Absorbance Measurement in Cuvettes Lying Longitudinal to the Light Beam

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Measurement in cuvettes with their longitudinal dimension parallel to the light beam, while under the influence of a centrifugal force, has many advantages that are not immediately apparent. This survey summarizes the advantages in comparison to cuvettes standing with their long axis perpendicular to the light beam: in the latter the pathlength is fixed by the geometry, but if the cuvette is lying longitudinal to the light beam the pathlength is given by the filling volume. Because of the dependence of the pathlength on the filling (reaction) volume when cuvettes are lying longitudinal to the light beam, the calculation formulas show that the calculation factors, both for enzyme and substrate determination, become independent of the reagent volume form. This is demonstrated by simple equations and confirmed in practice by use of the (Cobas) Bio centrifugal analyzer in assays for glucose and aspartate aminotransferase.

Additional Keyphrases: centrifugal analyzer · (Cobas) Bio system · spectrophotometry · analytical systems

Nowadays, most photometric determinations are done with use of a constant optical pathway, $b$, which is determined by the geometry of the cuvette and ordinarily is 1 cm. The precision of the light pathway for glass or quartz cuvettes is ~0.01 mm for the usual dimensions of either 0.5 or 1 cm. The pathlength for disposable, less-expensive plastic cuvettes seems to be specified to only ±0.05 mm.

For studies in the microlitre and millilitre ranges, there are special cuvettes that are so constructed that smaller reaction volumes can be used, commonly still with a 1-cm pathlength. Special cuvettes are available for use in measurements in the microlitre range as well as in the milliliter range.

In the method described here, absorbance is measured in cuvettes that are lying with the longitudinal dimension parallel to the light beam, a method that resembles the original Duboscq colorimetric method in that the optical pathlength can be varied by changing the filling volume. The larger the filling volume of the cuvette, the longer the optical pathlength for the light beam through the solution for which the absorbance is to be measured.

Principle of Measurement

Figure 1 shows diagrammatically the measurement of absorbance in cuvettes lying longitudinal to the light beam is shown. Passing the light beam longitudinally through the cuvette filled with the reaction mixture (sample + reaction solution) has several advantages, and offers greater flexibility for developing and optimizing purposes. These are discussed below.

**Independence of the result from possible errors in pipetting reaction solutions.** On the basis of the laws of Lambert and Beer in clinical chemical determination, the following general equation is used:

$$ A = a \times b_c \times c \times \frac{SV}{TV} $$

(1)

where

- $A$ = absorbance measured at wavelength $\lambda$
- $a$ = molar absorptivity at wavelength $\lambda$
- $c$ = concentration, in mol/L
- $b_c$ = pathlength of the cuvette, in cm
- $SV$ = sample volume
- $TV$ = total volume of reaction mixture

The advantage of measuring absorbance in cuvettes that are lying with their longitudinal axis parallel to the light beam can readily be shown by a simple analysis of error. Generally:

$$ c = \frac{A \times TV}{a \times b_c \times SV} $$

(2)

For cuvettes lying with their longitudinal axis perpendicular to the light beam, the total volume is calculated by

$$ TV = r^2 \times b_c $$

(3)

assuming a square cuvette with a cross section dimension of $r$.

The advantage of using cuvettes lying with their longitudinal axis parallel to the light beam is that the pathlength depends on the filling volume given by the total volume. Therefore, equation 2 simplifies to:

$$ c = \frac{A \times r^2}{a \times SV} $$

(4)

With cuvettes standing with their longitudinal axis perpendicular to the light beam, the light path $b_c$ is fixed and independent of the total filling volume of the cuvette. The equation would be

$$ c = \frac{A \times 1}{a \times b_c \times \frac{TV}{SV}} $$

(5)

Using simple equations for calculation of error for equations 4 and 5, an error is determined for a cuvette with a specified fixed pathway ($b_c$):

$$ dc = \frac{\partial c}{\partial A} dA + \frac{\partial c}{\partial b_c} db_c + \frac{\partial c}{\partial SV} dSV + \frac{\partial c}{\partial TV} dTV $$

(6)

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and for a cuvette with variable pathway:
\[
dc = \frac{dc}{DA} dA + \frac{dc}{DSV} dSV
\]
(7)

The relative error is therefore, for a fixed light path:
\[
\frac{dc}{c} = \frac{dA}{A} + \frac{db}{b} + \frac{dSV}{SV} + \frac{dTV}{TV}
\]
(8)

and for a variable light path:
\[
\frac{dc}{c} = \frac{dA}{A} + 2\frac{dr}{r} + \frac{dSV}{SV}
\]
(9)

On comparing equations 8 and 9, it is evident that an error in the total volume:

\[
TV = SV + DV + RV + SRV
\]
(10)

where
- \(SV\) = sample volume
- \(DV\) = diluent volume
- \(RV\) = reagent volume
- \(SRV\) = start reagent volume

is irrelevant to the result. Possible volume errors in pipetting diluent and reagent generally play no part.

The more reagent pipetted, the longer the pathway. This is shown in equation 3 by the mutual dependence of these factors.

Example: Assume that there was a pipetting error of 10% in the reagent volume, which does not absorb at the chosen wavelength. With cuvettes standing perpendicular to the light beam, the term \((1/b)(TV/SV)\) changes the dilution term \(TV/\text{SV}\) by 10% and the measured absorbance by 10%, because \(b\) is fixed.

With cuvettes lying with their long axis parallel to the light beam, the pathlength \(b\) depends on the filling volume. If the filling volume (= total volume) changes by 10%, the pathlength also changes by 10%, and the absorbance remains constant.

If the reagent absorbs at the chosen wavelength—for example in the determination of the aspartate aminotransferase (EC 2.6.1.1)—for all kinetic determinations (enzyme determination, fixation-time analysis, initial rate, and autoblinking procedures), any pipetting error in reagent volume plays no role. The great advantage of using cuvettes lying with their long axis in the light beam is that only an error in the pipetting of the specimen volume has to be considered. The influence of an error in the filling volume is of minor importance. An error in the pipetting volume of 1 \(\mu\)L corresponds to an error in the pathlength of 0.004 cm. Ordinarily the specimen volume varies between 3 and 25 \(\mu\)L, and the dispensing precision for it is always less than ±2%; the reagent volume is ordinarily between 80 and 150 \(\mu\)L, the dispensing precision less than ±1%.

However, if more absorbance measurements—not differences in absorbance measurements—are performed, the error in the filling volume naturally plays a role, but only in the same way as if the cuvette were standing with its long axis perpendicular to the light beam. Any error in the pipetting volumes of the specimen or reagent changes the relation between total volume and sample volume when cuvettes standing with their long axis perpendicular to the light beam are used, and changes the pathlength when cuvettes lying with their longitudinal axis parallel to the light beam are used.

From equation 9 the only disadvantage of the latter type of cuvette can be seen. An error in side length of the square cuvette has twice the effect on measurement with a variable optical pathway than with a fixed pathway. However, as the following estimate shows, the error is in either case relatively small.

If the length of the side of a square cuvette is 5 mm, a 1-cm light pathway corresponds to \(5 \times 5 \times 10 = 250 \text{ mm}^3\) (\(\mu\)L). An error of 0.01 mm produces a relative error of 0.4% (in comparison, for a cuvette with a fixed light path of 0.5 cm and the same error of ±0.01 mm, the relative error is only 0.2%).

The size of the calculation factor depends only on the sample volume used, not on the ratio of total volume to sample volume. With many enzymatic determinations, calculation requires a large factor, usually given by the equation

\[
\text{Activity, } U/L = \frac{1}{a \times b} \times \frac{TV}{SV} \times \frac{\Delta A}{\text{min}}
\]
(11)

where \(F^c = \frac{1}{a \times b} \times \frac{TV}{SV} = \) calculation factor for cuvettes with fixed light path.

For measurements with variable light path, substituting from equation 3 reduces equation 11 to:

\[
\text{Activity, } U/L = \frac{r^2}{a} \times \frac{1}{SV} \times \frac{\Delta A}{\text{min}}
\]
(12)

\(F^v = \frac{r^2}{a} \times \frac{1}{SV} = \) calculation factor for cuvettes with a variable light path.

It is well known that the larger the calculation factor, \(F^c\) or \(F^v\), and the smaller the resolution of the photometric system, the larger will be the error in the determination. An appropriate factor is derived by suitable choice of sample and reagent volumes and of light path. The following variables must be considered:

- range of linearity (depends on the ratio of \(SV\) to \(RV\))
- range of absorbance (depends on specific absorbance of reagents and linearity of photometer)
- most favorable range for measuring absorbance (depends on the optical-electronic system of the photometer)

Example 1: Aspartate aminotransferase determination

\[
SV = 50 \mu\text{L}
\]
\[
RV = 250 \mu\text{L}
\]
\[
350 \mu\text{L}
\]
\[
500 \mu\text{L}
\]
\[
a_{354\text{nm}} = 6.18 \times 10^3
\]

For cuvettes with a fixed light path, \(b_c = 1 \text{ cm}\):

\[
F^c_{RV=250\mu\text{L}} = 971
\]
\[
F^c_{RV=350\mu\text{L}} = 1295
\]
\[
F^c_{RV=500\mu\text{L}} = 1780
\]

For a variable light path with side \(r = 0.5 \text{ cm}\):

\[
F^v_{RV=250\mu\text{L}} = 809, b = 1.2 \text{ cm}
\]
\[
F^v_{RV=350\mu\text{L}} = 809, b = 1.6 \text{ cm}
\]
\[
F^v_{RV=500\mu\text{L}} = 809, b = 2.2 \text{ cm}
\]
With a variable light path, the calculation factor for all kinetic determinations is independent of the reagent volume used.

The only restriction is that the absorbance not be too high. With a constant factor (= 809), an optimal compromise between initial absorbance and linearity can be reached by a suitable choice.

Example 2: Glucose determination [hexokinase (EC 2.7.1.1)/glucose-6-phosphate dehydrogenase (EC 1.1.1.49)]

\[
\begin{align*}
SV &= 5 \ \mu L \\
RV &= 250 \ \mu L \\
350 \ \mu L \\
500 \ \mu L \\
\end{align*}
\]

For cuvettes with a fixed light path, \( b_c = 1 \) cm:

\[
\begin{align*}
F_{RV} &= 8.3 \\
F_{RV} &= 11.5 \\
F_{RV} &= 16.3 \\
\end{align*}
\]

For a variable light path with side \( r = 0.5 \) cm:

\[
\begin{align*}
F_{RV} &= 8.1, \ b = 1.02 \ cm \\
F_{RV} &= 8.1, \ b = 1.42 \ cm \\
F_{RV} &= 8.1, \ b = 2.02 \ cm \\
\end{align*}
\]

Because glucose determination by the hexokinase method is a reaction in which absorbance increases, a wider range of linearity can be obtained very easily by using a larger volume of reagent and keeping the calculation factor constant. This is not possible with cuvettes with a fixed light path.

Absorbance is independent of possible evaporation from the reaction mixture. Because the measured absorbance depends only on the product \( b \times c \), evaporation from the reaction mixture generally plays no part. The pathway, and thus the concentration, changes with evaporation, but the product remains unchanged:

\[
b_1 \times c_1 = b_2 \times c_2
\]

(13)

where \( b_1, c_1 \) are the concentration and pathway before evaporation and \( b_2, c_2 \) the concentration and pathway after evaporation.

In cuvettes with a fixed light path, this effect of evaporation causes errors (the solution concentrates, but \( b_c \) is constant).

No correction is necessary for the absorbance of the analysis blank and for analysis when measuring at different final volumes. Determinations are often performed with "interrupted" pipetting schemes. Absorbance is measured at intervals. This technique is applied mostly to "start reactions," in which the absorbance of the test blank, \( A(TB) \), is measured first in a mixture of reagent (but lacking the essential component) and sample. The specific reaction is then started by adding the essential component and the analysis absorbance, \( A(A) \), is measured:

Interrupted pipetting scheme

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>( \mu L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent lacking essential component</td>
<td>( a )</td>
</tr>
<tr>
<td>Sample</td>
<td>( b )</td>
</tr>
<tr>
<td>Mix well, measure ( A(TB) )</td>
<td></td>
</tr>
<tr>
<td>Reagent with essential component</td>
<td>( c )</td>
</tr>
<tr>
<td>Mix well, measure ( A(A) )</td>
<td></td>
</tr>
</tbody>
</table>

If cuvettes with a fixed pathway are used, \( A(TB) \) is measured with a reaction volume \((a + b) \ \mu L \) and \( A(A) \) with a reaction volume \((a + b + c) \ \mu L \). If \( c \) is not much smaller than \( a + b \), the absorbance measured first (the test blank) must be corrected for:

\[
A(TB)_{corrected} = A(TB) \times \frac{a + b}{a + b + c}
\]

(14)

This correction is unnecessary for measurements using cuvettes lying with their long axis parallel to the light beam, because with a change in filling volume the pathway of the light beam also changes.

Examples are: the determination of iron (\( c \) = solution of bathophenanthroline); the determination of triglycerides (\( c \) = glycerokinase suspension); and the determination of glucose (\( c \) = glucose-6-phosphate dehydrogenase solution).

Absorbance can be measured in the most favorable photometric range, by choice of appropriate filling volume of the reaction mixture in the cuvette. In general, photometric accuracy is not the same in all absorbance ranges. In previous apparatuses, which had no linear absorbance scales and measurement-range switches, the highest photometric accuracy was reached theoretically in the range 0.3 to 0.6 \( A \). This is no longer valid, because of the error-free working of amplifiers and improved instrumental sensitivity. Depending on the range, a reading that is within \( \pm 0.5\% \) of the true value, or 0.001 \( A \) (whichever is greater), is obtained with good photometers.

Generally, the best absorbance range for measurement is specified by the supplier of the photometer or determined by evaluating the change in coefficient of variation of an absorbance measurement as a function of measured absorbance.

The optimal absorbance range can be determined easily by varying the light path, which is done by varying the filling...
Fig. 3. Linearity of the relation between filling volume of a solution of NADH in water and absorbance
25 μL on the abscissa corresponds to 1 mm of light path

\[ A = a \times b \times c \]

or

\[ A = a \times \frac{FV}{r^2} \times c \]  \hspace{1cm} (15)

where \( FV \) = filling volume in the cuvette
\( r \) = side of the cuvette lying perpendicular to the light beam.

**Materials**

For checking linearity, the following substances in a water solution were used: uric acid (Merck, Darmstadt, G.F.R.; catalog number 413); \( \beta \)-nicotinamide-adenine dinucleotide (\( \beta \)-NADH), reduced form (Boehringer, Mannheim, G.F.R.; cat. no. 15142); and methyl orange (Merck, Darmstadt, G.F.R.; cat. no. 1322).

As test kits for the determination of glucose with hexokinase/glucose-6-phosphate dehydrogenase and the determination of aspartate aminotransferase according to the recommendations of the German Society for Clinical Chemistry, we used the commercially available test kits of F. Hoffmann-La Roche & Co. Ltd, Diagnostica.

**Procedures**

For varying the light path, different volumes, (25 to 400 μL) of the solutions of uric acid, NADH, and methyl orange were pipetted into the cuvettes. When working with 5-mm-square cuvettes, 25 μL corresponds to a light path of 1 mm. The light path was varied from 1 mm to 16 mm.

To confirm the independence of the calculation factor of the total volume, we performed the glucose and the aspartate aminotransferase determinations with use of 50 to 400 μL of reagents, and with 3 μL of specimen in the determination of glucose and 20 μL in the determination of the activity of aspartate aminotransferase.

**Results**

Figures 2–4 confirm that the light path in cuvettes lying with their longitudinal axis parallel to the light beam can indeed be varied from 1 to 16 mm. The system shows excellent linearity, even at 290 nm.

Confirmation of the independence of the calculation factor of total volumes is shown in Figures 5 and 6.

In Figure 5, at up to approximately 11 mmol/L (2 g/L), the volume of reagent could be varied from 50 to 400 μL without

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1 Shown for the first time at the 10th International Congress of Clinical Chemistry, Mexico City, Mexico, in February 1978.
altering the results. For concentrations of 22.2 (4 g/L) and 33.3 (6 g/L) mmol of glucose per liter, independence of the results of reagent volume is shown for 100 µL or greater volumes of reagent. Below 100 µL of reagent, insufficient NAD+ is present for determination of this glucose concentration.

The same is true of Figure 6, which shows the determination of the activity of the aspartate aminotransferase. For an activity of 800 U/L, 100 µL of reagent or more is required to provide enough NADH for the calculation factor to be independent of reagent volume. For lower activities, the independence could be shown very clearly.

Discussion

The enumerated advantages, and our confirmation of the variability of the light path in cuvettes lying longitudinal to the light beam and of the independence between calculation factor and total volume, show the superiority of this concept as compared with absorbance measured with centrifugal analyzers that incorporate cuvettes with a fixed light path.

Besides these advantages, there are others. For example, the light beam makes only the transition from cuvette wall to liquid to air. Consequently, reflection, scatter, absorption of the cuvette material, and refraction of light at the interface have to be considered only once.

A further advantage is that the solution to be tested is illuminated throughout the layer, and possible layer effects owing to flotation or sedimentation, such as may occur with centrifugal analyzers, only play a role by possibly changing the degree of light scattering by different distribution of particles. On the other hand, measurement of absorbance with cuvettes perpendicular to the light path is affected by possible layer effects within the cuvette, because of the fixed progress of the light path.