

Agilent ChemStation for UV-visible Spectroscopy

Understanding Your UV-visible Spectroscopy System



Agilent Technologies

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This handbook is for
A.09.xx revisions of the
Agilent ChemStation
software, where xx is a
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Understanding Your UV-visible Spectroscopy System

In This Book

This manual describes the data processing operations of the Agilent 8453 spectrophotometer and the Agilent ChemStation for UV-visible spectroscopy. It describes the processes and transformations that the spectrophotometric data undergo between the time that they are acquired in the spectrophotometer and their output on the Agilent ChemStation display or printer. The manual is designed to enable you to follow good laboratory practice (GLP) guidelines; using the information in the manual, you will be able to understand the data processing calculations from beginning to end and perform the data evaluation manually.

Chapter 1 “Overview of Data Processing” gives an overall summary of how data are acquired and processed.

Chapter 2 “Agilent ChemStation Registers and Views” explains the concepts of registers and views.

Chapter 3 “Tasks” describes the operation of the four Agilent ChemStation tasks.

Chapter 4 “Spectral Processing and Data Transformation” contains details of the operation of the mathematical functions, and describes how the wavelength data are extracted.

Chapter 5 “Evaluation” explains the Agilent ChemStation evaluation procedures.

Chapter 6 “Reports” contains descriptions of the Agilent ChemStation reports.

Chapter 7 “Interactive Math Functions” describes the mathematical functions that can be used interactively.

Chapter 8 “Configuration and Methods” explains configuration and describes the structure of the Agilent ChemStation software.

Chapter 9 “Verification” describes the process of system verification and describes each of the verification tests in detail.

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Overview of Data Processing

How data are acquired and processed in the spectrophotometer, transferred to, and then processed and reported by the Agilent ChemStation

Data Processing in the HP 8452 and Agilent 8453 Spectrophotometers

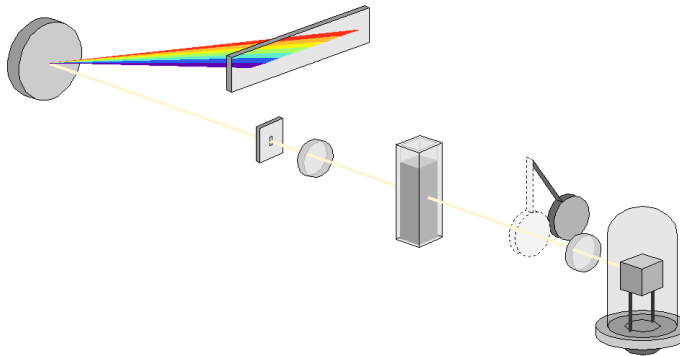
The following sections give an outline of how the HP 8452 and Agilent 8453 spectrophotometers evaluate and process spectra. All of these processes are implemented in firmware within the instrument. Since *raw* data is generally considered to be the actual values delivered by the measurement device to the controller (that is, the absorbance values), only an overview of the internal data processing is given here. Details of the algorithms are not provided.

HP 8452

The optical system of the HP 8452 is shown in Figure 1. The light source for the UV-visible and UV versions is a deuterium lamp. The light source for the Vis-SWNIR version is a tungsten lamp. The shutter serves simply as an optical on/off switch. The 328-element diode-array is a series of silicon photodiodes which are sensitive to light. Each photodiode is connected to a capacitor which is recharged at 100 msec intervals by the control electronics. When light falls on a photodiode its resistance changes proportional to the incident light and the capacitor is discharged. The amount of electricity required to recharge the capacitor at each recharge cycle is a measure of the light intensity.

Figure 1

Optical Path of the HP 8452 Spectrophotometer



The readout circuitry of the diode-array produces an analog signal which is fed to an analog-to-digital converter for conversion into the digital domain. The output from the A/D converter is a digital intensity spectrum expressed in counts.

Wavelength Calibration

Each photodiode is assigned to a particular wavelength (at 2 nm intervals with the UV-visible and Visible-SWNIR versions and 1 nm intervals with the UV version).

Dark current correction

When the shutter is closed and no light falls on the array there is still a low-level signal due to leakage from the capacitor. This is called the “dark current”. The dark current signal is continually measured and stored within the electronics. It is automatically subtracted from the signal obtained when the shutter is opened.

Variance

If an integration time of more than 100 msec is used, multiple spectra, dependent upon the selected integration time, are measured. The average of the spectra is calculated and, if requested, the variance of each data point is also calculated.

Reference Spectrum

The HP 8452 is a single beam spectrophotometer. To generate a sample spectrum two steps are required. First a *reference* intensity spectrum, I_0 , is measured. This is usually measured on the cuvette with solvent. This reference intensity spectrum is stored within the HP 8452.

Absorbance Spectra

For a sample, the intensity spectrum is measured and the absorbance spectrum is calculated using:

$$A = -\log((I_s - I_{sd}) / (I_0 - I_{0d}))$$

where

I_0 is the reference intensity measured with the blank

I_{0d} is the dark current measured immediately before the reference

I_s is the intensity measured with the sample

I_{sd} is the dark current measured immediately before the sample.

This absorbance spectrum together with the variance of each data point (if requested) is then transmitted to the controller.

Invalid data

The HP 8452 will *invalidate* measured values under certain circumstances. This means that the measured value is considered to be so unreliable that it is not usable. In the spectrum sent to the controller, the absorbance values at wavelengths which are invalidated are set to a special value which is recognized by the controller software and these values are not displayed. Reasons for invalid data are:

Negative Absorbances

Negative absorbance values are usually obtained because of an inappropriate blank. When negative absorbances are observed the intensity values measured are higher than expected and there is a danger that the linear range of the A/D converter will be exceeded, leading to conversion errors. The HP 8452 invalidates values lower than -0.05 to -0.1 A (the actual limit depends on the absolute intensity at the individual diodes).

Sample-related Dynamics

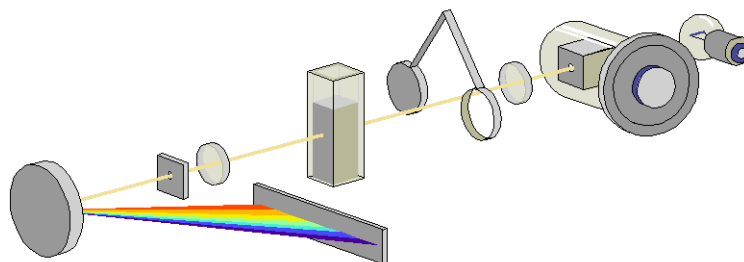
In some cases, where the sample is undergoing rapid absorbance changes with time, the difference between the signals obtained at the start and end of the integration time can be significant. If the sample points do not meet certain statistical criteria, the values are invalidated. The simplest statistical check is to invalidate the data if the measured intensity changes by more than 255 counts during the integration time.

Agilent 8453

The optical system of the Agilent 8453 is shown in Figure 2. It uses two light sources, a deuterium and a tungsten lamp. The shutter consists of two parts; one serves simply as an optical on/off switch, the second is a filter used to reduce stray light in the UV range. The 1024-element diode-array is a series of silicon photodiodes which are sensitive to light. Each photodiode is connected to a capacitor which is recharged at a constant interval by the control electronics. When light falls on a photodiode its resistance changes proportional to the incident light and the capacitor is discharged. The amount of electricity required to recharge the capacitor at each recharge cycle is a measure of the light intensity.

Figure 2

Optical Path of the Agilent 8453 Spectrophotometer



The readout circuitry of the diode-array produces an analog signal which is fed to an analog-to-digital converter for conversion into the digital domain. The output from the A/D converter is a digital intensity spectrum expressed in counts.

Dark Current Correction

When the shutter is closed and no light falls on the array there is still a low-level signal due to leakage from the capacitor. This is called the “dark current”. The dark current signal is continually measured and stored within the electronics. It is automatically subtracted from the signal obtained when the shutter is opened.

Stray Light Correction

In a standard measurement sequence, reference or sample intensity spectra are measured without and then with the stray light filter in the light beam. Without the filter the intensity spectrum over the whole wavelength range from 190-1100 nm is measured. The stray light filter is a blocking filter with 50% blocking at 420 nm. With this filter in place any light measured below 400 nm is stray light. This stray light intensity is then subtracted from the first spectrum to give a stray light corrected spectrum.

Variance

If an integration time of more than 100 msec is used, multiple spectra, dependent upon the selected integration time, are measured. The average of the spectra is calculated and, if requested, the variance of each data point is also calculated.

Reference Spectrum

The Agilent 8453 is a single beam spectrophotometer. To generate a sample spectrum two steps are required. First a “reference” intensity spectrum, I_0 , is measured. This is usually measured on the cuvette with solvent. This reference intensity spectrum is stored within the Agilent 8453.

Absorbance Spectra

For a sample, the intensity spectrum is measured and the absorbance spectrum is calculated (no stray light correction) using:

$$A = -\log((I_s - I_{sd}) / (I_0 - I_{0d}))$$

where

I_0 is the reference intensity measured with the blank

I_{0d} is the dark current measured immediately before the reference

I_s is the intensity measured with the sample

I_{sd} is the dark current measured immediately before the sample.

This absorbance spectrum together with the variance of each data point (if requested) is then transmitted to the controller.

When stray light correction is activated, I_s and I_0 are also corrected in the range 190 – 400 nm by subtracting the measured stray light:

$$A = -\log((I_s - I_{sd} - I_{s(\text{stray})}) / (I_0 - I_{0d} - I_{0(\text{stray})}))$$

where

$I_{0(\text{stray})}$ is the stray light intensity measured with the blank

$I_{s(\text{stray})}$ is the stray light intensity measured with the sample

Wavelength Calibration

The Agilent 8453 has a nominal sampling interval of 1 diode every 0.9 nm. Each spectrograph is calibrated in the factory using a series of emission lines from deuterium, zinc, argon and mercury lamps to obtain a calibration curve that relates each diode to a particular wavelength. The calibration parameters of this curve are stored within the ROM memory of each spectrograph. On site, this calibration can be checked by using the two emission lines from the deuterium lamp. With the aid of the calibration parameters, the firmware of the spectrophotometer determines absorbance values at 1 nm intervals and these values are then sent to the controller.

Invalid Data

The Agilent 8453 will *invalidate* a measured spectrum under certain circumstances. This means that the measured spectrum is considered to be so unreliable that it is not usable. In the spectrum sent to the controller the absorbance values at wavelength which are invalidated are set to a special value which is recognized by the controller software and these values are not displayed. Invalid data are produced when the sample is much more transparent than the blank.

Data Flow in the Agilent ChemStation

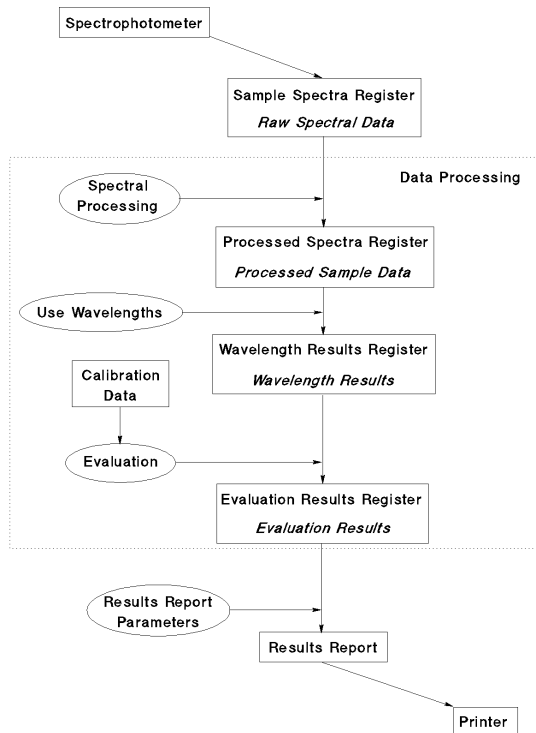
The data flow in the Agilent ChemStation software can be considered as two separate processes for samples and standards. The data flows are linked by the calibration data that is produced during the processing of standards and used in the evaluation of samples.

Data Flow for Samples

Figure 3 shows the flow of sample data through the Agilent ChemStation registers from the transfer of the sample absorbance values and statistics from the spectrophotometer to the Agilent ChemStation to the production of a printed report. For qualitative evaluations with no quantification, the calibration data is not included in the evaluation.

Figure 3

Data Flow for Samples

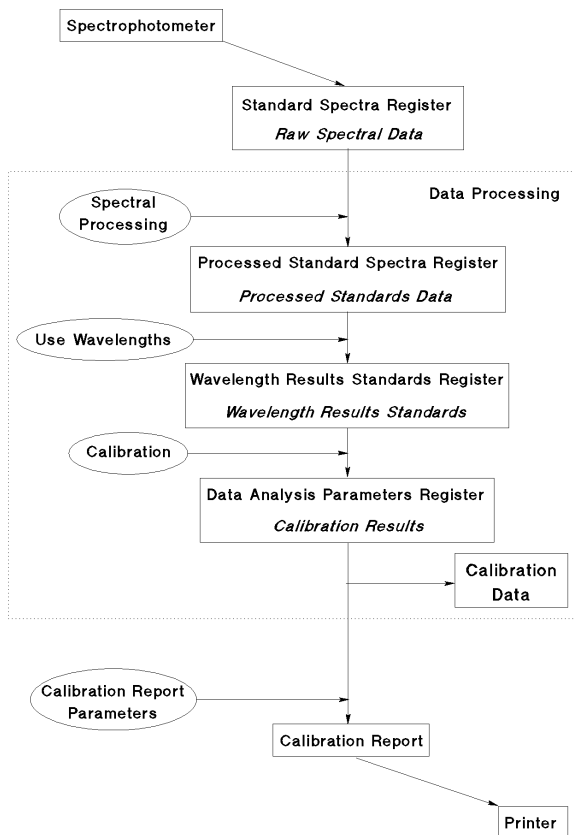


Data Flow for Standards

Data flow during the calibration process, which is concerned only with standard samples, is shown in Figure 4.

Figure 4

Data Flow for Standards



The calibration coefficients that are calculated during the calibration process are stored with the data analysis parameters in the Data Analysis Parameters register as part of the current method.

Once a calibration has been performed, and the calibration coefficients have been added to the Data Analysis Parameters register, the quantitative evaluation procedure uses the stored calibration data to perform a quantitative analysis on all subsequent samples that are measured using the current method. It is important to note that no data are destroyed during any of the data processing operations.

Agilent ChemStation Registers and Views

Concepts of registers and views, which are the
bases of data storage and display in the
Agilent ChemStation

Registers

A register is an area of computer memory where data are stored. The General Scanning software sets up several registers for different types of data; the most important registers are shown in Table 1.

Table 1

Agilent ChemStation Registers and Their Contents

Register	Contents
Configuration	System configuration data (see Chapter 8 "Configuration and Methods")
Sample System Parameters	Sampling system parameters to be used for measurements
Spectrophotometer Parameters	Spectrophotometer measurement parameter
Blank Spectrum	Last acquired blank spectrum
Raw Data (Sample Spectra)	Unprocessed data from sample measurements (usually absorbance data)
Raw Data (Standard Spectra)	Unprocessed data from standard sample measurements (usually absorbance data)
Math Result	Results of interactive math processing
Method Description	Definition of the method (see Chapter 8 "Configuration and Methods")
Data Analysis Parameters	Definitions and parameters of all data processing steps
Processed Sample Spectra	Spectral data from samples after spectral processing

Table 1

Agilent ChemStation Registers and Their Contents, continued

Processed Standard Spectra	Spectral data from standards after spectral processing
Wavelength Results	Function results from the specified wavelengths of the sample spectra
Wavelength Results Standards	Function results from the specified wavelengths of the standard spectra
Evaluation Results	Evaluation or analysis results
Evaluation Results Standards	Evaluation results that are used for calibration diagnostics

The registers which are specific to the standard samples and calibration data are used in the Quantification task only. During Quantification, the Data Analysis Parameters register includes the calibration coefficients generated during calibration process.

In addition, registers are set up for instrument parameters, method and automation parameters and analysis results. Temporary registers are also set up during data processing and during the execution of tasks.

Structure and Contents of Registers

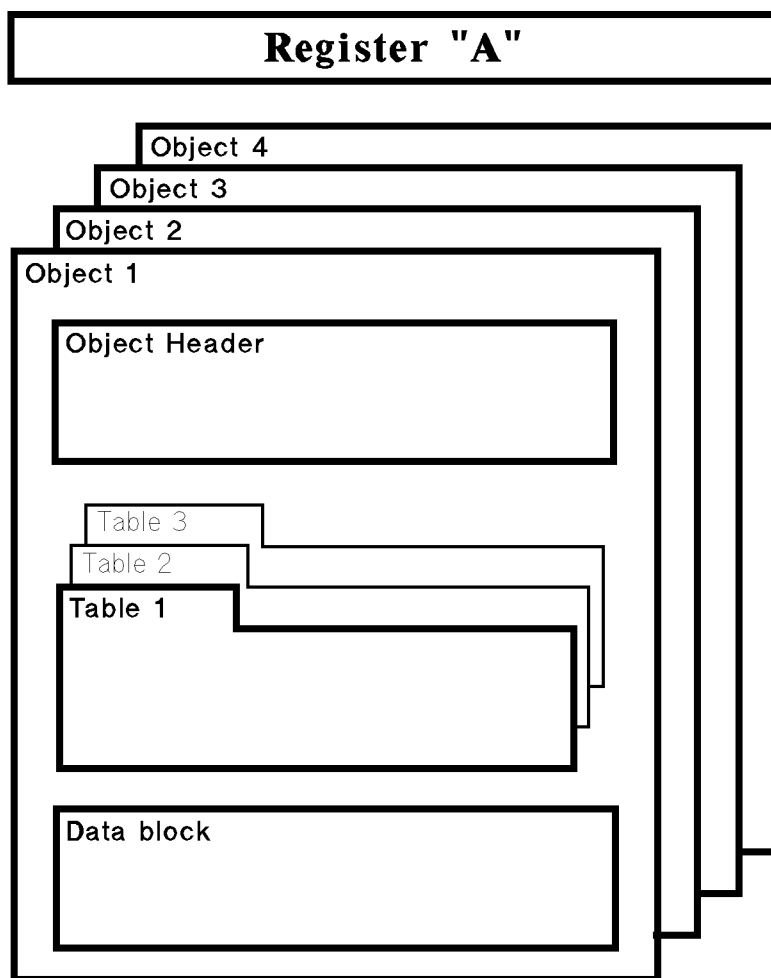
All registers have the same basic structure, which is similar to a database or card-index system. The fundamental unit of the register is the **object**, which is equivalent to a record in a database or a single card in a card index system. For example, a measured spectrum is an object in the raw data register. Each object has three parts:

- the **object header**, which contains static information about the object,
- a group of **tables**, which contain dynamic information about the object,
- the **data block**, which contains the object's data.

A graphical representation of the structure of a register is shown in Figure 5.

Figure 5

Register Structure



Data Structure of Spectra

The data structure of spectra is the same in the Samples register and the Math Result register:

- Header block, containing some or all of the following information:

Information about the sample:

the sample name,

Registers

solvent information (if available),
any available comment,
the operator name,
the data type (absorbance).

Information about the instrument on which the sample was run:

the identification of the type and firmware revision number,
the instrument identification number (HP 8452) or instrument serial number (Agilent 8453).

Information about the acquisition:

the date and time of acquisition,
the integration time,
the resolution,
cell path length and units,
cell temperature (if available).

- The Analytes Table, containing the information about the analytes. If no analyte information is available, this table is empty.
- The Data Block, containing the spectral information in a matrix of three rows:
 - the wavelength in ascending order,
 - the absorbance at that wavelength,
 - the standard deviation of the measurement, calculated from the variance data transferred from the spectrophotometer.

Views

Views are perspectives on the contents of registers. Each view displays the contents of the relevant registers in one or more windows; each window of the view displays a portion of the registers' contents.

Relationship Between Views and Registers

The Samples view and Math view display the contents of the Processed Samples register and Math Result register respectively:

- The Sample Spectra window and Math Result window are graphical representations of the Data Blocks from the respective registers.
- The Sample/Result table of the Samples view contains selected information from the Header block, together with the evaluation results. The Samples Table, accessible from the Sample/Result table, contains additional information from the Header block.

The Standards view displays the contents of the Processed Standard Spectra register.

Tasks

Parameters and operation of the four tasks:
Fixed Wavelengths, Spectrum/Peaks,
Ratio/Equation, and Quantification

Fixed Wavelength Task

For details of the calculations used in this task, see Chapter 5 “Evaluation”.

The Fixed Wavelength task allows the calculation of results from up to six wavelengths with or without background correction. The Fixed Wavelength parameters are shown in Table 2:

Table 2

Parameters for the Fixed Wavelength Task		
Parameter	Data Flow	Range of Values
Use Wavelength(s)	Sets the Used Wavelengths (see “Use Wavelengths” on page 37) to the selected values.	Up to six single wavelengths
Background Correction	Sets the Background Correction (see “Background Correction” on page 37) to the selected type.	none single reference wavelength subtract average over a range three-point drop line
Data Type	Sets the Spectral Processing (see “Spectral Processing” on page 36) to the selected type.	Transmittance Absorbance Derivative 1 Derivative 2 Derivative 3 Derivative 4
Display Spectrum	Sets the upper and lower wavelength limits for spectral display.	Instrument Settings

Spectrum/Peak

For details of the calculations used in this task, see Chapter 5 “Evaluation”.

The Spectrum/Peaks task determines the maxima and minima of the data points (y-values) in the defined wavelength range of the spectrum The last acquired spectrum is displayed annotated with the specified number of peaks and valleys, and the Sample Result table shows the wavelengths and absorbance values of the peaks and valleys. The All Peaks/Valleys button displays a list if the wavelengths and absorbances of all peaks and valleys.

The original spectrum is first derivatized (see “Derivative” on page 61) using a filter length of 5 and a polynomial degree of 3. The derivatized y-values are examined for transitions (change of sign); each transitional y-value is compared with its neighboring values, and if the neighboring values are more extreme, the transitional y-value is compared with the previous transitional y-value. If the difference of both values is greater than or equal to the sensitivity threshold, then the previous transitional point is stored as an extremum; if the difference is less than the sensitivity threshold, then neither point is stored.

The Spectrum/Peaks parameters are shown in Table 3:

Table 3

Parameters for the Peak / Valley Find Task		
Parameter	Data Flow	Range of Values
Data Type	Sets the Spectral Processing (see “Spectral Processing” on page 36) to the selected type.	Transmittance Absorbance Derivative 1 Derivative 2 Derivative 3 Derivative 4
Display Spectrum	Sets the upper and lower wavelength limits for spectral display	Instrument Settings
Number of Peaks	Sets the number of peaks reported.	0 to 19
Number of Valleys	Sets the number of valleys reported.	0 to 19

Ratio/Equation

For details of the calculations used in this task, see Chapter 5 “Evaluation”.

The Ratio/Equation task calculates a result based upon a specified equation. The equation can contain absorbances from up to six wavelengths plus sample weight and volume information. The following mathematical operators are available for use in an equation:

+	adds
-	subtracts
/	divides
*	multiplies
ABS(exp)	returns the absolute value of the expression
Ceil(exp)	rounds the expression up to the nearest integer
EXP(exp)	returns $e^{(\text{exp})}$
FLOOR(exp)	rounds the expression down to the nearest integer
LN(exp)	returns the natural logarithm (base e) of the expression
LOG(exp)	returns the logarithm (base 10) of the expression
SQR(exp)	returns the square of the expression
SQRT(exp)	returns the square root of the expression

The results of the equation are corrected for path length and dilution factor automatically.

The parameters for the Ratio/Equation task are shown in Table 4:

Table 4

Parameters for the Ratio/Equation Task

Parameter	Data Flow	Range of Values
Use Wavelength(s)	Sets the Used Wavelengths (see “Use Wavelengths” on page 37) to the selected values.	Up to six single wavelengths

Table 4

Parameters for the Ratio/Equation Task, continued

Parameter	Data Flow	Range of Values
Data Type	Sets the Spectral Processing (see "Spectral Processing" on page 36) to the selected type.	Transmittance Absorbance Derivative 1 Derivative 2 Derivative 3 Derivative 4
Equation Name	Sets the equation name.	Alphanumeric text
Equation	Sets the equation parameters.	Alphanumeric text
Unit	Sets the units for the result.	Alphanumeric text
Use sample weight and volume	Switches on the Weight and Volume entry fields in the Sample Information dialog box.	Checked (yes) or unchecked (no)
Prompt for sample information	Switches on the display of the Sample Information dialog box after sample measurement.	Checked (yes) or unchecked (no)
Display Spectrum	Sets the upper and lower wavelength limits for spectral display	Instrument Settings

Quantification

For details of the calculations used in this task, see Chapter 5 “Evaluation”.

The Quantification task calculates calibration coefficients from the measured data of a set of standards, then uses the calibration coefficients to determine the concentration of unknown samples. The equations used in the calculation of the calibration coefficients and the concentrations of unknown samples are given in Chapter 5 “Evaluation”. The parameters for the Quantification task are shown in Table 5:

Table 5

Parameters for the Quantification Task		
Parameter	Data Flow	Range of Values
Use Wavelength	Sets the Used Wavelengths (see “Data Transformation” on page 37) to the selected values.	Single wavelength value
Background Correction	Sets the Background Correction (see “Background Correction” on page 37) to the selected type.	none single reference wavelength subtract average over a range three-point drop line
Analyte name	Sets the analyte name.	Alphanumeric text
Calibration Curve Type	Sets the calibration curve type (see “Calibration Curve Types” on page 45.)	linear (Beer’s law) linear with offset quadratic quadratic with offset
Concentration unit	Sets the units of concentration; when the Concentration option is chosen, switches on the Concentration entry field in the Sample Information dialog box.	alphanumeric text

Table 5

Parameters for the Quantification Task, continued

Parameter	Data Flow	Range of Values
Weight & Volume unit	Sets the units of weight and volume; when the Weight & Volume option is chosen, switches on the Weight and Volume entry fields in the Sample Information dialog box.	alphanumeric text
Prompt for standard information	Switches on the display of the Standard Information dialog box after sample measurement.	Checked (yes) or unchecked (no)
Prompt for sample information	Switches on the display of the Sample Information dialog box after sample measurement.	Checked (yes) or unchecked (no)
Data Type	Sets the Spectral Processing (see "Spectral Processing" on page 36) to the selected type.	Transmittance Absorbance Derivative 1 Derivative 2 Derivative 3 Derivative 4
Display Spectrum	Sets the upper and lower wavelength limits for spectral display	Instrument Settings

Tasks

Quantification

Spectral Processing and Data Transformation

How math functions are used to process raw spectral data, and how wavelength data are extracted from processed data for evaluation

Spectral Processing

Absorbance

Absorbance is the default method of spectral storage in the UV-visible ChemStation. Absorbance spectra are calculated using the Absorbance function; for a complete description of the mathematical processes involved in calculating absorbance spectra, see “Absorbance” on page 60.

Transmittance

Transmittance spectra are calculated using the Transmittance function; for a complete description of the mathematical processes involved in calculating transmittance spectra, see “Transmittance” on page 61.

Derivative

First, second, third and fourth order derivative spectra can be calculated. The Derivative functions calculate the derivative of the data points (y-values) in the spectrum to the specified derivative order using a Savitsky-Golay algorithm with a filter length of 5 and a polynomial degree of 3. For a complete description of the Derivative function, see “Derivative” on page 61.

Data Transformation

Use Wavelengths

The Use Wavelengths function selects a sub-set of data from the full spectrum. The sub-set may be the processed spectral intensity value at a single wavelength, as in the Quantification task, or the processed spectral intensity values at up to six wavelengths as in the Fixed Wavelengths and Ratio/Equation tasks. The extracted value(s) may then be subjected to further modification by background correction to produce the final result.

Background Correction

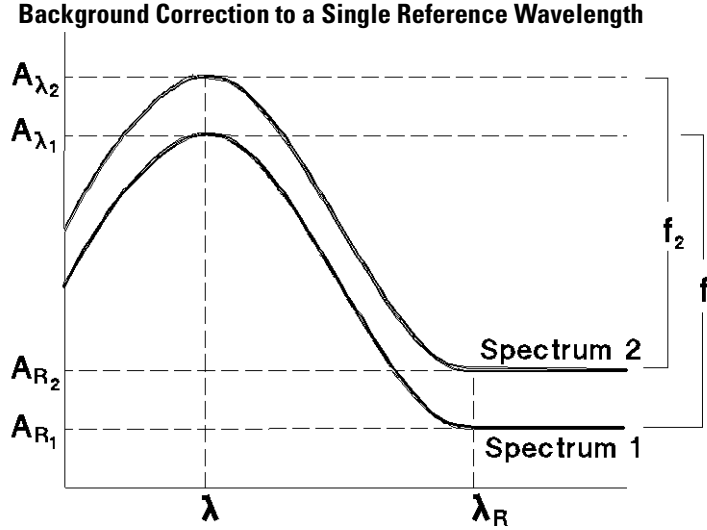
Background correction subtracts a calculated “background” value from the extracted spectral intensity value(s). There are three methods of calculating the background values:

- single reference wavelength
- subtract average over a range
- three-point drop line, used when two wavelengths are set

Single Reference Wavelength

The single reference wavelength method subtracts the spectral intensity value at a specified wavelength, as in Figure 6. The reference wavelength is usually selected at a point on the baseline beyond the sample absorbance.

Figure 6



Subtraction is carried out using equation (4.1):

$$f_{\lambda} = A_{\lambda} - A_{R_{\lambda}} \quad (4.1)$$

where

f_{λ} is the function result at wavelength λ

A_{λ} is the absorbance at wavelength λ

$A_{R_{\lambda}}$ is the absorbance at reference wavelength λ

The variances (if available) are treated according to equation (4.2):

$$\text{var}(f_{\lambda}) = \text{var}(A_{\lambda}) + \text{var}(A_{R_{\lambda}}) \quad (4.2)$$

where

$\text{var}(f_{\lambda})$ is the variance of the function result at wavelength λ

$\text{var}(A_{\lambda})$ is the variance of the absorbance at wavelength λ

$\text{var}(A_{R_{\lambda}})$ is the variance of the absorbance at reference wavelength λ

Subtract Average Over Range

The subtract average over range method uses the same calculation as for a single reference wavelength (see “Single Reference Wavelength” on page 37), but replaces the spectral intensity value at the single wavelength with the average intensity value over a specified wavelength range.

$$f_{\lambda} = A_{\lambda} - \left(\frac{(A_{R_1} + A_{R_2} + A_{R_3} + \dots + A_{R_n})}{n} \right)$$

where the terms are the same as for a single reference wavelength (see “Background Correction” on page 37).

Three-Point Drop Line

For a three-point drop-line background correction, the spectral intensity and variance values from two reference wavelengths are taken, giving A_{R_1} , A_{R_2} , $\text{var}(A_{R_1})$ and $\text{var}(A_{R_2})$. In this case, the reference wavelengths define a straight line (as in Figure 7) which is used to calculate $A_{R_{\lambda}}$ and $\text{var}(A_{R_{\lambda}})$ using equation (4.3) and equation (4.4).

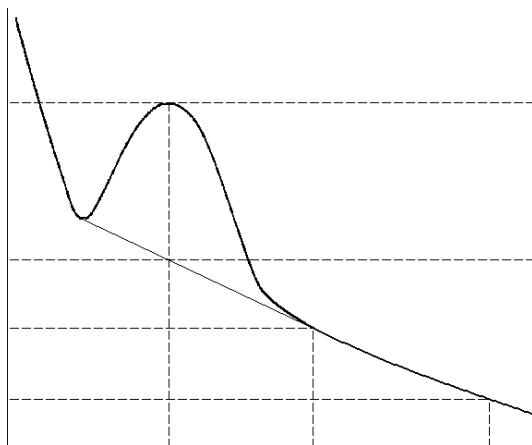
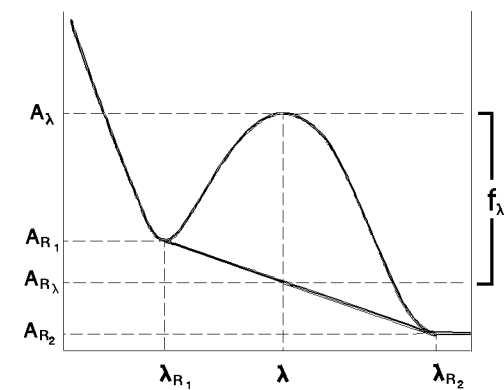
$$A_{R_{\lambda}} = \frac{1}{\lambda_{R_2} - \lambda_{R_1}} \{ (\lambda_{R_2} - \lambda) A_{R_1} + (\lambda - \lambda_{R_1}) A_{R_2} \} \quad (4.3)$$

$$\text{var}(A_{R_{\lambda}}) = \frac{1}{(\lambda_{R_2} - \lambda_{R_1})^2} \left\{ (\lambda_{R_2} - \lambda)^2 \text{var}(A_{R_1}) + (\lambda - \lambda_{R_1})^2 \text{var}(A_{R_2}) \right\} \quad (4.4)$$

where the terms are the same as for a single reference wavelength (see “Background Correction” on page 37). In Figure 7, the upper trace shows the spectrum before background correction, with the three-point drop line superimposed. The lower trace shows the spectrum after background correction.

Figure 7

Three-point Drop Line



Evaluation

The evaluation procedures available with the
Agilent ChemStation

Evaluation

This chapter describes the use of equations, and introduces quantification and explains the concepts that are used in the Quantification task of the software. It also describes the mathematical and statistical calculations that are used to produce a calibration curve for single component analysis and how the quantitative results are calculated using the calibration data.

Evaluation using an Equation

When an equation evaluation is specified, the analysis results are the results of the equation specified in the Equation Parameter dialog box. In the definition of the equation, you can use any result generated by the Use Wavelength(s) and stored in the Wavelength Results register and any of the other predefined variables: Dilution factor, Weight and Volume.

By default, the results are normalized to a 1 cm path length by dividing the function result by the value of the Path Length parameter in the Sample Spectra table:

$$f_{\text{corr}} = \frac{f}{l} \quad (5.1)$$

where

f_{corr} is the function result after path length correction

f is the uncorrected function result

l is the path length

Quantification

The Quantification task of the Agilent ChemStation software includes facilities for:

- measurement of calibration standards,
- calibration of a single analyte using the calibration standards,
- measurement of unknown samples, and
- quantification of the analyte in the unknown samples.

Beer-Lambert Law

The Beer-Lambert law states that the absorbance of a solute is directly proportional to its concentration:

$$A = Ecd \quad (5.2)$$

In equation (5.2):

A is absorbance

E is molar absorptivity or molar extinction coefficient ($\text{l.mol}^{-1}.\text{cm}^{-1}$)

c is analyte concentration (mol.l^{-1})

d is cell path length (cm)

To calculate the concentration of an unknown compound, the above equation can be solved for a given function result, f :

$$c = \frac{f}{Ed} \quad (5.3)$$

The reciprocal of the product of molar extinction coefficient and cell path length is often called the calibration coefficient, k .

$$c = kf \quad (5.4)$$

Calibration

Calibration is the correlation of the **function result** with known concentrations of sample. The basic assumption is that the variance in the

measured data is less than the variance in the standard concentrations. As a result of this correlation a **calibration curve** can be fitted to the data points and **calibration coefficients** can be calculated. The concentrations of unknown samples can then be calculated from the calibration coefficients and the function result of the samples.

Calibration Curve Types

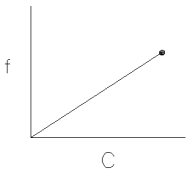
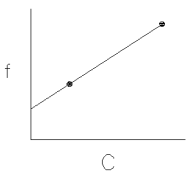
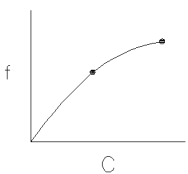
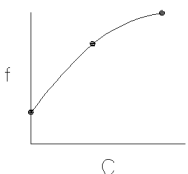
Real data may deviate slightly from the ideal linear relationship described in “Beer-Lambert Law” on page 44. The relationship between the function result and concentration may be described more accurately by adding an offset (a non-zero intercept) or a quadratic term (or both) to the equation.

The ChemStation provides four different calibration curve types, shown in Table 6. These calibration curve graphs are shown in the more traditional way with the concentration on the x-axes and the function results on the y-axes.

The Agilent ChemStation provides four different calibration curve types, shown in Table 6.

Table 6

Calibration Curve Types for Single Component Analysis

Curve Type	Curve	Equation
I Linear without zero offset		$c = k_1 f$
II Linear with zero offset		$c = k_0 + k_1 f$
III Quadratic without zero offset		$c = k_1 f + k_2 f^2$
IV Quadratic with zero offset		$c = k_0 + k_1 f + k_2 f^2$

c is the concentration

f is the function result

k_0, k_1, k_2 are calibration coefficients

Number of Standards

There is no fixed limit to the number of calibration standards that can be incorporated into a calibration; the more points that can be placed on the

curve, the more accurately the curve can be characterized. However, each of the calibration curve types requires a minimum number of standards of different concentrations (see Table 7).

Table 7

Minimum Number of Standards for Calibration Curves

Calibration Curve Type	Minimum Number of Standards
Linear, no zero offset (Beer's law)	1
Linear with zero offset	2
Quadratic, no zero offset	2
Quadratic with zero offset	3

Calibration Standards

If the absorbance of the analyte at the wavelength(s) you select strictly obeys Beer's Law, then one standard is sufficient to characterize the calibration curve (linear without zero offset). However, the use of two standards at different concentrations will confirm adherence to Beer's Law, or show up any irregularities. Two standards are necessary to characterize a linear calibration curve with zero offset. Addition of a third standard at a different concentration is sufficient to identify and characterize a non-linear calibration curve.

Calibration Curve Fits

The mathematical problem is to find the calibration coefficients in a given curve type which will allow the best determination of a future unknown sample.

The least squares method uses the analytical function data from Use Wavelengths of all standards to determine the calibration coefficients of the chosen calibration curve type by a least squares calculation.

If each i th data set (f_i, c_i) of the n standard data sets is expected to obey a function in the p coefficients, k_j , although the real (measured) values may cluster around the function because of statistical errors, ϵ_i , then the general equation is:

$$c_i = k_0 + k_1 f_i + k_2 f_i^2 + \epsilon_i \quad (5.5)$$

where

i is 1, 2, 3, ... n (the total number of standards)

The calibration coefficients, k_j , can be estimated using the least squares method, that is minimizing the sum of the squares of the errors, ε_i (the differences between the measured value and the calibration curve).

$$\sum_{i=1}^n (c_{\text{actual}_i} - c_{\text{calculated}_i})^2 = \sum_{i=1}^n \varepsilon_i^2 = \text{minimum} \quad (5.6)$$

In matrix notation:

$$C = Fk + \varepsilon \quad (5.7)$$

where

C is n -concentration column vector

F is $n \times p$ function result data matrix

k is p -calibration coefficient column vector

e is n -error column vector

The elements used in the calibration matrix F and the coefficient vector k are given in Table 8 with dimension p of the coefficient vector.

Table 8

Elements used in calibration matrix F

Curve Type	p	$F_{(i)\text{th row}}$			k		
I	1	f_i	—	—	k_1	—	—
II	2	1	f_i	—	k_0	k_1	—
III	2	f_i	f_i^2	—	k_1	k_2	—
IV	3	1	f_i	f_i^2	k_0	k_1	k_2

Example

Using curve type III, where $p = 2$, for 5 standards, $n = 5$:

$$c_1 = k_1 f_1 + k_2 f_1^2 + \varepsilon_1$$

through to

$$c_1 = k_1 f_5 + k_2 f_5^2 + \epsilon_5$$

That is:

$$\begin{bmatrix} c_1 \\ \dots \\ c_5 \end{bmatrix} = \begin{bmatrix} f_1 & f_1^2 \\ \dots & \dots \\ f_5 & f_5^2 \end{bmatrix} \begin{bmatrix} k_1 \\ k_2 \end{bmatrix} + \begin{bmatrix} \epsilon_1 \\ \dots \\ \epsilon_5 \end{bmatrix}$$

or, in matrix notation:

$$C = Fk + \epsilon$$

By defining F and k for each of the calibration curve types, the coefficients are given by the general equation:

$$k = (F^T F)^{-1} F^T C \quad (5.8)$$

where

F^T denotes the transpose of F

Example Continued:

$$\begin{bmatrix} k_1 \\ k_2 \end{bmatrix} = \left(\begin{bmatrix} f_1 & \dots & f_5 \\ f_1^2 & \dots & f_5^2 \end{bmatrix} \begin{bmatrix} f_1 & f_1^2 \\ \dots & \dots \\ f_5 & f_5^2 \end{bmatrix} \right)^{-1} \left(\begin{bmatrix} f_1 & \dots & f_5 \\ f_1^2 & \dots & f_5^2 \end{bmatrix} \begin{bmatrix} c_1 \\ \dots \\ c_5 \end{bmatrix} \right)$$

Calibration Results

This section contains the equations used in the calculations of the results given in the Calibration Table and the Calibration Results Summary.

Residual

$$e_i = C_{\text{actual}_i} - C_{\text{calculated}_i} \quad (5.9)$$

%Error

$$d_i = \frac{e_i}{C_{\text{calculated}_i}} \times 100 \quad (5.10)$$

where

e_i is the residual of the i^{th} standard (see “Residual” on page 49)

Std.Dev of Calibration

$$s = \sqrt{\frac{1}{(n-p)} \sum_{i=1}^n e_i^2} \quad (5.11)$$

where

e_i is the residual of the i^{th} standard

n is the number of standards

p is the number of coefficients in the equation of the calibration curve

Std.Dev of Coefficient k

$$s_{k_j} = s \sqrt{(F^T F)^{-1}_{jj}} \quad (5.12)$$

where

$(F^T F)^{-1}_{jj}$ is the j^{th} diagonal element of $(F^T F)^{-1}$

s is the standard deviation of calibration (see “Std.Dev of Calibration” on page 50)

Correl. Coeff (R^2)

$$R^2 = \frac{\sum_{i=1}^n (C_{\text{calculated}_i} - \bar{C})^2}{\sum_{i=1}^n (C_{\text{actual}_i} - \bar{C})^2} \quad (5.13)$$

In the case of curve types II and IV,

$$\bar{C} = \frac{1}{n} \sum_{i=1}^n C_i \quad (5.14)$$

In the case of curve types I and III, \bar{C} is set to zero.

Quantification of Unknown Samples

When the standards have been measured, and the calibration coefficients have been determined, the unknown concentration of a measured sample can be calculated simply by calculating C_{unk} in the equation of the calibration curve using the function result data, f_{unk} of the unknown sample:

$$C_{\text{unk}} = k_0 + k_1 f_{\text{unk}} + k_2 f_{\text{unk}}^2 \quad (5.16)$$

where k_0 , k_1 or k_2 may be zero depending on the calibration curve type (see Table 6 on page 46).

Reports

Format and contents of the reports that are available with the general purpose UV-visible software for the Agilent ChemStation

Reports

The general purpose UV-visible software for the Agilent ChemStation allows four reports to be generated.

- The Method Report is a report of the parameters of the current method.
- The Results Report is a report of the current evaluation results.
- The Automation Method Report is a report of the parameters of the current Automation Table.
- The Automation Results Report is a report of the current automation results.

The reports can be output directly to a printer or printed as a text file to a file with the extension .TXT. Reports that are printed to files are stored in the REPORTS subdirectory of the instrument's subdirectory.

In addition to the two formal reports, the UV-visible ChemStation software allows any selected window to be printed, and any view (all windows) to be printed. The windows and views are formatted as reports.

The appearance of the report is affected by two sets of parameters:

- The report configuration parameters control the margins, and indents for textual data. The annotation limit, which is the maximum number of spectra that are annotated, is also controlled by the report configuration parameters. If a spectrum plot contains less spectra than the annotation limit, all spectra are annotated. If the spectrum plot contains more spectra than the annotation limit, none of the spectra are annotated.
- The printer setup parameters control the target printer and the paper size and orientation.

The title of the report contains the task name together with the date and time of generation.

Method Report

The method report contains the information and parameters of the current method:

Method file	The method file name.
Information	The contents of the Method Information field of the Method Options and Information dialog box.
Operator	The Operator Name when the method was set up or modified.
Product	The Agilent ChemStation.

The Spectrophotometer parameters are extracted from the Instrument Parameters dialog box:

Instrument	The name of the instrument, from the configuration file.
Acquisition range	The instrument acquisition range from the Instrument Parameters dialog box.
Integration Time	The instrument integration time from the Instrument Parameters dialog box.
Std Deviation	Standard deviation is always Off.

The Data Analysis parameters are extracted from the parameters dialog box for the current task; some of the parameters reported depend on the task:

Data Type	(All Tasks) The data type from the parameters dialog box for the current task.
Display spectrum	(All Tasks) The limits of spectral display from the parameters dialog box for the current task.
Used Wavelength	(Fixed Wavelength and Quantification) The contents of the Used Wavelength(s) field(s) of the parameters dialog box for the current task.
Background Correction	(Fixed Wavelength and Quantification) The contents of the Background Correction field(s) of the parameters dialog box for the current task.
Find up to	(Spectrum/Peaks) Two lines showing the

	selected number of peaks and valleys taken from the Spectrum/Peaks Parameters dialog box.
Equation	(Ratio/Equation) The name and formula of the equation from the Equation Parameters dialog box.
Where	(Ratio/Equation) The explanation of the terms of the equation.

Results Report

The results report contains a header section with information about the method and data files, a graphical section and a results table. The graphical section and results table correspond with the Samples view for the selected task.

The header information consists of:

Method file	The method file name.
Information	The contents of the Method Information field of the Method Options and Information dialog box.
Data File	The data file name (including the full path name) and the date and time of creation.

In addition, the Results report for the Quantification task includes a summary of the calibration results:

Analyte name	The analyte name taken from the Quantification Parameters dialog box.
Calibration equation	The calibration equation calculated using the selected calibration curve type from the spectral data values extracted from the standard spectra.
Calibrated at	The date, time and operator name when the calibration was performed.

The last line of the report contains the current operator's name and a space for the operator's signature.

Automation Reports

The automation reports comprise an Automation Method report and an Automation Results Report.

Automation Method Report

The automation method report is a summary of the Automation Table. It contains the following information:

Operator	The name of the automation operator.
Method file	The automation method file name.
Measure Blank	The blank measurement sequence, taken from the Automation Setup dialog box.
Measure Standard	The standards and their concentrations, taken from the Automation Setup dialog box.
Measure Control	The control samples, their concentrations and the maximum permitted error, taken from the Automation Setup dialog box.
Measure Samples	The seed name of the samples, the number of samples and their dilution factor, taken from the Automation Setup dialog box.
Measurement sequence	The measurement sequence table, equivalent to the Automation Measurement Sequence dialog box.

Automation Results Report

The automation results report contains an appropriate Results Report (see “Results Report” on page 57) for each sample in the automated sequence. In addition, statistics of the sample results can be calculated:

- average value,
- minimum value,
- maximum value,
- standard deviation,
- % relative standard deviation.

Interactive Math Functions

Full explanations of the math functions,
including their operation at limiting conditions

Unitary Operations

The unitary mathematical functions operate on single spectra. If more than one spectrum is selected, the function operates on all selected spectra individually. If no spectra are selected, the operation is aborted and an error message is displayed.

Absorbance

Absorbance is the default method of spectral storage in the Agilent ChemStation. The Absorbance function uses logarithm and scalar multiply functions to transform transmittance spectra into absorbance spectra using equation (7.1). If the data are already absorbance, no processing is performed.

$$A = -\log_{10}\left(\frac{T}{100}\right) \quad (7.1)$$

where

A is absorbance

T is transmittance in percent

The variances of the spectrum (if available) are transformed by equation (7.2):

$$\text{var}(A) = \left(\frac{1}{T \cdot \ln 10}\right)^2 \text{var}(T) \quad (7.2)$$

Variance data are displayed as standard deviation data in the Tabular Data of Spectrum window. The availability of variance data depends on the integration time, set in the Spectrophotometer Parameter dialog box. Variance data are not available for integration times of less than 0.2 seconds (HP 8452A) or 0.1 seconds (Agilent 8453).

Transmittance

The Transmittance function uses exponential, reciprocal and scalar multiply functions to transform absorbance spectra into transmittance spectra using equation (7.3). The transmittance spectra are placed in the math result register.

$$T = 100 \cdot 10^{-A} \quad (7.3)$$

The variances, if available, are transformed by equation (7.4):

$$\text{var}(T) = (100 \cdot \ln 10 \cdot 10^{-A})^2 \cdot \text{var}(A) \quad (7.4)$$

All additional information, such as annotations, are preserved unchanged in the transmittance spectrum.

Derivative

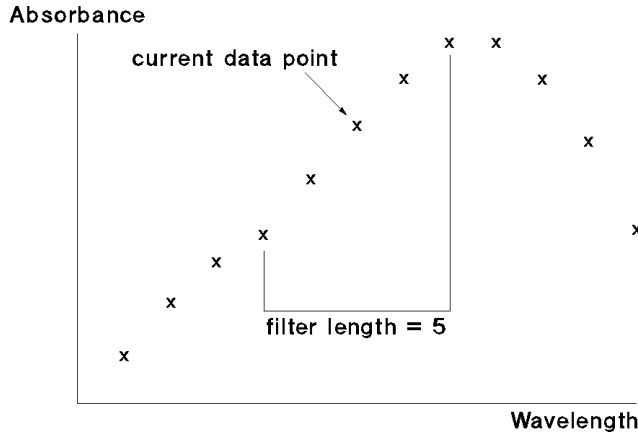
The derivative function calculates the derivative of the data points (y-values) in the spectrum using a Savitsky-Golay algorithm and places the resulting spectrum in the math result register.

Savitsky-Golay Algorithm

The Savitsky-Golay algorithm uses the Derivative Order, Filter Length and Polynomial Degree from the Derivative Parameter dialog box. For each data point in the spectrum, the calculation takes a set of data points equal to the filter length around the current data point, fits a curve of the specified polynomial degree, using a least squares fit. The fitted curve is then used to calculate the new value for the current data point, and the derivative of that point:

Figure 8

Filter length in the Savitsky-Golay algorithm



For each y-value, y_i ,

$$\text{deriv}(y_i) = \sum C_{kj} \cdot y_{i+j-1} \quad (7.5)$$

where

i is 1 ... data points-(filter length-1)

j is 1 ... filter length

k is 1 for smoothing, 2 for derivative

$\text{deriv}(y_1)$ corresponds to the $\left(\frac{(\text{filter length}-1)}{2} + 1\right)$ th value. For example,

if filter length is 5, then $\text{deriv}(y_1)$ becomes y_3 . For this reason, the length of

the spectrum is reduced by (filter length - 1) values;

$\left(\frac{(\text{filter length}-1)}{2}\right)$ values at the beginning of the spectrum and

$\left(\frac{(\text{filter length}-1)}{2}\right)$ values at the end of the spectrum are not processed. In

equation (7.5), C is the coefficient matrix:

$$C = N^{-1} \cdot F^T \quad (7.6)$$

where

Unitary Operations

N^{-1} is the inverse of N , the product of F^T and F

F^T is the transpose of F , the matrix of the powers of the polynomials

The matrix of the powers of the polynomials is generated using equation (7.7):

$$F_{ij} = k^{(j-1)} \quad (7.7)$$

where

i is 1 ... *filter length*

j is 1... *degree +1*

k is $i - \frac{\text{filter length} - 1}{2} - 1$

The y-values are multiplied by the reciprocal of the wavelength step at the end of the calculation:

$$y_i = \frac{y_i}{\text{step}} \quad (7.8)$$

where

step is the increment of the equidistant x-axis

For each variance value, $\text{var}(y_i)$,

$$\text{deriv}(\text{var}(y_i)) = \sum C_{kj}^2 \cdot \text{var}(y_{i+j-1}) \quad (7.9)$$

where

C is the coefficient matrix

i is 1 ... *data points - (filter length -1)*

j is 1 ... *filter length*

k is 1 for smoothing, 2 for derivative

The variance values are multiplied by the square of the reciprocal of the step at the end of the calculation:

$$\text{var}(y_i) = \frac{\text{var}(y_i)}{\text{step}^2} \quad (7.10)$$

If one of the values needed for the calculation is invalid, the resulting y-value or variance value is invalid.

All additional information, such as annotations, are preserved unchanged in the derivative spectrum.

Scalar Add

The scalar add function adds a constant value to each of the data points (y-values) in the spectrum and places the resulting spectrum in the math result register.

$$y_{\text{new}} = y + C \quad (7.11)$$

The scalar add function can be used to subtract a constant value by using a negative constant.

The variances (if available) and all other additional information, such as annotations, are preserved unchanged in the resulting spectrum.

Scalar Multiply

The scalar multiply function multiplies each of the data points (y-values) in the spectrum by constant value and places the resulting spectrum in the math result register.

$$y_{\text{new}} = y \times C \quad (7.12)$$

The scalar multiply function can be used to divide by a constant value by using the reciprocal of the desired divisor, $\frac{1}{C}$.

The variances (if available) are multiplied by the square of the constant value:

$$\text{var}(y_{\text{new}}) = \text{var}(y) \times C^2 \quad (7.13)$$

All other additional information, such as annotations, are preserved unchanged in the resulting spectrum.

Binary Operations

The binary mathematical functions, add and subtract, require two spectra for their operation. If no spectra are selected, or more than two spectra are selected, the operation is aborted and an error message is displayed.

Add

The add function adds two spectra together and places the result in the math result register. The spectrum selected first (spectrum A) is taken as the model and provides the wavelength range and resolution for the resulting spectrum. Spectrum A is first copied into the math result register, then the y-values (for example absorbance) from the second selected spectrum (spectrum B) are added to the y-values of the spectrum in the math result register as follows:

Spectra of Different Wavelength Resolutions

If the x-values of spectrum A do not completely agree with those of spectrum B

- the y-values of spectrum B are interpolated before adding them to the y-values of the resulting spectrum when the interval of the x-value in spectrum A is less than that of spectrum B.
- the y-values at intermediate x-values in spectrum B are ignored when the interval of the x-value of spectrum A is greater than that of spectrum B, and only the y-values from spectrum B at the x-values corresponding to spectrum A are added to the y-values of the resulting spectrum.

Spectra of Different Wavelength Ranges

If the x-range (wavelength range) of spectrum A is greater than the x-range of spectrum B, the extra y-values in spectrum A remain unchanged in the result.

If the x-range of spectrum A is less than the x-range of spectrum B, the extra y-values in spectrum B are ignored.

If there is no overlap between the x-ranges of the spectra, spectrum A remains unchanged.

Variances

If variances are available in both spectra, the values from the spectrum B are added to those in spectrum A in the same way as for the y-values.

If only spectrum A contains variances, the values remain unchanged in the result.

If only spectrum B has variances, they are ignored.

Additional information, such as annotations, are taken solely from spectrum A; all such items from spectrum B are ignored.

Subtract

The subtract function subtracts one spectrum from another and places the result in the math result register. The spectrum selected first (spectrum A) is taken as the model and provides the wavelength range and resolution for the resulting spectrum. Spectrum A is first copied into the math result register, then the y-values (for example absorbance) from the second selected spectrum (spectrum B) are subtracted from the y-values of the spectrum in the math result register as follows.

Spectra of Different Wavelength Resolutions

If the x-values of spectrum A do not completely agree with those of spectrum B:

- the y-values of spectrum B are interpolated before subtracting them from the y-values of the resulting spectrum when the interval of the x-value in spectrum A is less than that of spectrum B, and
- the y-values at intermediate x-values in spectrum B are ignored when the interval of the x-value of spectrum A is greater than that of spectrum B, and only the y-values from spectrum B at the x-values corresponding to spectrum A are subtracted from the y-values of the resulting spectrum.

Spectra of Different Wavelength Ranges

If the x-range (wavelength range) of spectrum A is greater than the x-range of spectrum B, the extra y-values in spectrum A remain unchanged in the result.

If the x-range of spectrum A is less than the x-range of spectrum B, the extra y-values in spectrum B are ignored.

Variances

If variances are available in both spectra, the values from the spectrum B are added to those in spectrum A in the same way as for the Add function (see “Add” on page 65).

If only spectrum A contains variances, the values remain unchanged in the result.

If only spectrum B has variances, they are ignored.

Additional information, such as annotations, are taken solely from spectrum A; all such items from spectrum B are ignored.

Configuration and Methods

Details of configuration, software structure and
method operation

System Configuration

The configuration of the Agilent ChemStation hardware and software to operate with the Agilent 8453 (or HP 8452A series) spectrophotometer and its accessories has three aspects.

- Configuration of the communications between the Agilent ChemStation and the other devices (spectrophotometer, temperature controller) that are connected with and controlled by it. This process is carried out using the Configuration Editor.
- Internal configuration of the software using the commands from the Config menu.
- Configuration of the sampling system using the commands from the Instrument menu.

Configuring the Hardware

The Configuration Editor, which is a stand-alone program, is used to configure the Agilent ChemStation for the spectrophotometers and other GPIB accessories that are connected with it. The Configuration Editor sets up the communication pathways that allow the Agilent ChemStation to control the spectrophotometer and other accessories that communicate via GPIB. The Configuration Editor builds the configuration information into the Microsoft® Windows™ WIN.INI file. The Editor is also used to configure the UV-visible ChemStation software's color scheme, and can be used to define the :default paths for the Agilent ChemStation software's files; configuration information for both the color scheme and paths are also included in WIN.INI.

Note that you can also redefine paths for the data files, method files and automation files (automation table and sample table) from within the Agilent ChemStation software.

The Agilent 8453 (or HP 8452A series) spectrophotometer and Agilent ChemStation undergo a dialog during start-up that results in the automatic configuration of the spectrophotometer. The following information is passed from the spectrophotometer to the Agilent ChemStation:

- The instrument type, firmware revision number and identification number

(HP 8452) or serial number (Agilent 8453).

- The sampling interval
- The time for which the instrument has been switched on.
- The lamp condition (on or off)
- The spectrophotometer status
- The availability of a blank spectrum

The Agilent ChemStation downloads the spectrophotometer parameters of the current method to the spectrophotometer when the method is loaded during start-up.

The Configuration File

The configuration file controls the system set-up at start-up. All information relating to the configuration of the Agilent ChemStation software is stored in the configuration file, and the information is transferred into the configuration register when the Agilent ChemStation is started. The file is updated (and the current configuration saved) by overwriting the current contents of the file with the contents of the configuration register when the Save Configuration check box is selected when the Agilent ChemStation software is closed down.

When you save the configuration using the Save Configuration check box, you also save the current size and position of all windows and tables in all views.

The configuration register contains the following information:

User interface

parameters Definitions of the sizes and positions of all graphics windows.

**Table templates
and**

descriptions Definitions of the format and contents of all tables.

System

variables The current sample name, whether the method has changed or not, system messages: information and error messages.

Acquisition

information Information about sampling systems, and the Configuration of Verification table.

Report

information Information for the formatting of reports.

Methods

A method is a set of parameters that defines the analysis of a sample. A method can define a simple analysis, such as measuring the sample in the cell and displaying the absorbance spectrum, or a complex one, such as introducing a sample using the specified sampling system, measuring the sample, performing spectral processing steps, calculating a result using a pre-defined equation and printing a report.

The Structure of Methods

The method definition optionally includes any or all of the following parameters:

- a description of the method.
- instrument and acquisition parameters:
 - parameters to prepare the configured sampling system to collect the sample.
 - the sample temperature and stirrer parameters of the Agilent 89090A temperature controller (if fitted and configured).
 - the acquisition parameters of the spectrophotometer.
 - the name of the data file in which the measured spectra will be stored automatically.
 - the parameters for the user interface display.
- the data analysis parameters:
 - the spectral processing parameters that are applied to the measured spectra.
 - parameters for the extraction of wavelength data.
 - the parameters necessary for the calculation of the final result, for example an equation or quantification parameters, depending on the method.

The contents of the method definition depends on the settings defined by the user and the functionality available in the selected task.

Method Files

Method files are stored in a subdirectory METHODS of the instrument directory (1 for example). They have the extension .M.

The Status of Methods

Methods can exist in two states:

- as the **current method**, which is the method that is loaded into memory,
- as a **stored method** on disk. The current method can be stored on disk by selecting Save Method As from the File menu. Any stored method can replace the current method in memory by selecting Load Method...from the File menu and specifying the method to be loaded.

The Contents of the Method

The definition of the current method is held in a register (see “Registers” on page 22 for a description of registers), which contains the following information:

- the date and time of the last update of the method.
- the product number and software revision number.
- the contents of the Method Information field of the Method Options and Information dialog box.
- The state of the Autosave Spectra to File check box and the contents of the adjacent field in the Method Options and Information dialog box.
- a table that is the core of the method containing the information that defines the method parameters:

data analysis parameters,
spectrophotometer parameters,
temperature controller parameters,
sampling system parameters,
report parameters,
automation parameters.

On Using a Method

When a method is loaded, the system is set up using the method parameters. The Measure command causes the method to be executed. On Using a Method shows the processes that occur, and the information that is input at each stage when a complete method is executed.

Sample Measurement

- 1 The configured sampling system collects the sample with the conditions specified in its parameter dialog box.
- 2 If the Wait for Temperature Ready check box in the sampling system's Setup dialog box is selected, the Agilent ChemStation checks the status of the Agilent 89090A Peltier temperature control accessory.
- 3 When the status of the temperature controller is Ready, the Agilent ChemStation initiates a measurement by the spectrophotometer of the sample in the cell using the parameters specified in the Spectrophotometer Parameters dialog box.
- 4 If the method is used in an Automation, and the Autosave Spectra to File check box in the Method Options and Information is selected, the spectra are appended to the defined file.

Data Analysis

- 1 The measured spectra undergo data analysis.

The sample spectra are processed using the parameters defined in the parameters dialog box for the selected task.

The data for the wavelength results are extracted from the processed spectra and the wavelength result is calculated.

If the Ratio/Equation task has been selected, the analysis result is calculated from the wavelength result using the equation parameters.

If the Quantification task has been selected, the analysis result is calculated from the wavelength result using the quantification parameters.

Printing the Report

- 1 The report is formatted according to the parameters in the Configure Report dialog box and printed according to the parameters in the Printer Control dialog box.

The WIN.INI File

During installation of the software and configuration of the system, three or more sections are added to the Microsoft® Windows™ initialization file, WIN.INI:

- | | |
|-----------------|---|
| [PCS] | contains the information about the instruments that are connected and the communications parameters. |
| [PCS,n] | contains the details of screen colors, data paths and file names for instrument number n. Each configured spectrophotometer has an entry, for example [PCS,1], [PCS,2] and so on. |
| [HPCHEM] | contains the access level and the encrypted password. |

You must not change any of the entries in these sections of the WIN.INI file. Any changes will give rise to unpredictable behavior, and may prevent the UV-visible ChemStation software from starting up.

The Directory Structure

During installation of the Agilent ChemStation software, a directory structure is set up that has a main directory with a minimum of four subdirectories. The default name for the main directory is HPCHEM; this directory and the HPCHEM\SYS and HPCHEM\UVEXE directories are appended to the PATH statement in the file AUTOEXEC.BAT during the installation process.

If you choose to use a different name for the main directory during installation, the name you choose is used in place of HPCHEM, HPCHEM\SYS and HPCHEM\UVEXE in the PATH statement. If you wish to change the name of the main directory after installation, you must also change the PATH statement in the file AUTOEXEC.BAT.

For an analytical system with one spectrophotometer, HPCHEM has four subdirectories:

- UVEXE contains software for the operation of the Agilent ChemStation software. UVEXE has five subdirectories:
 - HELPENU contains the on-line Help files. These files are necessary for the context-sensitive Help facilities of the Agilent ChemStation software.
 - LANGUAGE will contain files for local language support of the Agilent ChemStation software.
 - PICTURES contains the files for the graphical user interface. These files are also necessary for the operation of the Agilent ChemStation software.
 - SYSMACRO contains the system macro files. These files are necessary for the operation of the Agilent ChemStation software.
 - USERMACRO contains user-generated macro files.

You must not delete any of the files in the UVEXE or SYSMACRO subdirectories. If any of these files are missing or corrupted, the operation of your Agilent ChemStation software will be either unpredictable or impossible.

- 1 contains the parameter files and data files for the

The Directory Structure

analytical system configured as number 1. The files are held in five subdirectories.

AUTOMAT contains the saved automation tables.

DATA contains the sample spectra that have been saved on disk.

DIAGNOSE contains the results of verification tests.

METHODS contains the saved method files.

REPORTS contains the report files that have been saved on disk.

STDS contains the standard spectra that have been saved on disk.

If additional instruments are configured as numbers 2, 3, and so on, additional subdirectories are created for their parameter and data files.

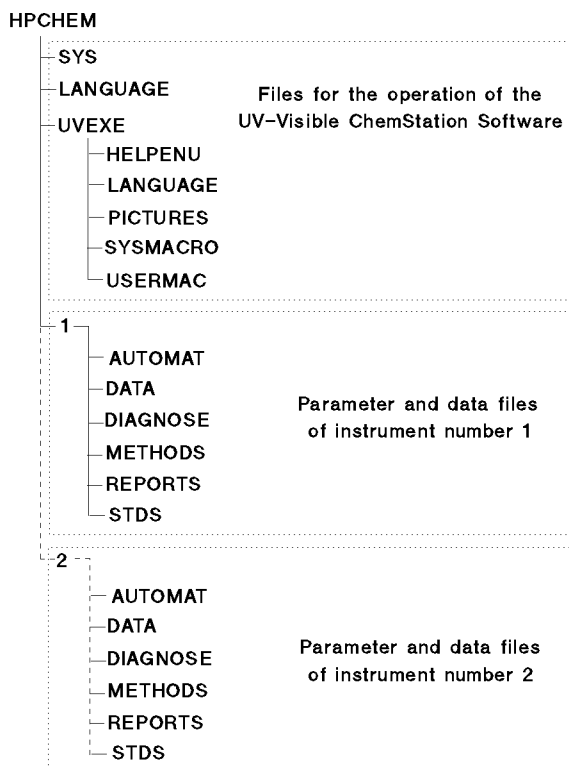
SYS contains software for the operation of the Agilent ChemStation software.

You must not delete any of the files in the SYS subdirectory. If any of these files are missing or corrupted, the operation of your Agilent ChemStation software will be either unpredictable or impossible.

LANGUAGE will contain files for local language support of the UV-visible ChemStation software.

Figure 9 shows the directory structure of the Agilent ChemStation software.

Figure 9

Directory Structure of the Agilent ChemStation Software**File Formats**

All standard files associated with the Agilent ChemStation, both software and data, are in binary format, and therefore cannot be accessed (neither viewed nor edited) using standard text editing programs. This ensures compatibility with the guidelines of good laboratory practice (GLP).

When report to file is selected, the report is saved in ASCII format with the extension .TXT; report graphics are saved as Windows metafiles (binary format) with the extension .WMF.

Verification

Verification is the process of evaluating your spectrophotometer to ensure that it complies with documented specifications

Configuration of Verification

The information about the spectrophotometer and the filters used for the verification that you enter into the Instrument Verification Parameter dialog box is transferred into the Configuration of Verification table and can be stored in the configuration file (see “System Configuration” on page 70) by selecting the Save Configuration check box in the Close dialog box when you exit from the Agilent ChemStation software. There is no necessity to edit the configuration parameters thereafter unless you change one of the verification filters.

The other information in the Configuration of Verification table is transferred into the table automatically. The values are dependent on the spectrophotometer that is connected; to conform to the requirements of good laboratory practice (GLP), these values cannot be edited.

Verification Tests

The instrument should be allowed to warm up for a minimum of 45 minutes before running a verification.

Wavelength Accuracy

An absorbance spectrum of an appropriate wavelength calibration standard is measured using 0.5 second integration time. This spectrum is converted into transmittance and the wavelength of specified peak minima are determined with the help of a spline function. The standards, reference wavelengths and permitted errors used for the wavelength accuracy test are shown in Table 9.

Table 9 Parameters for the Wavelength Accuracy Test

Spectrophotometer	Filter	Wavelengths Used (nm)	Permitted Error
Agilent 8453 190–1100 nm	NIST 2034 holmium oxide solution	241.11, 249.93, 278.07, 287.33, 333.42, 345.48, 361.24, 385.76, 416.45, 451.30, 467.89, 485.31, 536.81, 640.68*	±0.5 nm
HP 8452A Standard UV-visible 190–820 nm	NIST 2034 holmium oxide solution	241.1, 451.3, 640.8	±2 nm
HP 8452A #002 UV 190–510 nm	NIST 2034 holmium oxide solution	241.1, 361.3, 416.6	±1 nm
HP 8452A #003 Vis-SWNIR 470–1100 nm	McCrone	589.8, 807.8, 884.3	±2 nm

* The exact wavelengths of the bands varies with the spectral bandwidth (SBW) of the instrument. The Agilent 8453 has a nominal SBW of 1.5 nm over the wavelength range covered by holmium oxide solution (240–650 nm) determined either by the “Effective Bandwidth” method proposed by NIST¹ or by the toluene in hexane test proposed by the European Pharmacopoeia² (note that the actual typical value for 269/266 nm ratio for the Agilent 8453 is 1.75 which is equivalent to 1.45 nm SBW, whereas the specifications for the Agilent 8453 give a minimum value of 1.6 for the ratio). The actual wavelength values used for the Agilent 8453 are obtained by linear interpolation between the 2 nm and 1 nm values provided by NIST⁴ to obtain values for 1.5 nm SBW. The validity of this approach has been confirmed by NIST³.

References:

1. NBS Special Publication 260-66, Didymium Glass Filters for Calibrating the Wavelength Scale of Spectrophotometers, Venable W.H., Eckerle, K.L., October, 1979, p.70.
2. Pharmeuropa, Guide for the Technical Content of Monographs, Council of Europe, pp. 14-15, 1993.
3. Private communication, John Travis, NIST, 10 March, 1997.
4. NIST Certificate, Standard Reference Material 2034, Holmium Oxide Solution Wavelength Standard from 240 to 650 nm, Series No. 95, 1995.

The calculation includes an additional tolerance of ± 0.1 nm because of the uncertainty of determining the exact position of the peak. Upper and lower limits for the position of the measured peak are calculated from the used wavelength, the peak detection uncertainty and the permitted error:

$$\lambda_{\text{upper}} = \lambda_{\text{used}} + \lambda_{\text{permitted error}} + \lambda_{\text{uncertainty}} \quad (9.1a)$$

and

$$\lambda_{\text{lower}} = \lambda_{\text{used}} + \lambda_{\text{permitted error}} + \lambda_{\text{uncertainty}} \quad (9.1b)$$

The detected peak must lie within these two limits:

$$\lambda_{\text{lower}} \leq \lambda_{\text{measured}} \leq \lambda_{\text{upper}} \quad (9.2)$$

Photometric Accuracy

HP 8452A and Agilent 8453

The NIST SRM 930 E 10% transmittance, neutral density glass standard is used for the photometric accuracy test. The tolerance limits for the calibration value of the filter are increased by 1% per year (see the NIST documentation that was delivered with your filter), and this is allowed for in the calculation.

A spectrum is measured using the photometric accuracy filter with an integration time of 0.5 seconds. The absorbance value at 440 nm (UV and UV-visible instruments) or 635 nm (Vis-SWNIR instruments) is used to check the photometric accuracy. For HP 8452A spectrophotometers, the instrument tolerance of ± 0.005 AU is included in the result of the calculation performed by the software.

$$T_{\text{aging}} = \left(\frac{N_{\text{calib}}}{12} \right) \cdot T_{\text{nom}} \cdot 1\% \quad (9.3)$$

where

T_{nom} is the nominal transmittance of the photometric accuracy filter

N_{calib} is the age of the filter in months

T_{aging} is the aging tolerance of the filter in transmittance

$$A_{aging} = \log(e) \cdot \frac{T_{aging}}{T_{nom}} \quad (9.4)$$

where

T_{aging} is the aging tolerance of the filter in transmittance

e is the Euler constant

Substituting equation (9.4) into equation (9.3) gives equation (9.5):

$$A_{aging} = 0.00036191 \cdot N_{calib} \quad (9.5)$$

The permitted error for the verification is then:

$$\varepsilon = \sqrt{A_{aging}^2 + \varepsilon_{inst}^2} \quad (9.6)$$

where

ε_{inst} is the instrument tolerance

Finally, an upper and lower limit for the measured absorbance is calculated from:

$$A_{upper} = -\log(T_{nom}) + \varepsilon \quad (9.7a)$$

$$A_{lower} = -\log(T_{nom}) - \varepsilon \quad (9.7b)$$

The instrument passes the test if the measured absorbance, A_{meas} is within the upper and lower limits:

$$A_{lower} \leq A_{measured} \leq A_{upper} \quad (9.8)$$

Agilent 8453 Only

An alternative test may be used for the Agilent 8453 spectrophotometer using a potassium dichromate solution (57–63 mg/l) as specified by the European Pharmacopoeia:

Table 10 Parameters for the Agilent 8453 Photometric Accuracy Test

Spectrophotometer	Filter	Wavelengths Used	Photometric Accuracy Limit
Agilent 8453 190–1100nm	Potassium dichromate solution in 0.01 N sulfuric acid	235 nm	0.748 ±0.01 AU
		257 nm	0.865 ±0.01 AU
		313 nm	0.292 ±0.01 AU
		350 nm	0.640 ±0.01 AU

The absorbance values are normalized by the weight of potassium dichromate used:

$$\text{Normalized value} = \text{measured value} \times \frac{60.6}{\text{weight (mg)}} \times \frac{1}{\text{path length (cm)}}$$

Stray Light

The stray light is measured with an integration time of 0.5 seconds using a cut-off filter that transmits light at longer wavelengths and blocks light at shorter wavelengths. The transmittance at one or two wavelengths is used to check the stray light.

Table 11 Parameters for the Stray Light Test

Spectrophotometer	Filter	Wavelength(s) Used	Stray Light Limit
Agilent 8453 190–1100nm	NaNO ₂ (50 g/l)	340 nm	<0.03%
	NaI (10 g/l)	220 nm	<0.05%
	KCl (1.2%)	200 nm	<1%
HP 8452A Standard UV-Vis 190–820 nm	HOYA 056	220 nm	<0.05%
		340 nm	

Table 11 **Parameters for the Stray Light Test**

Spectrophotometer	Filter	Wavelength(s) Used	Stray Light Limit
HP 8452A #002 UV 190–510 nm	HOYA 056	220 nm 340 nm	<0.05%
HP 8452A #003 Vis-SWNIR 470–1100 nm	Schott RG850	530 nm 650 nm	<0.05%

The transmittance at the specified wavelengths must be lower than the stray light limit for the instrument to pass the wavelength accuracy test:

$$T_{\text{meas}} < \text{Stray Light Limit} \quad (9.9)$$

Resolution (Agilent 8453 only)

Resolution is measured by taking a blank spectrum on air (with nothing in the light path), then measuring the ratio of the absorbances at 269 nm and 266 nm in a 0.02% v/v solution of toluene in hexane. The resolution specification is:

$$\frac{\text{Abs}_{\text{max at 269}}}{\text{Abs}_{\text{min at 266}}} > 1.6 \quad (9.10)$$

Noise

Noise is measured by taking spectra of air (with nothing in the light path) with an integration time of 0.5 seconds over a run time of 60 seconds. An

RMS calculation of the noise is made at a single wavelength. Noise limits are shown in Table 12.

Table 12

Parameters for the Noise Verification Test		
Spectrophotometer	Wavelength Used	Noise Limit
Agilent 8453 190–1100nm	500 nm	<0.0002 AU
HP 8452A Standard UV-Vis 190–820 nm	500 nm	<0.0002 AU
HP 8452A #002 UV 190 –510 nm	340 nm	<0.0002 AU
HP 8452A #003 Vis-SWNIR 470–1100 nm	780 nm	<0.0001 AU

The RMS noise must be less than the specified value for the instrument to pass the noise test:

$$\sqrt{\frac{\sum (R - \bar{R})^2}{(n - 1)}} < \text{Noise Limit} \quad (9.11)$$

where

R is the measured absorbance value

\bar{R} is the average of the absorbance values to 5 points either side of R in time

n is the total number of absorbance values used

Baseline Flatness

Baseline flatness is measured by taking a spectrum of air (with nothing in the light path) with an integration time of 0.5 seconds. An RMS calculation of the

spectrum is made over the wavelength range of the instrument, as shown in Table 13.

Table 13

Parameters for the Baseline Flatness Test

Spectrophotometer	Wavelength Range Used	Baseline Flatness Limit
Agilent 8453	190–1100nm	<0.001 AU
HP 8452A Standard UV-Vis	190–820 nm	<0.001 AU
HP 8452A #002 UV	190–510 nm	<0.001 AU
HP 8452A #003 Vis-SWNIR	470–1100 nm	<0.0005 AU

The RMS value must be less than the specified value for the instrument to pass the baseline flatness test:

$$\sqrt{\frac{\sum (R - \bar{R})^2}{(n - 1)}} < \text{Baseline Flatness Limit} \quad (9.12)$$

where

R is the measured absorbance value

\bar{R} is the average of the absorbance values over the wavelength range

n is the total number of absorbance values used

Stability

Stability is measured by taking spectra of air (with nothing in the light path) with an integration time of 0.5 seconds and a cycle time of 5 seconds over a period of one hour. The stability is the difference between the maximum and

minimum absorbance readings at a specified wavelength as shown in Table 14.

Table 14

Parameters for the Stability Verification Test		
Spectrophotometer	Wavelength Used	Stability Limit
Agilent 8453 190–1100 nm	340 nm	<0.001 AU
HP 8452A Standard UV-Vis 190–820 nm	340 nm	<0.002 AU
HP 8452A #002 UV 190–510 nm	340 nm	<0.002 AU
HP 8452A #003 Vis-SWNIR 470–1100 nm	620 nm	<0.001 AU

The stability must be less than the specified value for the instrument to pass the stability test:

$$A_{\max} - A_{\min} < \text{Stability Limit} \quad (9.13)$$

The room temperature should remain constant to ± 1 °C during the stability test.

Spectrophotometer Selftests

The spectrophotometer diagnostic starts a series of self-tests that are essentially similar to the power-on self-tests (POST) carried out when the spectrophotometer is turned on. The diagnostic cycles through the self-tests continuously until it is aborted by choosing the Stop Spectrophotometer Diagnostic button.

HP 8452A Spectrophotometer

CPU test	The internal Z80 processor of the spectrophotometer is tested.
ROM checksum test	Each ROM is tested separately; the sum of the contents of all locations is compared with the value stored in the last two bytes of the ROM.
RAM test	The value in each location in RAM is saved, a new value is written into the location, read and compared, then the original value is restored (unlike the POST, this test is non-destructive; the contents of the RAM remain unchanged by the test.)
Communication test	The CPU writes to and reads from the registers of the GPIB controller, indicating that is functioning.
Hardware Multiplier test	A series of 8-bit \times 8-bit multiplication tests are performed, each test using different numbers.
Interrupt (9519A and 8051) test	The interrupt controller, AM9519A, is tested using a software-generated interrupt. If this test is passed, the ERROR, DIODE and FRAME interrupts are tested. This test checks the Z80 handshake with the 8051AH diode clocking controller.
Light bar indicator test	Each indicator lamp is turned on for 0.3 seconds, then

turned off.

More information on the individual self tests for the HP 8452A spectrophotometer is available from the *Interfacing and Programming Guide* for the HP 8452A spectrophotometer (part number 08452-90006), and additional information on the reporting of errors can be found in the *User's Guide* for the HP 8452A spectrophotometer (part number 08452-90008).

Agilent 8453 Spectrophotometer

Electronics self tests

The spectrophotometer electronics (CPU, RAM, ROM, &c.) are tested.

Dark Current test

Checks the residual dark current in the diode array when the shutter is used to block any incident light.

Lamp Intensity test

Checks the lamp intensity over the whole wavelength range against minimum limits.

Wavelength

Accuracy test

Uses the two deuterium emission lines at 486 nm and 656.1 nm to check the wavelength accuracy.

RMS Noise test

Checks the RMS noise over 60 seconds against a maximum limit.

RMS Baseline

Checks the baseline flatness over the wavelength range of the instrument.

Flow Test

The Flow Test diagnostic for the sipper and autosamplers measures the absorbance at a specified wavelength as sample replaces blank medium in the flow cell. The test pumps sample for 1.5 times the Pump time (set in the sampling system's Parameter dialog box), then plots a flow profile and calculates the estimated minimum pump time for a specified percentage absorbance to be reached. Default instrument parameters for the flow test are:

Table 15**Default Instrument Parameters for the Flow Test Diagnostic Test**

Spectrophotometer	Integration Time	Gain	Cycle Time	Run Time	Default Wavelength	Default Percent
Agilent 8453 190–1100 nm	0.5 s	Auto	1 s	Pump time × 1.5	340 nm	99.5
HP 8452A Standard UV-Vis 190– 820 nm	0.5 s	Auto	1 s	Pump time × 1.5	340 nm	99.5
HP 8452A #002 UV 190–510 nm	0.5 s	Auto	1 s	Pump time × 1.5	340 nm	99.5
HP 8452A #003 Vis-SWNIR 470–1100 nm	0.5 s	Auto	1 s	Pump time × 1.5	620 nm	99.5

At the end of the run, the averages of the first two and last two measurements are calculated; the difference between the two averages gives the 100% difference in absorbance:

$$A_{0\%} = \frac{A_1 + A_2}{2}$$

and

$$A_{100\%} = \frac{A_{n-1} + A_n}{2}$$

where

A is absorbance

n is the number of data points

The absolute absorbance value for the user-specified Percent objective is calculated by equation 9.13:

$$A_{\text{user}\%} = \frac{(A_{100\%} - A_{0\%}) \cdot \text{Percent}}{100} + A_{0\%} \quad (9.14)$$

where

Percent is the user-specified Percent objective

The flow profile is scanned to find the time, T_E , at which the measured absorbance exceeds the absolute absorbance for the user-specified Percent objective, $A_{\text{user}\%}$. This is the Estimated Minimum Pump Time.

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Agilent Technologies

In This Book

This manual describes the data processing operations of the Agilent 8453 spectrophotometer and the Agilent ChemStation for UV-visible spectroscopy. It describes the processes and transformations that the spectrophotometric data undergo between the time that they are acquired in the spectrophotometer and their output on the Agilent ChemStation display or printer. The manual is designed to enable you to follow good laboratory practice (GLP) guidelines; using the information in the manual, you will be able to understand the data processing calculations from beginning to end and perform the data evaluation manually.



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