. System suitability is designed to evaluate the components of the analytical system to show that the performance of the system meets the standards required by the method.

Method validation is performed once at the end of method development, whereas system suitability tests are performed on a given system periodically to determine its adequacy or effectiveness. System suitability requirements for chromatography systems have been well defined, though for UV-Visible spectroscopy, users have developed their own strategies for performing system suitability checks. For example:

A. Measure and calibrate using one standard with a concentration equal to 100 % of the expected component concentration. Then measure and quantify

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-----
Method Report                               Date 08/19/2013 Time 12:35:06
-----
*** Method report ***

Method File (modified)
Last Update      Date 8/19/2013 Time 12:34:26 PM
Operator         Juv-Visible Chemstation, Rev. B.04.03
Product          3
Number of Standards 3
Auxiliary Spectra 0
-----

Method Information:
Default Method

Method options:
Pre measure macro: none
Post measure macro: none
Store Spectra to File: none
Auto Analyze: off

Single Analysis

Sampling System:  sipper

Pump Direction:  Clockwise
Pump Time (s):   20
Wash Time (s):   0
Wait Time (s):   3
Sample Return (s): 0
Atr Segment (s): 0

Data Analysis:
Spectral Processing:
Absorbance
Use wavelength(s):
Single (nm):      430
Evaluation:       SCA
Calibrated at:    Date 8/19/2013 Time 10:22:56 AM
Operator:         J
Evaluation:       SCA
Calibrated at:    Date 8/19/2013 Time 10:22:56 AM
Operator:         J
Weighting Method: Least squares
Calibration Curve: C = k1 * A

Analyte Name      unit
-----
Yellow            mg/l

Method Report Parameter:
Information                on
Options/Info                on
Instruments/Acquisition    on
Data Analysis               on
Report                      off
Include Calibration Report  off

Calibration Report Parameter:
Standard Spectra            on
Processed Standard Spectra on
Path Length Table          on
Data Analysis Parameters   on
Coefficients                on
Used wavelength results    on
Calibration Table(s) of Analytes on
Curves                    on
Residual Spectra           on
Diagnostics                 on

Results Report Parameter:
Sample Information          off
Sample Spectra             off
Processed Sample Spectra   off
Used wavelength results    off
Evaluation Results         off
Statistical Information    off
Residual Spectra          off
Confirmation Results       off
Include Method Report      off
All Sample Spectra overlaid on
Summary                    on
Evaluation Result Statistics on
-----
*** End Method Report ***

```

Figure 5. Method documentation.

the standard and the standard diluted by a factor of two. The results of both samples should be within a specified percentage of the known concentration. Re-measuring the standard demonstrates the quality of the initial measurement. This can be implemented automatically in the Agilent Cary 8454 UV-Visible system by using the Automation function with a single standard and two control samples (standard and 50 % standard) and entering the acceptable error for the control samples. If a result for a control is outside the specified range it is automatically flagged in the results report.

B. Measure the standard and then a series of dilutions of the standard and calculate the extinction coefficient (absorbance/concentration) for each concentration. The values of the extinction coefficients should not vary by greater than a specified percentage. This can be implemented automatically in the Agilent Cary 8454 UV-Visible system by using the Automation function with up to three standards. On calibrating, the results of the calibration include the percent deviation of each standard from the calibration curve. This percentage error is identical to the deviation in the extinction coefficient of each standard.

Data quality

A properly validated system and analytical method does not guarantee that valid results will always be generated in routine use. To achieve valid results the prescribed procedures must be followed exactly by the operator. The Agilent Cary 8454 UV-Visible system provides features that help ensure this is done. ChemStation method files define the complete analysis as a global method. It is stored as a single file that, when loaded, sets all the parameters for data acquisition, data evaluation and calibration without operator interaction and thus eliminates the chance of error.

Global methods and Compliance

There are two different software packages available to meet workflow requirements for storing and protecting electronic records and application of electronic signature for compliance with Title 21 Code of Federal Regulations (CFR) Part 11.

Security Pack is a tool to help the user achieve 21 CFR Part 11 compliance and is available as an add-on to ChemStation UV-Vis. For security, two operation levels that are password protected are

included. At the manager level, global methods are developed, edited and stored. At the operator level, for routine testing, these global methods can be loaded and run. ChemStation is also able to use the features of OpenLAB ECM to help achieve 21 CFR Part 11 compliance. The Compliance Pack add-on targets a workflow that requires increased flexibility of access for different user groups to ChemStation method features and analysis parameters.

Standard operating procedures

For those procedures and actions that cannot be controlled by the system, standard operating procedures (SOPs) should be provided. SOPs are documented instructions that should be followed by the operator for a process to be considered valid. They can be divided into three types (Table 1):

- Maintenance of the Agilent Cary 8454 UV-Visible spectrophotometer
- Validation of the Agilent Cary 8454 UV-Visible system
- Correct operation

Table 1. Agilent Cary 8454 UV-Visible system SOP templates

Maintenance	Replacing the lamps
	Cleaning the source lens
Installation	Installing the Agilent Cary 8454 UV-Visible System
Validation	Validating the Agilent Cary 8454 Spectrophotometer
	Validating the General Purpose UV-Visible ChemStation Software
	Validating the Advanced ChemStation Software
Operation	Validating the Biochemical Analysis Software
	Measuring Sample Spectra Using Standard Cuvettes
	Measuring Sample Spectra Using the Sipper System

Error checks

Despite the above, errors can still occur. So wherever possible, it is advantageous to include built-in system checks on the quality of the results as they are being generated. Such tests can flag values that may be erroneous for any of the following reasons:

- The wrong sample may have been measured,
- The sample may have been contaminated,
- Measurements may have been made outside the linear range of the instrument.

An important part of the Agilent Cary 8454 UV-Visible system are those features that are inherent to the system, or that may be added to the method to ensure the quality of data obtained.

- **No measurement without blank:** The Agilent Cary 8454 UV-Visible spectrophotometer will not make a sample measurement if a blank measurement has not been made previously.
- **Voiding of erroneous data:** The Agilent Cary 8454 UV-Visible spectrophotometer can measure a spectrum in 0.1 second, but typically longer integration times of 0.5 or 1 second are used. This period is used to average several spectral measurements to improve the signal-to-noise ratio. In addition the standard deviations of the measurements are calculated. If there has been a problem which caused the measurement to vary during the integration time, such as a bubble, the standard deviation will be high. Valid samples should have no significant variation of absorbance during the integration time. The Agilent Cary 8454 UV-Visible spectrophotometer records as void any measurement that shows a high standard deviation, automatically eliminating erroneous measurements.

- **Statistics:** The Agilent Cary 8454 UV-Visible system has extensive statistical evaluation tools built-in to all stages of data processing from acquisition, through calibration to evaluation. These statistical values are important quality tools, indicating the precision of the measurements being made and are shown in Table 2.

Table 2. Quality statistics featured in Cary 8454 systems

General Purpose UV-Visible ChemStation
<ul style="list-style-type: none"> • Single component calibration: standard deviation of calibration, correlation coefficient, percent error of each standard from calibration curve.
Advanced UV-Visible ChemStation
<ul style="list-style-type: none"> • Standard deviation of each measurement. Specification of the standard deviation of calibration standards, for statistical evaluation of the calibration. • Single component calibration: standard deviation of calibration, correlation coefficient, uncertainty, percent error of each standard from calibration curve, leverage, Cook's Distance. • Single component quantification results, with 95% prediction interval. • Multicomponent analysis results, with 95% prediction interval relative fit error (describes the quality of the fit of the standards to the measurement spectrum, and indicates the incorrect sample identity or sample contamination).

- **Confirmation analysis:** The Agilent Cary 8454 UV-Visible system can evaluate a sample with up to four independent data analysis processes simultaneously. An important application of this capability is confirmation analysis. This technique uses wavelength to cross check the result at the analytical wavelength.

Confirmation analysis can detect for correct identity of the sample, contamination of the sample, decomposition of the sample, and measurement outside the linear dynamic range of the spectrophotometer. Samples not satisfying the requirements of the confirmation analysis are automatically flagged (Figure 5).

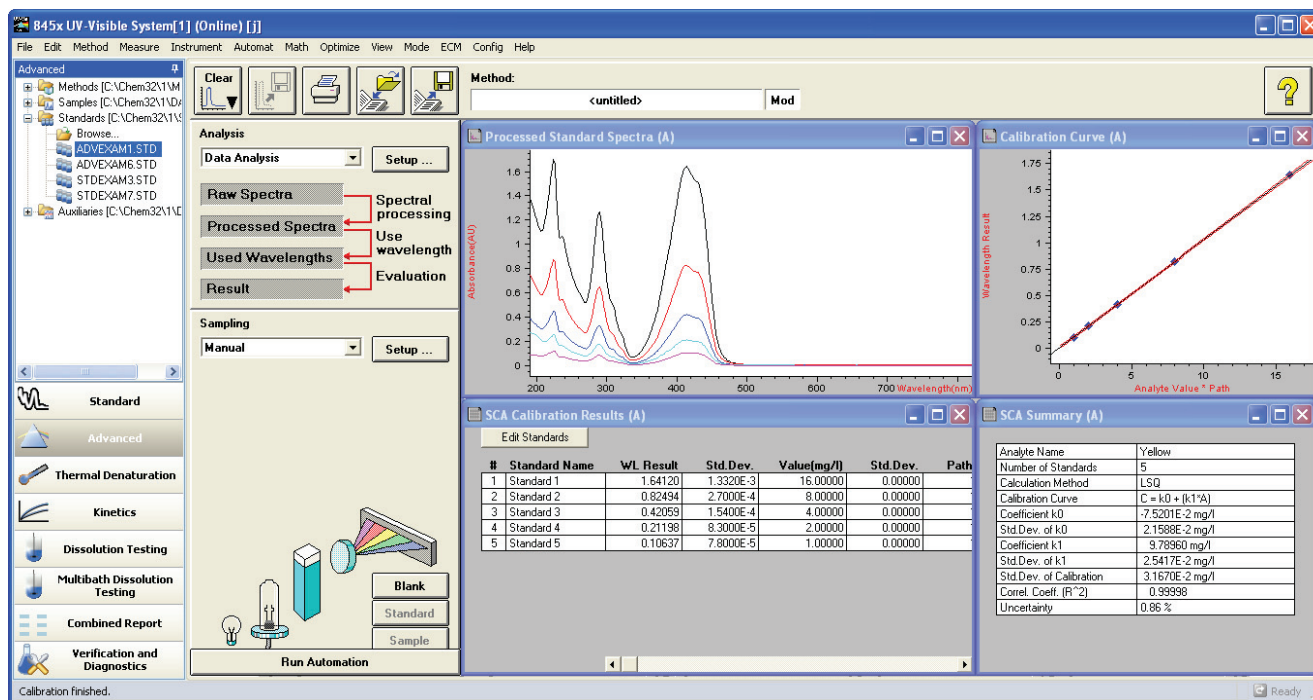


Figure 5. Confirmation analysis results (with a limit of 1 %)

Instrument Logbook

The Agilent Cary 8454 UV-Visible spectrophotometer has an internal logbook. This contains the results of all self-tests and any errors reported by the instrument. There is also access by the user to the logbook for noting instrument verification and any repairs performed on the instrument.

The process of generating raw data is fully documented electronically on disk. Each Agilent Cary 8454 UV-Visible spectrophotometer has a serial number and firmware revision number in firmware. This data is automatically read by the software each time the system is started and positively identifies the instrument with which measurements were made. Key information such as operator name, time, date, spectrophotometer serial number, and any other available information (for example, temperature if a Peltier temperature controller

is connected) is automatically appended to each measured spectrum and to result files. This information can be displayed on the screen but cannot be edited.

As part of the method, you can specify that all data should be automatically stored as it is acquired to ensure traceability of the results. Raw data is stored with all the annotation information described above. In addition, even if only one wavelength is used for the analysis, full spectral acquisition and storage can be specified so that all sample information is available for review.

Data storage

With the correct encoding and archiving practices, measurement data can be stored safely and for long periods of time. All of the Agilent Cary 8454 UV-Visible system data and method files are stored in binary format and these files cannot be edited.

References

1. Travis et al, Intrinsic Wavelength Standard Absorption Bands in Holmium Oxide Solution for UV-Visible Molecular Absorption Spectrophotometry, *J. Phys Chem. Ref Data* 34, 12 (2005)
2. Wavelength accuracy and reproducibility of the Agilent UV-Vis diode array using holmium oxide, *Agilent Application Note* 5991-3446EN (2013)

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