

Pyridine hemochromogen

HEME QUANTITATIONS FROM PYRIDINE HEMOCHROMOGEN SPECTRA

Hemochromogen formation

- Prepare a pyridine reagent mix by adding 3mL 1M NaOH and 6mL pyridine (use glass pipet) to 19mL H₂O in a glass container (28mL total volume).
- Put 1000μL of the pyridine reagent in a cuvet
- Add 35μL sample containing ≥5μM hemin.
- Add 18μL aqueous 15mM K₃Fe(CN)₆, mix well.
- Record the oxidized spectrum at 500-650nm. Repeat until stable.
- Add 2-5mg powdered Na₂S₂O₄ (sodium hydrosulfite, sodium dithionite).
- Mix well under the surface of the liquid mixture (minimize mixing with air).
- Record the reduced spectrum and repeat until stable.

Calculations

- For total heme (mM) subtract the absorbance readings at 540, 556 and 575nm in the oxidized spectrum from the corresponding readings in the reduced spectrum to get ΔA₅₄₀, ΔA₅₅₆ and ΔA₅₇₅. (For example, ΔA₅₄₀ = A₅₄₀ Na₂S₂O₄reduced - A₅₄₀ K₃Fe(CN)₆oxidized, etc.)
- Calculate [hemin, mM] in the mixture using the extinction coefficients:
 - 20.7/mM for ΔA₅₄₀ ((ΔA₅₅₆-ΔA₅₄₀)/20.7)
 - 32.4/mM for ΔA₅₇₅ ((ΔA₅₅₆-ΔA₅₇₅)/32.4)
- Factor in the dilution of the sample (30x or (1000+35+18)/35) and average the two results.
- For heme a, b, or c, multiply the difference of reduced minus oxidized absorbance at the five different wavelengths below and add the resulting five multiplication products.

Reference Information

From: Berry, E.A. and Trumpower, B.L. 1987. Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Analytical Biochemistry*. **161**: 1-15.

Table 4

Inverse matrix of extinction coefficients of pyridine hemochromes for calculating concentration (mM) from reduced minus oxidized absorbance at five different wavelengths.

Wavelength (nm):	540	549	558	588	620
Heme c:	-0.02778	0.04757	-0.01906	0.00084	-0.00157
Heme b:	-0.02943	-0.00088	0.04037	-0.00267	-0.00738
Heme a:	-0.02687	0.00456	0.00699	0.04353	-0.02820

Total heme may also be obtained by adding the results for hemes a, b and c for a sample. Authentic heme solutions with known concentrations may be used to check the method and also to help check that the calculations were performed correctly to obtain expected results.

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