Replication of “Comparison of Spectrophotometric Methods for the Determination of Carboxyhemoglobin in Postmortem Blood”

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Abstract

Our replicate of (Samuel, Kahl, Zaney, Hime, & Boland, 2021) has shown that the article accurately determined the accuracy and reproducibility of the methods for determination of carbon monoxide. We also found that the multicomponent spectroscopy from (Attia A. M., et al., 2015) as described in (Samuel, Kahl, Zaney, Hime, & Boland, 2021) did not work, but in collaboration with the authors we found the correct method and the original work was corrected. The multicomponent method from (Attia A. M., et al., 2015) did not accurately replicate the true values, but the other three methods all performed adequately within their calibration curves.

Zealand

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Intro

The article is structured so that we describe our deviations from the replication and the identified inconsistencies. Then follows a comparison of our data with the original article's (Samuel, Kahl, Zaney, Hime, & Boland, 2021) data and some additional data. The Materials and Methods section is very detailed, as to avoid any confusion. This is to facilitate direct replication of the experiments. Finally, we have examples of our calculations in which one will also find the constants we have used for calculations. The original lab book is available, but in Danish.

Deviation from replication

Saturation of blood

In the article they describe that the CO-gas is bubbled through the blood for 2 hours. For safety reasons we were reluctant to leave toxic gas running for so long. We instead used a balloon-method. The method is described in "Materials and methods". The evidence for saturation is available in "Additional data".

Cuvettes

We used Polystyrol/Polysterene cuvettes instead of quartz cuvettes since we work in the visual light range.

Case samples

We only had access to five case samples. These do not include Heat/Fire exposed samples or Challenging/Decomposed samples. Only "Normal" samples.

Identified inconsistencies

Inconsistency in Calibrators

We found the description of the calibration curve varied by the section in the original paper.

In (Samuel, Kahl, Zaney, Hime, & Boland, 2021) section Calibrators, it is described that must be made 20, 40, 60, 80 and 95%COHb from 0% and 100%.

In (Samuel, Kahl, Zaney, Hime, & Boland, 2021) section Linearity in Results, it is described that must be made a 5-point calibration curve with 20, 40, 60, 80 and 95%. Though an adjusted calibration curve for Derivative spectroscopy with 10, 20, 30, 40, 50 and 60%.

In (Samuel, Kahl, Zaney, Hime, & Boland, 2021) figure 1 they have used a 6-point calibration curve with 10, 20, 40, 60, 80 and 95%. The calibration curve for Derivative spectroscopy is here similar to the one described in the section Linearity in Results.

We chose to follow the instructions in figure 1, and thus our calibration curves are:

Derivative spectroscopy: 10, 20, 30, 40, 50, 60%

The three other methods: 10, 20, 40, 60, 80, 95%.

Inconsistency in wavelengths in method Multicomponent

In the section Multicomponent under Procedure (Samuel, Kahl, Zaney, Hime, & Boland, 2021) they wrote 500, 560, 577, 620 and 700 nm. In the formulas in section UV-Vis analysis of COHb in Introduction, 500, 569, 577 and 620 nm is used. This error has been corrected in the current version of the paper.

There is no description of the use of 700 nm, however upon contact with the authors this was found in (Attia A. M., et al., 2016). This has been corrected in the original papers current version.

Incorrect reference in method Multicomponent

(Attia A. M., et al., 2015) describes a wrong procedure, but has the constants mentioned in (Samuel, Kahl, Zaney, Hime, & Boland, 2021).

After contact with the corresponding author, we received the correct reference (Attia A. M., et al., 2016) which describes the purpose of the 700 nm measurement. But sample preparation is not consistent with the method described in (Samuel, Kahl, Zaney, Hime, & Boland, 2021).

Thus this study is carried out with the sample preparation from (Samuel, Kahl, Zaney, Hime, & Boland, 2021), the values for absorbance from (Attia A. M., et al., 2016) and the calculation procedure from (Attia A. M., et al., 2015).

The incorrect reference and description have been corrected in the original paper after contact with the authors and editors.

Inconsistency in wavelengths in method Two component plus multicomponent

In the introduktion (Samuel, Kahl, Zaney, Hime, & Boland, 2021) write that they measure at 418 and 432 nm. In the formulas in the section Procedures, they use 420 and 432 nm and they describe Ar as the ratio between 420 and 432 nm.

We chose to follow the instructions in procedures section of the paper.

Data comparison

We have included the original data from (Samuel, Kahl, Zaney, Hime, & Boland, 2021) for ease of comparison. We remind the reader that these are not our results, but a citation.

Original article

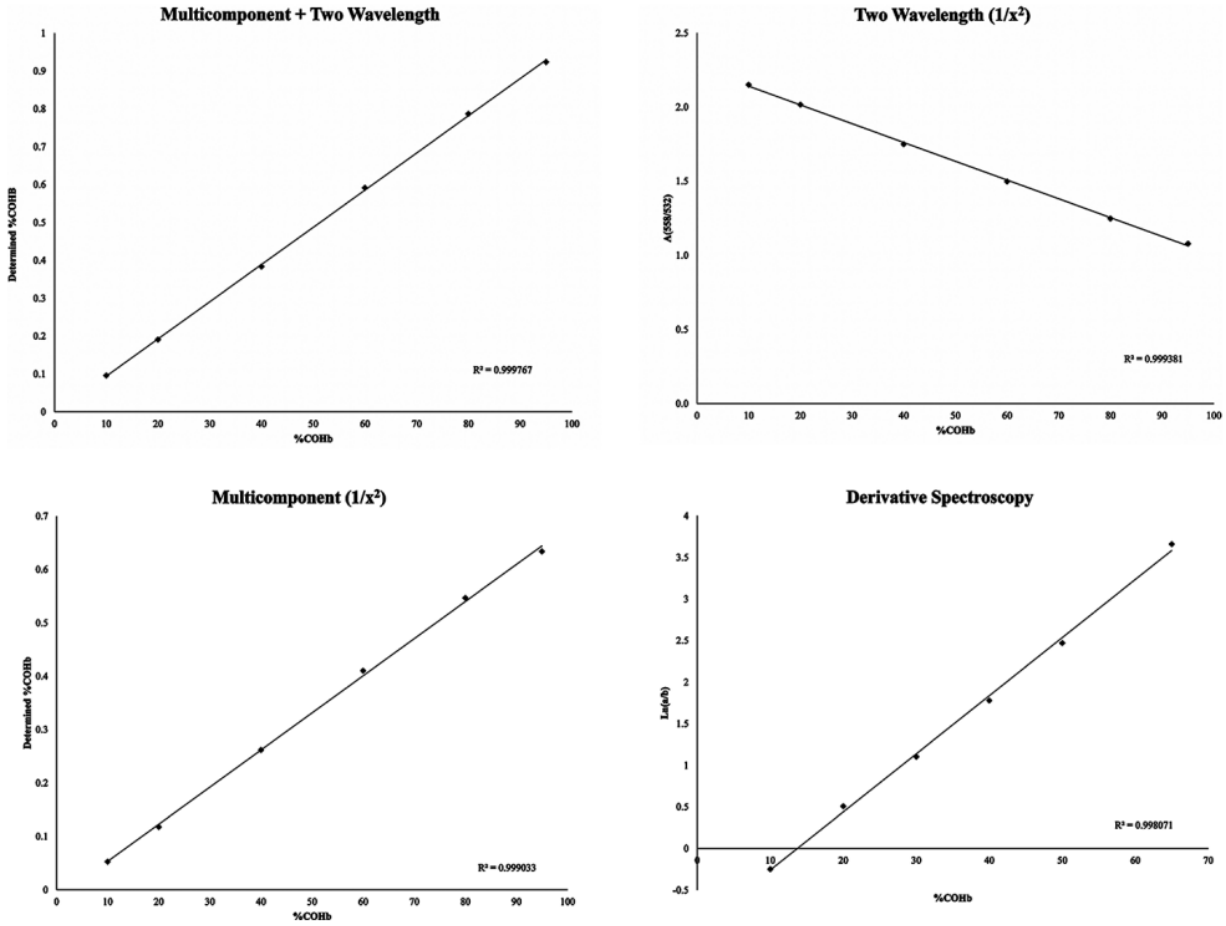

Figure 1: Calibration curves from (Samuel, Kahl, Zaney, Hime, & Boland, 2021)

	Bias		Within-run precision		Between-run precision	
Method	Low (%)	High (%)	Low (%)	High (%)	Low (%)	High (%)
Two-wavelength method	−1.2	−3.3	1.9	0.4	1.8	0.5
Multicomponent method	−10.5	1.2	1.3	0.4	6.7	3.5
Derivative spectroscopy method	−1.9	−5.6	2.0	1.6	3.3	5.3
Multicomponent + two-wavelength method	−1.2	−1.5	1.2	0.6	−3.1	1.5

Table 1: Precision and bias from (Samuel, Kahl, Zaney, Hime, & Boland, 2021)

Replication

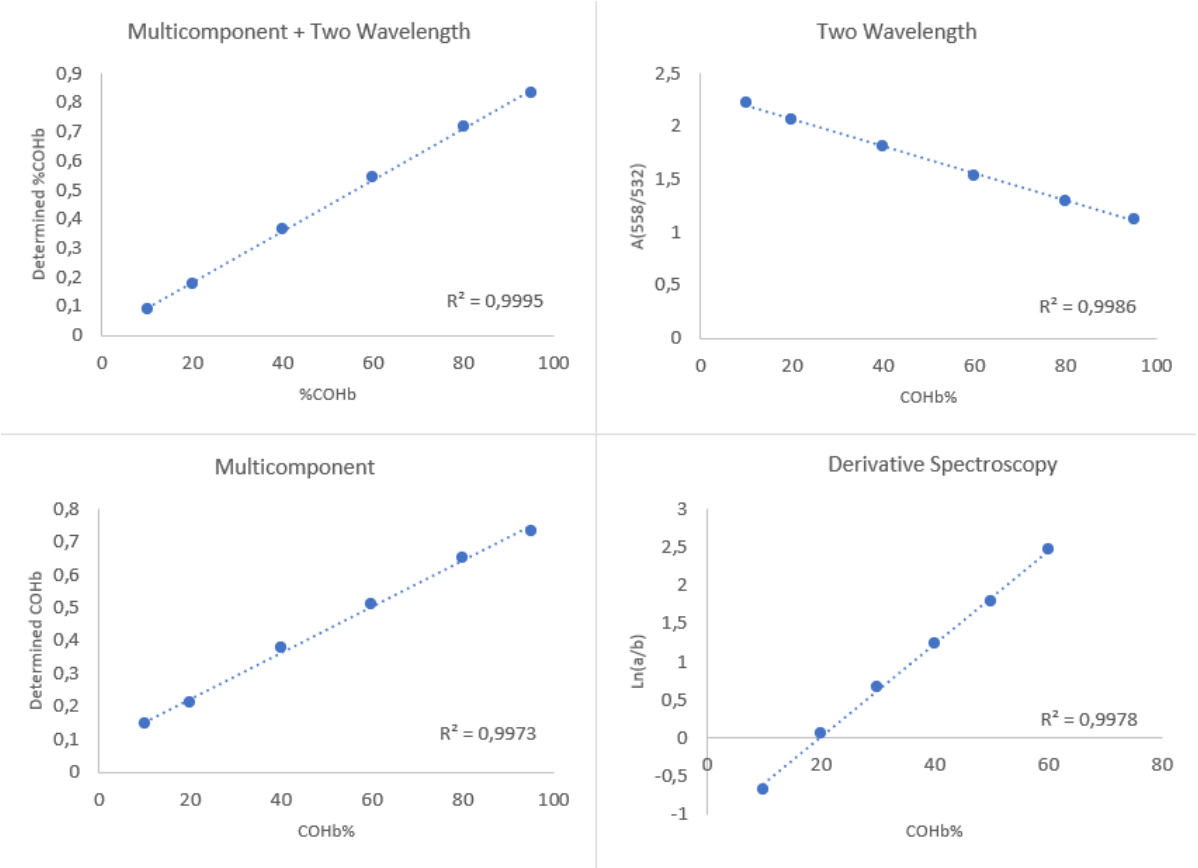

Figure 2: Standard curves for known concentrations. Two wavelength and derivative spectroscopy were used for calculating unknow samples. As multicomponent + two wavelength and multicomponent do not require an additional standard curve they are here for illustrative purposes.

	Bias			Within-run precision			Between-runs precision		
Method	Low (%)	Average (%)	High (%)	Low (%)	Average (%)	High (%)	Low (%)	Average (%)	High (%)
Two-wavelength	-0,1	-1,2	-2,6	0,1	0,6	2,0	0,4	0,8	1,5
Multicomponent	-0,8	-4,0	-11,6	0,1	0,2	0,6	0,3	1,2	2,3
Derivative spectroscopy	0,8	-2,6	-11,6	0,1	0,4	1,0	0,6	2,0	2,8
Two-wavelengths +Multicomponent	0,0	-1,2	-4,8	0,0	0,6	4,2	0,3	0,9	2,2

Table 2: Bias shows the deviation from the known values as measured by (Worm-Leonhard), indicating the highest, lowest and average deviation. Within run precision shows the precision within a triplicate for a single run, with the higher, lower and average standard deviation indicated. Between runs precision indicates the standard deviation between the independent replication carried out for each of the 5 samples, with the highest, lowest and average standard deviation listed. All values are in % carbon monoxide saturation.

We have managed to recreate all methods with standard curves that meet the requirement of linearity as described in the original article.

Two wavelengths: Here our curve is almost similar to the original work of (Samuel, Kahl, Zaney, Hime, & Boland, 2021).

Multicomponent: Here our curve looks almost similar to the original, here it is just shifted parallelly, ours being almost 0,1 higher.

Derivative spectroscopy: Our curve is flatter, our 60% is at 2,5 compared to the original's 3,7.

While this is not identical as the values can be spectrometer dependent it is irrelevant provided the values found are consistent with the known values.

Two-component + multi-component: Our curve is flatter than the original, our 95% is at 85% compared to the original's 95%.

Our table of precision and bias across methods is a little different than the original. Ours is based on 5 unknown case samples, instead of one low and high sample. Our bias is close to the bias in the experiments in the original article, although we have two samples that lie outside the calibration range. Our within-run precision looks like those in the original article, except for the highest deviation in Two-component + multi-component. Our between-runs precision has lower standard deviation.

Statistics for the unknown samples:

A statistical assessment of the difference between our found values and the assigned values from our source (Worm-Leonhard) was carried out using a t test assuming the standard deviations are different (as the source of the data is in fact different).

Sample	Assigned value (%COHb)	Two wavelenght	Multicomponent	Two-component plus multicomponent	Derivative spectroscopy
1	15,3	Yes	No	Yes	Yes
2	26,3	Yes	No	Yes	Yes
3	"Zero spike"	N/A	N/A	N/A	N/A
4	19,4	Yes	Yes	Yes	Yes
5	6,7	No	No	No	No

Table 3: Statistics for unknown samples. Are the samples within the 95% confidence interval like the assigned value?

Two wavelength, two component plus multicomponent, and derivative spectroscopy all consistently pass the test in 1,2, and 4, which are values within the calibration curve we have used. Sample 5 is consistently different across all methods, but is below the calibration curves, and it could potentially have been rectified using more calibrators. The zero spike, by its nature does not have a statistical material for the comparison.

Thus, we conclude that the quality of the different methods is adequately portrayed in the original. We also note that for accurate determination of low samples the used standard curve is not appropriate. We also note that as we have not displaced CO with a certified clean gas (nor did the original paper) there may simply be a low CO content in our “0” blood due to environmental background, which would naturally skew lower datapoints.

Additional data

CO saturation

We made an experiment to find the optimal saturation-time with the balloon-method, using the method Two wavelength.

We tested different treatment times (0 min; 15 min; 30 min; 60 min; 120 min and 24 h).

The purpose of this experiment was to test when we reached saturation.

The 0 min test was used as a comparison.

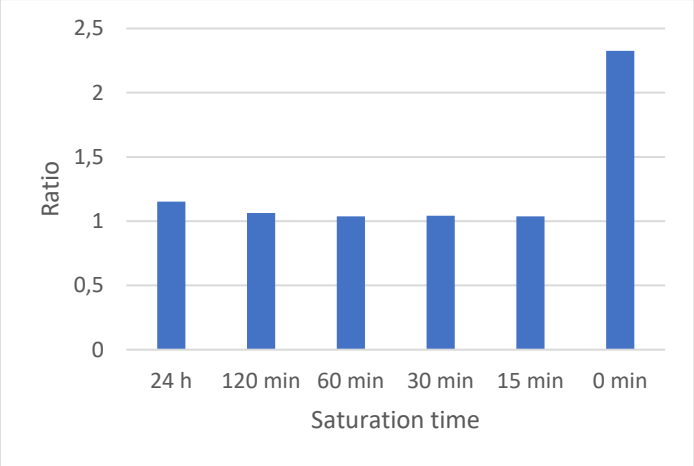

Figure 3: Saturation experiment. Shows the ratio (558/532 nm) with different treatment times, using the method Two wavelength.

As the figure shows, the difference is negligible after 15 min, and there is not a distinct pattern to the change at subsequent points in time. Thus, we concluded that after 15 min the blood was saturated.

We used 120 min saturation time, to make the closest replica possible under our different protocol.

Horse blood

As an addition to the replication, we investigated whether horse blood gave the same results as human blood. Horse blood is more easily available and safer to use. Furthermore, our source at the university (Worm-Leonhard) uses horse blood as standards.

We tried all four methods with horse blood and made a comparison.

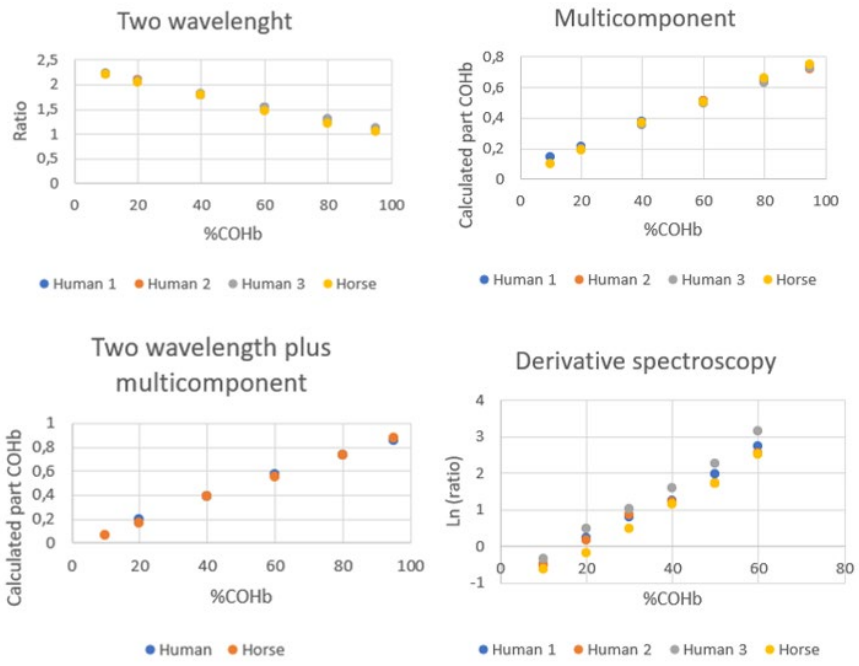

Figure 4: Horse blood experiment. Calibration curves made with horse blood.

As we can see, all of the calibration curves are very similar to each other. Therefore it is possible to replace the human blood with horse blood standards. Again we see a higher variation in the derivative spectroscopy, where both horse and human samples vary.

Effects of repeatedly freeze/thaw cycles

As an addition to the replication, we investigated the effects of repeatedly freezing and thawing. We suspected that repeated freeze/thaw cycles would change the samples. To verify this, we tested the same standards and two samples, every time we did a freeze/thaw cycle (5 times) and made a comparison with the fresh standards. Visually the standards/samples became more and more difficult to dissolve. Especially the low %COHb coagulated. We used the method Two wavelength.

	Slope	R^2
Fresh	-0,01389	0,998145
Freeze/Thaw 1.	-0,01342	0,999397
Freeze/Thaw 2.	-0,01237	0,992757
Freeze/Thaw 3.	-0,01179	0,995498
Freeze/Thaw 4.	-0,01167	0,991871
Freeze/Thaw 5.	-0,01078	0,978411

Table 4: Freeze/Thaw experiment. Slope and correlation for different numbers of freeze/thaw cycles.

The calibration curves changed with every freeze/thaw cycle. The linearity becomes worse and the curve's slope flattens out. Thus, we concluded that samples could not be frozen after treatment with sodium hydrosulfite.

Materials and methods

The lab book, with excel sheets and original data is available at:

https://app.labstep.com/sharelink/a0e6209c-2077-4607-9192-c235e6527bd0

Materials

Sodium dithionite, $Na_2S_2O_4$ (Lot.nr: STBB8194V; Art.nr: 157953-500G; Brand: Sigma-Aldrich)

Sodium carbonate, Na_2CO_3 (Lot.nr: 20.1861701.100; Art.nr: 1393.1000; Brand: Chemsolute)

Sodium hydroxide, NaOH (Lot.nr: 96-04-20; Art.nr: 1355.1000; Brand: Chemsolute)

Ammonium hydroxide, NH_4OH (Lot.nr: 296561703; Art.nr: 2672.1011; Brand: Chemsolute)

Potassium phosphate dibasic, K_2HPO_4 (Lot.nr: A1173199; Art.nr: 1.0599.1000; Brand: Emsure)

Potassium phosphate monobasic, KH_2PO_4 (Lot.nr: A830673; Art.nr: 1.04873.1000; Brand: Merck)

Potassium hexacyanoferrate(III), $K_3Fe(CN)_6$ (Lot.nr: A669173; Art.nr: 4973.0100; Brand: Merck)

Potassium cyanide, KCN (Lot.nr: 9639674; Art.nr: 4967; Brand: Merck)

Blank blood from a nonsmoker living in rural conditions

Horse blood

Blind unknown case samples

Cuvettes: Polystyrol/Polysterene; 10*10*45 mm; Ref. 67.741; Sarstedt

Centrifuge tubes, 13 mL: 101*16,5 mm; Polypropylen; Ref. 60.540.012; Sarstedt

Centrifuge tubes, 15 mL: 120*17 mm; Polypropylen; Ref. 62.554.502; Sarstedt

Instrumentation

Centrifuge: Eppendorf Centrifuge 5804

Cooling centrifuge: Hettich Zentrifugen Universal 30RF, with associated tubes

Spectrophotometer: Shimadzu UV-2600 UV-VIS spectrophotometer

Spectrophotometer settings

Two-wavelength, Multicomponent and Two-component plus multicomponent:

Photometric; Multipoint; Measuring mode: Absorbance; Slit width: 1,0 nm

Derivative spectroscopy:

Spectrum; Scan speed: Fast; Sampling interval: 0,1 nm; Slit width: 1,0 nm

Methods

The methods were carried out according to (Samuel, Kahl, Zaney, Hime, & Boland, 2021)s description of the original methods of (Katsumata, Aoki, M, Suzuki, & Yada, 1980) (two wavelength), (Parks & Worth, 1985) (derivative spectroscopy), (Rodkey, Hill, Pitts, & Robertson, 1979) (two component) and the corrected version of the method for multicomponents described in deviations from replication.

Calibrators

Add 25 mg sodium hydrosulfite to 1 mL blank blood in a 13 mL centrifuge tube, mix. Use the "balloon-method" to saturate the blood with CO-gas. Make sure the regulation valve on the gas flask is closed and open the main valve. The piece of pipe from the main valve to the regulation valve will be filled with CO-gas. Close the main valve again. Place a balloon on a hose, open the regulation valve and fill the balloon with gas. Place the balloon on the top of the centrifuge tube, taped firmly and place it in a shaker. Let stand for 2 hours. This is the 100% COHb blood.

Figure 5: Pictures of the setup of the balloon method.

The 0% COHb blood: Add 25 mg sodium hydrosulfite to 1 mL blank blood in a 13 mL centrifuge tube, mix.

Now mix the calibrators from the 0% and 100% COHb blood.

Calibrators for Two-wavelength, Multicomponent and Two-component plus multicomponent:

10, 20, 40, 6 0, 80 and 95%.

Calibrators for Derivative spectroscopy:

10, 20, 30, 40, 50 and 60%.

Calibrators are used the same day.

Two-wavelength

Make a working solution with 0,1% sodium carbonate and sodium hydrosulfite. Use 0,8 mg sodium hydrosulfite pr. mL 0,1% sodium carbonate (eg.: 32 mg to 40 mL). This solution should be used the same day.

Transfer 2,5 mL solution to a 1 cm cuvette.

Add 10 µL sample.

Add 200 µL 5 M sodium hydroxide.

Cap the cuvette and invert repeatedly to mix.

Let stand for 5 min.

Zero the UV/VIS spectrophotometer with DI-water and measure at 532 nm and 558 nm.

The ratio (558 nm/532 nm) is correlated to %COHb.

Derivative spectroscopy

Add 25 mg sodium hydrosulfite to 10 mL 230 mM ammonium hydroxide in a 15 mL centrifuge tube.

If you have many samples, then make a working solution with 625 mg sodium hydrosulfite, 4,135 mL 25% ammonium hydroxide and DI-water in a 250 mL measuring flask and transfer 10 mL to 15 mL centrifuge tubes.

Add 10 µL sample, cap and invert repeatedly to mix.

Let stand for 30 min.

Transfer to a 1 cm cuvette.

Zero the UV/VIS spectrophotometer with 25 mg sodium hydrosulfite to 10 mL 230 mM ammonium hydroxide and make a scan between 390 and 450 nm.

Find the second derivative of the spectra (with 3 nm steps) and the amplitudes 410-420 nm and 435-445 nm.

The ratio of the amplitudes (410-420 nm/435-445 nm) is correlated to %COHb.

Multicomponent

Add 5 mL ice-cold DI-water to a 15 mL centrifuge tube.

Add 30 µL sample and whirlymix.

Centrifuge at 4600 RCF in 10 min (Eppendorf Centrifuge 5804).

Transfer the supernatant to a 1 cm cuvette.

Zero the UV/VIS spectrophotometer with DI-water and measure at 500, 569, 577, 620 and 700 nm.

700 nm is not used in the calculation. It is used to estimate light scattering, if the value is higher than 0,009, the sample is discarded.

This method does not use a calibration curve like the methods Two wavelengths and Derivative spectroscopy, but some fixed values. You will find the math and values in the section “Calculation examples”.

The curve is the calculated COHb content correlated to %COHb.

Two-component plus multicomponent

Phosphate buffer (pH 6,85):

Make 1 L 0,1M potassium phosphate dibasic solution, dissolve 17,6 g in DI-water.

Make 1 L 0,1M potassium phosphate monobasic solution, dissolve 13,6 g in DI-water.

Add 0,1M potassium phosphate dibasic solution to the 0,1M potassium phosphate monobasic solution, until pH=6,85.

Add 600 µL phosphate buffer (pH=6,85) to 6 mL DI-water.

Add 50 µL sample and mix.

Let stand for 10 min.

Make a working dilution with phosphate buffer (pH=6,85) and sodium hydrosulfite. Use 0,78 mg sodium hydrosulfite pr. mL phosphate buffer (ex.: 39 mg to 50 mL). This solution should be used the same day.

Transfer 2,3 mL working solution to 1 cm cuvette.

Add 200 µL sample/phosphate buffer solution.

Cap the cuvette and invert repeatedly to mix.

Zero the UV/VIS spectrophotometer with the working solution.

Measure at 420 nm and 432 nm.

This method does not use a calibration curve like the methods Two wavelengths and Derivative spectroscopy, but some fixed molar attenuation coefficients. The method to find them, will be described below. You will find the math in the section “Calculation examples”.

The curve is the calculated COHb content correlated to %COHb.

Molar attenuation coefficients:

Add 200 µL blank blood from a nonsmoker to 30 mL DI-water. Mix.

Centrifuge at 5 °C and 1500 cfu for 15 min (Hettich Zentrifugen Universal 30RF).

The supernatant is the primary dilution.

Make a secondary dilution: dilute 10 mL primary dilution 10 times with DI-water in a 100 mL measuring flask.

The remaining primary dilution is added 3-4 mg potassium hexacyanoferrate(III) and 2-3 mg potassium cyanide. Mix and let stand for 2 hours.

Measure absorbance at 540 nm to find the concentration of CNMetHb.

Saturate 10 mL (2*5 mL) of the secondary dilution with CO-gas with the “balloon-method”.

Add 10 mg sodium hydrosulfite to 1 cm cuvettes (I used 8 cuvettes - saturated/untreated secondary dilution in triplicate and 2 cuvettes for “zero”)

Add 2,5 mL saturated secondary dilution, untreated secondary dilution and DI-water to the cuvettes.

Cap the cuvettes and invert repeatedly to mix.

Let stand for 15 min.

Zero the UV/VIS spectrophotometer with sodium hydrosulfite/DI-water dilution.

Measure at 420 nm and 432 nm.

Calculation examples

Two-wavelength

The calibration curve’s equation is used to calculate the concentration of %COHb with the ratio (A_{558}/A_{532}) of the unknown sample.

Derivative spectroscopy

If your spectrophotometer does not have a setting that can calculate the second derivative, then find it like this:

Formula: $D_\lambda = \frac{A_{\lambda-stepsize} - 2\times A_\lambda + A_{\lambda+stepsize}}{Stepsize^2}$

We used a 3 nm stepsize.

Find the amplitudes (difference between highest and lowest D_λ) for 410-420 nm and 435-445 nm.

The calibration curve’s equation is used to calculate the concentration of %COHb with the ratio of the two amplitudes (410-420 nm/435-445 nm) from the unknown sample.

Multicomponent

Formulas:

$$C_{SHb} = \frac{A_{620} - 0{,}46241784 \times A_{500} + 0{,}10425144 \times A_{569} + 0{,}066173573 \times A_{577}}{19{,}28380181}$$

$$C_{MetHb} = \frac{7{,}587561597 \times A_{500} - 2{,}1061484 \times A_{569} - A_{577} - 24{,}17452346 \times C_{SHb}}{56{,}06121255}$$

$$C_{COHb} = \frac{A_{569} - 2{,}186651145 \times A_{500} + 15{,}72636593 \times C_{MetHb} + 6{,}117605743 \times C_{SHb}}{2{,}726668607}$$

$$C_{oxyHb} = \frac{A_{500} - 5{,}279 \times C_{COHb} - 9{,}067 \times C_{MetHb} - 6{,}502 \times C_{SHb}}{5{,}154}$$

$$C_{Hb} = C_{SHb} + C_{MetHb} + C_{COHb} + C_{oxyHb}$$

$$Part\ COHb = \frac{C_{COHb}}{C_{Hb}}$$

Ex.:

$$C_{SHb} = \frac{0{,}075 - 0{,}46241784 \times 0{,}298 + 0{,}10425144 \times 0{,}379 + 0{,}066173573 \times 0{,}408}{19{,}28380181} = 0{,}000192$$

$$C_{MetHb} = \frac{7{,}587561597 \times 0{,}298 - 2{,}1061484 \times 0{,}379 - 0{,}408 - 24{,}17452346 \times 0{,}000192}{56{,}06121255} = 0{,}018733$$

$$C_{COHb} = \frac{0{,}379 - 2{,}186651145 \times 0{,}298 + 15{,}72636593 \times 0{,}018733 + 6{,}117605743 \times 0{,}000192}{2{,}726668607} = 0{,}008495$$

$$C_{oxyHb} = \frac{0{,}298 - 5{,}279 \times 0{,}008495 - 9{,}067 \times 0{,}018733 - 6{,}502 \times 0{,}000192}{5{,}154} = 0{,}01592$$

$$C_{Hb} = 0{,}000192 + 0{,}018733 + 0{,}008495 + 0{,}01592 = 0{,}04334$$

$$Part\ COHb = \frac{0{,}008495}{0{,}04334} = 0{,}195997 \approx 19{,}6\%$$

Two-component plus multicomponent

Formulas for fixed values:

$$C_{CNMetHb} = \frac{A_{540}}{\varepsilon \times l} = \frac{A_{540}}{11000 \times 1}$$

$$\varepsilon_{COHb} = \frac{A}{l \times C_{CNMetHb}}$$

$$\varepsilon_{Hb} = \frac{A \ - \ (f\varepsilon_{COHb} \times l \times C_{CNMetHb})}{(1 - f\varepsilon_{COHb}) \times l \times C_{CNMetHb}}$$

$$f\varepsilon_{COHb} = \ 1\% \ in \ human \ nonsmoker \ blood$$

$$F_1 = \frac{\varepsilon_{Hb}(432)}{\varepsilon_{Hb}(420)}$$

$$F_2 = \frac{\varepsilon_{COHb}(432)}{\varepsilon_{Hb}(420)}$$

$$F_3 = \frac{\varepsilon_{COHb}(420)}{\varepsilon_{Hb}(420)}$$

Formulas for standards and samples:

$$A_r = \frac{A_{420}}{A_{432}}$$

$$\%COHb = \frac{1 - (A_r \times F_1)}{A_r \times (F_2 - F_1) - F_3 + 1}$$

Ex. for fixed values:

$$C_{CNMetHb} = \frac{0{,}55333333}{11000 \times 1} = 0{,}000050303 \ mol/L$$

$$\varepsilon_{COHb}(420) = \frac{0{,}973}{1 \times 0{,}000050303} = 19342{,}77108$$

$$\varepsilon_{COHb}(432) = \frac{0{,}211666667}{1 \times 0{,}000050303} = 4207{,}831325$$

$$\varepsilon_{Hb}(420) = \frac{0{,}524 \ - \ (1 \times 0{,}01 \times 0{,}000050303)}{(1 - 0{,}01) \times 1 \times 0{,}000050303} = 10522{,}07825$$

$$\varepsilon_{Hb}(432) = \frac{0{,}669666667 \ - \ (1 \times 0{,}01 \times 0{,}000050303)}{(1 - 0{,}01) \times 1 \times 0{,}000050303} = 13404{,}61847$$

$$F_1 = \frac{13404{,}61847}{10522{,}07825} = 1{,}273952$$

$$F_2 = \frac{4207{,}831325}{10522{,}07825} = 0{,}399905$$

$$F_3 = \frac{19342{,}77108}{10522{,}07825} = 1{,}838303$$

Ex. for standards and samples:

$$A_r = \frac{0{,}496}{0{,}428} = 1{,}158879$$

$$\%COHb = \frac{1 - (1{,}158879 \times 1{,}273952)}{1{,}158879 \times (0{,}399905 - 1{,}273952) - 1{,}838303 + 1} = 0{,}25732 \approx 25{,}7\%$$

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