Application note

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Unlocking Advanced Calculation Capabilities in Insight™ Pro for UV-Visible Spectroscopy

Introduction

UV-Visible spectroscopy is a highly useful analytical technique utilized in a wide array of application spaces, including quality control checks and fundamental research. In this method of analysis, UV-Visible light is used to probe electronic transitions within an analyte. Consequently, these spectra are specific to the analyte measured, and can provide a wide range of both qualitative and quantitative information about the sample. Often, the spectrum is reported as absorbance; according to Beer's law, the absorbance is directly proportional to the analyte concentration, providing a non-destructive method of quantification.

While concentration determination is the most common use of UV-Visible spectroscopy, this technique can also be used for a variety of other analyses as well (e.g., binding equilibria, kinetic analysis, etc.). In many of these circumstances, further data analysis beyond Beer's law is needed, and this often requires additional calculations. These analyses can range anywhere from simple conversions to complex fitting procedures. The Thermo Scientific™ Insight™ Pro software used to run the Thermo Scientific™ Evolution™ UV-Visible spectrophotometers allows for extensive custom calculations within acquired data sets. This specialized software can help users carry out data analyses without needing to export spectra into separate programs. Herein the different types of calculations available within the Insight Pro software are outlined, and examples included.

Equation Builder vs Advanced Calculation

Within the Insight Pro software, there are two main functions which allow for custom calculations: the additional results/equation builder, and advanced calculation. Both functions allow for the use of basic math operators (addition, subtraction, multiplication, and division), in addition to other functions. However, it should be noted that the calculations included within the additional results can only be applied to one sample at a time. Analyses which require data from more than one sample cannot be carried out through these additional calculations, however they can be performed using the advanced calculation function.

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Additional Results

When initially setting the instrument parameters, the ability to calculate additional results can be enabled. By switching on the "Calculate Additional Results" toggle (Figure 1a), the ability to select and/or generate equations is enabled. A group of pre-set methods are available to choose from, such as calculating the concentration of various dyes. Custom equations can also be generated through the Equation Builder function (Figure 1b). Table 1 outlines the available calculations and syntax used for pre-built and customizable equations. User-defined equations can also be generated using basic math functions as described earlier. By convention, to define the collected data (e.g., absorbance, reflectance, etc.) at a specified wavelength, the syntax "Y(w1)" is used, where w1 is the wavelength, as shown in Figure 1a.

Once the samples are measured, the equations included through the additional results function will display in the measurement table following collection of the raw data. This information can also be included in the report. Figure 1a shows an example of what can be included in additional results. Herein, three calculations are included: one to report the raw absorbance measured at 630 nm; one to provide a pass/fail result based on the absorbance at 630 nm subtracted by the absorbance at 700 nm; and one to report the peak width at half the maximum.

The peak width calculations may not be as common as a background or impurity correction, however they can provide useful qualitative information. Broadening of UV-Visible absorption bands can often occur as a result of interactions with the local environment. Consequently, in some environments, like quality control, it can be helpful to determine the peak width for comparison to the peak width under different experimental conditions. By convention, the peak width is typically reported as the "full width at half maximum" (FWHM), though it can be reported as the full width at the baseline as well. As finding the peak width manually for each sample can be time consuming, the Insight Pro software can be used instead to automatically report this value.

In the equation builder, pre-built functions can be accessed in the "Type" drop down menu or can be manually entered using the "User Defined" calculation type if the appropriate syntax is known (Figure 1b). For example, to use the "PWidth" pre-built function, the syntax "PWidth(700,500,50)" was used as shown in Figure 1a. This will allow the software to determine the width of the peak found between 700 nm and 500 nm at 50% of the peak maximum value, as described in Table 1. In the additional results table, the equation name can be defined as well as the appropriate units. Figure 2 demonstrates how the additional results are displayed within the measurement screen using simulated data.

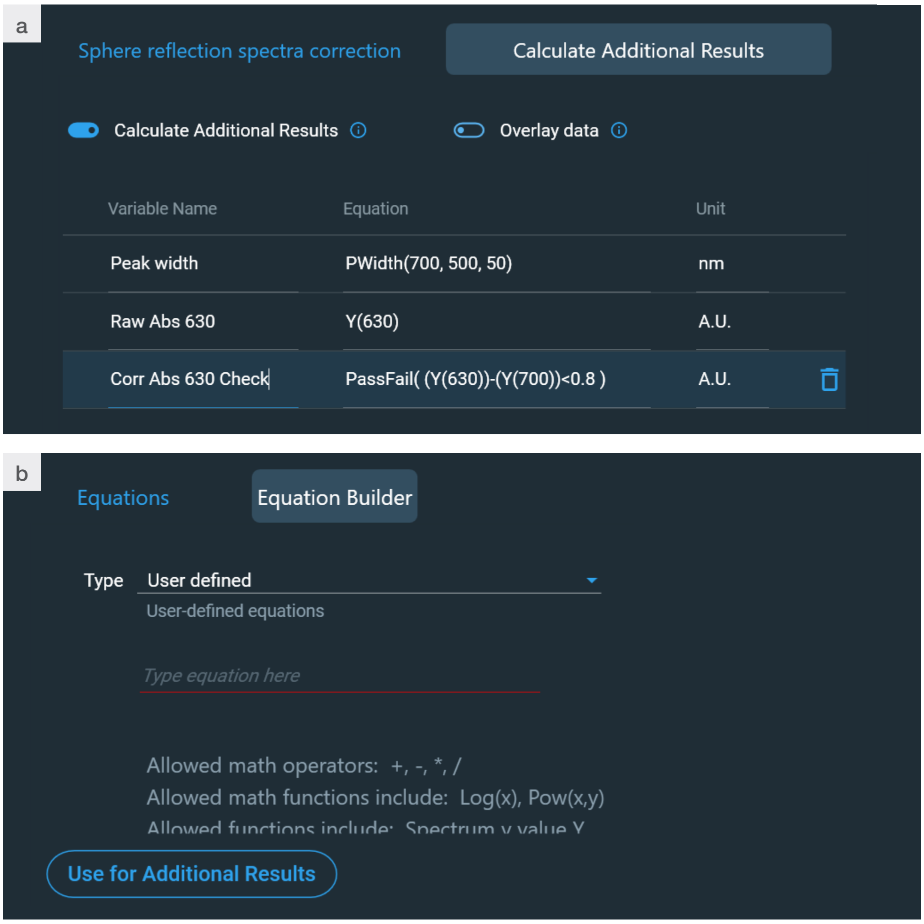


Figure 1. (a) Additional results calculations table and (b) equation builder within the Insight Pro software.

Calculation Name	Definition	Input logic
Basic Math Functions	Returns the measured signal (i.e. absorbance, reflectance, transmittance, etc.)	Y(w1)
	Returns the measured signal multiplied by a defined factor (k)	k*Y(w1)
	Returns the quotient of the measured signal at two different wavelengths (w1 and w2)	Y(w1)/Y(w2)
	Returns the sum of the signal at one wavelength and the signal at a second wavelength scaled by k.	Y(w1)+k*Y(w2)
	Returns the log (base 10) of the signal collected at the defined wavelength	Log(Y(w1))
	Returns the signal collected at the specified wavelength (w1) after correction using a single-point baseline correction (w2)	Y(w1,w2)
	Returns the signal collected at the specified wavelength (w1) after correction using a sloping-baseline. The sloping-baseline correction is defined as w2 and w3.	Y(w1,w2,w3)
	Returns the signal at the specified wavelength (w1) raised to the "p" power.	Pow(Y(w1),p)
Area	Returns the area under the curve for a defined wavelength range. No baseline correction is applied.	Area(w1,w2)
	Returns the area under the curve for a defined wavelength range (w1 and w2). A sloping baseline is applied and is defined by w3 and w4.	Area (w1,w2,w3,w4)
PMax	Returns the maximum signal intensity (i.e. absorbance, reflectance, transmittance, etc.) within a defined wavelength range	PMax(w1,w2)
PMax-PMin	Returns the difference between the maximum signal (defined by w1 and w2) and the minimum signal (defined by w3 and w4)	PMax(w1,w2)-PMin(w3,w4)
Pmax/PMin	Returns the maximum signal (defined by w1 and w2) divided by the minimum signal (defined by w3 and w4)	Pmax(w1,w2)/Pmin(w3,w4)
PLoc	Returns the peak maximum location within a defined wavelength range	PLoc(w1,w2)
PWidth	Returns the width of a peak at a defined percent of the maximum peak intensity over a defined wavelength range	PWidth(w1,w2,k)
PassFail	Returns a Pass/Fail result based on a given expression	PassFail(expression) Ex: PassFail(Y(260)> 0.5 AND Y(260)<0.8)

Table 1. Calculations available in the equation builder within the additional results settings.

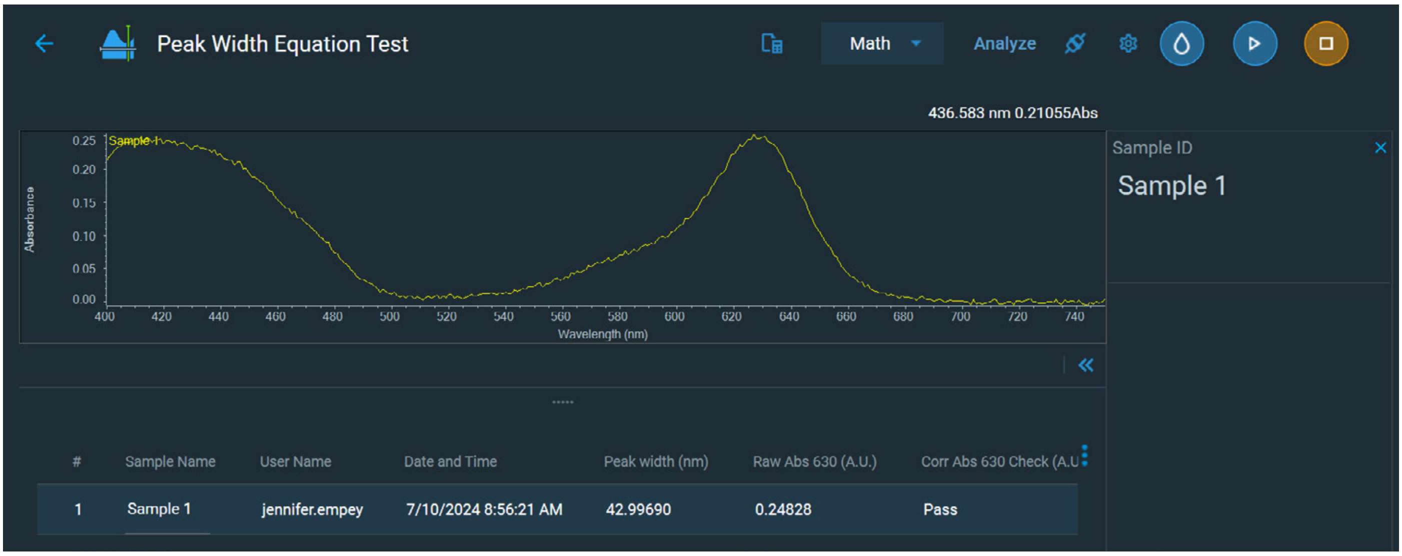


Figure 2. Measurement window displaying the peak width calculation, the raw absorbance at 630 nm, and a Pass/Fail test. The data reported here is simulated to demonstrate the software capabilities.

Advanced Calculation

While the additional results function can be helpful for analyzing a single sample at a time, it cannot be used for calculations involving more than one sample measurement in the equation. For these circumstances, advanced calculation can be used instead. This feature within Insight Pro is not a part of the instrument settings, but as an analysis tool available following data collection.

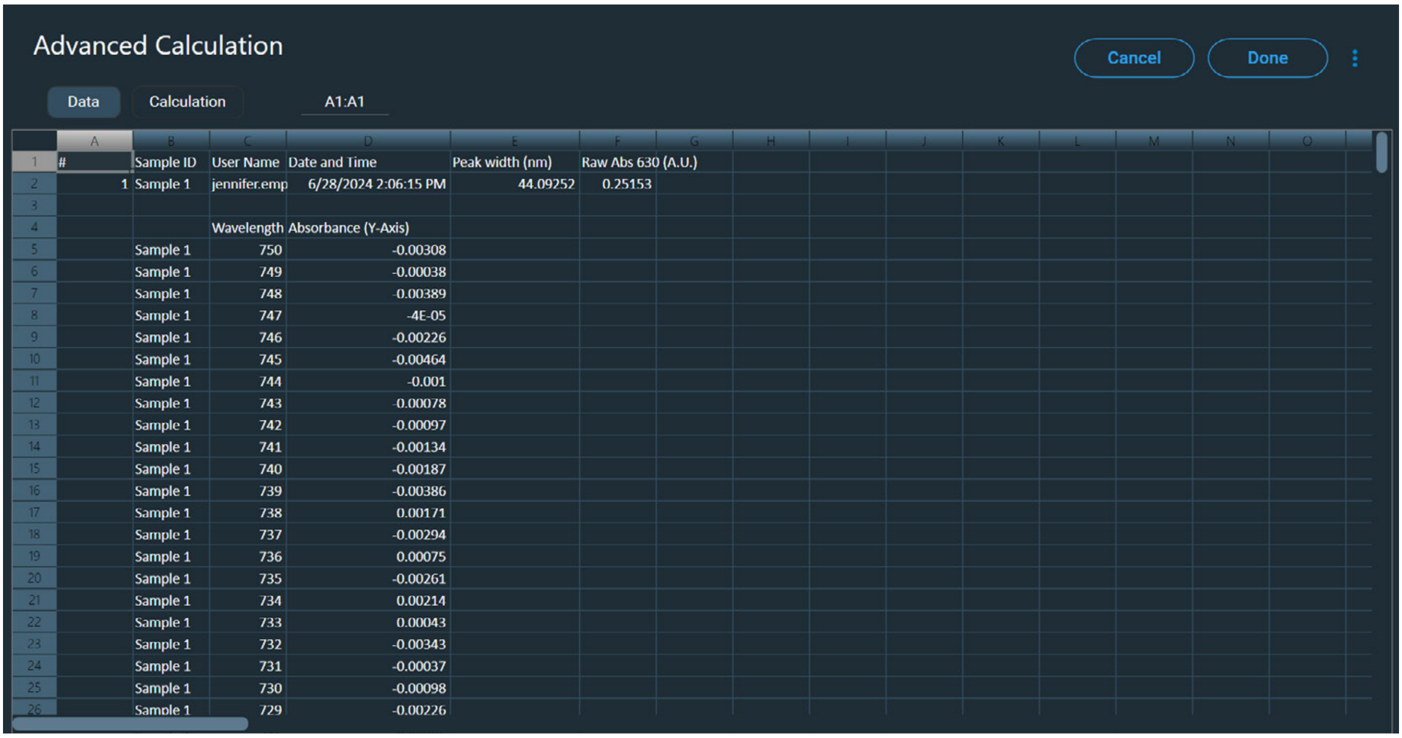


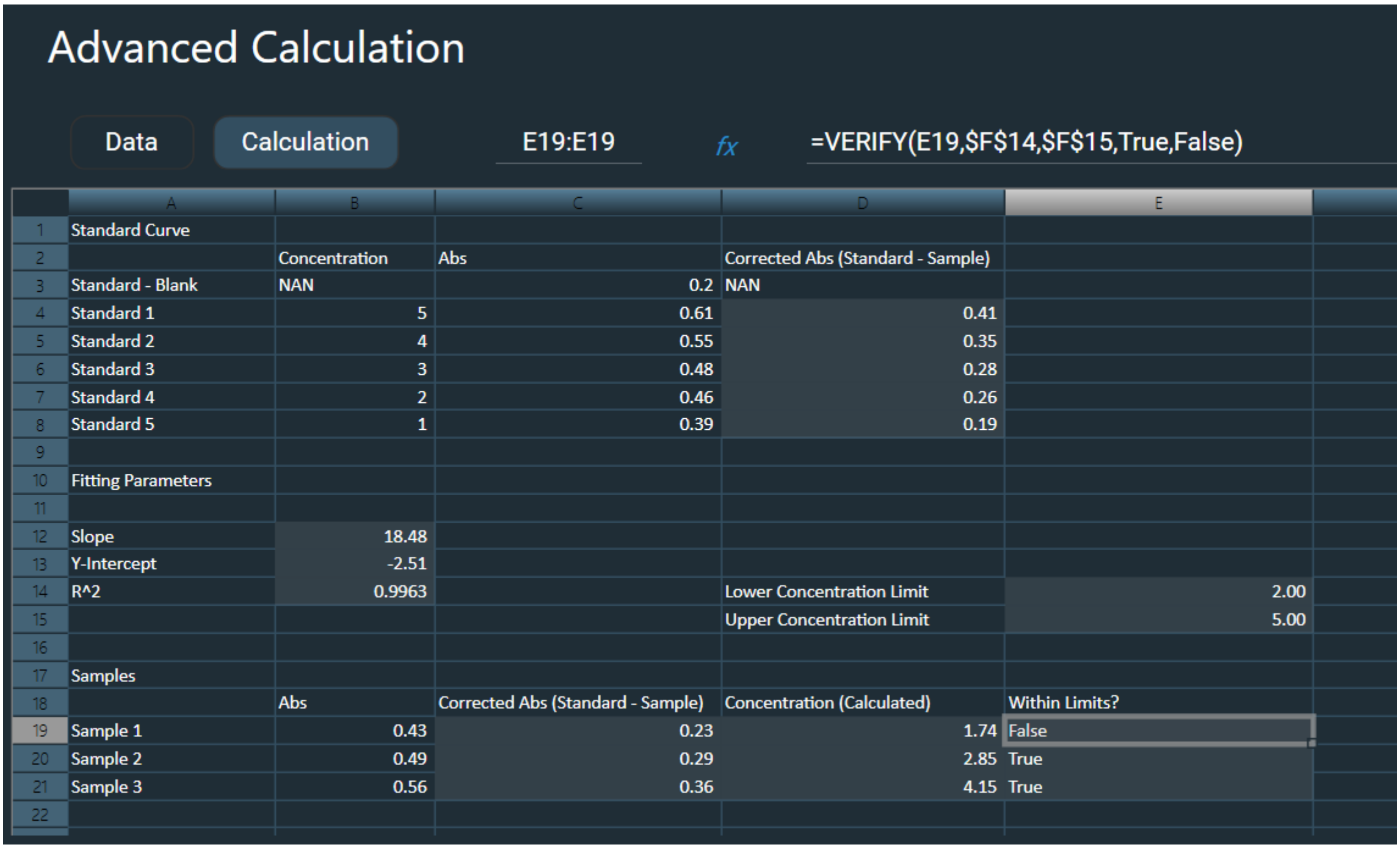
Figure 3. Example Data sheet in advanced calculation with simulated data.

The advanced calculation function consists of two spreadsheet tabs: the “Data” spreadsheet and the “Calculation” spreadsheet. The former includes the raw data collected for each sample measured in the experiment, as well as details recorded in the measurement table. This includes results for all calculations included in the additional results setting. Figure 3 includes an example of what the data typically looks like in this tab. It is important to note that while data can be copied from this sheet, it cannot be altered. The Insight Pro software prevents users from changing any cell in the “Data” sheet, regardless of if the security software (Thermo Scientific™ Security Suite software package) is in use.

Within the “Calculation” sheet, cells can be edited by the user and equations can be included as well. Beyond basic math (i.e., addition, subtraction, multiplication, and division), Table 2 includes a list of available formulas and allowed syntax. Much like other spreadsheet software, cells within the sheet can be referenced within equations. The available equations cover a wider range of calculations than can be covered through the additional results options, providing a tool for more complicated analyses. As mentioned earlier, this functionality allows the user to generate equations using data collected from more than one sample. This can be particularly useful when special corrections are needed between the sample and a given standard or blank solution.

Calculation Name		Definition	Input logic
Basic Math Functions	**Sum**	Returns a sum of the defined values	SUM(value 1:value 2) OR SUM(value 1, value 2, …)
	Average	Returns the average of the defined values	AVERAGE(value 1:value 2) OR AVERAGE(value 1, value 2, …)
	Stdev	Returns the standard deviation for the defined list of values	STDEV(value 1, value 2, …) OR STDEV(value 1:value 2)
	Sqrt	Returns f(x) for the following equation: $f(x)=\sqrt{(x)}$	SQRT(value)
	Count	Returns the number of values included	COUNT(value 1:value 2) OR COUNT(value 1, value 2, …)
	Min	Returns the minimum value within the defined list of values	MIN(value 1, value 2, …) OR MIN(value 1:value 2)
	Max	Returns the maximum value within the defined list of values	MAX(value 1, value 2, …) OR MAX(value 1:value 2)
	Abs	Returns the absolute value of the defined value	ABS(value)
	Exp	Returns f(x) for an exponential equation as shown below: $f(x)=e^x$	EXP(value)
	Log	Returns f(x) for an logrithmic equation with the defined base as shown below: $f(x)=log_{base}(x)$	LOG(value,base)
	Log10	Returns f(x) for an logrithmic equation with the base 10 as shown below: $f(x)=log_{10}(x)$	LOG10(value)
	Power	Returns f(x) for the following equation: $f(x)=x^p$ where "p" is the defined power	POWER(value,power)
Rounding Functions	**Round**	Returns the defined value rounded to a number of defined decimal places	ROUND(value, number of decimal places)
	Ceiling	Rounds the defined value up to a multiple of a defined significant value	CEILING(value, significant value) Ex: CEILING(0.233,2) = 2 CEILING(2.233,2) = 4
	Floor	Rounds the defined value down to a multiple of a defined significant value	FLOOR(value, significant value) Ex: FLOOR(0.233,2) = 0 FLOOR(2.233,2) = 2
Trigonometric Functions	**sin**	Returns f(x) for the following equation: $f(x)=sin(x)$	SIN(value)
	Asin	Returns f(x) for the following equation: $f(x)=sin^{-1}(x)$	ASIN(value)
	cos	Returns f(x) for the following equation: $f(x)=cos(x)$	COS(value)
	Acos	Returns f(x) for the following equation: $f(x)=cos^{-1}(x)$	ACOS(value)
	tan	Returns the f(x) for the following equation: $f(x)=tan(x)$	TAN(value)
	Atan	Returns the f(x) for the following equation: $f(x)=tan^{-1}(x)$	ATAN(value)
	Atan2	Returns f(x) for the following equation: $f(x)=tan^{-2}(x)$	ATAN2(value)
Fitting Functions	**Linear**	Returns a specified fitting parameter ("m", "c", "r") or an anticipated value (x') for a linear function based on the defined x and y values for the fit.	LINEAR(x values, y values, "k") where "k" can be: x' - returns y value for a given x value "m" - returns slope of linear function "c" - returns y-intercept of linear function "r" - returns coefficient of determination (R^2)
	LinearZ	Returns a specified fitting parameter ("m", "c", "r") or an anticipated value (x') for a linear function based on the defined x and y values for the fit. This fit forces the y-intercept to be 0.	LINEARZ(x values, y values, "k") where "k" can be: x' - returns y value for a given x value "m" - returns slope of linear function "r" - returns coefficient of determination (R^2)
	Quadratic	Returns a specified fitting parameter ("a", "b, "c", "r") or an anticipated value (x') for a quadratic function ($f(x) = ax^2+bx+c$ based on the defined x and y values for the fit.	QUADRATIC(x values, y values, "k") where "k" can be: x' - returns y value for a given x value "a","b","c" - return the polynomial coefficients "r" - returns coefficient of determination (R^2)
	QuadraticZ	Returns a specified fitting parameter ("a", "b, "c", "r") or an anticipated value (x') for a quadratic function ($f(x) = ax^2+bx+c$ based on the defined x and y values for the fit. This fit forces the "c" coefficient of the polynomial to be 0.	QUADRATICZ(x values, y values, "k") where "k" can be: x' - returns y value for a given x value "a","b" - returns polynomial coefficients "r" - returns coefficient of determination (R^2)
Miscellaneous	**Verify**	Evaluates if a specified value falls within or outside of a set of defined limits. If the value is within the limits, expression 1 is evaluated. Otherwise, expression 2 is evaluated.	VERIFY(value, lower limit, upper limit, expression 1, expression 2)
	Mod	Returns the remainder after the input value is divided by the defined divisor.	MOD(value, divisor)
	Percent	Returns the quotient of a specified value divided by 100. This value is scaled by a specified scaling factor	PERCENT(value, scaling factor) Ex: PERCENT (35,35) = (35/100)*35
	Copy	Returns the value included in the specified spreadsheet cell found in the "Data" sheet.	'Data'!cell Ex: 'Data'!A2

Table 2. Calculations available within advanced calculation function.



Advanced Calculation

Data | Calculation | E19:E19 | fx | =VERIFY(E19,F14,F15,True,False)

	A	B	C	D	E
1	Standard Curve				
2		Concentration	Abs	Corrected Abs (Standard - Sample)	
3	Standard - Blank	NAN	0.2	NAN	
4	Standard 1	5	0.61	0.41	
5	Standard 2	4	0.55	0.35	
6	Standard 3	3	0.48	0.28	
7	Standard 4	2	0.46	0.26	
8	Standard 5	1	0.39	0.19	
9					
10	Fitting Parameters				
11					
12	Slope	18.48			
13	Y-Intercept	-2.51			
14	R^2	0.9963		Lower Concentration Limit	2.00
15				Upper Concentration Limit	5.00
16					
17	Samples				
18		Abs	Corrected Abs (Standard - Sample)	Concentration (Calculated)	Within Limits?
19	Sample 1	0.43	0.23	1.74	False
20	Sample 2	0.49	0.29	2.85	True
21	Sample 3	0.56	0.36	4.15	True
22					

Figure 4. Example "Calculation" sheet with arbitrary data. Included are examples of the LINEAR and VERIFY functions outlined in Table 2. Note: NAN means "not a number".

Simple fitting (i.e., linear or quadratic functions) is also possible within advanced calculations. While the "Quant" application is sufficient for fitting data generated through traditional standard curve analyses, the fitting capabilities in advanced calculation can be used when the absorbance needs to be fit as a function of parameters outside of concentration (e.g., pH). It can be also used if the data needs to be corrected using the absorbance of a standard prior to preparation of the standard curve. For example, Figure 4 includes an example data set with arbitrary absorbance values, mimicking a sample in which the overlapping absorbance from an impurity needs to be corrected prior to creation of the standard curve. Here, the absorbance of a standard "Blank" sample is subtracted from the absorbance collected for each subsequent standard, providing a corrected absorbance.

Fits to a linear function are also demonstrated in Figure 4. Under "Fitting Parameters" the slope and Y-intercept are calculated using the LINEAR function outlined in Table 2. This was performed using the standard concentration and corrected absorbance described in the "Calculation" spreadsheet (Figure 4). The coefficient of determination (r^2) can be calculated using the LINEAR function as well to ensure the fit function used is appropriate. It can often be useful to report these parameters when fitting data, particularly in regulated environments where meticulous record-keeping is of high importance. Using the LINEAR function once again (see Table 2 for required syntax), the absorbance can be converted to concentration.

Note, Figure 4 also includes grayed out cells, indicating these cells are locked. Security features like this are available to protect against editing cells and sheets; such features are outlined in the Insight Pro User Guide. The Insight Pro software also allows the user to create templates based on the selected instrument settings, making experimental set-up faster for future measurements. If calculations are included in the advanced calculation sheet, then these calculations will also be included in the associated experiment template. As a note, only the input values and equations from the "Calculations" sheet will be included in the template. No values from the "Data" sheet will be retained in the template.

Setting Pass/Fail Criteria

In quality assurance/quality control environments, a quick Pass/Fail display is often needed for reporting purposes. There are multiple options for this form of reporting through Insight Pro. These options are available within the additional results settings, as a function in advanced calculation, and as limitations for samples measured using the Quant application.

Additional Results

First, the equation builder within the additional results settings includes a pre-built Pass/Fail value (Table 1). This function allows the user to input an expression defining the limits the measured value (i.e. absorbance, reflectance, etc.) must meet to be considered passing. Typically, this expression involves defining a single wavelength which needs to within two bounds. If the measured value is within the defined limits, the measurement table will display a "Pass" result for the corresponding sample. Otherwise, a "Fail" result will be displayed for the sample. Figure 1a includes an example Pass/Fail expression in which the absorbance measured at 700 nm is subtracted from the absorbance at 630 nm and must be below 0.8 to pass. This mimics a situation in which the Pass/Fail criteria is dependent on a corrected absorbance rather than the raw absorbance of the sample.

Advanced Calculation

If using the advanced calculation option, the VERIFY function can be utilized as a Pass/Fail check. This equation allows the user to check if a value falls within a set of specified upper and lower limits. The value to be tested and the limits can either be input as numeric values or as cells within the spreadsheet. This can be helpful if the result of an equation needs to meet a given criteria rather than if the raw absorbance needs to be within a set of defined boundaries.

Unlike the PassFail function in additional results, the VERIFY equation allows the user to also define a set of If/Then expressions as well (see Table 2 for details on the required syntax). These expressions can be a statement (i.e. True/False) or can be different equations. Figure 4 includes an example use of this function. In this example, the concentration calculated using the LINEAR function is compared against a set of limits. Here, "True" (expression 1) was selected to denote falling within the limits while "False" (expression 2) indicates the value is outside of the limits.

Instrument Settings: Control Limits

Finally, control limits can also be set within the Quant application. This application allows the user to generate a standard curve using solutions of known concentration. The Insight Pro software is then able to fit this data to a user-defined function (e.g., linear, quadratic, etc.). When samples of unknown concentration are measured, the generated fit (standard curve) is used to automatically calculate the analyte concentration.

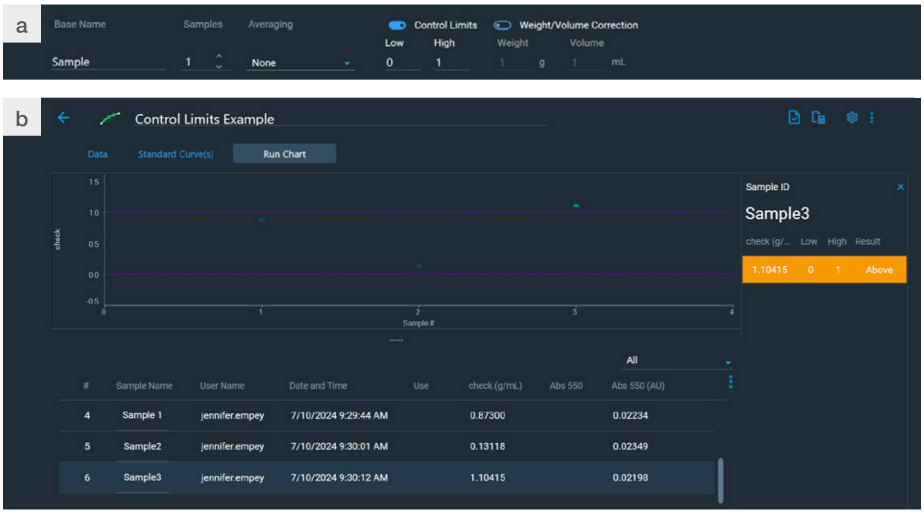


Figure 5. (a) Control limit settings and (b) example standard curve measurement screen displaying control limits and sample Pass/Fail results. Data included was simulated to demonstrate the software capabilities.

In some circumstances, like quality control environments, the unknown sample must meet a specific concentration requirement before it can be used downstream. To aid in this determination, “control limits” can be set in the instrument settings (Figure 5a). Once lower and upper concentration limits are set the software will display either “Pass”, “Above” or “Below” in the measurements run chart. Figure 5b includes results for a simulated data set in which the concentration of one sample is higher than the control limits set. For ease of visualization, dashed lines are also included in the run chart to define the bounds for acceptable concentration values. Note, this capability is also possible when selecting biological applications which utilize a standard curve (e.g., the Protein Bradford application).

Summary

As there are multiple different uses of UV-Visible spectroscopy across many fields, there exists a diverse set of data analysis procedures commonly employed. The examples included herein outline a few of the many possible methods for performing these calculations through the Insight Pro software. These includes simple fitting procedures and establishing pass/fail expressions, which can be useful for both research environments and quality. The array of capabilities in the additional results and advanced calculation functions provide a large list of possible analyses without requiring the data be exported into a separate software.

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