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K Witt[1], H U Wolf[2], C Heuck[3], M Kammel[1], A Kummrow[1] and J Neukammer[1]

[1] Physikalisch-Technische Bundesanstalt (PTB), Abbestrasse 2–12, 10587 Berlin, Germany
[2] Gesellschaft zur Erstellung von Fachgutachten (GEFG), Lisztstraße 10, 89231 Neu-Ulm, Germany
[3] Institut für Laboratoriumsdiagnostik, Heinrich-Heine-Universität, Moorenstrasse 5, 40225 Düsseldorf, Germany

Abstract

Haemoglobin concentration in blood is one of the most frequently measured analytes in laboratory medicine. Reference and routine methods for the determination of the haemoglobin concentration in blood are based on the conversion of haeme, haemoglobin and haemiglobin species into uniform end products. The total haemoglobin concentration in blood is measured using the absorbance of the reaction products. Traceable absorbance measurement values on the highest metrological level are a prerequisite for the calibration and evaluation of procedures with respect to their suitability for routine measurements and their potential as reference measurement procedures. For this purpose, we describe a procedure to establish traceability of spectral absorbance measurements for the haemiglobincyanide (HiCN) method and for the alkaline haematin detergent (AHD) method. The latter is characterized by a higher stability of the reaction product. In addition, the toxic hazard of cyanide, which binds to the iron ion of the haem group and thus inhibits the oxygen transport, is avoided. Traceability is established at different wavelengths by applying total least-squares analysis to derive the conventional quantity values for the absorbance from the measured values. Extrapolation and interpolation are applied to get access to the spectral regions required to characterize the Q-absorption bands of the HiCN and AHD methods, respectively. For absorbance values between 0.3 and 1.8, the contributions of absorbance measurements to the total expanded uncertainties (95% level of confidence) of absorbance measurements range from 1% to 0.4%.

1. Introduction

Absorption measurements are applied in different domains covering, amongst others, material research, e.g. characterization of materials used for lithography [1], kinetic measurements [2], DNA concentration measurements [3] and protein quantification [4]. To ensure comparability and uniformity of measurements of chemical and biological analytes, traceability and appropriate uncertainty analysis is a basic requirement. The influence of calibration of flame atomic spectrophotometers and molecular absorption

spectrophotometers on the combined uncertainties for chemical measurements was studied when determining the accuracy of copper content in low carbon steel [5] and copper and manganese in water [6]. Depending on the copper concentration in water, the calibration uncertainty ranged from 0.56% to 3% [6]. In addition to absorbance calibration, wavelength calibration and stray light control are important [7].

Neutral density filters certified by national metrology institutes are generally used for the calibration of absorbance or transmittance to establish traceability. Since the accuracy of transmittance values and the stability of such filters directly

influence the result of chemical or biological concentration measurements, an international key comparison of the Consultative Committee on Photometry and Radiometry was performed covering the wavelength range from 380 nm to 1000 nm [8]. Often, calibration of biochemical analysers by neutral density filters cannot be established since various manufacturers incorporate specific absorption cells in their instruments. Hence, solutions of defined spectral characteristics must be used to obtain traceable photometric measurements [9].

Reliable and comparable interlaboratory results are particularly important for medical diagnosis, since concentration limits of analytes may be essential for diagnosis and for selection and monitoring of therapy. Quantification of biomolecules and establishment of traceability are challenging because of the complex matrices associated with bio-measurements. In many cases, traceability to SI units is currently not possible. To achieve comparability of results, according to ISO 17511 the calibration hierarchy and metrological traceability refer to an international conventional reference measurement procedure and international conventional calibrators (see figure 3 in [10]).

Haemoglobin measurements are important, e.g. for the monitoring of pregnant women and for anaemic patients to initiate transfusions and to control the quality of blood products intended to increase the erythrocyte concentration in such patients. For the determination of haemoglobin in blood a variety of methods are used. A primary reference measurement procedure would be highly recommended for their calibration. Unfortunately, at present no primary reference measurement procedure for haemoglobin exists. The internationally accepted conventional reference measurement procedure is the haemiglobincyanide (HiCN) method [11–15]. The method is based on the conversion of all haemoglobin variants in blood to HiCN, the determination of the spectral absorbance at 540 nm and calculation of the concentration.

The HiCN method has several disadvantages, i.e. the limited stability of the international conventional calibrator, the toxicity of the conversion reagents and the lack of availability of HiCN reagents in a number of developing countries. Alternative methods are, therefore, preferably used for routine applications [16–19]. With respect to metrology, the alkaline haematin detergent (AHD) method [20, 21] is of particular interest because of the high stability of the reaction product, lower toxicity and the availability of crystalline, stable chlorohaemin, which is suited as a primary calibrator [22]. In addition, verdoglobin is also converted to haemin when applying the AHD method, a variant which is not accessible with conversion by HiCN reagents. Currently, comparison experiments between the HiCN and AHD methods are being carried out at PTB to derive the degree of equivalence and to develop a primary reference measurement procedure for the determination of total haemoglobin concentration. These experiments include investigations of reaction kinetics and of influencing quantities like bilirubin background absorption and light scatter of lipids in normal and pathological samples.

In this paper, we describe the procedure to establish traceability of absorbance values in the spectral regions relevant for haemoglobin concentration measurements.
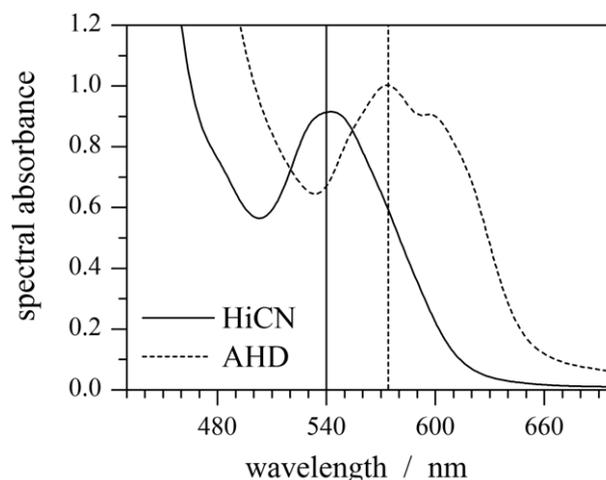

Figure 1. Absorption spectra of HiCN and AHD. The respective wavelengths of 540 nm and 574 nm used for the determination of the total haemoglobin concentration are indicated by vertical lines.

Absorbance values of neutral density filters, the reference values of which were assigned with a primary national reference instrument, were measured in the range between 0.03 and 2.0 for the spectrophotometer used to determine haemoglobin concentrations in blood samples. In addition to the total least-squares (TLS) analysis we also included least-squares (LS) fitting to derive the traceability constants by linear regression. The latter method was also applied, since in clinical or biomedical laboratories generally analyses based on TLS are not yet established and TLS results converge to LS results when uncertainties of the measurand Y and the independent quantity X significantly differ. High-accuracy measurements of haemoglobin concentrations applying gravimetric dilution and two different chemical conversion reagents (i.e. HiCN and AHD) to obtain stable end products are presented. The contribution of the spectrophotometer calibration to the combined uncertainty in total haemoglobin measurements is analysed.

2. Experimental details

2.1. Determination of haemoglobin concentration from absorbance measurements

Both HiCN and AHD methods rely on the measurement of the spectral absorbance. The absorption wavelengths of 540 nm and 574 nm are selected to optimize sensitivity and specificity. Spectra of the absorbance of the Q-bands are shown in figure 1; the respective measurement wavelengths of 540 nm and 574 nm are indicated by vertical lines. The mass concentration $\beta(\mathrm{Hb(Fe)})$ of haemoglobin is calculated from the conventional quantity value of the decadic absorbance $A(\lambda)$ by

$$\beta(\mathrm{Hb(Fe)}) = \frac{A(\lambda) \cdot M(\mathrm{Hb(Fe)})}{d \cdot \varepsilon(\lambda) \cdot \phi}. \tag{2.1}$$

The symbols $M(\mathrm{Hb(Fe)})$ and d represent the molar mass of the monomeric haemoglobin Hb(Fe) and the layer thickness of the rectangular spectrophotometric cuvette; $\varepsilon(\lambda)$ is the

Table 1. Conventional quantity values of the spectral transmittance and absorbance of optical reference filters used for back-tracing. Uncertainties are given with an expansion factor $k = 2$ corresponding to a level of confidence of about 95%. The values were measured with the primary reference instrument at PTB using a spectral half-width of 0.7 nm.

Filter identification		$\lambda = 540$ nm		$\lambda = 546$ nm		$\lambda = 570$ nm		$\lambda = 574$ nm		$\lambda = 580$ nm	
(a)						$\tau_f(\lambda) \pm 2u(\tau_f(\lambda))$					
SRM 2031,	45-90	0.933 00	0.002 88	0.933 00	0.002 88	0.933 00	0.002 88	0.932 00	0.004 80	0.933 00	0.002 88
SRM 2031,	45-30	0.333 00	0.001 48	0.336 00	0.001 49	0.344 00	0.001 51	0.346 00	0.002 53	0.348 00	0.001 52
SRM 1930,	30-1514	0.316 00	0.001 42	0.317 00	0.001 43	0.308 00	0.001 40	0.304 00	0.002 31	0.299 00	0.001 37
SRM 2031,	45-10	0.130 00	0.000 74	0.131 00	0.000 74	0.137 00	0.000 77	0.138 00	0.001 28	0.140 00	0.000 78
SRM 1930,	10-1514	0.104 00	0.000 62	0.104 00	0.000 62	0.099 00	0.000 60	0.097 10	0.000 98	0.094 90	0.000 58
SRM 930d	3-096	0.029 60	0.000 22	0.029 80	0.000 23	0.028 60	0.000 22	0.028 10	0.000 36	0.027 70	0.000 21
SRM 930d	1-096	0.009 33	0.000 08	0.009 42	0.000 09	0.008 91	0.000 08	0.008 80	0.000 13	0.008 58	0.000 08
(b)						$A_f(\lambda) \pm 2u(A_f(\lambda))$					
SRM 2031,	45-90	0.030 12	0.001 34	0.030 12	0.001 34	0.030 12	0.001 34	0.030 58	0.002 24	0.030 12	0.001 34
SRM 2031,	45-30	0.477 56	0.001 93	0.473 66	0.001 92	0.463 44	0.001 91	0.460 92	0.003 17	0.458 42	0.001 90
SRM 1930,	30-1514	0.500 31	0.001 95	0.498 94	0.001 95	0.511 45	0.001 97	0.517 13	0.003 29	0.524 33	0.001 99
SRM 2031,	45-10	0.886 06	0.002 46	0.882 73	0.002 45	0.863 28	0.002 43	0.860 12	0.004 04	0.853 87	0.002 42
SRM 1930,	10-1514	0.982 97	0.002 58	0.982 97	0.002 58	1.004 36	0.002 61	1.012 78	0.004 37	1.022 73	0.002 64
SRM 930d	3-096	1.528 71	0.003 29	1.525 78	0.003 29	1.543 63	0.003 31	1.551 29	0.005 54	1.557 52	0.003 33
SRM 930d	1-096	2.030 12	0.003 95	2.025 95	0.003 94	2.050 12	0.003 97	2.055 52	0.006 63	2.066 51	0.004 00

molar decadic spectral absorption coefficient of the reaction product to which all haemoglobin variants are converted. The character ϕ denotes the volume fraction of the blood sample in the measurement solution. In medical diagnostics, the haemoglobin concentration is mostly given in $g\,dL^{-1}$ for historical reasons. On the other hand, in some medical subject areas [23], the haemoglobin concentration is listed in $g\,L^{-1}$. In this paper, we use the latter recommendation, i.e. the unit $g\,L^{-1}$ for $\beta(Hb(Fe))$. The decadic absorbance $A(\lambda)$ and the volume fraction ϕ are dimensionless quantities and the unit is 1. If the layer thickness is given in cm and the molar mass in $g\,mol^{-1}$, the molar decadic spectral absorption coefficients have the units $L\,mol^{-1}\,cm^{-1}$. The molar mass $M(Hb(Fe))$ amounts to $16\,114.5\,g\,mol^{-1}$ [24] and the values for the extinction coefficients are $\varepsilon^{HiCN}(\lambda = 540\,nm) = 11\,000\,L\,mol^{-1}\,cm^{-1}$ [25] and $\varepsilon^{AHD}(\lambda = 574\,nm) = 6945\,L\,mol^{-1}\,cm^{-1}$ [22]. The corresponding non-expanded uncertainties amount to $u(M) = 16\,g\,mol^{-1}$, $u(\varepsilon^{HiCN}) = 20\,L\,mol^{-1}\,cm^{-1}$ and $u(\varepsilon^{AHD}) = 19\,L\,mol^{-1}\,cm^{-1}$.

For routine applications, the measurement of the absorption at one wavelength, automated or manual preparation of the measurement solution and the use of a single-beam spectrophotometer or a filter-based instrument are adequate to derive haemoglobin concentrations $\beta(Hb(Fe))$. When determining reference values, however, we control the volume fraction ϕ gravimetrically and apply a double-beam spectrophotometer to achieve higher accuracy.

2.2. Establishing traceability and specifications of reference filters

To allow high-accuracy measurements of the spectral absorbance of the Q-bands of HiCN and AHD, traceability was established in the two spectral regions covering 537 nm to 549 nm and 569 nm to 581 nm. Corresponding wavelength scans were performed for the neutral density filters used as primary standard to derive the conventional quantity value A

for the spectral absorbance

$$A(\lambda) = C_0(\lambda) + C_1(\lambda)a(\lambda) \qquad (2.2)$$

from the measured absorbance values a. The constants $C_0(\lambda)$ and $C_1(\lambda)$ were determined using a metal-on-quartz filter set (SRM 2031), two glass filters from an SRM 930d set and two filters from an SRM 1930 set, provided by the National Institute of Standards and Technology. The total heights and widths of the filters are about 30 mm and 10 mm. The flatness over the central 5 mm × 20 mm clear aperture is $<\lambda$ at 633 nm and the parallelism is specified to be less than 0.1 mrad. The absorbance of these filters was measured regularly (usually every two years) using the national reference instrument (see section 2.3 and [8]) of the Physikalisch-Technische Bundesanstalt. The conventional quantity values of the spectral transmittance of the filters were found to be stable since the first assignment of values in 1983 (SRM 2031) and in 1992 (SRM 930d and SRM 1930). For wavelengths necessary for haemoglobin absorbance measurements the conventional quantity values of the spectral transmittance recently measured are listed in table 1(a). The absolute values of the expanded ($k = 2$) uncertainties of the regular transmittance

$$2u(\tau_f) = \frac{0.3}{100} \cdot (1 - \log_{10}\tau_f(\lambda)) \cdot \tau_f(\lambda) \qquad (2.3)$$

were estimated according to the entry in the 'Calibration and Measurement Capabilities' table of the key comparison database of the International Bureau of Weights and Measures (BIPM). These uncertainties are included in table 1; the level of confidence corresponds to about 95%. The factor of 0.3 in (2.3), being characteristic of the instrument used, has to be replaced by 0.5 for the wavelength of 574 nm since the transmittance at this particular wavelength was measured using a different spectrophotometer. The absorbance values $A_f(\lambda)$, calculated from the transmittance

$$\tau_f = 10^{-A_f(\lambda)}, \qquad (2.4)$$

Table 2. Measured mean values of the absorbance of optical filters used for back-tracing. These values were obtained with the spectrophotometer used for the determination of haemoglobin concentration. Combined uncertainties are given according to equation (2.8) for a single repeat measurement with an expansion factor $k = 2$ corresponding to a level of confidence of about 95%.

Filter identification		$\lambda = 540$ nm		$\lambda = 546$ nm		$\lambda = 570$ nm		$\lambda = 574$ nm		$\lambda = 580$ nm	
						$a_f(\lambda) \pm 2u(a_f(\lambda))$					
SRM 2031,	45-90	0.030 63	0.000 16	0.030 62	0.000 15	0.030 49	0.000 09	0.030 49	0.000 11	0.030 47	0.000 11
SRM 2031,	45-30	0.474 70	0.000 64	0.472 31	0.000 64	0.461 24	0.000 75	0.459 50	0.000 58	0.456 41	0.000 59
SRM 1930,	30-1514	0.500 39	0.000 40	0.498 92	0.000 16	0.512 93	0.001 78	0.517 16	0.001 23	0.524 26	0.001 12
SRM 2031,	45-10	0.883 78	0.000 83	0.879 54	0.000 89	0.860 72	0.001 16	0.857 91	0.000 90	0.853 16	0.000 91
SRM 1930,	10-1514	0.985 50	0.000 79	0.982 47	0.000 31	1.005 49	0.002 82	1.012 27	0.001 87	1.023 18	0.001 65
SRM 930d	3-096	1.529 87	0.000 92	1.526 20	0.000 43	1.545 18	0.002 32	1.550 36	0.001 39	1.557 45	0.000 93
SRM 930d	1-096	2.028 63	0.001 20	2.023 81	0.000 60	2.048 34	0.003 01	2.055 02	0.001 86	2.064 12	0.001 22

are given in table 1(*b*). The uncertainties of the transmittances and the absorbance values are related by the derivative $|d\tau_f/dA_f|$ as proportionality factor [26], i.e. $u(\tau_f) = |d\tau_f/dA_f| \cdot u(A_f)$. Taking the relation (2.4) between the transmittance and absorbance into account to calculate the derivative $|d\tau_f/dA_f| = \tau_f \cdot \ln(10)$, the uncertainty of the absorbance values in table 1(*b*) is obtained as

$$2u(A_f) = \frac{1}{\tau_f \cdot \ln(10)} \cdot 2u(\tau_f). \qquad (2.5)$$

The approach given in (2.3) for the estimation of the uncertainties of the transmittance corresponds to the generalized model for the variance function [27]

$$(u(A_f))^2 = (\alpha_1 + \alpha_2 \cdot A_f)^J \qquad (2.6)$$

describing the uncertainties for the absorbance values. Replacing $u(\tau_f)$ in (2.3) by the expression following from (2.5) and using (2.4), it can be shown that

$$u(A_f) = \left(\frac{0.3}{2 \cdot 100} \cdot \frac{1}{\ln(10)} + \frac{0.3}{2 \cdot 100} \cdot \frac{1}{\ln(10)} \cdot A_f \right). \qquad (2.7)$$

It follows that the coefficients are given by $\alpha_1 = \alpha_2 = 1.5 \times 10^{-3}/\ln(10)$ and $J = 2$, corresponding to a constant contribution α_1 and a term $(\alpha_2 \cdot A_f)$ linearly increasing with increasing absorbance values. The parameters α_1 and α_2, being proportional to the factor 0.3 in (2.3), account for the characteristics of the specific spectrophotometer used.

2.3. Description of spectrophotometers

The spectrophotometer used at PTB to assign reference values to the filters is described in the key comparison K6 of the Consultative Committee for Photometry and Radiometry [8]. The set-up is a self-built system based on a Jobin Yvon HRD1 single-pass double monochromator with a focal length of 0.6 m capable of performing calibrations of spectral transmittance and reflection in the wavelength range between 200 nm and 20 µm. In the visible spectrum, transmittance filters are calibrated for the transmittance in the range between 1 and 10^{-6}. Versatile measurement schemes can be realized concerning parameters such as beam diameter, spectral band-width and divergence.

For the calibrations of the filters in the visible spectral range from 380 nm to 800 nm a 1200 lines mm^{-1} grating was

used. The spectral band-width was set to 0.7 nm (FWHM) and the uncertainty of the wavelength adjustment amounted to 0.5 nm. The detection system was a photomultiplier detector (EMI 9558) and the determination of the spectral transmittance was accomplished at perpendicular incidence with a slightly convergent beam of 2 mm width and 12 mm height at the centre of the samples. The temperature was controlled and amounted to $23\,°C \pm 1\,°C$.

Various influences are accounted for in the evaluation of the measurement uncertainty. Stray light is reduced by using the double-monochromator system to a level of typically 10^{-6} at 220 nm. False light is eliminated by using appropriate cut-on filters. The linear signal drift of the temperature-controlled halogen lamp in the visible is less than 10^{-4} and is further reduced by a time-symmetric measurement technique. The linearity of the detection system is determined by separate measurements using a two-lamp technique. For the multiplier detector typical corrections are found to be less than 10^{-3}. Standard deviations are 2×10^{-4} or less for the measurements at various wavelengths and optical densities of the calibrated filters.

Traceability of absorbance values was established for the instrument (Cary 6000i, Agilent Technologies, CA, USA) used to derive haemoglobin concentrations from absorption measurements. For the ultraviolet and visible spectral ranges of the double-beam spectrophotometer two monochromator gratings with 1200 lines mm^{-1} serve to select the required wavelengths. Transmitted light is detected by an R928 photomultiplier tube (Hamamatsu, Shizuoka, Japan). The photometric accuracy is specified as 0.000 25 at an absorbance of 0.3 and the specified stability within 1 h is <0.0002. The specified stray light is <0.000 07% in transmission measurement at 370 nm applying a 50 mg L^{-1} NaNO$_2$ solution. The spectral half-width was set to the same value as was used for the assignment of reference values for the filter transmittances, i.e. to 0.7 nm. Also the size of the elliptically shaped beam (2 mm width and 12 mm height) approximately corresponded to the beam size of the reference spectrophotometer. The temperature was stabilized to $23\,°C \pm 0.1\,°C$ by the internal heated/cooled cuvette holder. The traceability was established for absorbance values below $A = 2$, since the absorbance values of HiCN and AHD solutions are below this absorbance in the wavelength range of interest. For absorbance values below $A = 2$ the specified linearity of the instrument is <0.0014 and the

noise is 0.0001 for a signal averaging time of 1 s. The wavelength accuracy, specified as <0.08 nm, was controlled by a low-pressure mercury lamp at 546.1 nm. The centre of gravity of the recorded spectral emission line deviated from the expected wavelength by about 0.05 nm. This deviation is an order of magnitude lower compared with the wavelength accuracy of 0.5 nm specified for the reference values of the filters and used in the uncertainty calculations in the following section.

2.4. Measurement procedure and uncertainty estimation for measured absorbance values a_f

Each filter was measured at two randomly selected positions of the six positions of the sample beam of the multi-cell holder to account for position-dependent influences on the traceability constants $C_0(\lambda)$ and $C_1(\lambda)$. Wavelength scans were performed and repeated 50 times at each position, yielding absorbance values $a_{f,j,\ell}(\lambda)$ and $a_{f,\tilde{j},\ell}(\lambda)$. The index f $(1 \leqslant f \leqslant 7)$ serves for the filter identification, j and $\tilde{j}$ $(j \neq \tilde{j}; 1 \leqslant j \leqslant 6; 1 \leqslant \tilde{j} \leqslant 6)$ characterize the position of the cuvette holder and ℓ $(1 \leqslant \ell \leqslant 50)$ stands for the repeat measurement. This protocol allows us to access uncertainty contributions caused by the tuning of the gratings, by insertion of the filter in a specific holder position and by using different positions of the cuvette holder.

The combined uncertainty $u_c^2(a_f(\lambda))$ of the average absorbance $a_f = (1/2)(a_{f,j} + a_{f,\tilde{j}})$ is written as a sum of two terms,

$$u_c^2(a_f(\lambda)) = u^2(a_f(\lambda)) + \left| \frac{\Delta a_f(\lambda)}{\Delta \lambda} \right|^2 u^2(\lambda), \quad (2.8)$$

the second of which accounts for the wavelength-dependent contribution. Using the standard deviations $s(a_{f,j,\ell}(\lambda))$ and $s(a_{f,\tilde{j},\ell}(\lambda))$ for single repeat measurements $a_{f,j,\ell}(\lambda)$ and $a_{f,\tilde{j},\ell}(\lambda)$ at a defined position as well as the standard deviation $s(a_{f,j}(\lambda); a_{f,\tilde{j}}(\lambda))$ of both average values $a_{f,j}$ and $a_{f,\tilde{j}}$, the combined uncertainty is given by

$$u_c^2(a_f(\lambda)) = \left(\frac{1}{2}\right)^2 s^2(a_{f,j,\ell}(\lambda)) + \left(\frac{1}{2}\right)^2 s^2(a_{f,\tilde{j},\ell}(\lambda))$$
$$+ s^2(a_{f,j}(\lambda); a_{f,\tilde{j}}(\lambda))$$
$$+ \left\{ \frac{a_f(\lambda + \Delta\lambda/2) - a_f(\lambda - \Delta\lambda/2)}{\Delta \lambda} \right\}^2 u^2(\lambda). \quad (2.9)$$

The slope of the absorbance values as a function of the wavelength was calculated from the measured values choosing $\Delta\lambda = 2$ nm and the uncertainty of the wavelength was $u(\lambda) = 0.5$ nm. To validate the uncertainty estimation according to (2.9), for a selected filter the absorbance was measured 50 times by removing and inserting the filter at the same and at all six positions of the cuvette holder to estimate contributions to the uncertainty eventually caused by mechanical displacements. The standard deviation of measurements when removing and inserting a filter at the same position is less than 6×10^{-5} and only slightly larger compared with the repeat measurements without moving the neutral density filter (5×10^{-5}). On the other hand, the
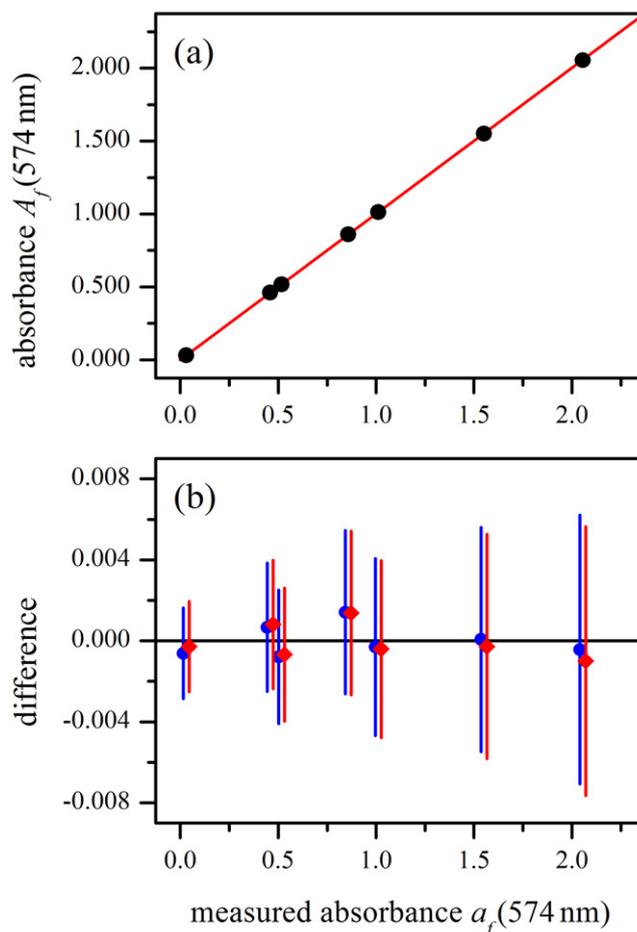

Figure 2. (a) Spectral absorbance $A_f(574 \text{ nm})$ of reference filters, measured with the primary reference procedure, plotted versus the spectral absorbance $a_f(574 \text{ nm})$ determined with the photometer for haemoglobin measurements. (b) Differences between the absorbance $A_f(574 \text{ nm})$ of the reference filters and the calculated values for LS (blue) and TLS (red) analyses. The uncertainties represent the expanded uncertainties $2u(A_f(574 \text{ nm}))$. To facilitate the comparison of LS and TLS results, the associated X-axes are shifted by -0.015 and $+0.015$, respectively.

variance at different positions is about an order of magnitude larger and amounts to 3×10^{-4} at $A \approx 1$. This term, i.e. $s^2(a_{f,j}(\lambda); a_{f,\tilde{j}}(\lambda))$, is the most important for all wavelengths and a minor contribution to the combined uncertainty is due to the wavelength uncertainty.

The results of all measurements of the filter absorbance values are listed in table 2. The uncertainties for a single repeat measurement, calculated according to (2.9) and multiplied by the expansion factor of $k = 2$, are also given in table 2. The level of confidence corresponds to approximately 95% as in tables 1(a) and (b).

3. Data analysis

3.1. Determination of traceability constants

The conventional quantity values of the absorbance $A_f(\lambda)$ were plotted against the measured average absorbance $a_f(\lambda)$ for all wavelengths measured (see table 2). Linear regression

Table 3. Constants C_0, C_1 for the calculation of conventional quantity values for absorbance and expanded uncertainties derived by TLS and LS analyses. The expansion factor $k = 2.571$ corresponds to 5 degrees of freedom and a level of confidence of about 95%.

Wavelength /nm	Analysis	$C_0(\lambda) \pm ku(C_0(\lambda))$		$C_1(\lambda) \pm ku(C_1(\lambda))$	
540	TLS	0.000 13	0.001 52	1.000 24	0.002 03
540	LS	0.000 36	0.001 90	0.999 97	0.002 47
546	TLS	−0.000 14	0.001 49	1.001 14	0.001 98
546	LS	0.000 28	0.001 86	1.000 68	0.002 40
570	TLS	0.000 02	0.001 54	1.000 60	0.002 29
570	LS	0.000 06	0.002 20	1.000 25	0.002 91
574	TLS	0.000 36	0.002 49	1.000 55	0.003 34
574	LS	0.000 71	0.003 15	1.000 11	0.004 06
580	TLS	0.000 01	0.001 52	1.000 73	0.002 03
580	LS	0.000 00	0.001 94	1.000 69	0.002 45

was applied for the determination of the traceability constants. An example is shown in figure 2, where the data for the wavelength of 574 nm are depicted. The measurement results are indicated as dots. The straight line (figure 2(*a*)) was calculated from a TLS regression. The TLS method [28] was applied to obtain the best straight line in terms of its slope $C_1(\lambda)$ and intercept $C_0(\lambda)$ and to get a reliable uncertainty determination of these two parameters. The advantage of TLS is its independence of the choice of assignment of $A_f(\lambda)$ and $a_f(\lambda)$ to the X- and Y-axes and that the uncertainties $u(X)$ and $u(Y)$ are accounted for in the calculation of the best straight line. In addition to TLS, we also applied an LS fitting procedure to derive the traceability constants. The LS method was included since the uncertainties of the reference absorbance values $u(A_f)$ significantly exceeded the combined uncertainties $u_c(a_f(\lambda))$ for the measured average absorbance values. Under such conditions, TLS converges towards LS results. This is demonstrated in figure 2(*b*), where the differences between the conventional quantity values $A_f(\lambda)$ and the values calculated by LS (blue dots) and TLS (red diamonds) are plotted as a function of the measured absorbance $a_f(\lambda)$. The associated X-values $a_f(\lambda)$ were displaced by −0.015 and +0.015 to enable the comparison of LS and TLS. The uncertainties included in figure 2(*b*) reflect the expanded uncertainties $2u(A_f(574\,\text{nm}))$ given in table 1(*b*). Close agreement between TLS and LS calculations was also observed for the other wavelengths (see table 3) for the specific data sets $a_f(\lambda)$, $A_f(\lambda)$ used to establish traceability. This close agreement is due to the fact that $u(A_f(574\,\text{nm})) \gg u_c(a_f(\lambda))$. Hence, when deriving the traceability constants $C_0(\lambda)$ and $C_1(\lambda)$, given in table 3 along with their expanded uncertainties, only minor contributions are due to $u_c(a_f(\lambda))$. The expansion factor $k = 2.571$ covers a level of confidence of about 95% and follows from the fitting of straight lines to 7 pairs of data values in each case, which results in 5 degrees of freedom. The expanded uncertainties derived by TLS are slightly smaller than the uncertainties obtained by LS analyses. Taking the estimated uncertainties into account, all values of the bias $C_0(\lambda)$ and the slope $C_1(\lambda)$ are consistent with zero and one. It follows that the absorbance values measured with the spectrophotometer (Cary 6000i, Agilent Technologies)
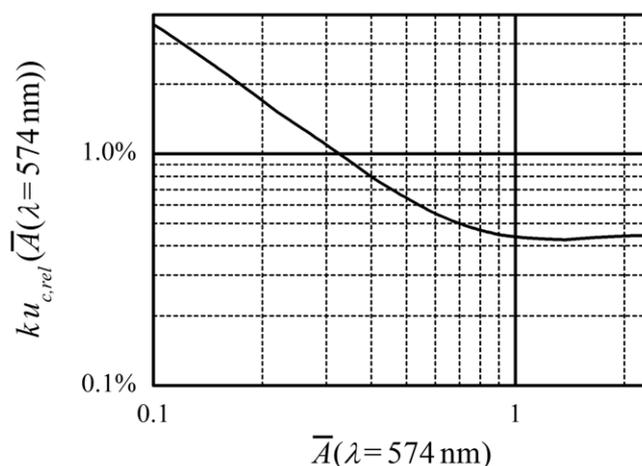

Figure 3. Calculated combined uncertainty (95% confidence level) as a function of the conventional quantity value of the absorbance $\bar{A}(574\,\text{nm})$. The expansion factor varies from $k \approx 2.7$ to $k \approx 2.4$ between $\bar{A}(574\,\text{nm}) = 0.1$ and 2.0, since the effective degrees of freedom change.

used in our experiments for haemoglobin determination are in accordance with the values that are measured with the national reference instrument.

3.2. Uncertainties of haemoglobin concentrations associated with absorbance measurements

The haemoglobin concentration $\beta(\text{Hb}(\text{Fe}))$ in blood is derived from average conventional quantity values of the absorbance $\bar{A}(\lambda)$. These values are obtained from (2.2) using the mean value $\bar{a}(\lambda)$ of the absorbance, measured for a dilution with a defined volume fraction ϕ of the sample in the measurement solution. The absorbance of the haemoglobin solution is measured with respect to a second cuvette with the pure conversion solution in the reference beam. Two cuvette pairs were used for the same haemoglobin dilution to account for uncertainty contributions when measuring at different positions of the cuvette holder and for influences of different cuvettes. Similar to the filter measurements, the main contribution to the uncertainties arises from the different positions of the holder, and the repeated insertion/removal in the spectrophotometer is covered by the variance determined from 20 repetitive wavelength scans. Typically, uncertainties $u(\bar{a}(\lambda)) = u(a(\lambda))/\sqrt{20}$ range between 2×10^{-5} and 3×10^{-5}.

The combined uncertainty $u_c^2(\bar{A}(\lambda))$ of the absorbance is calculated according to the usual procedure for uncertainty propagation [26] from the uncertainties of the calibration constants and the measured absorbance:

$$
\begin{aligned}
u_c^2(\bar{A}(\lambda)) &= \left(\frac{\partial \bar{A}}{\partial C_0(\lambda)}\right)^2 u^2(C_0(\lambda)) + \left(\frac{\partial \bar{A}}{\partial C_1(\lambda)}\right)^2 u^2(C_1(\lambda)) \\
&\quad + \left(\frac{\partial \bar{A}}{\partial \bar{a}(\lambda)}\right)^2 u^2(\bar{a}(\lambda)) \\
&= u^2(C_0(\lambda)) + (\bar{a}(\lambda))^2 u^2(C_1(\lambda)) + (C_1(\lambda))^2 u^2(\bar{a}(\lambda)).
\end{aligned}
$$
(3.1)

The result for the wavelength $\lambda = 574$ nm is depicted in figure 3 to demonstrate the behaviour of the combined relative uncertainty $ku_{c,rel}(\bar{A}(\lambda = 574 \text{ nm}))$ as a function of the absorbance $\bar{A}(\lambda = 574 \text{ nm})$. The uncertainties given in table 3 for the TLS analysis and $u(\bar{a}(\lambda)) = 10^{-4}$ were used. The expansion factor was derived by calculating the effective degrees of freedom by the Welch–Satterthwaite formula [26, clause G.4],

$$\nu_{eff} = \frac{u_c^4(\bar{A}(\lambda))}{\frac{u^4(C_0(\lambda))}{5} + \frac{(\bar{a}(\lambda))^4 u^4(C_1(\lambda))}{5} + \frac{(C_1(\lambda))^4 u^4(\bar{a}(\lambda))}{19}}. \quad (3.2)$$

It follows that the degrees of freedom and hence the expansion factor k vary as a function of the absorbance, e.g. at the wavelength $\lambda = 574$ nm between $k = 3.8$ for small absorbance values and $k = 2.5$ for high absorbance values. Since the t-distribution is defined for integers only, the expansion factor is linearly interpolated to calculate the continuous uncertainty function $ku_{rel}(\bar{A}(\lambda = 574 \text{ nm}))$ displayed in figure 3. The degrees of freedom correlated with the uncertainties $u(C_0(\lambda))$ and $u(C_1(\lambda))$ amount to $\nu = 5$, as discussed above. For the degrees of freedom associated with the measured spectral absorbance $\bar{a}(\lambda)$ we chose $\nu = 19$, since for haemoglobin measurements 20 repeat measurements were performed for a specific measurement suspension.

For low values of absorbance the main contribution to uncertainty arises from $u(C_0(\lambda))$, whereas for high absorbance values $u(C_1(\lambda))$ becomes increasingly important. For absorbance values of 0.1, the expanded relative uncertainty amounts to 3.5%; for high absorbance the uncertainty approaches the limit

$$u_{rel}(\bar{A}(\lambda)) \xrightarrow[\bar{a}(\lambda) \to \infty]{} u_{rel}(C_1(\lambda)). \quad (3.3)$$

According to the DIN standard for total haemoglobin determination in blood [15], the expanded uncertainty of absorbance measurements is required to be less than 1% to provide reference measurement values of concentrations. It follows from figure 3 and corresponding observations for the other wavelengths (i.e. 540 nm, 546 nm, 570 nm and 580 nm) that the measurement solution should be prepared so that the spectral absorbance $a(\lambda)$ exceeds 0.3. This can be done by adjusting the volume fraction ϕ, i.e. the volume of the blood sample to be added to the conversion solution. The maximum value for the absorbance acceptable for haemoglobin concentration measurements is generally not limited by the sensitivity of the photometric detection but by the chemical reaction of the conversion. For accurate haemoglobin concentration measurements it is essential that the conversion reaction has reached its end point. For high volume fractions the reaction kinetics may be delayed and the conversion can be incomplete. Preliminary results indicate that for absorbance values between 0.3 and 1.8, adjusted by preparing measurement solutions with suitable volume fractions from the same blood sample, haemoglobin concentrations are in good agreement. Hence the range for absorbance values recommended for dilution series for both HiCN and AHD methods is $0.3 \leqslant \bar{a}(\lambda) \leqslant 1.8$ at the respective wavelength of 540 nm or 574 nm.

4. Accurate determination of haemoglobin concentrations

A typical example for the determination of haemoglobin concentration is shown in figure 4. Subsequent to gravimetric dilution of the sample in the HiCN or AHD conversion reagent, the solution was centrifuged for 15 min at a relative acceleration of approximately $1000 \cdot g_n$. This is in agreement with the requirement in DIN 58931 [15], i.e. the numerical value of the product of the relative centrifugal acceleration and the centrifugation time (given in minutes) was $\geqslant 15\,000$. The measurement solutions were pipetted in cuvettes with 1 cm absorption path length. After a conversion time of 90 min the absorbance of the haemoglobin solutions was measured against a cuvette containing the respective conversion reagents. The influence of evaporation was negligible, as was deduced from comparison experiments at different times using non-closed containers. Centrifugation was applied to avoid enhanced extinction due to light scatter from cell fragments or remaining white blood cells. The spectral dependences of the conventional quantity value of the spectral absorbance $\bar{A}(\lambda)$ for HiCN and the AHD complex are depicted in figures 4(a) and (b). These values were obtained according to (2.2). The wavelength dependence of the traceability constants $C_0(\lambda)$ and $C_1(\lambda)$ was linearly interpolated between 540 nm and 546 nm and extrapolated to 539 nm and 548 nm to analyse the data for the HiCN protocol. A second-order polynomial interpolation/extrapolation was used for the AHD method. The volume fractions amount to $\phi = 0.012\,780 \pm 0.000\,025$ and $\phi = 0.018\,865 \pm 0.000\,037$ (expansion factors $k = 2$), resulting in absorbance values from 1.17 to 1.19 and from 1.08 to 1.11, respectively. The wavelength ranges cover the maxima of the Q-bands. The wavelengths of 540 nm and 574 nm, used to derive the haemoglobin concentration, are indicated as vertical lines in figure 4. The differences $\bar{A}(\lambda) - \bar{a}(\lambda)$ between conventional quantity values and the measured values of the absorbance are plotted in figures 4(c) and (d) as a function of the wavelength. Typically, the differences are of the order of 10^{-3} only. The agreement between measured values and conventional quantity values of absorbance is a consequence of the values derived for the bias $C_0(\lambda)$ and the slope $C_1(\lambda)$ being consistent with zero and one, respectively.

The expanded uncertainties for the conventional quantity values $\bar{A}(\lambda)$ and the measured values $\bar{a}(\lambda)$ are compared in figures 4(e) and (f). The expansion factors were derived as described above by calculating the effective degrees of freedom according to (3.2). For an absorbance of about $\bar{A}(\lambda) \approx 1$ the uncertainty $u(\bar{A}(\lambda))$ contributes to the combined uncertainty in haemoglobin measurements similarly to the other components listed in table 4. For absorbance values $A(\lambda) < 1$ the contribution of $u(A(\lambda))$ increases (see figure 3) and represents the major contribution at $A(\lambda) \approx 0.3$. Hence, the accuracy of haemoglobin concentration measurements for small absorption values could be improved by reducing the uncertainty of the absorbance of the reference filters. For both procedures, the uncertainties $ku(\bar{A}(\lambda)) \approx 4 \times 10^{-3}$ significantly exceed the precision or reproducibility of the measurement $ku(\bar{a}(\lambda)) < 10^{-4}$ by more than one order of magnitude.
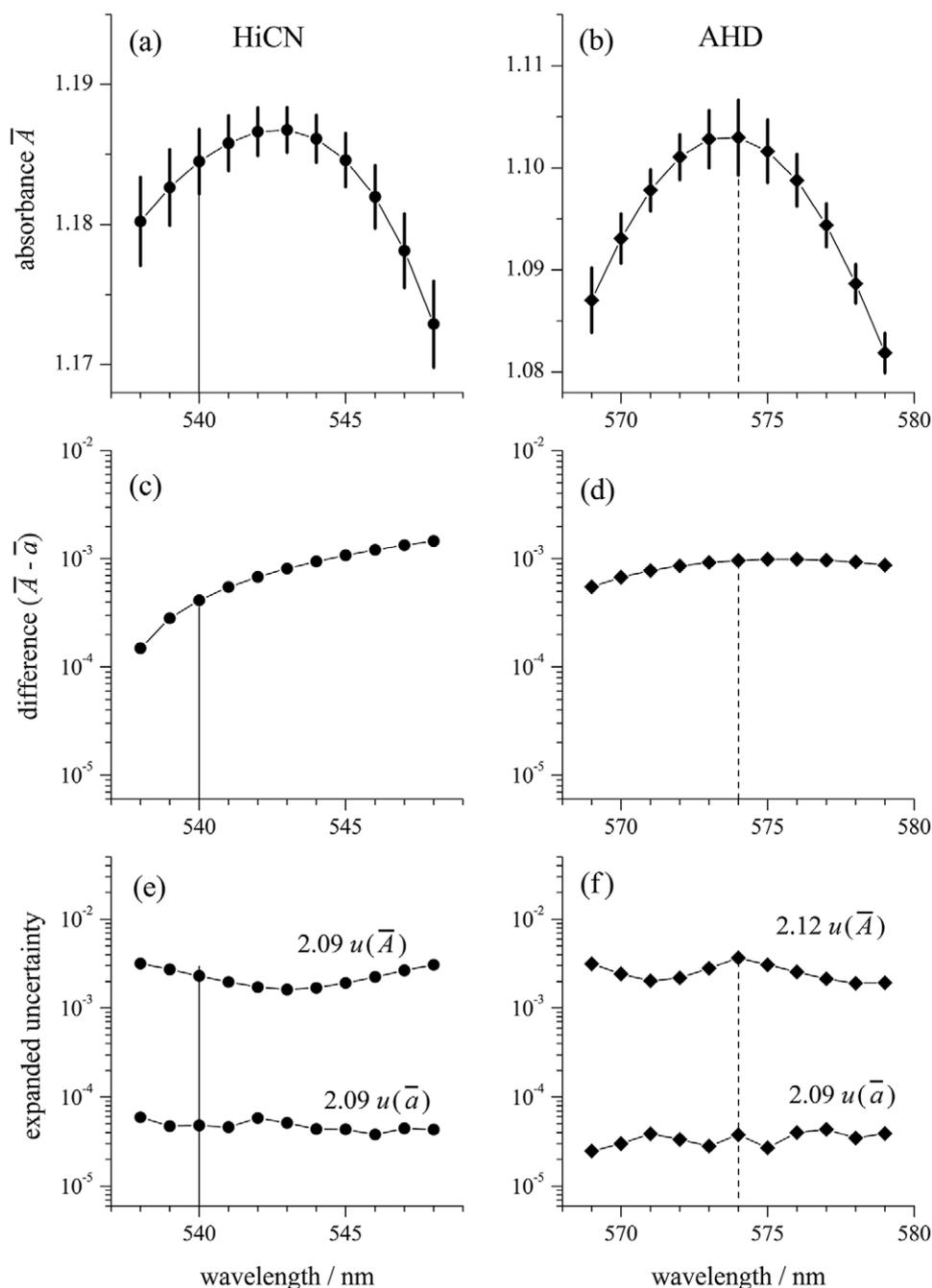
Figure 4. Absorbance spectra of blood samples after conversion into HiCN (*a*) and using the AHD conversion reagent (*b*). Differences between the average conventional quantity values $\bar{A}$ and the average of the measured values $\bar{a}$ of the absorbance for the HiCN (*c*) and the AHD method (*d*). Comparison of the expanded uncertainties of the conventional quantity values $ku(\bar{A})$ and measured values $ku(\bar{a})$ for both conversion methods (*e*), (*f*).

The measurement of the absorption spectrum reveals that the absorption maximum for the AHD complex is at the wavelength of 574 nm, which is slightly less than the value of 575 nm reported in the literature [21]. The haemoglobin concentrations (see table 4)

$$\beta_{\text{HiCN}}(\text{Hb(Fe)}) = (135.8 \pm 0.7)\,\text{g}\,\text{L}^{-1} \qquad (4.1)$$

and

$$\beta_{\text{AHD}}(\text{Hb(Fe)}) = (135.7 \pm 1.0)\,\text{g}\,\text{L}^{-1} \qquad (4.2)$$

obtained with the HiCN and AHD procedures (level of confidence $\approx$95%) are in excellent agreement. This

observation was confirmed for a larger set (50) of blood samples, indicating that both methods are suitable for the determination of reference measurement values.

5. Summary and outlook

Our experiments and the analyses described in this paper served to establish the traceability of absorbance measurements in the vicinity of the Q-bands of HiCN and the AHD complex. The relation between the conventional quantity value of the absorbance A_f of the reference filters and the

Table 4. Comparison of the haemoglobin concentration in blood determined by the HiCN and AHD procedures. All quantities required for the calculation are included. Expanded uncertainties ($k = 2$) are listed.

Quantity	Value	$100\times$ Relative ($k = 2$) uncertainty
$\beta_{HiCN}(Hb(Fe))$	135.76 g L^{-1}	0.54
$A_{HiCN}(\lambda = 540 \text{ nm})$	1.184 37	0.20
ϕ_{HiCN}	0.012 780	0.20
$\varepsilon_{HiCN}(\lambda = 540 \text{ nm})$	$11\,000 \text{ L mol}^{-1}\text{ cm}^{-1}$	0.36
$\beta_{AHD}(Hb(Fe))$	135.66 g L^{-1}	0.72
$A_{AHD}(\lambda = 574 \text{ nm})$	1.102 95	0.32
ϕ_{AHD}	0.018 865	0.20
$\varepsilon_{AHD}(\lambda = 574 \text{ nm})$	$6945 \text{ L mol}^{-1}\text{ cm}^{-1}$	0.54
d	10 mm	0.20
M	$16\,114.5 \text{ g mol}^{-1}$	0.20

measured absorbance a_f is linear. The calibration constants (bias C_0 and slope C_1) were determined by least-squares (LS) and total least-squares (TLS) analyses. No significant differences for both LS and TLS approaches were observed. This is explained by the fact that the reproducibility or precision $u(a_f(\lambda))$ of the measured values a_f is more than one order of magnitude smaller than the accuracy $u(A_f(\lambda))$.

Using the traceability constants to derive the conventional quantity value of the absorbance $\bar{A}(\lambda)$ from the measured absorbance $\bar{a}(\lambda)$, we determined the haemoglobin concentration of a blood sample using two different conversion solutions. Values derived by the HiCN method and the AHD procedure are in good agreement. However, at present both methods are not sufficiently evaluated to serve as a primary reference measurement method according to the definitions in ISO 17511 [10]. In particular, the HiCN method uses an international conventional calibrator [29], which does not fulfil the requirements of a primary calibrator. On the other hand, the calibrator of the AHD method, chlorohaemin, can be produced with high purity and possibly used as a primary calibrator in the traceability chain. The next steps to develop a primary reference measurement procedure include the determination of the spectral absorbance of the AHD complex with higher accuracy, the measurement of the kinetics for the conversion reaction and the thorough investigation of influences that unspecifically contribute to absorbance. Significant perturbations are expected in blood samples exhibiting pathologically high concentrations of bilirubin, lipids or leukocytes. In each case the spectral characteristic is modified due to additional light absorption or light scatter. Consequently, a higher order or primary reference measurement procedure must rely on the determination of absorption spectra rather than on the measurement at a single wavelength. This implies the establishment of traceability for absorbance values in a larger spectral range.

Acknowledgments

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