INTRODUCTION:

The term colorimetry originates from the times when measurements were done by comparing the color of a component under investigation with a standard color by the eye. While this principle is still the basis of modern techniques, of course the measurement with the eye has been replaced by the measurement of photons with a photon detector. From this the term photometry came about. This technique is covered by a more generic term called spectrometry, which refers to all techniques that are based upon the production or interaction of electromagnetic radiation with matter. The concerned matter may produce or absorb electromagnetic radiation, from which the terms emission and absorption spectrometry originate. Further spectroscopic techniques are characterized according to the type of electromagnetic radiation involved, which may range from X-ray to radio-frequency, or in other words: which part of the spectrum is used. Nowadays spectrometric methods are the most applied analytical techniques in the world. Particularly the radiation in the UV (180-380 nm), Visible (380-750 nm) and Infra-red (750-40000 nm) is most commonly used.

Applikon analyzers apply absorption spectrometry in the visible range, in which we can talk about photons. That’s why this technique is referred to as absorption spectro-photometry. Since not a spectrum range is applied, but rather one wavelength (or color!), the term is often called absorption-photometry. But luckily also the term colorimetry is still widely in use.

THEORY:

a) Beer’s Law.

Colorimetric measurements are performed by beaming a ray of light of a certain wavelength through a reaction solution. This is depicted in the following picture.

Here a parallel beam with radiant power $P_0$ is passed through an absorbing solution with thickness “$b$”. As it passes through the solution it is attenuated by the absorption of the reaction solution, i.e. by interaction between the photons and the absorbing components which are present in the solution with concentration “$c$”. The remaining radiant power of the beam is $P_r$, the radiant power absorbed is $P_a$.

The extent to which this phenomena takes place is expressed in Transmittance “$T$” and Absorbance “$A$”. Where $T$ is the fraction of incident radiation that is passed through (transmitted) by the solution: (The term Absorption, the fraction of incident radiation that is absorbed by the solution, so Absorption = $P_a/P_0$, is not so much in use)

$$T = P_r / P_0$$

$$A = - \log T = \log P_0 / P_r$$

A (Absorbance) is the negative logarithm of the transmittance: (An older term not officially used anymore is E “Extinction”)

Transmittance is usually expressed in percentage of the measured radiant power through a solution with no absorbing components present. This is normally the solvent, often water, which is then said to have a transmittance of 100%. The transmittance of a solution with absorbing components then has a certain value less than 100%. As a consequence the absorbance value will increase as the transmittance decreases. This value has no unit, but is yet often expressed in Absorbance Units (AU). If for instance a solution with absorbing components has a transmittance compared to the solvent of 10 %, the absorbance will have a value of: $A = \log (100/10) = 1$ AU or 1000 mAU (milli AU).
As can be expected, the absorbance of a solution is in relation with the concentration of the absorbing components in the reaction solution "c", their capacity to absorb the radiation of a particular wavelength "\( \varepsilon_{\lambda} \)" and the path length that the beam of radiation travels through the solution “b”. This relation is known as a statement of:

**Beer’s law**  \( A = \varepsilon_{\lambda} \cdot b \cdot c \)

Note that \( \varepsilon_{\lambda} \), also known as the ‘Molar absorptivity’ or ‘Molar extinction coefficient’, is very much dependent on the wavelength of the beam of radiation. This becomes apparent when looking at a typical wavelength absorbance spectrum of a solution in which Beer’s law normally is applied, i.e. the UV-Visible range of approx. 200 - 800 nm.

![Absorbance spectrum](image)

Clearly in this spectrum the absorbance varies very much as a function of wavelength. Obviously the most sensitive analysis method would be attained when the measurement takes place at the wavelength where the absorbance is maximal, \( \lambda_{\text{max}} \).

(Also at other wavelengths in the spectrum may be measured. This would be advantageous if the sample has already an own color at \( \lambda_{\text{max}} \). This would be rarely the case however.)

b) **Limitations.**

Apart from instrumental and chemical deviations there is a real limitation of Beer’s law; it describes the absorption behavior of fairly dilute solutions only. At high concentrations the interactions between the absorbing components are increased to the point where their ability to absorb a certain wavelength is altered. This is not only limited to the absorbing components (color dye) themselves, but may also be caused by other present components at higher concentrations, particularly electrolytes. Moreover this may also have a severe effect on the refractive index of the reaction solution, which in turn will influence the molar extinction coefficient of the color dye.

The main instrumental limitation concerns the light that is applied: In order to comply with Beer’s law theory, monochromatic light should be applied. However in practice, this is approached by means of a continuous light source (with a broad emission spectrum) in combination with a monochromator or filter. This will result in a beam of light with a certain bandwidth. The choice of bandwidth is a compromise between instrumentation costs and performance; A narrower bandwidth is achieved by placing greater demands on instruments design. On the other hand a broader bandwidth will result in a bigger deviation from Beer’s law. Among other causes like chemical deviations and other instrumental imperfections, this is the most important reason for deviation from the linearity of Beer’s law.

This becomes evident when Beer’s law is taken for a number of wavelengths with each wavelength having a different extinction coefficient. Consider a situation with only two wavelengths, n and m:

\[
A = \text{LOG} \frac{(P_0^n + P_0^m)}{(P_r^n + P_r^m)} \Rightarrow A = \text{LOG} (P_0^n + P_0^m) - \text{LOG} (P_0^n \cdot 10^{-\varepsilon_{m,b,c}} + P_0^m \cdot 10^{-\varepsilon_{m,b,c}})
\]

This function is not a linear one. Only if \( \varepsilon_n = \varepsilon_m \) the equation simplifies to:

\[ A = \varepsilon \cdot b \cdot c \]
However, if the used radiation does not comprise a spectral region in which the absorbance changes a lot as a function of wavelength, the deviations are acceptable. This is illustrated at right.

For colorimetric analysis the demand for very narrow bandwidth light (< 10 nm) is not high since most spectra in the visible range have a smooth path with wide peaks.

Another important instrumental limitation is the noise level. The noise in the photon detector signal reading limits the range of measurement. Luckily the lowest concentration range, at low absorbance, is at 100% transmission, i.e. a highest detector signal.

<table>
<thead>
<tr>
<th>Transmission % (⇔ signal)</th>
<th>Absorbance mAU (⇔ concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>1</td>
<td>2000</td>
</tr>
<tr>
<td>0.1</td>
<td>3000</td>
</tr>
</tbody>
</table>

This means that at low concentration, small absorbance deviations can be detected. However some noise is always present, so there is a limit to what extent deviations can be measured. When the concentration increases, the transmission will go down and the noise will go up. Eventually the noise in relation to the signal is so big that this will limit the range at high concentration.

The relation of signal and noise is depicted below. Here the noise is related to the Absorbance signal (not the Transmission) which is after all related to the concentration searched for.

At concentration zero a remaining absolute error, i.e. instrumental noise, exists. When related to the absorbance difference ($\Delta A$, see 4)Analysis Methods) at concentration zero, which is normally zero, the relative error is very big. The relative error then quickly decreases as $\Delta A$ increases while the absolute error does not change significantly. Between point a and b the absolute error increases evenly with $\Delta A$, which means that the relative error is quite constant.

Beyond point b, the absolute error increases more steadily because the transmission, i.e. the amount light measured by the photon detector, is rapidly becoming less. This causes the relative error to increase as well, and soon the relative error is so big that a good measurement is not possible anymore.

The limitation of noise is of course dependent on the quality of the components and the design of the photometer. Important aspects are: Stability of the light source and the signal processing unit, elimination of stray light and electro-magnetic interference from the surroundings.

A typical usable absorption range is 0 to 2000 mAU.

Chemical deviations refer to those phenomena concerning the color forming reactions, see 2c) Selectivity, such as incompleteness of the reaction, dissociation of the absorbing component and interference from competing reactions.

Despite these limitations, colorimetry is generally a sensitive analysis method. If the selectivity towards other components is sufficient, often measurement in the low $\mu$g/l (ppb) range is possible.
c) **Selectivity.**

Just occasionally the component that is searched for, the analyte A, also is the absorbing component in the sample solution. Usually an additional reaction with a color reagent R has to take place to form a color dye C, which is an indicator for the analyte: \[ A + R \rightarrow C \]

There are numerous reagents known that are able to form a color dye. Sometimes a secondary reaction must take place with another reagent (e.g. an oxidator or reductor) to form the final color dye or to form a more stable product: \[ C + R' \rightarrow C' \]

Normally the colorimetric method concerns a color development. However in some applications the method concerns a color decrease. In these cases typically a colored reagent is added to the sample first. This initial color is measured. The analyte in the sample reacts, sometimes only after the addition of a second reagent, resulting in a consumption of colored reagent and the formation of a non-colored component (N), and therefore in a decrease of color: \[ C + R'' \rightarrow C'' + A \rightarrow N \]

So the de-coloration is a measure for the concentration in this case.

Often all these reactions take place under specific circumstances, for instance at a certain pH. This circumstance may be created by the addition of a buffer. To enhance the formation of the color dye it is usually necessary to keep the temperature at a reasonable level; normally room temperature is sufficient. At low temperatures the formation may be very slow or not taking place at all. High temperatures may have a negative effect as the color dye may (increasingly) disintegrate.

Ideally the color reaction is specific for the concerning analyte so that undesirable reactions will not interfere the analysis. At least a higher selectivity of the reagent towards the analyte over other components is necessary. However, this is not always the case. Many color reagents form color dyes with various components which will have a contribution to the absorbance at the same wavelengths moreover. When the interfering component (I) is present in significant concentration, measures must be taken to attain a selective analysis method.

One of them can be a change in composition of the reaction solution, for instance to change the pH to a level where reaction with the analyte is favorable against interfering reactions. Another would be to perform a preceding reaction with the interfering component to block out, or mask, its influence on the color formation.

This is realized by the addition of a masking reagent (M) which forms a non-interfering component NI.

\[
\text{Masking step: } A + I + M \rightarrow A + NI \\
\text{Color formation step: } A + NI + R \rightarrow C + NI
\]

**EXAMPLE:**

The determination of Silica with the color reagent Molybdate. Soluble Silica reacts with the Molybdate ion in an acid solution to form a green-yellow colored Silicomolybdic acid complex that in turn is converted to a blue complex by reduction with ascorbic acid. Present Phosphate ions will also form a colored complex, even more readily than Silica. Oxalic acid is added to minimize this phosphate interference because it de-composes the Phosphate complex. At the same time a catalytic acceleration of the color development of the Silicomolybdic blue complex is achieved.

\[
\begin{align*}
\text{Si} & \quad + \quad \text{PO}_4^{3-} \quad + \quad 2 \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \quad + \quad x \quad \text{H}_3\text{O}^+ \\
& \quad \Rightarrow \quad [\text{Si} \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]_\text{complex} \quad + \quad [\text{PO}_4^{3-} \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]_\text{complex} \\
[\text{Si} \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]_\text{complex} & \quad + \quad [\text{PO}_4^{3-} \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]_\text{complex} \quad + \quad \text{H}_2\text{C}_2\text{O}_2 \\
& \quad \Rightarrow \quad \text{PO}_4^{3-} \quad + \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \quad + \quad [\text{Si} \quad (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]_\text{complex}
\end{align*}
\]

The absorption of this Silica complex can be measured at a wavelength of 880 nm.

Altogether colorimetric analysis need careful tuning to make a robust and selective method possible. On the other hand, the implementation of colorimetric analysis is fairly straightforward, as described in the next paragraph.
3) HARDWARE:

a) Platforms and modules:

Colorimetric analysis in the Applikon Analyzers (ADI2040, ADI2019, ALERT Colorimeter) can be performed by means of a Cuvette module or a LED Dipping Probe, see Hardware & Installation Part of the manuals. In both modules a LED is used as a light source. A LED emits a light spectrum over a limited range (also called ‘bandwidth’), depending on the type and which is in some cases limited enough to realize acceptable calibration lines.

The following wavelengths are available:

<table>
<thead>
<tr>
<th>LED</th>
<th>Wave length (nm)</th>
<th>Color reaction solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PURPLE BLUE</td>
<td>430</td>
<td>Yellow</td>
</tr>
<tr>
<td>BLUE</td>
<td>470</td>
<td>Yellow</td>
</tr>
<tr>
<td>BLUE GREEN</td>
<td>505</td>
<td>Red</td>
</tr>
<tr>
<td>AQUA GREEN</td>
<td>520</td>
<td>Red</td>
</tr>
<tr>
<td>GREEN</td>
<td>565</td>
<td>Blue</td>
</tr>
<tr>
<td>AMBER</td>
<td>590</td>
<td>Blue</td>
</tr>
<tr>
<td>ORANGE</td>
<td>610</td>
<td>Red</td>
</tr>
<tr>
<td>RED</td>
<td>650</td>
<td>Green</td>
</tr>
<tr>
<td>INFRA RED</td>
<td>875</td>
<td></td>
</tr>
</tbody>
</table>

A more dedicated range can be realized in combination with an interference filter, which has a typical bandwidth of less than 10 nm. See illustration aside of a 430 nm LED (which has a very big bandwidth) in combination with a 405 nm filter. This option is only available in the cuvette module.

Other advantages of the cuvette module over the LDP are:

- Reduction of air-bubble problem (glass cuvette is not hydrophobic),
- Better Chemical inertness (only glass and PTFE),
- Less temperature drift (LED source is stabilized and not immerged in reaction solution),
- Longer light path (3 cm) (which gives higher sensitivity, see Beer’s Law),
- Thermostat included in the module (to ensure that samples are measured at room temperature).

As illustrated in the picture beside, the light of the source is guided through a collimating lens before it passes through the cuvette. Optionally an additional (interference) filter may be installed, which are available for many wavelengths in the visible spectrum. So the combination of the applied LED and the applied filter determine the ultimate wavelength and intensity. The selection of those are dictated by the concerning application. On the other side of the cuvette the beam is caught by a lens and then detected by a photon-diode which converts the light in an electric signal. This signal is connected to a Sensor Card LED probe.
Note:
- The cuvette module and the LDP are used with the same sensor card.
- The minimal volume is necessary to perform measurements, i.e. to immerse the light path; for the cuvette minimal 8 ml, for the LDP minimal 20 ml.

Whether or not a filter should be included is very much dependant on the application. The effect of the inclusion of a filter is illustrated with the following examples:

**Dichromate:**

A comparison is made between a LED 430 nm in a LDP (light path 1 cm) and a Cuvette (light path 3 cm) and a cuvette with 405 filter. The very big bandwidth of the 430 nm LED used in a steep part of the absorption spectrum of Dichromate, results in a very curved calibration line, i.e. Beer’s law is not obeyed. An enormous improvement is realized when a filter is included although also applied in a steep part of the spectrum. Only then the (3 times) higher sensitivity potential of the 3 cm light path of the cuvette is demonstrated. Just at higher concentration at approx. an absorbance of 2500, a non-linearity is evident.

**Nickelsulfate:**

The same comparison here, however now with a reasonable narrow bandwidth LED and now measuring in a flat part of the spectrum of Nickel-Sulfate. It clearly shows in the pictures that Beer’s law is obeyed for all options. A minimal improvement is gained when a filter is applied.
b) Other hardware

All sorts of sampling devices may be applied, while addition of reagents can normally be done with sufficient accuracy by means of low flow tubing pumps. In some cases addition with a burette is necessary to meet chemical resistance requirements or in case very little reagent volumes are required. The effect of volumetric errors on the accuracy of the analysis is not very critical, provided that the reagent volume is significantly smaller in relation to the sample volume.

This is best illustrated with an example (see for dilution factor PARAMETERS):
Suppose we take a 10 ml sample and add 2 reagents, each 1 ml. Suppose we measure a concentration of 100:

\[
\text{In the reaction solution a concentration is measured of } \quad 100 \times \frac{10}{12} = 83.3. \\
\text{(because of dilution with reagents.)}
\]

This is corrected with the dilution factor (the volumes have to be inserted in the analyzer);

\[
\text{This means: } \quad 83.3 \times \frac{12}{10} = 100.
\]

Now suppose we make an error of 1 ml in the sample volume (11 ml):

\[
\text{In the reaction solution a concentration is measured of } \quad 100 \times \frac{11}{13} = 84.6. \\
\text{This is still corrected with the dilution factor; } \quad \text{This means: } \quad 84.6 \times \frac{12}{10} = 101.5.
\]

So, despite the fact that we make a profound error of 10 % in the sample volume, only a 1.5 % error on the final result is caused.

In the same example the effect of a 10 % error (too little) on the reagent additions is illustrated:

\[
\text{In the reaction solution a concentration is measured of } \quad 100 \times \frac{10}{11.8} = 84.7. \\
\text{This is still corrected for with the set volumes; } \quad \text{This means: } \quad 84.7 \times \frac{12}{10} = 101.7.
\]

So, a 1.7 % error on the final result is caused.

c) Pre-conditioning

Colorimetric measurements require (ideally) that the sample is not turbid by particles of some kind or is otherwise unclear. (The colorimetric method applied in the Applikon Analyzers however, can correct for some turbidity or own color of the sample, see 4 a) Analysis procedure.) This explains why filtration is the most obvious operation in a possible pre-conditioning system. Other operations may be necessary as well, e.g. cooling, pressure reduction, just like it could be applied in any other application when necessary. Because colorimetric methods are often very sensitive, i.e. measuring in a low concentration range (ppb levels), dilution is also an obvious operation. There are numerous ways to realize dilution, all of them will have a negative effect on the accuracy/repeatability however.
4) ANALYSIS METHODS

a) Procedure

There is no special analysis method module for colorimetric measurement other than a Drift Controlled Measurement (DCM) of a sensor input, in this case a LED input. The calculation of the analysis result however, requires a particular result processing method involving a calibration line.

An analysis Time Program would typically have the following structure:

<table>
<thead>
<tr>
<th>Start</th>
<th>Clean vessel</th>
<th>take sample</th>
<th>add misc. Reagents</th>
<th>initial meas. $A_0$</th>
<th>add color Reagents</th>
<th>Color development</th>
<th>Final meas. $A_1$</th>
<th>result processing</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After rinsing the cuvette or reaction vessel and taking a sample, one or more miscellaneous reagents, like buffer or masking agent, may be added after which an initial absorbance measurement takes place. This initial absorbance is subtracted later from the final measured absorbance; in this way interfering factors are eliminated such as sample own color or turbidity, miscellaneous reagents own color and refractive index variations.

The final measurement takes place after addition of color reagent. Next a color development takes place, with a certain pace, depending on the color reaction.

Note that this way of setting up the analysis method is very powerful, as is demonstrated in the following:
Both absorbencies are now processed in the following way:

$$A_1 - A_0 = \Delta A = \log \frac{T_{100\%}}{T_1} - \log \frac{T_{100\%}}{T_0} = \log \frac{T_0}{T_1}$$

This absorbance $\Delta A$ is now used in the calibration line, as well in the calculation of the eventual analyte concentration. This means that the absolute absorbance readings are not important, rather the difference in reading is taken into account. In this way turbidity, refractive index and own colors are cancelled out.

b) Calibration.

Colorimetry is a relative method, therefore a prior calibration is needed before quantitative measurement can be performed. This is done by feeding the analyzer different standard solutions and analyze them in the same way as the sample.
The readings of the standard solutions are averaged and used in a least square regression method to derive a calibration line. This is often a linear function, but also curved lines could be used if deviations from Beer’s law are evident. In this case the curve is often described with a polynomial function.

Once the calibration line is determined, analysis of the sample may be performed. In the result processing routine the concentration now has to be calculated from the measured $\Delta A$. This can be simplified if the calibration line is derived for concentration as a function of absorbance, so reverse to the normal notation.
When for example a third grade polynomial function is used (See further):

Normal notation: \[ \Delta A = C_0 + C_1 \cdot [c] + C_2 \cdot [c]^2 + C_3 \cdot [c]^3 \]

Finding \([c]\) for a measured \(\Delta A\), three solutions are possible which can be found with mathematical operations (iteration). A reverse notation makes the derivation of \([c]\) simple:

Reverse notation: \([c] = C'_0 + C'_1 \cdot \Delta A + C'_2 \cdot \Delta A^2 + C'_3 \cdot \Delta A^3\)

In principle the calibration must be performed just once. Fouling or other variations in the light intensity, do not effect the shape of the line, nor does variation in sample or reagent coloration. Alterations in volumes do effect the calibration line so they should not change. But even if sample volume has changed (e.g. with pipette replacement) this can simply be corrected for in the calculation of the result.

A possible regular correction of the line may be performed for the offset. This is useful in case that one or more reagents contain a trace of analyte (which cannot be avoided sometimes), or when the color reagent which is added after the initial absorbance has an unstable own color. This correction may be done automatically by performing an analysis of a blank or standard solution. With this value the offset of the calibration line can be corrected. However, by way of precaution and as a way for validation, a validity check of the offset value should be done (by means of an Conditional Action in the result processing item) in order to avoid erroneous analysis results for the sample.
For this reason a complete automated calibration is not recommended, nor is it necessary.

5) PARAMETERS:

A distinction in analyzer types is present for the concerning parameters:

- **ADI2040:**
  - No dedicated colorimetric analysis module: DCM + freely programmable result processing, use inverse calibration line notation.

- **ADI2019HD:**
  - Automated calibration module + dedicated result processing using the obtained calibration line.

- **ADI2019 Special:**
  - Manual calibration line insertion + dedicated result processing using the calibration line.

- **ALERT Colorimeter:**
  - Manual calibration line insertion + dedicated result processing using the calibration line.

a) **Volume Constants:** (2019HD, 2019 Special, ALERT Colorimeter; UTILITY Constants menu)

- The 2019HD automatic calibration routine, delivers a calibration line which is normalized for volumetric aspects, i.e. which is corrected for dilution effects of reagent additions. This means that in the calculation of the result derived from the calibration line, the dilution factor is used to correct for dilution:

  \[ \text{Dilution factor} = \frac{\text{Sample volume}}{\text{Sample volume} + \text{Reagent volume}} \]

  The use of the volume constants in the ADI2019HD is mandatory.

- The ADI2019 Special and ALERT Colorimeter also use a calibration line. Optionally the volume constants can be used for the dilution factor. This depends on the applied calibration line. If a normalized calibration line is applied the actual values for the volume constants must be used. A normalized calibration line is obtained in a 2019HD calibration program (and can be used in other analyzers.

  If a dedicated calibration line is inserted, i.e in which volume effects have been calculated in, this is expressed in the coefficients of the calibration line. Now no dilution factor is necessary; in this case fill in for the sample volume 1 and for the reagent volume 0.

  A dedicated calibration line is obtained by feeding discrete standard solutions of different concentration and derive the calibration line from the analyzed absorption differences which are not corrected for dilution effects.
b) **Slope factor** (2019HD, 2019 Special, ALERT Colorimeter; UTILITY Constants menu)

For colorimetric method with a color development a positive slope in the calibration line is anticipated. For methods with de-coloration a calibration line with negative slope is anticipated. This is solved in the concerning analyzers by using a slope factor; positive for color development, negative for de-coloration. This will alter the processing of the measurement data as follows:

- **Positive slope:** The initial measurement ($A_0$) is subtracted from the final measurement ($A_1$).
- **Negative slope:** The final measurement ($A_1$) is subtracted from the initial measurement ($A_0$). This will result in a normal positive calibration line again.

**c) Number of standards:**

This parameter is only available in the automatic calibration routine of the ADI2019HD (CALIB Macro), wherein the concerning standard concentrations are created by adding a concentrated standard solution in a blank solution (in the reaction vessel or cuvette) with a burette. The concentration of the used standard in the burette must be inserted in a constant.

Notes:
- The obtained calibration line from the ADI2019HD is a normalized line (see Volume Constants). Therefore it may be used in other analyzers if the actual volume are inserted as constants.
- The maximum concentration created with the burette is 10% of the concentrated standard solution in the burette.
- The theory about standard concentrations and calibration lines as described here, can also be used when standards are created manually and calibration lines are calculated outside the analyzer (normalized or dedicated).

For the choice of standard concentration points the following approach is statistically favorable:

- **If a linear calibration line is expected just the blank and 100% values (i.e. with zero analyte concentration and the maximal expected concentration) is needed.** Multiple analysis of the standard that are averaged will gain more reliability of the calibration line.

The calibration line can be noted as: $\Delta A = C_0 + C_1 \cdot [c]$  
(Offset + Slope * Concentration)

Where the Slope takes account for the molar extinction coefficient, the path length and dilution factors, and the Offset takes account for possible contributions of the color reagent and blank solution.

- **If a non-linear line or curve is expected more values are necessary.**

To describe a second order polynomial curve with the formula: $\Delta A = C_0 + C_1 \cdot [c] + C_2 \cdot [c]^2$, at least 3 point are needed.

To describe a third order polynomial curve with the formula: $\Delta A = C_0 + C_1 \cdot [c] + C_2 \cdot [c]^2 + C_3 \cdot [c]^3$, at least 5 point are needed.

An optimal distribution of the standard concentrations as a function of the number of calibration points is given in the following table:

<table>
<thead>
<tr>
<th>number \ standard</th>
<th>0 %</th>
<th>25 %</th>
<th>35 %</th>
<th>50%</th>
<th>65 %</th>
<th>75 %</th>
<th>100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

If for example a calibration line has to be determined between 0 and 1000 µg/l, the following standards will gain most statistically valuable data: 0, 250, 350, 500, 650, 750 and 1000 µg/l. So the use of up to 7 points on the line is possible to obtain maximal information and therefore the most appropriate calibration line. Of course also here the repetition of the calibration points analysis will also lead to a more reliable calibration curve.
d) Drift factor

The development of the color takes some time (usually minutes) to reach a steady state. This is dependent on the specific reaction rate for the concerning substances, which is also influenced by temperature and concentrations.

- ADI2040:
  With the Drift Controlled Measurement a Drift factor and a Drift Interval can be set in order to take the absorbance reading when it has stabilized. The lower the drift factor and the higher the drift interval, the more stable the signal must be before it is taken. Normally 1-3 mAU and 10 sec res. are good settings for accurate measurements. When high analysis frequency is required, a higher drift factor may be used.

- ADI2019HD/Special/ALERTColorimeter: MEASURE macro
  Also here a drift controlled measurement is applied, but only the Drift factor can be set. The Drift Interval is not a parameter and is fixed on 10 sec.

The drift control can be ignored by choosing a high factor (e.g. 1000 mAU), this means that the drift factor is immediate met, and the absorbance reading is taken.

e) Wait time

Before the drift control is started, optionally an initial wait period can be respected. In general a short waiting time (normally 30 s) is applied to allow the photometer to stabilize and for the color reaction to get started.

Furthermore the wait time may be used for a previous reaction (to the color reaction) which progress cannot be detected with color absorbance measurement.

Also in some special applications where a steady state situation is not reached, the wait time is used (instead of a drift control) after which the absorbance reading is taken. In this case the drift factor should be chosen very high to ignore the drift control (see Drift factor).

With all analyzers the Wait Time can be set in seconds:
ADI2040, DCM item, ADI2019HD/Special/ALERTColorimeter: MEASURE macro

f) Offset correction (2019HD, 2019 Special, ALERT Colorimeter; OPUTPUT macro)

To automatically correct for color change of reagents, own color of sample or fouling of the cuvette, a regular standard analysis may be performed. The result of this standard analysis can be used to correct the offset of the calibration line. The concentration of the standard should be set as a constant. This option can be put ON of OFF.

In the ADI2040 an automatic offset correction is also possible in a result processing item.