Spectrophotometric evaluation of hemolysis in plasma by quantification of free oxyhemoglobin, methemoglobin, and methemalbumin in presence of bilirubin

Christian Heckl1,2* | Alexander Lang1,2 | Adrian Rühm1,2 | Ronald Sroka1,2 | Thomas Duffield3,4 | Michael Vogeser4 | Michael Paal4

1Laser-Forschungslabor, LIFE-Center, University Hospital, LMU Munich, Germany
2Department of Urology, University Hospital, LMU Munich, Germany
3Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK
4Institute of Laboratory Medicine, University Hospital, LMU Munich, Germany

*Correspondence
Christian Heckl, Laser-Forschungslabor, LIFE-Center, University Hospital, LMU Munich, Fraunhoferstraße 20, 82152 Planegg, Germany. Email: christian.heckl@med.uni-muenchen.de

Funding information
DFG Graduiertenkolleg GRK 2274; Hans-Fischer-Gesellschaft e.V.

Abstract
Severe intravascular hemolysis leads to the simultaneous presence of free heme pigments (oxyhemoglobin, methemoglobin, and methemalbumin) and bilirubin in human plasma. Standard spectrophotometric methods used to assess in vivo hemolysis inadequately address this complex analytical situation. Thus, we propose a novel quantification algorithm to ensure the highest analytical specificity. A corresponding second-derivative fitting algorithm was validated according to the guideline of bioanalytical method validation from the European Medicines Agency using plasma specimens (n = 1759) spiked with different concentrations of oxyhemoglobin and methemoglobin. The results were compared to standard spectrophotometric quantification methods described by Harboe, Noe, and Fairbanks. Based on the second-derivative method, simultaneous quantification of oxyhemoglobin and methemoglobin/methemalbumin in samples with total bilirubin concentrations ≤4.9 mg/dL (83.8 μmol/L) provided robust results (inaccuracy ≤20%, imprecision ≤16%). Analyzing UV/VIS spectra of plasma from patients with confirmed severe intravascular hemolysis evidenced an underestimation of up to 33% for the combined free heme pigment content. The employed second-derivative algorithm allows for automated and highly specific quantification of the free heme pigment content in diluted human plasma, which cannot be realized with standard spectrophotometric evaluation methods. An Excel-based tool readily applicable to clinical datasets accompanies this manuscript.

Abbreviations: EMA, European Medicines Agency; LLOQ, lower limit of quantification.
INTRODUCTION

Various hematological and non-hematological diseases may cause hemolysis in vivo. Intravascular hemolysis is defined by the premature destruction of red blood cells and the release of their contents into the bloodstream, with adverse clinical consequences. Numerous medical conditions can cause intravascular hemolysis, including immune-mediated hemolytic anemia, thrombotic microangiopathy, osmotic and mechanical hemolysis. Free heme pigments and bilirubin that are present in the plasma upon hemolysis can be used for diagnostic purposes. In circulation, free hemoglobin is immediately captured by the scavenger protein haptoglobin and delivered to the reticuloendothelial system for degradation [1]. However, in the case of pronounced hemolysis, the available haptoglobin is rapidly depleted and, consequently, the “naked” free hemoglobin in circulation is exposed to oxidative conditions and transformed into methemoglobin [2]. This process reduces the binding affinity of heme to globin, which is why free heme is increasingly bound by albumin, resulting in the formation of methemalbumin [3]. The entire process results in a brownish discoloration of the plasma (see Figure 1).

In clinical practice, one primarily attempts to quantify free hemoglobin in human plasma to diagnose and assess the course of acute and chronic hemolysis, especially when serum haptoglobin is depleted or an acute phase response (with a reactive increase of haptoglobin) may obscure intravascular hemolysis. In recent reports, it was highlighted that free heme pigments (including oxyhemoglobin, methemoglobin, and methemalbumin) act as a disease-driving compound, with the sequela of adverse pathophysiologic reactions, including inflammation, vascular disease, renal impairment, and lung injury [4–6]. Given that free heme pigments are an independent

---

**KEYWORDS**
derivative spectrophotometry, free hemoglobin, hemolysis, methemalbumin, methemoglobin, oxyhemoglobin

---

![Diagram of intravascular hemoglobin metabolism](image)
predictor of outcome, free hemoglobin is increasingly quantified in critical illness, for example, in patients with severe sepsis and extracorporeal support [7, 8]. Besides, free hemoglobin serves as a routine quality control marker of packed erythrocytes for transfusion to assess storage-related hemolysis [9].

As confirmed by proficiency testing (also termed external quality assessment) results in 2020, most clinical laboratories use spectrophotometric evaluation procedures for free hemoglobin quantification [10]. These methods are intended to quantify oxyhemoglobin using discrete wavelengths in the Soret, alpha, and beta bands of heme. Various spectrophotometric multi-wavelength methods are described in the literature, including those of Harboe [11], Noe [12], Fairbanks [13], and Kahn [14]. Blood plasma analysis can also be performed by Raman spectroscopy, using an excitation wavelength in the near-infrared region to record the Raman spectra [15, 16]. All these methods are susceptible to interferences introduced by hyperbilirubinemia and hypertriglyceridemia of varying degrees; moreover, they all have in common that they do not adequately address the presence of methemoglobin and methemalbumin that contribute to the total free heme pigment content.

Previously, we have described a mathematical approach to process primary UV/VIS-spectrometric data by second-derivative fitting for oxyhemoglobin quantification [17]. While interference by bilirubin – which must systematically be expected in patients with in vivo hemolysis – was overcome, the presence of methemoglobin or methemalbumin was not reported in the analysis. Knowledge about the presence of methemoglobin or methemalbumin in the specimens provides an added clinical value, given that severe intravascular hemolysis must be assumed in this case. Consequently, a further improvement of this data processing approach becomes necessary to provide accurate analysis for the latter compounds too.

The main objective of this continued optimization work was, therefore, to develop and validate a robust second-derivative spectrophotometry evaluation method for the simultaneous quantification of free total heme pigment content in different redox states in human plasma, comprising oxyhemoglobin, methemoglobin, methemalbumin, and bilirubin. As with standard spectrophotometric methods, the corresponding assay should be as simple as possible and provide a test result within minutes.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Potassium ferricyanide (K₃[Fe(CN)₆]), hemin chloride, dimethyl sulfoxide (DMSO), and Pur-A-Lyzer™ maxi dialysis tubes (MCWO 12–14 kDa) were obtained from Sigma–Aldrich (St. Louis, MI, USA). Buffered scattering-free solution for spectrophotometric analysis (Bioanalytic, Umkirch, Germany) and disposable 10 mm transmission polystyrene semi-micro cuvettes were used (Sarstedt, Nümbrecht, Germany).

2.2 | Sample preparation

In the following paragraphs, the preparation of the measured samples and the measurement device used is described in detail. Besides, an explanation of the data analysis including the developed second-derivative fitting method, and the method validation protocol are described.

For method validation, leftover anonymized icteric citrate plasmas from routine patient care were used. Non-icteric citrate plasma pools with minimal free hemoglobin contents were prepared from carefully freshly drawn citrate plasmas from three healthy volunteers who signed a declaration of consent. Until spiking, all specimens were stored at −80°C protected from light to avoid degradation.

For the preparation of stock solutions, EDTA-whole blood was sampled from a healthy donor who signed a consent form. Free hemoglobin was isolated from whole blood by an osmotic shock [18]. Methemoglobin was formed from fresh hemolysate by incubation with a four-fold molar excess of potassium ferricyanide at 22°C for 15 minutes, followed by removal of the oxidant by equilibrium dialysis. Total hemoglobin was quantified with the XN-1000™ hematology analyzer (Sysmex, Kōbe, Japan). Evaluation with molar extinction coefficients [19, 20] revealed ≥95% oxyhemoglobin content for the fresh hemolysate and ≥96% for the methemoglobin dialysate, which is why the fresh hemolysate is referred to as oxyhemoglobin and the dialysate as methemoglobin.

Serial dilution with distilled water yielded 10x concentrated oxyhemoglobin and methemoglobin stock solutions (up to 20 000 mg/L). These stock solutions were prepared independently for calibrators and quality controls. Hemin, representing a spectral surrogate for methemalbumin [21, 22], was diluted in nitrogen-purged DMSO-water (80:20, %/%), and immediately subjected to spectrophotometric analysis.

To prepare spiked calibrator sets for oxyhemoglobin and methemoglobin and corresponding quality control sets, one volume of the hemoglobin stock solutions was mixed with nine volumes of citrate plasma thus obtaining a total hemoglobin concentration ranging from 25, 50, 100, 250, 500, 1500, and 2000 mg/L. Total bilirubin was quantified using the colorimetric diazo-method (Roche Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany) from a control sample prepared with distilled water.
2.3 Spectrophotometry

Spiked citrate plasmas (including control samples) were diluted 1:11 with buffered, scattering-free solution and UV/VIS absorption spectra of 350 to 700 nm were recorded with the U-1900 spectral photometer (Hitachi High-Technologies, Tokyo, Japan) using semi-micro cuvettes and the following photometer settings: step size 0.5 nm, scan speed 200 nm/min, spectral bandpass 4 nm. The spectrometer used, has a wavelength accuracy of ±0.5 nm and photometric accuracy of ±0.004 of the absorbance value. Spectral data sets were evaluated with the second-derivative fitting method and its refinements as described in this article and with the multi-wavelength evaluation methods of Harboe [11], Noe [12], and Fairbanks (evaluation A2) [13] for diluted samples.

2.4 Data analysis

2.4.1 Second-derivative fitting algorithm for quantification of total free heme pigments

The total free heme pigment content was determined with a second-derivative fitting algorithm, as previously described for oxyhemoglobin quantification [17]. For this purpose, reference absorption spectra were recorded for pure oxyhemoglobin and pure methemoglobin in distilled water and normalized to the maximum absorbance at the Soret band at 415 and 405 nm, respectively (see Figure 2). Reference absorption spectra were also recorded for pure hemin in nitrogen-purged DMSO-water (80:20, %/%), where hemin served as a spectral surrogate for methemalbumin [21, 22]. However, due to equivalent spectral signatures, equimolar solutions of methemoglobin and hemin were not distinguishable by the fitting algorithm. Therefore, all spiking experiments in plasma were done exclusively with oxyhemoglobin and methemoglobin.

To perform the fitting algorithm, a suitable wavelength range must be defined which includes the relevant discriminating features in the absorption spectra of the heme pigments to be distinguished. It was set to 380 to 650 nm to cover the heme specific spectral information in the Soret, alpha, and beta bands. The quantitative amounts of the free heme pigment oxyhemoglobin and methemoglobin/methemalbumin were determined by minimizing the mean square of the second derivative of the residual spectrum obtained after simultaneous subtraction of the reference spectra $A_{oxyHb}(\lambda)$ and $A_{metHb}(\lambda)$ of oxyhemoglobin and methemoglobin/methemalbumin from the input specimen spectrum with iteratively adjusted parameters $a_{oxyHb}$ and $a_{metHb}$ (see Equation (1) and Figure 3).

$$\left( \frac{\lambda_{\text{max}}}{\lambda_{\text{min}}} \right)^2 \left( \frac{d^2}{d\lambda^2} \left( A_{\text{raw}}(\lambda) - a_{oxyHb} A_{oxyHb}(\lambda) - a_{metHb} A_{metHb}(\lambda) \right) \right)^2 \rightarrow \text{minimum}$$

To calculate the second derivative spectrum, a Savitzky–Golay filter [23] with a window size of 7 nm was applied to the measured spectrum, and the second derivative was then calculated from a third-order polynomial approximation of the resulting filtered spectrum.

Finally, the absolute concentrations of oxyhemoglobin and methemoglobin/methemalbumin were derived from the spectral amplitudes $a_{oxyHb}$ and $a_{metHb}$ by comparison with the reference absorption spectra previously obtained on pure solutions of each heme species with known molarity. The absolute concentration of bilirubin was subsequently calculated from the final residual spectrum using the spectral value at 460 nm ($\pm 1.5$ nm) after suitable background correction (by subtracting the absorption value at 700 nm multiplied by a factor of 4.03). The resulting value is then divided by a conversion factor of $6.61 \times 10^{-2}$ to obtain the absolute bilirubin concentration in mg/dL. Both factors were derived from a bilirubin calibration method previously established with the same equipment [17].

2.4.2 Spectral analysis for refined determination of free heme pigment portions

The second-derivative fitting algorithm described in the preceding section provided reliable results for the
combined free heme pigment concentration (oxyhemoglobin plus methemoglobin/methemalbumin) in plasma, but only approximate results for the two individual constituents. To determine the contributions separately with satisfactory precision, the ratio of the two concentrations must be determined in addition to the total free heme pigment concentration. For this purpose, the precise position of the absorption maximum in the Soret band region was analyzed. The rationale for this approach is that, depending on the proportion of the two spectral constituents, the position of the effective absorption maximum in the Soret band region varies from 415 nm (for 100% oxyhemoglobin) to 405 nm (for 100% methemoglobin/methemalbumin) (see Figure 4A).

In principle, the position of the absorption maximum can be determined directly from the recorded raw spectra, but the short-wavelength background absorption and the bilirubin absorption (peaking at 460 nm) must be sufficiently weak compared to the absorption of the heme pigments in the Soret band region to avoid false results. Therefore, this analysis was additionally performed on the sum of the two spectral contributions of the heme pigments obtained from the second-derivative fitting as described in section 2.4.1.

The dependence of the methemoglobin/methemalbumin fraction $f_{\text{metHb}}$ on the wavelength position $\lambda$ of the absorption maximum (see Figure 4B) can be approximately described by the custom
phenomenological relationship defined by Equation (2). The model parameters $a$ and $b$ were adjusted for optimal agreement with experimental data obtained on controlled mixtures of oxyhemoglobin and methemoglobin/methemalbumin.

$$f_{\text{metHb}}(\lambda) = c \cdot \ln \left( \frac{1}{a \cdot (\lambda - b)} - 1 \right) + d \quad (2)$$

with:

$$c = \left( \ln \left( \frac{1}{a \cdot (\lambda_{\text{min}} - b)} - 1 \right) \right)^{-1} \quad (3)$$

$$d = \left( 1 - \ln \left( \frac{1}{a \cdot (\lambda_{\text{max}} - b)} \right) \right)^{-1} \quad (4)$$

The parameters $c$ in Equation (3) and $d$ in Equation (4) were chosen such that the fit-function $f_{\text{metHb}}(\lambda)$ yields 1, i.e. a methemoglobin fraction of 100%, when the maximum of the absorbance spectrum is located at 405 nm (represented by $\lambda_{\text{min}}$) and 0, that is, an oxyhemoglobin fraction of 100%, when the maximum is located at 415 nm (represented by $\lambda_{\text{max}}$). Positions of intensity maxima at wavelengths shorter than 405 nm and longer than 415 nm determined from the absorption spectra are assigned to 100% methemoglobin and 100% oxyhemoglobin, respectively.

### 2.5 Method validation

The quantification method described in this article was validated following the current guideline of bioanalytical method validation from the European Medicines Agency (EMA) [24] concerning the calibration curve, limits of quantification, inaccuracy, and imprecision. The EMA guideline requires that back-calculated concentrations of standards and quality controls should be within ±15% of the nominal value, except for the lower limit of quantification (LLOQ) where it should be within ±20% [24]. Before spiking of patient control plasma samples with oxyhemoglobin and methemoglobin, the pre-existing total free heme pigment concentration in the samples was determined as background from 1:11 diluted specimens using the second-derivative method described in section 2.4.1. This pre-existing free heme pigment concentration was subtracted from all corresponding results, those derived with the second-derivative method described in this article and those obtained with the standard methods of Harboe [11], Noe [12], and Fairbanks [13].

Independent sample sets were used to validate the fitting algorithm as follows: The first two sample sets were used to calibrate and test the fitting algorithm on a carefully drawn blood plasma set with minimized endogenous heme pigment background and no spectral interference from bilirubin. Subsequently, the fitting algorithm was expanded on a training set with icteric specimens and finally cross-validated with an independent quality control test set. Thus, a total of 1759 samples were measured comprising the calibration data sets for oxyhemoglobin and methemoglobin and the independent quality control data sets, as described in detail below.

#### 2.5.1 Oxyhemoglobin and methemoglobin calibration

Calibration sets of pure oxyhemoglobin and methemoglobin for the determination of the conversion factors were prepared by spiking a non-icteric citrate plasma pool on four separate days with six replicates each, giving final concentrations of 25, 50, 100, 250, 500, 1000, 1500, and 2000 mg/L (384 samples in total).

#### 2.5.2 Non-icteric plasma pool – quality controls

A separate non-icteric plasma pool was spiked simultaneously with oxyhemoglobin and methemoglobin at seven mixing ratios (0:100, 10:90, 25:75, 50:50, 75:25, 90:10, 100:0; %/%) to obtain six final concentrations of total hemoglobin (25, 50, 100, 250, 500, 1000 mg/L) on three separate days with five replicates each (630 samples in total). The sample-set without the spectral interference of bilirubin was used to establish the second-derivative fitting algorithm for determining absolute pigment concentrations of oxyhemoglobin and methemoglobin/methemalbumin.

#### 2.5.3 Native patient samples – quality control training set

Native patient citrate plasmas with ascending total bilirubin concentrations of 0.2 to 42.2 mg/dL (3.4–721.8 μmol/L) were individually spiked with oxyhemoglobin and methemoglobin at seven mixing ratios (0:100, 10:90, 25:75, 50:50, 75:25, 90:10, 100:0; %/%) to obtain six final concentrations of total hemoglobin (25, 50, 100, 250, 500, 1000 mg/L) were prepared in each case. This preparation and subsequent characterization were performed on three separate days with five replicates each (630 samples in total). Samples were grouped into
the following categories: non-icteric (total bilirubin <1.0 mg/dL, n = 230), mildly icteric (total bilirubin ≥1.0 to 4.9 mg/dL, n = 205), and strongly icteric (total bilirubin ≥5.0 mg/dL, n = 195). The sample-set was used as a training set to optimize the second-derivative fit and the analysis of the free heme pigment portion described in sections 2.4.1 and 2.4.2.

2.5.4 | Native patient samples – quality control test set

Native patient citrate plasmas with ascending total bilirubin concentrations of 0.3 to 22.7 mg/dL (5.1–388.3 μmol/L) were individually spiked with oxyhemoglobin and methemoglobin at seven mixing ratios (0:100, 10:90, 25:75, 50:50, 75:25, 90:10, 100:0; %/%). Samples with five different concentrations of total hemoglobin (25, 50, 100, 250, 500 mg/L) (non-icteric n = 15, mildly icteric n = 71, strongly icteric n = 29) were prepared in each case. This sample set was used for testing and therefore used blinded concerning total bilirubin and the spiking concentrations of oxyhemoglobin and methemoglobin.

2.6 | Assessment of hemolysis in patient care

UV/VIS spectra from native patient care plasma samples (n = 16) with elevated free hemoglobin concentrations ≥50 mg/L as determined with the standard multi-wavelength evaluation method of Harboe [11] were screened for distinct spectral blue shifts of the heme Soret band position and crosschecked with clinical history and laboratory results demonstrating intravascular hemolysis. These spectra were evaluated using the second-derivative method described in section 2.4.1, a previously described oxyhemoglobin second-derivative fitting method [17], and also with the spectrophotometric method of Noe [12] and Fairbanks [13]. The results of the different methods were evaluated by comparing the average inaccuracy of all patient samples from all methods.

2.7 | Statistical evaluation

All obtained results are presented as inaccuracy (Equation (5)) and imprecision (Equation (6)) according to the specification of the EMA guidelines [24].

\[
\text{Inaccuracy} = \frac{\text{mean result} - \text{reference}}{\text{reference}} \quad (5)
\]

\[
\text{Imprecision} = \frac{\text{standard deviation}}{\text{reference}} \quad (6)
\]

Data analysis of all recorded UV/VIS-spectra and statistical analysis were done with Matlab (R2018b, MathWorks, Natick, Massachusetts, USA). The results of the three quality control data sets are presented in boxplots. The box shows the middle 50% of the data points and the line inside the box represents the median values. Data points that lie outside the box up to a maximum of 1.5 times the box length (2.7σ) are shown as whiskers, whereas data points with a larger distance are shown as outliers by crosses.

3 | RESULTS

3.1 | Method validation

3.1.1 | Oxyhemoglobin and methemoglobin calibration

Using the methods described in section 2.2, the calibration range of 25 to 2000 mg/L for oxyhemoglobin and 25 to 1000 mg/L for methemoglobin gave an R² ≥ 0.99 for linear regression fitting for both analytes. Intra- and inter-day imprecision and inaccuracy were ≤1.8%. Conversion factors of 7.03 × 10⁻⁴ per mg/L at 415 nm and 8.80 × 10⁻⁴ per mg/L at 405 nm were obtained for oxyhemoglobin and methemoglobin, respectively.

3.1.2 | Non-icteric plasma pool – quality controls

Combined oxyhemoglobin and methemoglobin quality controls prepared by spiking a non-icteric plasma pool (see section 2.5.2) resulted in within- and between-run inaccuracy and imprecision values ≤14% for the quantification of total hemoglobin (sum of oxyhemoglobin and methemoglobin/methemalbumin) using the second-derivative method described in section 2.4.1 (see Table 1 and, for comparison with Harboe, Figure 5). In contrast, the evaluation algorithms of Harboe, Noe, and Fairbanks showed a deviation of up to 35% for the total hemoglobin content, depending on the methemoglobin proportion.

3.1.3 | Native patient samples – quality control training set

When investigating the samples described in section 2.5.3, for total bilirubin concentrations of up to 4.9 mg/dL
The second-derivative method described in section 2.4.1 yielded inaccuracy and imprecision values ≤20% for the quantification of total hemoglobin, whereas the standard methods of Harboe, Noe, and Fairbanks over- and underestimated the total hemoglobin content by up to 138% (see Table 2 and Figure 6 for the comparison with Harboe). For samples with bilirubin concentrations of >5 mg/dL inaccuracy and imprecision values for the second-derivative fit were found to exceed the specifications of ≤20% for the LLOQ and ≤15% as proposed by the EMA guideline [24].

It was also examined whether the position of the maximum absorption in the Soret band region is consistent with the determined proportion of oxyhemoglobin and methemoglobin/methemalbumin, or whether that proportion could be refined, that is, determined more precisely, by a detailed analysis of this spectral position. For this purpose, the two constituent proportions were plotted against the wavelength position of the absorption maximum in the Soret band (see Figure 7). The maximum position was determined in two ways: once directly from the UV/VIS raw spectra (blue dataset) and once from the weighted sum of the reference spectra of the two constituents as derived from the second-derivative method described in section 2.4.1 (red dataset). The model parameters \( a \) and \( b \) in Equation (2) were iteratively determined to obtain the best fit of each dataset as follows: For the analysis based on the reference spectra of the two constituents derived from the second-derivative fitting (red dataset): \( a_1 = 1.81 \times 10^{-7} \text{ nm}^{-1} \), \( b_1 = 404.23 \text{ nm} \) \( (R^2 = 0.90) \).

For the direct analysis of the UV/VIS raw spectra (blue dataset): \( a_2 = 4.83 \times 10^{-2} \text{ nm}^{-1} \) and \( b_2 = 404.26 \text{ nm} \) \( (R^2 = 0.81) \). Based on the resulting two calibration curves, the contributions of oxyhemoglobin and methemoglobin/methemalbumin were determined with inaccuracy and imprecision of ≤16% and ≤30%, respectively.

### Table 1: Non-icteric plasma pool – quality controls, comparative analysis

| Total hemoglobin (mg/L) | Fitting algorithm | Harboe   | Noe    | Fairbanks |
|-------------------------|------------------|----------|--------|-----------|
| 25                      | 14/3.0           | -35/2.9  | -22/3.7| -32/6.9   |
| 50                      | 5.4/2.4          | -32/1.9  | -26/2.3| -28/3.2   |
| 100                     | 3.1/1.9          | -30/1.6  | -27/1.8| -22/3.0   |
| 250                     | 0.9/2.7          | -29/2.1  | -29/2.2| -19/2.3   |
| 500                     | 0.8/2.1          | -28/1.2  | -29/1.1| -18/1.4   |
| 1000                    | -6.4/2.5         | -30/1.2  | -32/1.1| -21/1.5   |

**Note:** Mean values for within-run inaccuracy and imprecision of the total hemoglobin concentration (oxyhemoglobin and methemoglobin/methemalbumin) measured on three different days on quality control samples with different mixing ratios of oxyhemoglobin and methemoglobin (see section 2.5.2) are displayed for the refined second-derivative fitting method described in this article and the evaluation procedures of Harboe, Noe, and Fairbanks.

### Figure 5
Results of the total free heme concentrations in Table 1 from the known values as boxplots, according to the second-derivative fitting algorithm (blue) and the evaluation method of Harboe (red). The control plasma set had no spectral interferences of bilirubin.

(83.8 \( \mu \text{mol/L} \)), the second-derivative method described in section 2.4.1 yielded inaccuracy and imprecision values ≤20% for the quantification of total hemoglobin, whereas the standard methods of Harboe, Noe, and Fairbanks over- and underestimated the total hemoglobin content by up to 138% (see Table 2 and Figure 6 for the comparison with Harboe). For samples with bilirubin concentrations of >5 mg/dL inaccuracy and imprecision values for the second-derivative fit were found to exceed the specifications of ≤20% for the LLOQ and ≤15% as proposed by the EMA guideline [24].

It was also examined whether the position of the maximum absorption in the Soret band region is consistent with the determined proportion of oxyhemoglobin and methemoglobin/methemalbumin, or whether that proportion could be refined, that is, determined more precisely, by a detailed analysis of this spectral position. For this purpose, the two constituent proportions were plotted against the wavelength position of the absorption maximum in the Soret band (see Figure 7). The maximum position was determined in two ways: once directly from the UV/VIS raw spectra (blue dataset) and once from the weighted sum of the reference spectra of the two constituents as derived from the second-derivative method described in section 2.4.1 (red dataset). The model parameters \( a \) and \( b \) in Equation (2) were iteratively determined to obtain the best fit of each dataset as follows: For the analysis based on the reference spectra of the two constituents derived from the second-derivative fitting (red dataset): \( a_1 = 1.81 \times 10^{-7} \text{ nm}^{-1} \), \( b_1 = 404.23 \text{ nm} \) \( (R^2 = 0.90) \).

For the direct analysis of the UV/VIS raw spectra (blue dataset): \( a_2 = 4.83 \times 10^{-2} \text{ nm}^{-1} \) and \( b_2 = 404.26 \text{ nm} \) \( (R^2 = 0.81) \). Based on the resulting two calibration curves, the contributions of oxyhemoglobin and methemoglobin/methemalbumin were determined with inaccuracy and imprecision of ≤16% and ≤30%, respectively.

### 3.1.4 Native patient samples – quality control test set

In agreement with the results of quantifying total hemoglobin from the training specimen set described in section 2.5.3, inaccuracy and imprecision values ≤16% were obtained with the second-derivative method for all samples described in section 2.5.4 with total bilirubin concentrations ≤4.9 mg/dL (83.8 \( \mu \text{mol/L} \)), while total hemoglobin was over- and underestimated by up to ≤171% when using the formulas of Harboe, Noe, and Fairbanks (see Table 3 and, for comparison with Harboe, Figure 8A).
By evaluating the maximum position in the Soret band region, based on the weighted reference spectra of the two constituents derived from the second-derivative method and the UV/VIS raw spectra, respectively, the oxyhemoglobin and methemoglobin content of the samples could be derived separately with inaccuracy and imprecision $\leq 21\%$ and $\leq 47\%$.

The evaluation of total bilirubin based on the second-derivative method as described in section 2.4.1, in comparison with reference measurements using a clinical chemistry analyzer, yielded inaccuracy and imprecision values of $\leq 13\%$ and $\leq 17\%$ for all samples tested with bilirubin concentrations $\geq 1.5 \text{ mg/dL (25.7} \mu\text{mol/L)}, respectively, (see Figure 8B).

### Table 2: Native patient samples – quality control training set, comparative analysis

| Total bilirubin (mg/dL; $\mu$mol/L) | Total hemoglobin (mg/L) | Fitting algorithm | Harboe | Noe | Fairbanks |
|-----------------------------------|------------------------|-------------------|--------|-----|-----------|
| Non-icteric $<1; <17.1$ (n = 230) | 25                     | −8.7/9.4          | −40/20 | −26/24 | −3.3/21   |
|                                   | 50                     | −5.5/10           | −35/19 | −30/21 | −11/16    |
|                                   | 100                    | −3.0/11           | −32/20 | −30/21 | −15/16    |
|                                   | 250                    | 4.4/6.9           | −26/19 | −26/21 | −12/12    |
|                                   | 500                    | 4.8/5.3           | −26/18 | −27/20 | −14/11    |
|                                   | 1000                   | 1.7/7.1           | −27/17 | −28/18 | −15/9.7   |
| Mildly icteric $\geq1.0–4.9; \geq17.1–83.3$ (n = 205) | 25                     | −20/14           | 52/66  | 138/112 | −37/41    |
|                                   | 50                     | −9.9/13           | −4.4/46 | 26/68  | −26/34    |
|                                   | 100                    | −8.7/9.9          | −12/23 | 7.4/31  | −19/14    |
|                                   | 250                    | −0.2/11           | −8.9/44 | 1.5/61  | −10/17    |
|                                   | 500                    | −5.5/13           | −10/29 | 0.7/40  | −20/17    |
|                                   | 1000                   | −4.0/9.8          | −26/20 | −26/22 | −17/12    |
| Strongly icteric $\geq5.0; \geq85$ (n = 195) | 25                     | −306/327          | 266/448 | 654/554 | −235/444  |
|                                   | 50                     | −21/72            | 228/158 | 409/225 | −36/103   |
|                                   | 100                    | −14/64            | 121/137 | 218/176 | −3.2/70   |
|                                   | 250                    | −3.4/22           | 77/47  | 130/58  | −18/25    |
|                                   | 500                    | −28/14            | −22/28 | −4.1/38 | −37/17    |
|                                   | 1000                   | −23/13            | −25/20 | −17/23 | −27/14    |

**Note:** Mean values for within-run inaccuracy and imprecision of the total hemoglobin concentration (oxyhemoglobin and methemoglobin/methemalbumin) of blended oxyhemoglobin and methemoglobin quality controls are displayed for the refined second-derivative fitting method described in this article and the evaluation procedures of Harboe, Noe, and Fairbanks for three independent measurement series on samples grouped into three bilirubin concentration ranges.

### Figure 6
Calculated deviations of the total free heme concentrations in Table 2 from the known values as boxplots, according to the second-derivative fitting algorithm (blue) and the evaluation method of Harboe (red). Evaluation of the quality control training set with varying concentrations of bilirubin.

By evaluating the maximum position in the Soret band region, based on the weighted reference spectra of the two constituents derived from the second-derivative method and the UV/VIS raw spectra, respectively, the oxyhemoglobin and methemoglobin content of the samples could be derived separately with inaccuracy and imprecision $\leq 21\%$ and $\leq 47\%$.

The evaluation of total bilirubin based on the second-derivative method as described in section 2.4.1, in comparison with reference measurements using a clinical chemistry analyzer, yielded inaccuracy and imprecision values of $\leq 13\%$ and $\leq 17\%$ for all samples tested with bilirubin concentrations $\geq 1.5 \text{ mg/dL (25.7} \mu\text{mol/L)}, respectively, (see Figure 8B).

### 3.2 | Assessment of hemolysis in patient care

Applying the optimized second-derivative method in this project to raw UV/VIS spectrophotometric data obtained from routine native patient care samples with confirmed severe intravascular hemolysis (n = 16) revealed spectral signatures of methemoglobin/methemalbumin (see Figure 9 showing the evaluation of a non-icteric (A) and an icteric (B) sample). The residual spectrum resulting during the second-derivative fitting, however, showed no
prominent signatures at the oxyhemoglobin and methemoglobin/methemalbumin Soret band maxima, confirming adequate subtraction of the heme pigment fractions determined by the fitting algorithm. In com-
parison, when using the previously described oxyhemoglobin fitting algorithm [17] and the standard spectrophotometric method of Harboe [11], Noe [12], and Fairbanks [13], the total heme pigment content of these samples was underestimated by at least 33% compared to the algo-

4 | DISCUSSION

In the present study, we developed and validated a
refined spectrophotometric second-derivative fitting
method to quantify the total free heme pigment content
in human plasma comprising oxyhemoglobin on the one
hand and (the spectrally indistinguishable) methemoglo-
bin and methemalbumin on the other hand. Compared
to the standard spectrophotometric evaluation methods,
the presented method enables a far more detailed assess-
ment of hemolysis with a reliable and fully automated
procedure. An Excel-based quantification tool for direct
evaluation of UV/VIS raw spectra accompanies this man-
uscript for rapid method implementation.

Standard spectrophotometric methods based on the
evaluation of signals at discrete wavelengths are inade-
quate concerning the determination of methemoglobin
and methemalbumin. For example, methods designed to
specifically quantify oxyhemoglobin using the spectral

![Figure 7](image)

**FIGURE 7** Determination of the hemoglobin ratio from the
wavelength position of the absorption maximum in the Soret band
region. Depicted are the maximum positions determined from the
weighted sum of the two spectral constituents derived by the
second-derivative fitting method (red data points) and from the
measured raw spectrum (blue data points) as boxplots. The Soret
band maximum position of pure oxyhemoglobin at 415 nm (solid
gray line) and that of pure methemoglobin/methemalbumin at
405 nm (dotted gray line) are also indicated as vertical
lines (n = 420)

| Total bilirubin (mg/dL; μmol/L) | Total hemoglobin (mg/L) | Inaccuracy (%)/Imprecision (%) |
|--------------------------------|-------------------------|--------------------------------|
| Non-icteric <1; <17.1 (n = 15) | 25                      | -12/16 -39/38 -19/44 -8.0/104 |
|                               | 50                      | 10/9.9 -12/23 -1.4/25 2.2/41   |
|                               | 100                     | 4.4/3.8 -20/17 -15/21 2.7/29   |
|                               | 250                     | 4.6/3.5 -39/4.1 -40/4.7 -23/4.4 |
|                               | 500                     | 7.8/5.9 -26/14 -27/16 -12/11   |
| Mildly icteric ≥1.0–4.9; ≥17.1–83.3 (n = 71) | 25 | 0.4/14 78/83 171/144 13/91 |
|                               | 50                      | 8.8/8.4 44/58 96/89 15/48      |
|                               | 100                     | 8.0/8.8 5.0/27 25/39 5.5/26    |
|                               | 250                     | 7.6/8.5 -13/21 -6.1/25 -11/15   |
|                               | 500                     | 9.0/7.7 -14/18 -10/19 -7.1/11   |
| Strongly icteric ≥5.0; ≥85 (n = 29) | 25 | 15/13 611/289 1004/451 -96/154 |
|                               | 50                      | 3.1/5.8 237/141 407/218 -22/54  |
|                               | 100                     | 3.1/11 109/42 195/71 32/45     |
|                               | 250                     | 6.3/2.0 42/29 83/40 -21/13     |
|                               | 500                     | 2.4/7.1 2.1/19 20/24 -17/14    |

**TABLE 3** Native patient samples – quality control test set, blinded comparative analysis

Note: Mean values for within-run inaccuracy and imprecision of the total hemoglobin concentration (oxyhemoglobin and methemoglobin/methemalbumin) of blended oxyhemoglobin and methemoglobin quality controls are displayed for the refined second-derivative fitting method described in this article and the evaluation procedures of Harboe, Noe, and Fairbanks for three measurement series on samples grouped into three bilirubin concentration ranges.
absorption signal at the alpha and beta band of oxyhemoglobin, such as the method of Kahn [14], do not provide any information about the presence of methemoglobin or methemalbumin. Even methods that utilize the strong molecular absorption of the three heme molecules at the Soret band (410–415 nm), e.g. Harboe [11], Noe [12], Fairbanks [13], lead to incomplete characterization of the free heme pigment content because the contributions of oxyhemoglobin, methemoglobin, and methemalbumin contained in the spectral signal cannot be separated by distinct wavelength evaluation due to their spectral overlap. Based on the standard multi-wavelength spectrophotometric methods, methemoglobin and methemalbumin can only be determined separately from oxyhemoglobin by a combination of non-equivalent methods, e.g. by combined evaluation with the methods of Kahn and Harboe. However, such an analytical approach would pose several pitfalls. Samples must be examined multiple times, precise quantitative results can, thus, not be obtained, and a strong background absorption from bilirubin, as caused by hyperbilirubinemia, is known to falsify spectral evaluations based on the Soret band [25]. In contrast, continuous spectral absorbance scans combined with derivative evaluations can solve such analytical challenges [26].

The second-derivative method described here was established and optimized with normalized pure spectra of oxyhemoglobin and methemoglobin. During the fit procedure, these pre-determined oxyhemoglobin and methemoglobin reference spectra were simultaneously subtracted with iteratively adjusted weights from a raw UV/VIS spectrum in the spectral range 380 to 650 nm until the mean squared second derivative of the residual spectrum was minimized. For methemalbumin, the spectral signature of molecular hemin was used as a spectral surrogate [21, 22]. Based on that, however, methemoglobin and methemalbumin were not distinguishable in diluted samples. Therefore, only the sum of both analytes can be reported.

The method was comprehensively validated according to the specifications proposed by the EMA guideline of bioanalytical method validation [24]. In blending experiments, up to a bilirubin concentration of 4.9 mg/dL (83.8 μmol/L), the second-derivative method complied with the EMA guideline, while the standard methods of Harboe, Noe, and Fairbanks violated the EMA specifications.

In a second refinement step, the analysis of the position of the absorption maximum in the Soret band region provided an average deviation of the individual heme
pigment fractions of ≤21%. This additional analysis provided more accurate results when evaluating the maximum position of the weighted sum of the reference spectra of the two constituents as derived from the second-derivative fitting, as opposed to evaluating the raw UV/VIS spectra. Heme pigment differentiation is of added value in direct comparison to a previously described oxyhemoglobin fitting algorithm that also complied with the specifications proposed by the current EMA guidelines [17].

The performance of the second-derivative fitting method was evaluated by examining raw UV/VIS spectra of native patient care samples with confirmed severe intravascular hemolysis and a blue-shift of the Soret band. The analysis revealed the spectral signature of methemoglobin and methemalbumin in addition to that of oxyhemoglobin, indicating that the total heme pigment content (oxyhemoglobin plus methemoglobin and methemalbumin) was underestimated by up to 33% when using standard evaluation methods. Precise quantification of the different circulating free heme pigments can be valuable for clinical assessment. The presence of methemalbumin is indicative of rapid erythrocyte breakdown and thus provides further information about the severity and dynamic of hemolysis, regardless of its etiology. Although methemalbumin cannot be distinguished from methemoglobin with the second-derivative method presented in this article, the quantitative determination of the sum concentration of these two heme pigments is still valuable for diagnostic purposes.

Besides the evaluation of intravascular hemolysis or hemorrhage, further applications for the presented method are conceivable. The analysis method used in this project could be used for a more comprehensive evaluation of the quality of packed red blood cell units, where standard spectrophotometric methods may systematically under-report the degree of hemolysis due to storage-related in vitro methemoglobin formation [27]. Another potential application for the second-derivative method described in this article is spectrophotometric cerebrospinal fluid analysis for the diagnosis of subarachnoid hemorrhage [28]. Spectral contributions of oxyhemoglobin and methemoglobin will be eliminated by subtracting an optimized weighted sum of reference spectra, such that the contribution of the target measurand bilirubin that is produced solely in vivo remains for further analysis.

In conclusion, we propose a refined second-derivative fitting method for the simultaneous quantification of oxyhemoglobin, methemoglobin/methemalbumin, and bilirubin. Using a Microsoft Excel macro that accompanies this manuscript, the method can directly be applied in clinical practice. Raw UV/VIS spectra are readily evaluated and results are immediately reported. The present method allows for highly reliable quantification of the total free heme pigment content in plasma, along with the separate quantification of oxyhemoglobin, methemoglobin/methemalbumin, even in case of a spectral interference from bilirubin.

**ACKNOWLEDGMENTS**
This work was supported by the Hans-Fischer-Gesellschaft e.V. (Munich, Germany) and the DFG Graduiertenkolleg GRK 2274. We thank Regina Scherer-Burić for her scientific contribution. This manuscript is part of the inaugural thesis of C. Heckl to be submitted at the Medical Faculty of the LMU Munich, Germany. Open Access funding enabled and organized by ProjektDEAL.

**CONFLICT OF INTEREST**
The authors declare no potential conflict of interest.

**AUTHOR CONTRIBUTION**
All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

**DATA AVAILABILITY STATEMENT**
Research data are not shared.

**ETHICS STATEMENT**
According to the local Institutional Review Board guidelines the study is exempt from review.

**ORCID**
Christian Heckl https://orcid.org/0000-0002-7418-7176
Michael Paal https://orcid.org/0000-0001-8831-0238

**REFERENCES**
[1] D. J. Schaer, P. W. Buehler, A. I. Alayash, J. D. Belcher, G. M. Vercellotti, *Blood* 2013, 121, 1276.
[2] B. Faitre, P. Menu, P. Labrude, C. Vigneron, *Artif. Cells Blood Substit. Immobil. Biotechnol.* 1998, 26, 17.
[3] T. R. Kelly, R. L. Klein, J. M. Porquez, G. M. Homer, *Ann. Surg.* 1972, 175, 15.
[4] M. T. Gladwin, T. Kansias, D. B. Kim-Shapiro, *J. Clin. Invest.* 2012, 122, 1205.
[5] D. R. Janz, L. B. Ware, *J. Intensive Care* 2015, 3, 20.
[6] M. Frimat, I. Bouhadhay, L. T. Roumenina, *Toxins (Basel)* 2019, 11, 660.
[7] M. Adamzik, T. Hamburger, F. Petrat, J. Peters, H. de Groot, M. Hartmann, *Crit. Care* 2012, 16, R125.
[8] J. R. Neal, E. Quintana, R. B. Pike, J. D. Hoyer, L. D. Joyce, G. Schears, *J. Extra Corpor. Technol.* 2015, 47, 103.
[9] S. O. Sowemimo-Coker, *Transfus. Med. Rev.* 2002, 16, 46.
[10] Instand: Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V. Ringversuch: Hämatologie 16 - Freies Hämoglobin (236), 2020.
SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Heckl C, Lang A, Rühm A, et al. Spectrophotometric evaluation of hemolysis in plasma by quantification of free oxyhemoglobin, methemoglobin, and methemalbumin in presence of bilirubin. J. Biophotonics. 2021;14:e20200461. https://doi.org/10.1002/jbio.202000461