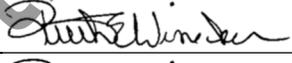# SOP 075 – Carbon Monoxide Detection by Second Derivative and Absorbance Spectrophotometry

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Carbon Monoxide Detection by Second Derivative Spectrophotometry		**075**	
	Revision:	**Revision Date/Initials:**	
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	Added section 8 – STAT Analysis 12.1 – updated/clarified reporting rules	07/20/2015 – MSF 03/28/2017 - MSF	
Approving Authority Name	**Approving Authority Signature**	**Approval Date**	
Ruth E. Winecker, Ph.D.	*[signature]*	04/08/2015	
Ruth E. Winecker, Ph.D.	*[signature]*	06/27/2016	
Ruth E. Winecker, Ph.D.	*[signature]*	12/06/2017	

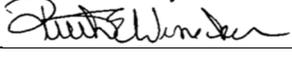# SOP 075 – Carbon Monoxide Detection by Second Derivative and Absorbance Spectrophotometry

1. **Introduction and Principle of Assay**

   1.1. Carbon monoxide binds to hemoglobin forming carboxyhemoglobin which prevents the binding of oxygen. In deaths associated with fire, inhalation of car exhaust or situations involving a faulty heater, carboxyhemoglobin concentrations may exceed 50% SAT. Deaths may occur at lower concentrations in the young, the elderly or in individuals with significant natural disease (e.g. COPD, heart disease).

   1.2. This method is designed to detect the presence of carbon monoxide in blood by UV-Vis spectrophotometry. The initial screen uses the second derivative of the absorption spectrum to measure the amount of carboxyhemoglobin (418 nm) and reduced hemoglobin (430 nm) in the unknown specimen. These values are compared to experimentally derived values for known specimens and the percent of saturated carboxyhemoglobin is determined.

   1.3. To confirm the results derived from the initial screening test, the specimens are analyzed a second time and the absorbance at four wavelengths (510, 540, 555, and 578 nm) are measured to ascertain the amount of carboxyhemoglobin and reduced hemoglobin. These values are used to calculate the percent of saturated carboxyhemoglobin.

2. **Specimens**

   2.1. Whole or hemolyzed Blood

      2.1.1. For derivative, typically 0.012 mL – additional specimen may be required to compensate for specimens with a lower percentage of hemoglobin.

      2.1.2. For absorbance, typically 0.05 mL– additional specimen may be required to compensate for specimens with a lower percentage of hemoglobin.

   2.2. Spleen – if sufficient bloody fluid cannot be obtained from the vial containing the spleen, a homogenate can be prepared by squeezing bloody fluid from the spleen into a secondary vial. This Homogenate shall be recorded via the chain-of-custody and used for analysis. If no fluid can be extracted, report as "Specimen Unsuitable" with the report comment "Specimen matrix was unsuitable for analysis due to low percentage of hemoglobin".

   2.3. Charred Blood

      2.3.1. Weigh approximately 0.5g of charred blood into a 16x100mm test tube and record the weight for the chain-of-custody.

2.3.2. To start, add 100µL DiH2O to the blood and vortex mix for 30 seconds. If all of the water is absorbed, add an additional 100µL and repeat vortex.

2.3.3. Centrifuge for 5 minutes at 2000 x g (3000 rpm).

2.3.4. Tilt test tube to aliquot the resulting fluid for analysis – larger aliquot sizes may be required due to a lower percentage of hemoglobin.

3. **Reagents and Materials**

   3.1. Water (HPLC grade)

   3.2. Concentrated Ammonium Hydroxide

   3.3. Sodium Hydrosulfite

4. **Standards, Controls, and Solutions**

   4.1. Reducing Reagent-Make fresh on the day of use.

      4.1.1. In a 250 mL graduated cylinder, place 249 mL of deionized water. Add 1 mL of concentrated ammonium hydroxide with a micropipette. Seal with glass stopper and invert three time to mix.

   4.2. Cvc 233 – CO-Oximeter Calibration Verification Controls
         *verify that controls have not reached their expiration date and that the correct lot numbers appear on the standard & control worksheet.

      4.2.1. Level 1 ~ 68% saturated

      4.2.2. Level 2 ~ 4% saturated

      4.2.3. Level 3 ~ 18% saturated

5. **Equipment and Special Supplies**

   5.1. 16x125 screw top test tubes with caps

   5.2. Macro pipette (5mL)

   5.3. Macro pipette disposable tips

   5.4. Micro pipette (20uL and 100uL)

   5.5. Micro pipette disposable tips

5.6. Parafilm

5.7. Kim Wipes

5.8. Pasteur Pipettes

5.9. Bulb

5.10. Small spatula

5.11. 10mm UV cuvette

6. **Instrumentation and Parameters**

6.1. Instrument………………...HP 8454 UV-Visible Spectrophotometer

6.2. Method 1…………………deriv.m (wavelength measured: 394 – 445 nm)

6.3. Method 2……………….A.m. (wavelength measured: 450-603 nm)

7. **Procedure – Non-STAT CO Analysis (See Section 8)**

7.1. Open the UV-Vis software using the icon on the desktop.

7.2. Turn on the HP 8453 Spectrophotometer.

7.3. Check status of the lamps (on/off) – From the menu bar select "Instruments" – "Lamp(s)…".

7.4. Verify that both lamps are "On" – change lamp status to "On" if needed using on-screen prompts.

7.5. Allow the lamps to warm up for at least 45 minutes.

7.6. Perform an instrument "self-test".
   7.6.1. Select "Mode" and then "Verification and Diagnostics" from the menu bar.

   7.6.2. In the diagnostics screen, select "Start" (lower left) and click "OK".

   7.6.3. Wait a few minutes for the system test to complete.

   7.6.4. View the results and verify that all parameters have passed.

	Specification	Measured	Result
Filter and shutter test	< 500 msec	200 msec	Passed
Dark current test	0..12000 cts	2863..2988 cts	Passed
Min. intensity (190nm - 220nm)	> 2000 cts	60098 cts	Passed
Min. intensity (220nm - 350nm)	> 5000 cts	38524 cts	Passed
Min. intensity (350nm - 500nm)	> 2000 cts	21438 cts	Passed
Min. intensity (500nm - 950nm)	> 4000 cts	31039 cts	Passed
Min. intensity (950nm - 1100nm)	> 200 cts	746 cts	Passed
Wavelength at 486.0nm	485.5..486.5 nm	486.293 nm	Passed
Wavelength at 656.1nm	655.6..656.6 nm	656.336 nm	Passed
RMS Noise	< 0.0002	0.00007	Passed
RMS Baseline flatness	< 0.001	0.000054	Passed

7.6.5.

    7.6.5.1.   If any of the parameters do not pass acceptance criteria, wait another 15 minutes and retry. If there are still non-passing parameter(s), contact the instrument chemist.

7.6.6.   Save the "self-test" to the instrument and print the diagnostic report to include with batch data.

    7.6.6.1.   Save Self-Test Results to hard drive:

        7.6.6.1.1.   From the menu bar, select "File" – "Save" – "Save Self-Test Results".

        7.6.6.1.2.   Save file to: C:\CHEM32\1\DIAGNOSE using the date of test as the file name (e.g. 053014.SFT).

    7.6.6.2.   From the menu bar, select "File" – "Print" – "Print Self-Test Results". Alternatively, "cnt+alt+print screen" can be used to copied into Microsoft paint – save as a bitmap (.bmp) into the load folder (to be printed later when on PC connected to printer).

7.7.   Select "File - Load Method…" from the tool bar menu. A dialog box will appear.

7.8.   Select the "deriv.m" method from the C:\Chem32\1\methods\ directory.

7.9.   Create and name a new folder with the assay full load number in C:\Chem32\1\Data

7.10.   Prepare 16x125 screw top test tubes for controls (3), blank, and each case by labeling it with the control name or specimen number.

7.11.   Pipette 10 mL of reducing reagent into each tube.

    7.11.1.   Note: The blank is used for blanking the instrument and for diluting specimens into the linear UV-Vis range of 0.3-1.0 absorbance (A_{max}).

7.12. To each control tube (Levels 1, 2, and 3), add 12 µL of the appropriate control with a micropipette. Seal the top of each ampoule with Parafilm for later use.

7.13. Pipette a quantity of specimen (begin with 12 µL) into the tubes prepared in step 7.10 with a micropipette.

7.14. Add about 100 mg of sodium hydrosulfite (equivalent to 1 heaping small red spatula-full) to each tube. Cap each tube and invert 3-4 times to dissolve.

7.15. Let sit for 2 minutes.

7.16. Transfer about 4 mL of the blank to a clean quartz cuvette with a Pasteur pipet. Clean outside of cuvette with KimWipes and flick with finger to remove bubbles. **NOTE**: Inspect the cuvette for scratches, marks and cleanliness prior to use.

7.17. Insert the cuvette into the spectrophotometer and press the "Blank" button in the lower left corner of the screen with the mouse.

7.18. The spectrophotometer will read the blank and record the spectrum. Close the blank spectrum by pressing the "X" in the upper right corner of the spectrum window.

7.19. Prepare the controls/samples for analysis as follows:

   7.19.1. **Controls & Samples:** Transfer about 4mL of the sample to a quartz cuvette with a Pasteur pipet. Note: For control level 3, transfer approximately 2mL of control followed by 2mL of Blank into the cuvette for analysis.

7.20. Insert the cuvette into the spectrophotometer and click the "Sample" button in the lower left corner of the screen with the mouse. Verify that all peaks and valleys are smooth, and that the A_{max} falls between 0.3 and 1 abs units. If not, adjust the dilution or aliquot more specimen as needed and repeat.

7.21. A dialog box will appear after the spectrophotometer has read the sample. The absorbance spectrum will appear in the upper half of the screen.

7.22. Click the "Analyze" button. The computer will calculate the second derivative of the absorbance spectrum.

7.23. Click directly on the derivative curve so that it is highlighted. Select "File" – "Export selected data as" – "CSV format".

7.24. Name the sample with the last five digits of the S# as appropriate - plus add an "**ad**". Name the controls lev1ad, lev2ad, or lev3ad. Save in the in C:\Chem32\1\Data Ex: lev1ad or 13084ad. Select "**OK**".

7.25. Select the "Clear" button in the upper left corner of the screen and select "Samples."

    7.25.1. Rinse cuvette with three volume equivalents of reducing reagent between uses.

7.26. Repeat steps 7.19 through 7.25 for all controls and specimens.

7.27. Select "**File**" from the menu bar of the UV-Visible program. Select "**Load Method…**" from the "**File**" pull-down menu. A dialog box will appear.

7.28. Select the "**abs.m**" method from the C:\uv-vis\methods directory. Select "**OK**".

7.29. Prepare 16x125mm screw-top tubes for each control, blank, and specimen by pouring 10mL of reducing reagent into each tube and labeling with the control level, blank, or case number.

7.30. Pipette 100uL of control levels 1 and 2 and 50µL of level 3 into the appropriate labeled tubes prepared in step 7.29. Add nothing to the blank.

7.31. Pipette 50 µL of specimens into the appropriate labeled tubes prepared in step 7.29 .

7.32. Using the small spatula, add about 100mg of sodium hydrosulfite (1 heaping spatula full) to each tube. Cap each tube and invert 3-4 times to dissolve. Let sit for 2 minutes.

7.33. Transfer about 4 mL of the blank reagent to a quartz cuvette. Insert the cuvette into the spectrophotometer and press the "**Blank**" button in the lower left corner of the screen.

7.34. The spectrophotometer will read the blank and record the spectrum. Check that the displayed spectrum is devoid of smooth peaks. Close the blank spectrum by pressing the "**X**" in the upper right corner of the spectrum window.

7.35. Transfer about 4mL of the control or sample to a quartz cuvette.

7.36. Insert the cuvette into the spectrophotometer and press the "**Sample**" button in the lower left corner of the screen.

7.37. The absorbance spectrum will appear in the upper right quadrant of the screen.

7.38. Verify that all peaks and valleys are smooth and that the A_{max} falls between 0.3 and 1 abs units. If the absorbance range is too high or low dilute with blank or add more specimen respectively and repeat steps 7.36 through 7.38.

7.39. Click directly on the absorbance curve so that it is highlighted. Select "**File**" – "**Export selected data as**" – "**CSV format**".

7.40. Name the sample as the last 4-5 numbers of the S# plus add an "**a**" (Ex: lev1a or 13803a). Save in the in C:\Chem32\1\Data. Select "**OK**".

7.41. Rinse cuvette with three volume equivalents of Di Water between specimens.

7.42. Repeat steps 7.35 through 7.41 for all controls and specimens.

7.43. Once all the controls and specimens have been saved, use windows file explorer to copy the newly created datafiles into the load folder created in step 7.9.

7.44. Transfer the load folder containing the newly acquired data to S:\toxicology\Instrument Data\up\Data

7.45. Open the Excel worksheet "**co_analysis_uv-vis02_ (+date)**" found in the **S:\toxicology\Instrument Data\uv\Data** folder.

7.46. Fill in the analyst, date of assay, load number, S and T numbers for the control or specimen to be analyzed.

7.47. The box under **Specimen data file**, enter a data file name control or specimen to be analyzed. (Ex: lev1a or ####a).

7.48. Select "**View**" from the menu bar. Select "**macros**" (ALT F8). A dialog box will appear.

7.49. Choose "**Derivative**" and press "**run**". This macro will calculate the % saturation for the control or specimen for the Second Derivative and Absorbance Spectrophotometry methods. Check that all controls values are acceptable and that the % saturation by both methods does not vary more than the acceptance limits set in section 11..

7.50. Click the "Printer" icon to print report.
7.51. Repeat steps 7.46 - 7.50 for all datafiles.

8. **Procedure – Preliminary Autopsy Diagnostic (PAD) CO**

   8.1. Follow steps 7.1 to 7.9

   8.2. Analyze the listed specimens following the steps outlined in section 7:

   8.2.1. Blank
   8.2.2. PAD Specimen
       8.2.2.1. **Note**: Since the PAD specimens will not have a specimen number (S#), use the following naming convention for the datafiles (if there are multiple PAD specimens name sequentially): PAD1_'date'AD and PAD1_'date'A - (e.g. PAD1_041718ad, PAD2_041718ad, etc.)

   8.3. Once data is collected, analyze with the Excel worksheet **"co_analysis_uv-vis02_ (+date)"** found in the **S:\toxicology\Instrument Data\uv\Data** folder.

   8.4. Take results to any available Toxicologist and await instructions.

9. **Procedure – STAT CO Analysis**

   9.1. Follow steps 7.1 to 7.9

   9.2. Analyze the listed specimens following the steps outlined in section 7:

   9.2.1. Blank
   9.2.2. Control level 1
   9.2.3. STAT Specimen

   9.3. Once data is collected, analyze with the Excel worksheet **"co_analysis(+date)"** found in the **S:\toxicology\Instrument Data\uv\Data** folder.

   9.4. Take results to any available Toxicologist and await instructions.

10. **Calculations**

    10.1.1. The calculations below are performed by the "co_analysis" spreadsheet.

    10.1.2. Carbon Monoxide Percent Saturated (Second Derivative):

    10.1.2.1. % Sat = {[LN (HbCO/Hb)] – Intercept} / Slope

       10.1.2.1.1. % Sat = carbon monoxide percent saturated

       10.1.2.1.2. HbCO = distance between the apex and valley of the carboxyhemoglobin peak (mm)

10.1.2.1.3. Hb = distance between the apex and valley of the reduced hemoglobin peak (mm)

10.1.2.1.4. Intercept = experimentally derived intercept (-2.133)

10.1.2.1.5. Slope = experimentally derived slope (0.0707)

10.1.3. Carbon Monoxide Percent Saturated (Absorbance):

10.1.3.1. $\% \text{ Sat} = \dfrac{(A540 – A510) – 0.62(A555 – A510)}{(A555 – A510) - 0.42\,(A578 – A510)}$

10.1.3.1.1. % Sat = carbon monoxide percent saturation

10.1.3.1.2. A510 = the absorbance at wavelength 510 nm
10.1.3.1.3. A540 = the absorbance at wavelength 540 nm

10.1.3.1.4. A555 = the absorbance at wavelength 555 nm

10.1.3.1.5. A578 = the absorbance at wavelength 578 nm

11. **Quality Control**

11.1. The spectra appearance, wavelength, and absorbance are compared with previous assays to insure instrument performance. The carbon monoxide saturation of the three control levels are evaluated as follows:

11.1.1. Level 1: >55 %SAT

11.1.2. Level 2: 1-10 %SAT

11.1.3. Level 3: 14-24 %SAT

11.2. If any discrepancy occurs, a senior scientist or toxicologist must be contacted. He/she will evaluate the performance of the instrument and determine what remedial action will be taken if any.

12. **Assessment of Sample Data**

12.1. Specimens from decomposed bodies may have degraded or insufficient hemoglobin to obtain valid spectra causing an interference with the assay.

12.1.1. If during analysis (see 7.38) you are not able obtain an $A_{max} > 0.3$ due to insufficient hemoglobin, report as "Specimen Unsuitable" due to low hemoglobin.

12.1.2. Absorbance spectra should be compared to the control spectra and evaluated for shift. If a shift has occurred, refer the specimen to the palladium chloride - CO by diffusion method SOP-074.

12.2. Comparisons of results between the initial and confirmation analysis shall be assessed as follows: (non-conforming specimens shall be repeated).

12.2.1. Averaged results $\geq 20\%$ SAT must not vary more than 25% (Max/Min).

12.2.2. Averaged results $< 20\%$ SAT must not vary more than 5% SAT.

12.2.3. If the results to not meet Max/Min criteria, refer the specimen to the palladium chloride - CO by diffusion method SOP-074.

13. **Reporting**

13.1. All results from both second derivative and absorbance analysis (initial and confirmation) will be averaged and truncated to the nearest whole number. Record results both on the printed report and in Toxlog.

13.1.1. For averaged results falling between 5% and 60% CO saturation, the average of the initial and confirmation analysis shall be reported.

13.1.2. Averaged results less than 5% CO saturation shall be reported as "Less than 5% SAT".

13.1.3. Averaged results greater than 60% CO saturation shall be reported as "Greater than 60% SAT".

14. **Preparation of Load**

14.1. After completing all data generation and reviewing for corrections, the analyst will assimilate the data in the following order:

14.1.1. Load assignment sheets, followed by any additional notes to file pertaining to load.

14.1.2. Load specimen sheet.

14.1.3. Chain-of-Custody.

14.1.4. Aliquot Chain-of-Custody.

14.1.5. Standard and control worksheet (Copy of Packaging Insert for positive controls".

14.1.6. Print out of instrument self-test (with load number, date, and initial).

14.1.7. Data printouts for all controls and case work.

14.1.8. The Load Checklist should be initialed and dated to acknowledge completion of load.

14.1.9. The finished data package will be placed in the data review box in room 2401 (Toxicology Office).

15. **References**

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15.3. Parks and Worth. Carboxyhemoglobin Determination by Second-Derivative Spectroscopy. Clin. Chem. 31/2:279-281. 1985.

15.4. Widdop, Brian. Analysis of Carbon Monoxide. Ann Clin Biochem 39:378-391. 2002