Pilot study CCQM-P201: “Quantification of Total Haemoglobin in Blood”

Authors

Claudia Swart[1], Paulo José Beltrão[2], Sandra Mara Naressi Scapin[2], Youssef Bacila Sade[2], Marcelo Neves de Medeiros[2], Ji-Seon Jeong[3], Youxun Jin[4], Yahui Liu[4], Liuxing Feng[4], Xiaoyun Gong[4], Lulu Feng[4], Michael Winchester[5], Lee Yu[5], Cristian Arsene[1], Alexander Schulze[1], Rüdiger Ohlendorf[1], Christine Brauckmann[1]

Affiliations

[1]PTB – Physikalisch-Technische Bundesanstalt, Braunschweig, Germany

[2]INMETRO - National Institute of Metrology Standardization and Industrial Quality, Rio de Janeiro, Brasil

[3]KRISS - Korea Research Institute of Standards and Science, Daejeon, South Korea

[4]NIM China - National Institute of Metrology, Beijing, China

[5]NIST- National Institute of Standards and Technology, Gaithersburg, USA

Abstract

Under the auspices of the Protein Analysis Working Group (PAWG) of the Comité Consultatif pour la Quantité de Matière (CCQM) a pilot study, CCQM-P201, was organised. Five National Metrology Institutes (NMIs) participated in this pilot study and provided a total of eleven measurement results using six different methods based on three different measurement principles. The purpose of this pilot study was to develop measurement capabilities for the accurate quantification of intact haemoglobin (Hb) whereby measurement results could be made directly traceable to the SI. The choice of protein resulted from its ability to be quantified via different measurement principles providing different routes of traceability. Hb also provided a model of an intact protein with a complex tetrameric structure which is often measured via quantification of a specific subunit or the incorporated haem group. To achieve appropriate results for the chosen measurand, it was necessary to distinguish between the whole protein and fragments of the tetrameric structure, such as dimers or monomers. The participants were encouraged to investigate several of the measurement methods available. This was considered particularly advantageous in this instance as various methods applied to the same analyte may assist in identifying possible previously unknown biases or interferences of the various methods. Hb is being used as a model system to build capacity in methods for the quantification of proteins with a molar mass ≤ 100 kDa in biologically fluids in a concentration range > $1 \cdot 10^6$ pmol/g.

This was the first pilot study for the quantification of an inherent protein in blood with all the natural variations that protein shows in a clinical sample. The different methods applied by the participants included isotopic dilution (ID) organic mass spectrometry (MS) of specific peptides after tryptic digestion, species-specific ID inductively coupled plasma mass spectrometry (ICP-MS), post-column ID ICP-MS, quantification via total iron after chromatographic isolation of Hb and acid digestion as well as two optical methods. One of the latter was the reference method proposed by the International Committee for Standardisation in Haematology (ICSH) based on the conversion of Hb to hemiglobincyanide (HiCN), the other used the conversion of Hb with alkaline haematin detergent both followed by the measurement of spectroscopic absorbance.

Considering the use of very different measurement techniques for the quantification of total Hb, the results are in good agreement with a reference value with corresponding expanded uncertainty of (120.6 ± 1.2) mg/g calculated as DerSimonian-Laird mean. However, the observed differences between the results are not completely reflected in the reported measurement uncertainties. The calculated interlaboratory standard deviation or Tau value (dark uncertainty) was approximately 3 % whilst the average reported measurement uncertainty was approximately half this (1.4 %). A possible reason for this large difference may lie in the underestimation of any necessary conversion factors and their uncertainty contributions, between the target analyte measured and the requested measurand.

Introduction

The mass fraction of haemoglobin (Hb) in blood has been measured for well over a century and today it is still one of the most frequently requested blood tests. Normal cell functioning relies on a steady supply of oxygen. Hb is the oxygen transporting component of blood, therefore, determining its concentration was one of the first blood tests developed. A reduction in the oxygen carrying capacity of blood is referred to as anaemia. The condition and its severity can be monitored by the blood Hb concentration. Although not a disease in itself, anaemia can indicate the presence of a long list of other diseases. High levels of Hb, however, usually indicates polycythaemia, which can lead to heart failure, heart attack or stroke.

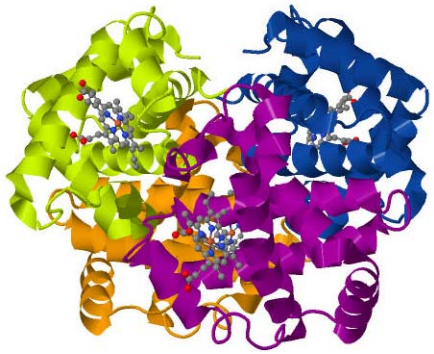
In 1958, a panel from the Haemoglobin Standard, Division of Medical Sciences, National Research Council, concluded that the best spectroscopic method for the quantification of Hb involved its conversion to hemiglobincyanide (HiCN) followed by measurement of its spectroscopic absorbance at 540 nm [1]. Further work by the International Committee for Standardisation in Haematology (ICSH) established the molar mass of Hb enabling the direct determination of Hb mass concentration via the Beer-Lambert law [2]. In 1966, the ICSH prepared a reference solution of HiCN [3]. A preparation of this was accepted as the first World Health Organisation (WHO) international HiCN reference preparation. In 1996, on the basis of continual experimental studies, the reference method and the specifications for the international standard were modified [4]. However, the photometric determination of HiCN still remains the reference method for Hb quantification and the ICSH standard is used to assign values to secondary HiCN reference solutions.

Although the HiCN method has been used for routine Hb determinations, it is often impractical for laboratories because of the toxicity of potassium cyanide used for derivatisation, and restrictions on its use and disposal. An alternative method is the alkaline haematin detergent (AHD575) method [5]. However, the ICSH recommend that if other methods of analysis are used for routine measurements (e.g. determination of oxyhaemoglobin, haemiglobinazide, iron determination), it should be adjusted to obtain comparability with the HiCN method. Due to the reference method and calibrator used, the concentration of Hb is typically defined as the sum of oxygenated haemoglobin, deoxygenated haemoglobin, carboxyhaemoglobin and haemiglobin. However, greater consideration needs to be given as to how Hb measurement results from any reference measurement procedure can be used to establish traceability to the SI.

In the context of the CCQM-PAWG, Hb is of particular interest as it can be used to assess different methods and measurement principles for determining its concentration in blood. Metalloproteins are particularly important in medical diagnosis as they represent around 30 % of the whole proteome. As Hb is an iron containing metalloprotein both speciation based (iron) and peptide-based isotope dilution mass spectrometry methods can be used to provide SI traceable measurement results. However, the analytical targets (iron and/or peptide sequences) are not the measurand and consideration needs to be given in assessing any uncertainty contributions associated with the necessary conversions from the measured target to the final measurand. It is essential that NMIs can provide reliable reference values which are not method dependent and are not biased by the different Hb forms such haemoglobin, haemiglobin, the genetic variants such as HbA2, HbA0 etc and which provide information on the measurand required by the end user.

The aim of this pilot study is to demonstrate a laboratory's capability to quantify reliably/traceably an intact protein with a complex tetrameric structure. To achieve this, it was necessary to distinguish between the whole protein and fragments of the tetrameric structure, such as dimers or monomers. As this was a pilot study, participants were encouraged to investigate several of the measurement methods available. This was considered particularly advantageous in this instance as various methods applied to the same analyte may assist in identifying possible hitherto unknown biases or interferences of the various methods.

Measurand(s)



Human haemoglobin

Mean molecular weight: 64458 g/mol

Expected mass fraction range: 100-150 g/kg

Monomer average: 141 amino acids. molecular weight 16115 g/mol

Structure: tetrameric

CAS number: 9008-02-0

Study material

Whole blood was purchased from the German Red Cross (DRK). The samples were prepared using a single donor blood unit which had tested negative for Anti-HIV-1&2, Anti-HCV, HBsAg, Syphilis, HCV NAT, HIV NAT, HBV NAT, HAV NAT and ParvoB19 NAT. To remove cell fragments, the blood was transferred to a 500 mL PFA bottle directly after receipt and frozen over night at - 21 °C. The blood was then allowed to thaw in the refrigerator at 4 °C. For homogenisation the bottle was rotated for about 2 h at ca. 4 °C. To remove the cell fragments the blood was then centrifuged at 20000 g for 1 h at 4 °C. The supernatant was separated from the pellet containing the cell fragments and again homogenised for about 2 h at ca. 4 °C. Aliquots of 0.5 mL were then pipetted into separate 1.5 mL Eppendorf LoBind tubes. Between each sampling the remaining blood was rotated for 2 min to avoid the formation of a gradient in the bottle. The samples were then freeze-dried before distribution to the participants.

Homogeneity

In accordance to ISO Guide 35 the samples were checked for homogeneity and stability issues [6,7]. The measurements were performed using UV/Vis. The homogeneity of Hb in the sample material was tested in five different tubes (WB_B3_21, WB_B3_32, WB_B3_45, WB_B3_76 and WB_B3_86). For the analysis, four aliquots were prepared from each tube and each aliquot was measured three times, respectively (figure 1).

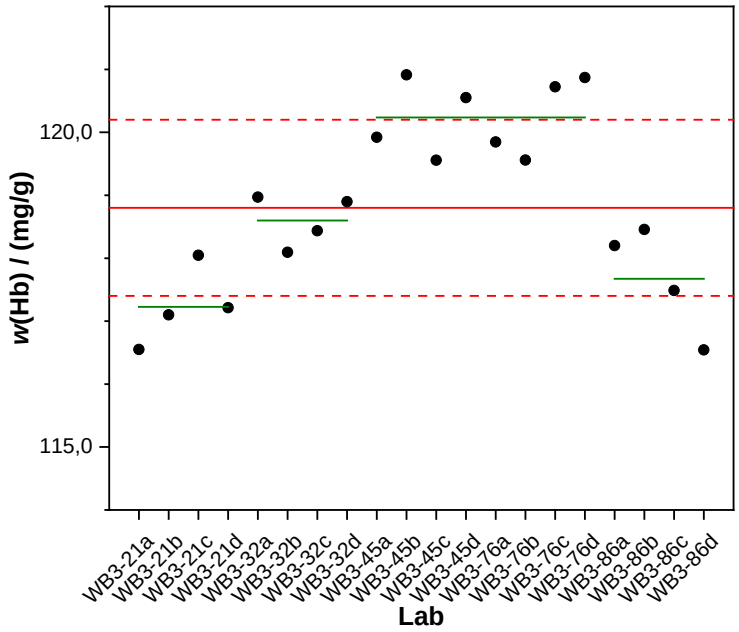


Figure 1: Homogeneity of Hb in lyophilised whole blood samples measured with UV/Vis.

By means of one-way analysis of variants, the between bottle/tubes uncertainty u_{bb} caused by inhomogeneity was calculated. Therefore, the variance among and within (s_{among} and s_{within}) the tubes was evaluated (equation 1 + 2) and the "difference" is u_{bb}. (n_0 = effective number of subsamples, k = number of tubes, n_i = number of aliquots per tube):

$$s^2_{\text{within}} = \frac{1}{n-k}\sum_{i=1}^{k}\sum_{j=1}^{n_i}(w_{ij} - \overline{w}_i)^2 \quad (1)$$

$$s^2_{\text{among}} = \frac{1}{k-1}\sum_{i=1}^{k} n_i(\overline{w}_i - \overline{w})^2 \quad (2)$$

$$u^2{}_{\text{bb}} = s^2{}_{\text{bb}} = \frac{s^2_{\text{among}} - s^2_{\text{within}}}{n_0} \quad (3)$$

$$n_0 = \frac{1}{k-1}\left[\sum_{i=1}^{k} n_i - \frac{\sum_{i=1}^{k} n_i^2}{\sum_{i=1}^{k} n_i}\right] \quad (4)$$

Table 1 summarises the uncertainty contribution due to homogeneity.

Table 1: Homogeneity results for lyophilised blood.

	in mg/g	**in %**
s_{within}	0.71	0.6
s_{among}	2.3	1.8
u_{bb}	1.1	0.9

F	10.6
F_{crit}	3.06
p-value	0.0003

Based on these results the material seems to be sufficiently homogeneous to be used in the pilot study. However, it turned out that the variation caused by the UV/Vis measurement was larger than the variations measured in the study material. Therefore, a more precise method would be required to characterise a material used in a potential KC.

Stability

Both short-term and long-term stability was assessed for the study material using LC-ICP-MS. Short-term stability was assessed at room temperature and 4 °C, to assess stability for the conditions used during sample transport (figure 2). As degradation was observed at both temperatures within a week, it was decided to send the samples at -20 °C.

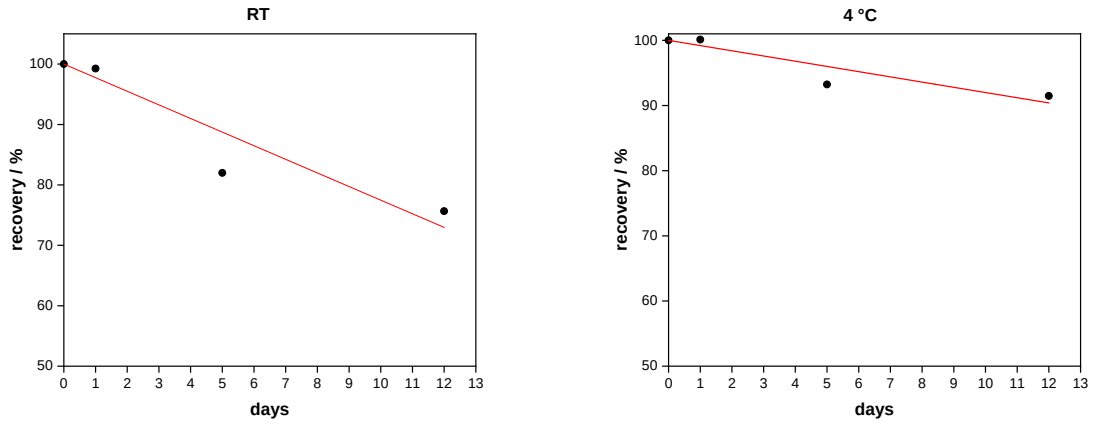


Figure 2: Short-term stability of Hb in lyophilised whole blood samples at: left: room temperature; right: 4 °C.

Long-term stability was tested at -20 °C, using an isochronous measurement design, with the reference temperature being -80 °C. Within the measurement uncertainty the samples were stable for at least half a year (figure 3).

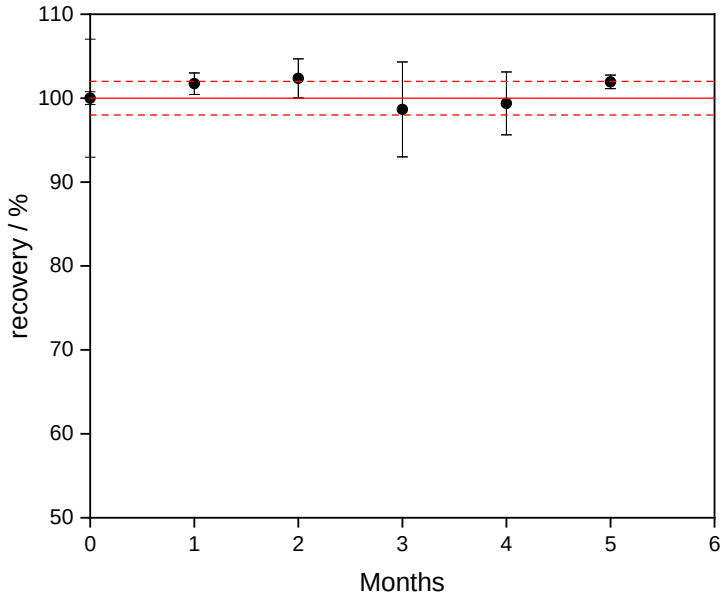


Figure 3: Long-term stability of Hb in lyophilised whole blood samples at -20 °C. Error bars denote the uncertainty *u*(*w*(Hb)) for a coverage factor of *k* = 1.

The slope of the trendline is smaller (0.17 mg/g/months) than the uncertainty of the slope (0.22 mg/g/months). Thus, the material is deemed stable for the duration of the study.

Instructions and Participants

The samples were shipped to the participants on cool packs at -20 °C with specific instructions that they should be stored at -80 °C immediately on receipt. The shipping details are given in table 2. The reconstitution had to be performed according to the technical protocol provided to each partner together with the samples and via email and samples should be measured immediately after reconstitution. The minimum amount of sample to be used was 20 µL and laboratories were requested to analyse at least three aliquots per vial.

Together with the samples, a technical protocol was sent to all participants of the interlaboratory comparison, providing information about the properties of the samples, recommendations for sample handling and the recommended procedure for reconstitution and to check for losses and correct for evaporation effects during storage. The sample bottles were accompanied by an individual table compiling the masses of the empty tubes, tubes with blood sample and tubes with freeze-dried blood sample needed to calculate a correction factor between original blood sample and reconstituted freeze dried samples. Annex I shows the technical protocol of the pilot study CCQM P201.

Table 2: Shipping details of the samples for P201

Lab code	Samples sent	Samples received
1	-	-
2	2019-04-15	2019-04-18
3	2019-04-03 Again 2019-05-06	First shipment thawed finally received: 2019-05-09
4	2019-04-03 Again 2019-05-06	First shipment thawed finally received: 2019-05-09
5	2019-04-03 Again 2019-05-06	First shipment thawed finally received: 2019-05-08

Methods

The participants were free to use any suitable method but were asked to include a full description of their measurement procedure. Potential methods were e.g. LC-ICP-MS, LC-MS/MS, Raman, optical spectroscopy after derivatisation. Participants were permitted to submit results from more than one method that had an expected relative expanded measurement uncertainty U_{rel} of less than 3 %. The measurement principle, methods and analytical targets used by the participants are listed in Table 3.

Table 3: Instrumentation/methods and calibration strategies used by the participants as reported Some laboratories used more than one method indicated by _subnumber.

Lab code	Sample preparation	Sep.	Detection method
1_1	U-^{15}N-Hb spike, tryptic digestion, desalting using SPE	LC	MS/MS peptides: VGAHAGEYGAEALER, MFLSFPTTK and TYFPHFDLSHGSAQVK
1_2	^{54}Fe-Hb spike, dilution 1:100	SEC	ICP-MS, m/z 54, 56
2_1	Addition of AHD-conversion reagent	-	Absorbance at 574 nm
2_2	tryptic digestion, desalting using SPE	C18	MS/MS, 5 point calibration peptides: VGAHAGEYGAEALER, MFLSFPTTK and TYFPHFDLSHGSAQVK
3	Addition of AHD-conversion reagent	-	Absorbance at 574 nm
4_1	Reaction with $K_3Fe(CN)_6$+KCN+KH_2PO_4+detergent	-	Absorbance at 540 nm
4_2	Labelled peptides added to diluted samples, tryptic digestion, desalting	C18	MS/MS peptides: VGAHAGEYGAEALER, MFLSFPTTK
4_3	Labelled Hb added to samples, tryptic digestion, desalting	C18	MS/MS peptides: VGAHAGEYGAEALER, MFLSFPTTK
4_4	Addition of ^{54}Fe, HNO3 digestion	-	ICP-HRMS
4_5	Dilution	LC	Postcolumn ICP-HRMS
5	HNO_3 digestion, standard addition	-	ICP-MS

Results

Participants were requested to determine the mass fraction *w*(Hb) of Hb in human blood.

The results of the comparison are listed in Table 4 and the graphical evaluation is shown in Figures 4 to 7. The detailed report of each participant is given in Annex II.

Table 4: Results as submitted by the participants. Some laboratories used more than one method indicated by _subnumber.

Lab code	Method	*w* (Hb) mg/g	*u*(*w* (Hb)) mg/g	*U*(*w* (Hb)) mg/g	*k*
1_1	LC-MS	120.1	1.2	2.5	2.0
1_2	LC-ICP-MS	117.4	0.55	1.1	2.0
2_1	AHD	124.9	1.6	3.2	2
2_2	LC-MS/MS	126.8	5.3	10.6	2
3	AHD	124.36	0.85	1.82	2.14
4_1	HiCN	119.89	1.02	2.04	2
4_2	peptides	120.69	1.75	3.50	2
4_3	IDMS	121.17	1.91	3.82	2
4_4	ID-ICP-MS total Fe	115.5	1.0	2.1	2.0
4_5	ID-ICP-HRMS PC	115.3	1.2	2.4	2
5	ICP-MS	127.8	3.0	6.9	2.31

With

w (Hb) = reported result of the participant for total Hb

u(*w* (Hb)) = associated uncertainty of the result for total Hb

U(*w* (Hb)) = expanded uncertainty of the result for total Hb

k = coverage factor used to calculate the expanded uncertainty

Calculation of the Reference Value (RV)

A reference value (RV) was established using consensus values that were evaluated based on the participants' data [8,9]. Therefore, the arithmetic mean $\bar{w}_{\mathrm{A}}$ (eq. (8)), the median $\bar{w}_{\mathrm{M}}$ (eq. (10) +(11)), the uncertainty weighted mean $\bar{w}_{\mathrm{UWM}}$ (eq. (13)) and the DerSimonian-Laird mean $\bar{w}_{\mathrm{DL}}$(eq. (16) +(17)) were calculated along with their associated uncertainties $u(\bar{w}_{\mathrm{A}})$ (eq. (9)), $u(\bar{w}_{\mathrm{M}})$ (eq. (12)) ,$u(\bar{w}_{\mathrm{U}})$ (eq. (15)) and $u(\bar{w}_{\mathrm{DL}})$ (eq. (18)) for all participants.

$$\bar{w}_{\mathrm{A}} = \frac{1}{N}\sum_{i=1}^{N} w_i \tag{8}$$

$$u(\bar{w}_{\mathrm{A}}) = \sqrt{\frac{1}{N\cdot(N-1)}\sum_{i=1}^{N}(w_i - \bar{w}_{\mathrm{A}})^2} \tag{9}$$

$$\bar{w}_{\mathrm{M}} = \frac{1}{2}\left(w_{N/2} + w_{N/2+1}\right) \quad N \text{ even} \tag{10}$$

$$\bar{w}_{\mathrm{M}} = w_{(N+1)/2} \quad N \text{ odd} \tag{11}$$

$$u(\bar{w}_{\mathrm{M}}) = \sqrt{\frac{\pi}{2N}} \cdot 1.483 \cdot \mathrm{med}(|w_i - \bar{w}_{\mathrm{M}}|) \tag{12}$$

$$\bar{w}_{\mathrm{UWM}} = \frac{\sum_{i=1}^{N}\frac{w_i}{u^2(w_i)}}{\sum_{i=1}^{N}\frac{1}{u^2(w_i)}} \tag{13}$$

The data set was checked for consistency by means of the chi-squared test. χ^2_{obs} was calculated according to eq. (14).

$$\chi^2_{\mathrm{obs}} = \sum_{i=1}^{N}\left(\frac{w_i - \bar{w}_{\mathrm{UWM}}}{u(w_i)}\right)^2 \tag{14}$$

Details of the Chi-squared test applied to all participants results is shown in Table 5. For comparison a chi-squared distribution with a 95 percentile and (*N*-1) = degrees of freedom $\chi^2_{0.05,N-1}$ was used.

Table 1: Results of chi-squared test applied for the results of all participants and for the results of the NMIs. Values rounded to yield integer numbers.

	N	χ^2_{obs}	$\chi^2_{0.05,N-1}$	mutually consistent?
all participants	11	90.0	14.1	no

Due to the observed mutual inconsistency of the data, $u(\bar{w}_{\mathrm{UWM}})$ was corrected using the eq. (15).

$$u(\bar{w}_{\mathrm{UWM}}) = \sqrt{\frac{\chi^2_{\mathrm{obs}}}{N-1}\left(\sum_{i=1}^{N}\cdot\, u^2(w_i)\right)} \tag{15}$$

$$\bar{w}_{\mathrm{DL}} = \sum_{i=1}^{N} \bar{w}_i \cdot w_i \tag{16}$$

$$\bar{w}_i = \frac{\left(u_i^2+\lambda\right)^{-1}}{\sum_{j=1}^{N}\left(u_i^2+\lambda\right)^{-1}} \text{ with } \lambda \text{ the interlaboratory variance} \quad (17)$$

$$u(\bar{w}_{\mathrm{DL}}) = \sqrt{\sum_{i=1}^{N}\left(\frac{\bar{w}_i{}^2(w_i-\bar{w}_{\mathrm{DL}})^2}{(1-\bar{w}_i)}\right)} \quad (18)$$

In figure 4 to 7 all calculated consensus values with their associated uncertainties and the results as submitted by the partners for P201 are illustrated. The consensus values were calculated from all participants' results. The calculations of the different reference values and their associated uncertainties have been validated independently by at least two different persons in independent evaluations.

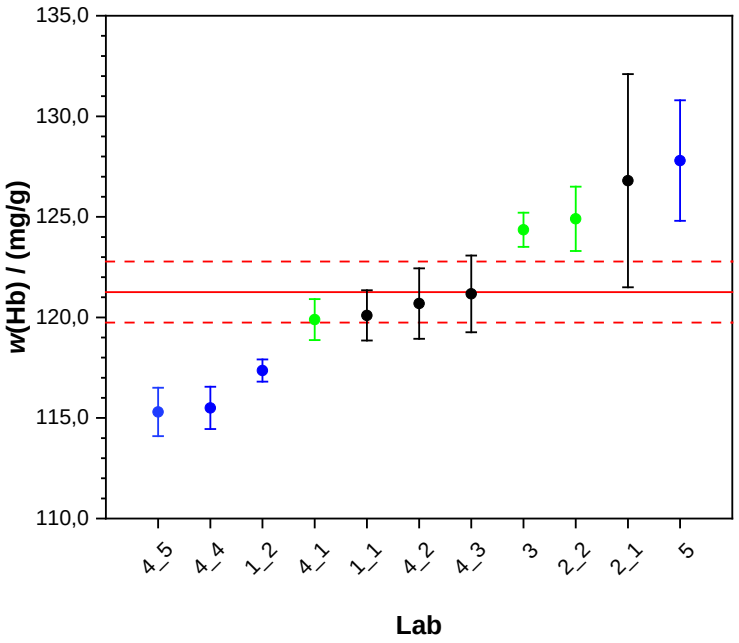


Figure 4: Hb mass fraction *w*(Hb) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Hb)) for a coverage factor of *k* = 1 as reported. The red line shows the arithmetic mean of all participants: $\bar{w}_{\mathrm{A}}$ = (121.3 ± 1.3) mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\bar{w}_{\mathrm{A}})$ associated with the arithmetic mean. Blue dots represent the results using ICP-MS, green dots the ones using optical spectrometry and black the results using MS/MS.

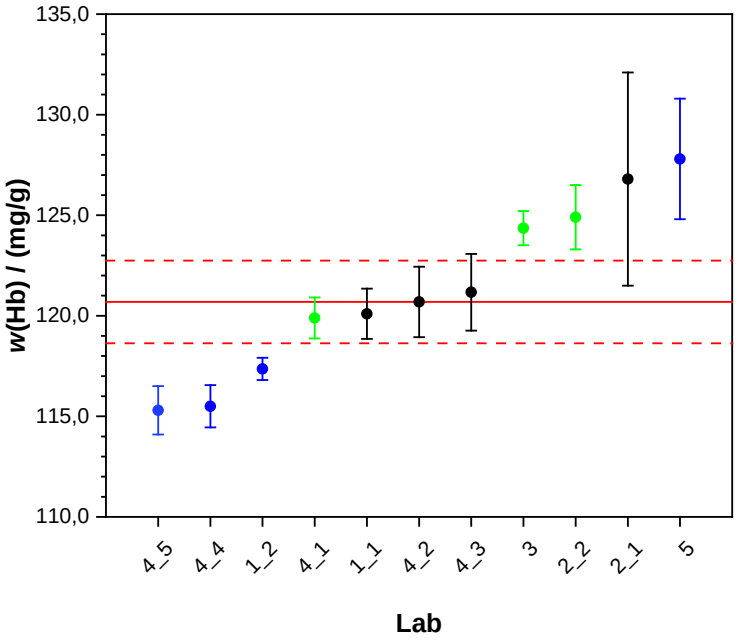


Figure 5: Hb mass fraction *w*(Hb) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Hb)) for a coverage factor of *k* = 1 as reported. The red line shows the median of all participants: $\bar{w}_{\mathrm{M}}$ = (120.7 ± 2.1) mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\bar{w}_{\mathrm{M}})$ associated with the median. Blue dots represent the results using ICP-MS, green dots the ones using optical spectrometry and black the results using MS/MS.

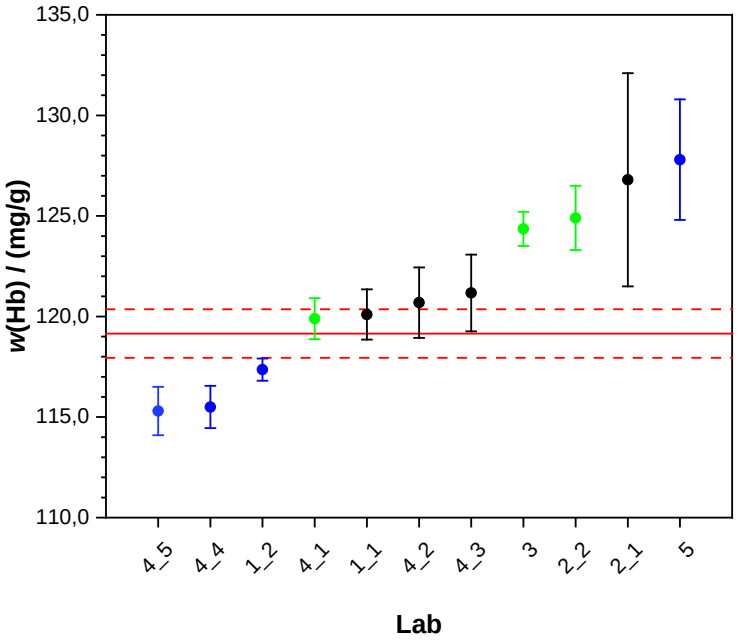


Figure 6: Hb mass fraction *w*(Hb) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Hb)) for a coverage factor of *k* = 1 as reported. The red line shows the uncertainty weighted mean of all participants: $\bar{w}_{\mathrm{UWM}}$ = (119.3 ± 1.0) mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\bar{w}_{\mathrm{UWM}})$ associated with the uncertainty weighted mean. Blue dots represent the results using ICP-MS, green dots the ones using optical spectrometry and black the results using MS/MS.

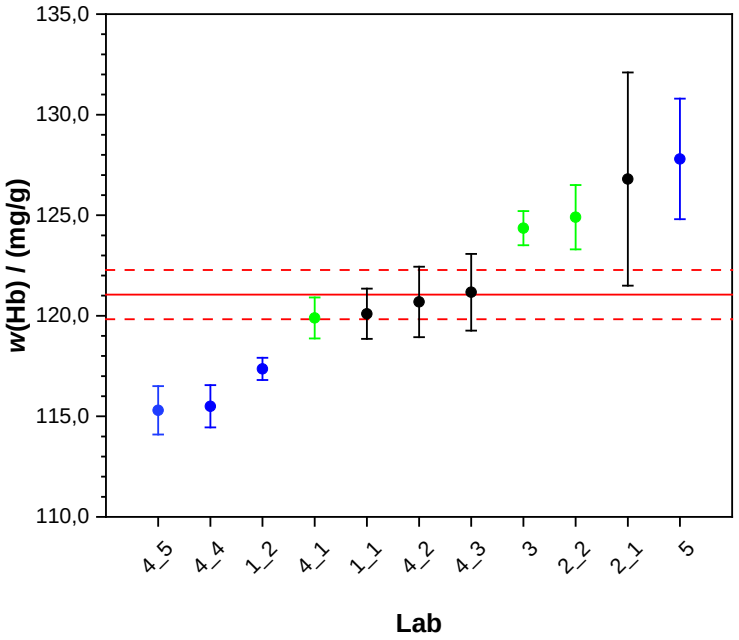


Figure 7: Hb mass fraction *w*(Hb) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Hb)) for a coverage factor of *k* = 1 as reported. The red line shows the DerSimonian-Laird mean of all participants: $\bar{w}_{DL}$ = (120.6 ± 1.2) mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\bar{w}_{DL})$ associated with the uncertainty weighted mean. Blue dots represent the results using ICP-MS, green dots the ones using optical spectrometry and black the results using MS/MS.

Degrees of Equivalence (DoE)

The degree of equivalence (DoE) (d_i) of a result w_i equals its deviation from the consensus value, which are in our case four different possible values. For all these values the d_i with its corresponding uncertainty $u(d_i)$ as well as the normalized error (E_n) were calculated according to GUM using the error propagation with the eq. (19) – (22) [10].

$$d_i = w_i - w_{ref} \quad (19)$$

$$u(d_i) = \sqrt{u^2(w_i) + u^2(\bar{w}_{ref})} \quad (20)$$

$$E_n = \frac{d_i}{U(d_i)} \quad (21)$$

Except for DerSimonian-Laird where $u(d_i)$ is calculated as:

$$u(d_i) = \sqrt{u^2(w_i) + \lambda - u^2(\bar{w}_{DL})} \quad (22)$$

The results are summarised in table 6 – 9 and plotted in figure 8 – 11. When E_n is between 0 and 1, the results and consensus values are in agreement within their estimated uncertainties. The calculations of the DoEs of the different approaches and their according uncertainties have been validated independently by at least two different persons in independent evaluations.

Table 2: Mass fractions *w*(Hb) and their associated combined and relative expanded uncertainties *u*(*w*(Hb)) and u_{rel}, resp., using a coverage factor *k* = 1 as reported by the participants. Degrees of equivalence d_i and their associated expanded uncertainty *U*(d_i) were calculated according to equation (19) and (20). A coverage factor of *k* = 2 was used to calculate *U*(d_i)= *k* · *u*(d_i). E_n was calculated according to equation (21). As consensus the arithmetic mean of all participants $\bar{w}_A$ was applied.

Consensus value: $\bar{w}_A$= (121.3 ± 1.3) mg/g					
Lab code	(*w* (Hb) ± *u*(*w*(Hb))/ mg/g	u_{rel}	d_i(A)	*U*(d_i)	E_n
1_1	120.1 ± 1.2	1.0 %	-1.2	3.6	0.32
1_2	117.4 ± 0.55	0.5 %	-3.9	2.8	1.39
4_1	119.89± 1.02	0.9 %	-1.4	3.3	0.42
4_2	120.69 ± 1.75	1.4 %	-0.6	4.4	0.13
4_3	121.17 ± 1.91	1.6 %	-0.1	4.6	0.02
4_4	115.5 ± 1.0	0.9 %	-5.8	3.3	1.73
4_5	115.3 ± 1.2	1.0 %	-6.0	3.5	1.69
5	127.8 ± 3.0	2.3 %	6.5	6.5	1.00
3	124.36 ± 0.85	0.7 %	3.1	3.1	1.00
2_1	124.9 ± 1.6	1.3 %	3.6	4.1	0.88
2_2	126.8 ± 5.3	4.2 %	5.5	10.9	0.51

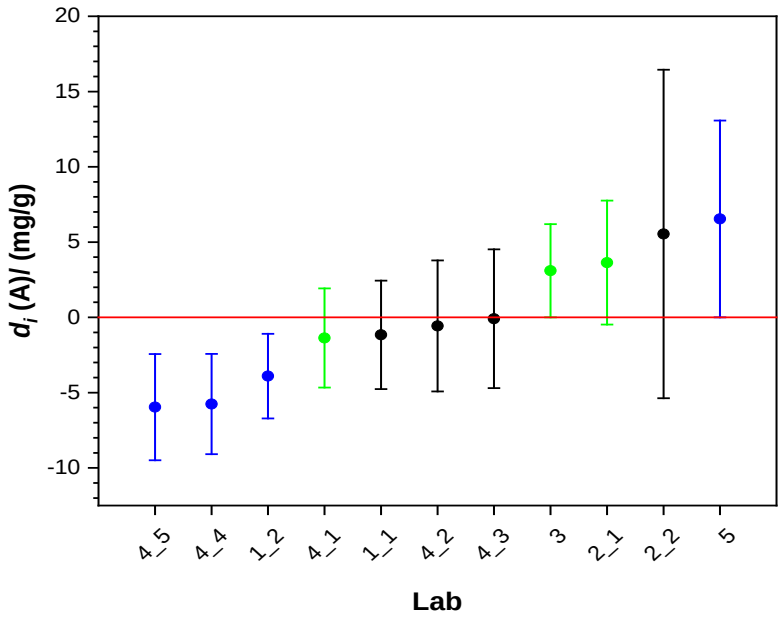


Figure 8: Graphical representation of the equivalence statements related to the arithmetic mean of all participants $\bar{w}_A$ – DoE-plot of the data reported by the P201 participants according to table 4. Blue dots represent the DoE d_i(A) of the results using ICP-MS, green dots the ones of using optical spectrometry and black of the results using MS/MS. The error bars denote the expanded uncertainty associated with the degree of equivalence *U*(d_i) according to eq. (20). calculated by applying a coverage factor of *k* = 2. using *U*(d_i) = *k* · *u*(d_i). Results enclosing zero with their uncertainty interval are considered to be consistent with $\bar{w}_A$.

Table 7: Mass fractions *w*(Hb) and their associated combined and relative expanded uncertainties *u*(*w*(Hb)) and u_{rel}. resp., using a coverage factor *k* = 1 as reported by the participants. Degrees of equivalence d_i and their associated expanded uncertainty *U*(d_i) were calculated according to equation (19) and (20). A coverage factor of *k* = 2 was used to calculate *U*(d_i)= *k* · *u*(d_i). E_n was calculated according to equation (21). As consensus the median of all participants $\bar{w}_M$ was applied.

Consensus value: $\bar{w}_M$= (120.7 ± 2.1) mg/g					
Lab code	(*w* (Hb) ± *u*(*w*(Hb)) / mg/g	u_{rel}	d_i(M)	*U*(d_i)	E_n
1_1	120.1 ± 1.2	1.0 %	-0.6	4.8	0.12
1_2	117.4 ± 0.55	0.5 %	-3.3	4.3	0.78
4_1	119.89± 1.02	0.9 %	-0.8	4.6	0.17
4_2	120.69 ± 1.75	1.4 %	-0.0	5.4	0.00
4_3	121.17 ± 1.91	1.6 %	-0.5	5.6	0.09
4_4	115.5 ± 1.0	0.9 %	-5.2	4.6	1.12
4_5	115.3 ± 1.2	1.0 %	-5.4	4.8	1.13
5	127.8 ± 3.0	2.3 %	7.1	7.3	0.98
3	124.36 ± 0.85	0.7 %	3.7	4.4	0.82
2_1	124.9 ± 1.6	1.3 %	4.2	5.2	0.81
2_2	126.8 ± 5.3	4.2 %	6.1	11.4	0.54

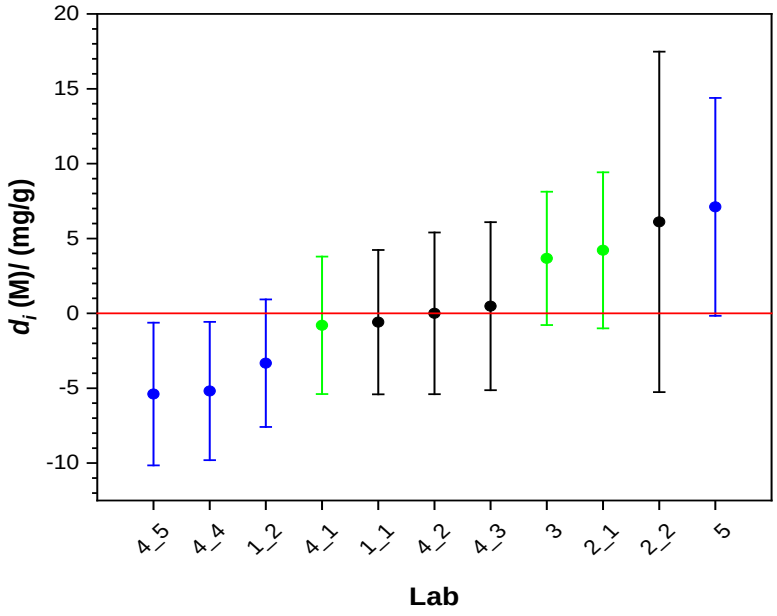


Figure 9: Graphical representation of the equivalence statements related to the median of all participants $\bar{w}_M$ – DoE-plot of the data reported by the P201 participants according to table 4. Blue dots represent the DoE d_i(M) of the results using ICP-MS, green dots the ones of using optical spectrometry and black of the results using MS/MS. The error bars denote the expanded uncertainty associated with the degree of equivalence *U*(d_i) according to eq. (20) calculated by applying a coverage factor of *k* = 2. using *U*(d_i) = *k* · *u*(d_i). Results enclosing zero with their uncertainty interval are considered to be consistent with $\bar{w}_M$.

Table 8: Mass fractions *w*(Hb) and their associated combined and relative expanded uncertainties *u*(*w*(Hb)) and u_{rel}. resp., using a coverage factor of *k* = 1 as reported by the participants. Degrees of equivalence d_i and their associated expanded uncertainty *U*(d_i) were calculated according to equation (19) and (20). A coverage factor of *k* = 2 was used to calculate *U*(d_i)= *k* · *u*(d_i). E_n was calculated according to equation (21). As consensus the uncertainty weighted mean of all participants $\bar{w}_{UWM}$ was applied.

Consensus value: $\bar{w}_{UWM}$= (119.3 ± 1.0) mg/g					
Lab code	(*w* (Hb) ± *u*(*w*(Hb))/ mg/g	u_{rel}	d_i(UWM)	*U*(d_i)	E_n
1_1	120.1 ± 1.2	1.0 %	0.8	3.1	0.26
1_2	117.4 ± 0.55	0.5 %	-1.9	2.2	0.89
4_1	119.89± 1.02	0.9 %	0.6	2.8	0.22
4_2	120.69 ± 1.75	1.4 %	1.4	4.0	0.36
4_3	121.17 ± 1.91	1.6 %	1.9	4.2	0.45
4_4	115.5 ± 1.0	0.9 %	-3.8	2.8	1.35
4_5	115.3 ± 1.2	1.0 %	-4.0	3.0	1.31
5	127.8 ± 3.0	2.3 %	8.5	6.3	1.36
3	124.36 ± 0.85	0.7 %	5.1	2.5	2.03
2_1	124.9 ± 1.6	1.3 %	5.6	3.7	1.52
2_2	126.8 ± 5.3	4.2 %	7.5	10.8	0.70

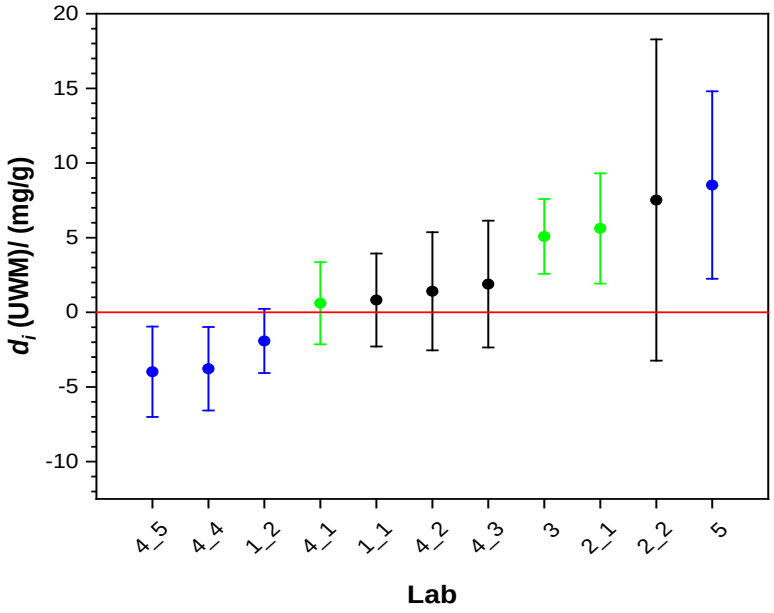


Figure 10: Graphical representation of the equivalence statements related to uncertainty weighted mean of all participants $\bar{w}_{UWM}$ – DoE-plot of the data reported by the P201 participants according to table 4. Blue dots represent the DoE d_i(UWM) of the results using ICP-MS, green dots the ones of using optical spectrometry and black of the results using MS/MS. The error bars denote the expanded uncertainty associated with the degree of equivalence *U*(d_i) according to eq. (20) calculated by applying a coverage factor of *k* = 2. using *U*(d_i) = *k* · *u*(d_i). Results enclosing zero with their uncertainty interval are considered to be consistent with $\bar{w}_{UWM}$.

Table 9: Mass fractions *w*(Hb) and their associated combined and relative expanded uncertainties *u*(*w*(Hb)) and u_{rel}. resp., together with a coverage factor of *k* = 1 as reported by the participants. Degrees of equivalence d_i(DL) and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (19) and (22). A coverage factor of *k* = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. E_n was calculated according to equation (21). As consensus the DerSimonian-Laird mean of all participants $\bar{w}_{DL}$ was applied.

Consensus value: $\bar{w}_{DL}$= (120.6 ± 1.2) mg/g					
Lab code	(*w* (Hb) ± *u*(*w*(Hb))/ mg/g	u_{rel}	d_i(DL)	$U(d_i)$	E_n
1_1	120.1 ± 1.2	1.0 %	-0.5	7.0	0.07
1_2	117.4 ± 0.55	0.5 %	-3.2	6.6	0.49
4_1	119.89± 1.02	0.9 %	-0.7	6.8	0.10
4_2	120.69 ± 1.75	1.4 %	0.1	7.4	0.01
4_3	121.17 ± 1.91	1.6 %	0.6	7.5	0.08
4_4	115.5 ± 1.0	0.9 %	-5.1	6.8	0.75
4_5	115.3 ± 1.2	1.0 %	-5.3	6.9	0.77
5	127.8 ± 3.0	2.3 %	7.2	8.8	0.82
3	124.36 ± 0.85	0.7 %	3.8	6.7	0.56
2_1	124.9 ± 1.6	1.3 %	4.3	7.2	0.60
2_2	126.8 ± 5.3	4.2 %	6.2	12.4	0.50

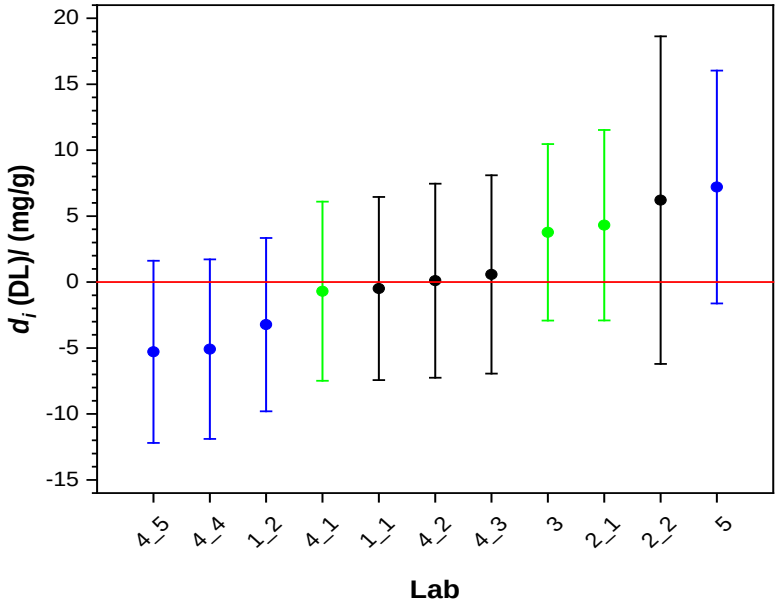


Figure 11: Graphical representation of the equivalence statements related to the DerSimonian-Laird mean $\bar{w}_{DL}$ – DoE-plot of the data reported by the P201 participants according to table 4. Blue dots represent the DoE d_i(DL) of the results using ICP-MS, green dots the ones of using optical spectrometry and black of the results using MS/MS. The error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (22) calculated by applying a coverage factor of *k* = 2. using $U(d_i) = k \cdot u(d_i)$. Results enclosing zero with their uncertainty interval are considered to be consistent with $\bar{w}_{DL}$.

Discussion and Conclusions

Considering the use of very different measurement techniques for the quantification of total Hb, the results are in very good agreement. Three results were obtained using an optical method after chemical conversion. These methods mainly detect the haeme group incorporated in the protein. The approach of quantifying Hb via specific peptides and detection with molecular MS take all proteins containing the specific amino acid sequence of the peptide into account regardless of the conformation and/or binding status of the protein. ICP-MS methods using chromatographic separation before detection, however, can selectively separate the tetrameric form of Hb. Dimers or other oligomers as well as Hb bound to other proteins will be separated by the chromatography.

It is useful to consider the different approaches that would be used in assigning a reference value in any future key comparison. As the reported results comprised many different measurement principles and procedures, it is not surprising that the reported uncertainties vary between method and laboratories. While there are considerations that need addressing in using any estimator, the observed differences between the results are not reflected in the reported measurement uncertainties. This is best represented by the DerSimonian-Laird treatment of the data. The calculated interlaboratory standard deviation or Tau value (dark uncertainty) was approximately 3 % whilst the average reported measurement uncertainty was approximately half this (1.4 %). A possible reason for this large difference may lie in the underestimation of any necessary conversion factors and their uncertainty contributions between the target analyte measured and the requested measurand. Lab 1, for examples, has shown that the differences in their results obtained by LC-MS in comparison to LC-ICP-MS were due to Hb bound to haptoglobin. This accounts for approximately 2.5 % in whole blood (and about 100 % in plasma or serum). Therefore, a detailed and unambiguous description of the measurand is required before proceeding to a key comparison..

Lab 5 reported problems with the homogeneity of the samples. It must be stressed that the whole blood samples tend to sediment quite quickly. Therefore, they must be shaken while drawing the subsamples. This will be made clearer in the technical protocols in the future.

Core Competencies and How Far Does the Light Shine Statement

The aim is to demonstrate the capability to quantify reliably an intact protein with a complex tetrameric structure. It will be necessary to distinguish between the whole protein and fragments of the tetrameric structure such as dimers or monomers. The present pilot study will not serve this purpose. CMC claims cannot be derived from this.

References

[1] Division of Medical Sciences, National Research Council 1958 Hemoglobin Standard Science **127** (3311) 1376-1378

[2] Bnumitzer, G. 1964 The molecular weight of human haemoglobin. In erythrocytometric methods and their standardization Bibl. Haemat. Fasc **18** 59-60

[3] Russel J., Eilers M.D. 1967, Notification of Final Adoption of an International Method and Standard Solution for Hemoglobinometry Specifications for Preparation of Standard Solution Amer. J. Clin. Pathol. **47**(2) 212-214

[4] Zwart A. et al. 1996 Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobinocyanide standard (4th edition) J Clin Pathol **49**(4) 271–274

[5] Heuck. C. C. et al. 2008 The Alkaline Haematin Detergent (AHD(575)) Method for the Determination of Haemoglobin in Blood - a Candidate Reference Measurement Procedure. Clin. Lab. **54**(7-8) 255-272

[6} ISO Guide 35:2017(E). Reference materials - Guidance for characterization and assessment of homogeneity and stability. International Organization of Standardization. 2017-08. fourth edition.

[7] Rienitz. O. et al. 2012 CCQM-K87 "Mono-elemental Calibration Solutions" Metrologia **49**

[8] CCQM Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence version 10

[9] Sachs. L. Angewandte Statistik 1997 Springer Berlin 978-3-662-05746-9

[10] -JCGM 100:2008 Evaluation of measurement data — Guide to the expression of uncertainty in measurement BIPM First edition September 2008

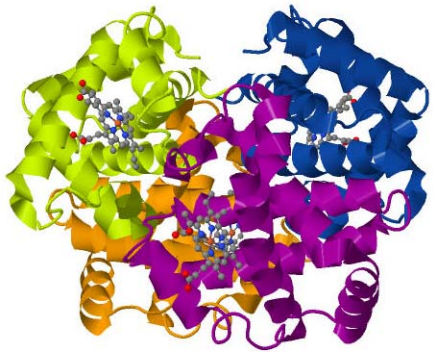
Annex I

Technical protocol

Introduction

Haemoglobin is an important marker for anaemia. It is a Fe-containing protein in the red blood cells responsible for the transport and storage of oxygen. Low levels of haemoglobin indicate anaemia. a condition in which a body is undersupplied with oxygen. causing fatigue and weakness. It can be caused by a loss of blood or a severe infection such as e.g. malaria. High levels of haemoglobin usually are a sign for polycythaemia which can lead to heart failure. heart attacks or strokes. Haemoglobin abnormalities result in very serious hereditary diseases. such as sickle-cell anaemia and thalassemia. Therefore. blood preservations are all investigated regarding their haemoglobin content. According to the French Health Insurance (Sécurité Sociale) refund statistics. haematology laboratory tests that include haemoglobin analysis occupy the first place regarding the number of blood biomarker tests. It accounts for 32 million of tests per year with a total cost of 237 million Euros. Although the HiCN method. conversion of haemoglobin to cyanmethaemoglobin. recommended by the World Health Organisation (WHO) has been used for routine haemoglobin determinations. it is often impractical for laboratories because of the toxicity of potassium cyanide used for derivatisation and its restrictions on the use and disposal. Furthermore. only a very limited number of reference materials are available such as the reference material BCR-522 for the determination of the total haemoglobin content containing cyanmethaemoglobin isolated from bovine blood. While it serves well for the calibration of the HiCN method recommended by the World Health Organisation (WHO). it shows significant differences to freshly prepared samples from human erythrocytes in other methods. Another reference material is the Japanese reference material JCCRM-912-2 which indicates two different concentrations. one obtained with the HiCN method and another one obtained with blood counters. Therefore. it is essential that NMIs can provide reliable reference values which are not method dependent and are not biased by the different haemoglobin forms such methaemoglobin. haemoglobin and genetic variants such as HbA_2. HbA_0 etc.

Measurand



Human haemoglobin

Mean molecular weight: 64458 g/mol

Expected concentration range: 100-150 g/kg

Monomer average: 141 amino acids. molecular weight 16115 g/mol

Samples

Participants will be provided with three Eppendorf tubes (Protein LoBind) containing lyophilised whole blood received from 0.5 mL fresh blood each. The blood has been frozen and

centrifuged to remove cell fragments and facilitate homogenisation. The samples were prepared using a single donor blood unit which was tested negative for Anti-HIV-1&2. Anti-HCV. HBsAg. Syphilis. HCV NAT. HIV NAT. HBV NAT. HAV NAT. ParvoB19 NAT. Since no known test method can offer complete assurance that infectious agents are absent from this material. it should be handled and disposed according to the recommendations of the concerned local and national legislation and regulations for potentially infectious human blood specimen.

The homogeneity of the samples was determined with u_{bb} = 1 %. The material was found to be homogenous down to the minimum sample amount of 20 µL and stable at -20 °C for at least three months. The stability will be further monitored during the study.

Sample handling

The samples will be shipped to the participants on cool packs at -20 °C (please control the temperature indicator) and should be stored at -80 °C by the participants. The samples should be measured immediately after reconstitution. For reconstitution of the sample allow the tube to reach ambient temperature. Please weigh the tubes immediately after opening the bags. Weigh them together with their labels. Tap the bottom of the tube to dislodge most of the adhering material from the cap. Carefully open the cap and add 0.4 mL ultrapure water. Close the cap and weigh the tube again. Homogenise the sample for 1 h on a vortex shaker and also by inversion to ensure that all material still attached to the cap is also dissolved. Short centrifugation will help to remove every sample from the cap. Analyse the sample immediately after reconstitution. Please make sure that the sample is well homogenised before taking aliquots. The minimum amount of sample to be used is 20 µL. Depending on the method used delipidation might be necessary. At least three aliquots should be analysed of each sample.

For calculation of results the difference in weight between original blood sample and reconstituted sample should be considered. The weight of the samples before lyophilisation you can find in the tables below: (all values were given specific for each sample of each partner)

Sample: WB B3-XX	Mass / g	Air buoyancy correction	Corrected masses:	
Empty tube (m_1)			m_{tube}	
Before lyophilisation (m_2)			$m_{blood.2}$	
After lyophilisation				

Measurement techniques and methods

The participants are free to use any suitable method which provides traceability to the SI. but are asked to include a full description of their measurement procedure. Potential methods are e.g. isotope dilution in combination with LC-ICP-MS. LC-MS/MS or Raman as well as optical spectroscopy after derivatisation. When specific peptides are used for the quantification. it is recommended to choose them from the α-chain as this chain is preserved in most common haemoglobin variants. Participants may obtain results with more than one method and report them separately. Note that the relative expanded measurement uncertainty U_{rel} associated with your result should not exceed 3 %. You are asked to determine the following quantities:

Mass fraction *w* (Hb) of haemoglobin in human blood in g/kg

Reporting

The deadline for the submission of results is 30 September 2019. Please send your report via E-mail using the template that will be provided to you. Please report all your results (including the values of each aliquot) in terms of a mass fraction *w* in g/kg. Please report also all the masses of all samples at the time of opening the tubes for the first time. Please refer to section 6 to do this.

Please calculate uncertainties for all the results reported according to the GUM. Please. report also your sources of traceability along with a description of the method(s) you used.

If you need further assistance or encounter any kind of problem. please contact the organisers.

Potential reference material

- JCCRM 912: "Certified Reference Material for Total Hemoglobin Measurement".
  3 concentration levels
- BCR-522: haemoglobincyanide in bovine blood lysate
- IRMM/IFCC-467: purified protein HbA_0 with KCN.
  total haemoglobin as additional information

References

Brauckmann C. et al.. Preparation and characterisation of 57Fe enriched heamoglobin spike material for species-specific isotope dilution mass spectrometry. J Anal Atom Spectrom. (2016); 31:1846–57.

Frank C. et al.. Comparison of potential higher order reference methods for total haemoglobin quantification - an interlaboratory study. Anal. Bioanal. Chem. (2017). 409(9). 2341-2351.

Arsene C. G. et al.. Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. Anal Chem. (2009); 80(11). 4154–60.

Heuck. C. C. et al. The Alkaline Haematin Detergent (AHD(575)) Method for the Determination of Haemoglobin in Blood - a Candidate Reference Measurement Procedure. Clin. Lab. (2008). 54(7-8). 255-272.

BIPM. Evaluation of measurement data—guide to the expression of uncertainty in measurement. JCGM 100:2008.

Evaluation of measurement data – Guide to the expression of uncertainty in measurement. JCGM 100:2008.

Frank Spieweck. Horst Bettin. *Methoden zur Bestimmung der Dichte von Festkörpern und Flüssigkeiten*. PTB Bericht W-46. ISBN 3-89429-132-X. Braunschweig. 1998.

Roland Nater. Arthur Reichmuth. Roman Schwartz. Michael Borys. Panagiotis Zervos. *Dictionary of Weighing Terms. A Guide to the Terminology of Weighing*. Springer. ISBN 978-3-642-02013-1. Berlin. 2009.

Checking for correcting reconstitution effects

Within this “Technical Protocol” you should have received a table summarizing all tubes enclosed in your parcel together with the masses of the empty tubes m_{tube} and the masses of the blood in these tubes $m_{blood.2}$.

These masses were determined from the apparent masses (weighing values) of the empty tube m_1 and the tube containing the according blood m_2 determined at a time t_1 and t_2. respectively. Since the ambient conditions (relative humidity of the air φ. air pressure p and air temperature ϑ) were different at these times (t_1 and t_2). according air buoyancy correction factors $K_{i,j}$ depending on the time j and the density of the weighed material i (PP in case of the tube. ρ_{tube}. and in case of blood. $\rho_{blood.2}$) were calculated to convert the apparent masses m_1 and m_2 into the masses m_{tube} and $m_{blood.2}$.

$$m_{\text{tube}} = K_{tube.1} \cdot m_1$$

$$K_{\text{tube.1}} = \frac{1 - \frac{\rho_{\text{air.1}}}{\rho_{\text{cal.1}}}}{1 - \frac{\rho_{\text{air.1}}}{\rho_{\text{tube}}}}$$

$$\rho_{\text{air.1}} = \frac{0.348444\ \frac{\text{kg/m}^3}{\text{hPa}} \cdot p_1 - \phi_1 \cdot \left(0.252\ \frac{\text{kg/m}^3}{\text{°C}} \cdot \vartheta_1 - 2.0582\ \frac{\text{kg}}{\text{m}^3}\right)}{273.15 + \frac{1}{\text{°C}} \cdot \vartheta_1}$$

$$m_{\text{blood.2}} = K_{\text{blood.2}} \cdot \left(m_2 - \frac{m_{\text{tube}}}{K_{\text{tube.2}}}\right)$$

$$K_{\text{tube.2}} = \frac{1 - \frac{\rho_{\text{air.2}}}{\rho_{\text{cal}}}}{1 - \frac{\rho_{\text{air.2}}}{\rho_{\text{tube}}}} \quad \text{and} \quad K_{\text{blood.2}} = \frac{1 - \frac{\rho_{\text{air.2}}}{\rho_{\text{cal}}}}{1 - \frac{\rho_{\text{air.2}}}{\rho_{\text{blood.2}}}}$$

$$\rho_{\text{air.2}} = \frac{0.348444\ \frac{\text{kg/m}^3}{\text{hPa}} \cdot p_2 - \phi_2 \cdot \left(0.252\ \frac{\text{kg/m}^3}{\text{°C}} \cdot \vartheta_2 - 2.0582\ \frac{\text{kg}}{\text{m}^3}\right)}{273.15 + \frac{1}{\text{°C}} \cdot \vartheta_2}$$

The following parameters were used to perform the calculations above: ρ_{tube} = 946 kg/m^3.

$\rho_{blood.2}$ = 1052 kg/m^3. and ρ_{cal} = 8000 kg/m^3.

Before sampling the first aliquot from a tube. you are asked to weigh the tube (including label and cap) at the time t_3 yielding its apparent mass m_3. while also collecting the corresponding ambient conditions (relative humidity of the air φ_3. air pressure p_3 and air temperature ϑ_3). This way you are able to observe even minor losses due to evaporation and are also able to correct for them. Please note: Directly before the weighing. you should open the cap of the tube and tighten it immediately afterwards to equilibrate the pressure inside and outside the tube. To calculate the correction. please follow the step-by-step recipe:

Step 1: Calculate the air density $\rho_{air.3}$

$$\rho_{air.3} = \frac{0.348444\ \frac{kg/m^3}{hPa} \cdot p_3 - \phi_3 \cdot \left(0.252\ \frac{kg/m^3}{°C} \cdot \vartheta_3 - 2.0582\ \frac{kg}{m^3}\right)}{273.15 + \frac{1}{°C} \cdot \vartheta_3}$$

Step 2: Calculate the air buoyancy correction factor of the tube $K_{tube.3}$

$$K_{tube.3} = \frac{1 - \frac{\rho_{air.3}}{\rho_{cal.3}}}{1 - \frac{\rho_{air.3}}{\rho_{tube}}}$$

Step 3: Calculate the air buoyancy correction factor of the blood $K_{blood.3}$

$$K_{blood.3} = \frac{1 - \frac{\rho_{air.3}}{\rho_{cal.3}}}{1 - \frac{\rho_{air.3}}{\rho_{blood.3}}}$$

Step 4: Calculate the mass $m_{blood.3}$ of the blood at the time t_3 before sampling the first aliquot from the tube

$$m_{blood.3} = K_{blood.3} \cdot \left(m_3 - \frac{m_{tube}}{K_{tube.3}}\right)$$

Step 5: Calculate the difference in mass Δm

$$\Delta m = m_{blood.3} - m_{blood.2}$$

Step 6: Calculating the correction factor f_{blood}. Please report this corrected mass fraction w_2.

$$w_2 = f_{blood} \cdot w_3 \quad \text{with} \quad f_{blood} = \left(1 + \frac{\Delta m}{m_{blood.2}}\right)$$

When setting up an uncertainty budget please use the following standard uncertainties (type B. normal distribution. coverage factor k = 1) associated with the mass of the empty tube m_{tube} and with the mass of the blood $m_{blood.2}$. respectively: $u(m_{tube})$ = 0.0002 g and $u(m_{blood.2})$ = 0.0001 g.

The following table summarizes all the symbols used throughout the equations above.

Symbol	Unit	Quantity	Comment
m_{tube}	g	Mass of the empty tube (corrected for air buoyancy)	Individually listed for every tube no. in the table sent to each participant
$m_{blood.2}$	g	Mass of the blood before lyophilisation (corrected for air buoyancy)	Individually listed for every tube no. in the table sent to each participant; determined immediately after bottling in the pilot laboratory (Lab 1)
$m_{blood.3}$	g	Mass of the blood after reconstruction (corrected for air buoyancy)	To be determined prior to sampling the first aliquot in the participant's laboratory
Δm	g	Mass difference (loss) of the blood (corrected for air buoyancy)	Difference between $m_{blood.2}$ and $m_{blood.3}$; determined prior to sampling in the participant's laboratory
m_1	g	Apparent mass (reading of the balance) of the empty tube	Determined in the pilot laboratory (Lab 1); used to calculate m_{tube}
m_2	g	Apparent mass (reading of the balance) of the sum of the empty tube and the blood	Determined in the pilot laboratory (Lab 1) immediately after bottling; used to calculate $m_{blood.2}$
m_3	g	Apparent mass (reading of the balance) of the sum of the empty tube and the sample/calibration blood	Determined in the participant's laboratory prior to sampling; used to calculate $m_{blood.3}$
w_2	g/kg	Mass fraction of Hb	Value corrected for reconstitution effects; calculated from w_3
w_3	g/kg	Mass fraction of Hb	Value actually measured in the participant's laboratory
f_{blood}	1	Factor to correct the measured mass fraction for reconstitution effects	To be calculated by the participant
$K_{tube.1}$	g/g	Air buoyancy correction factor	Valid for the tube material (PP) at the time of the determination of m_1
$K_{tube.2}$	g/g	Air buoyancy correction factor	Valid for the tube material (PP) at the time of the determination of m_2
$K_{tube.3}$	g/g	Air buoyancy correction factor	Valid for the tube material (PP) at the time of the determination of m_3
$K_{blood.2}$	g/g	Air buoyancy correction factor	Valid for the blood at the time of the determination of m_2
$K_{blood.3}$	g/g	Air buoyancy correction factor	Valid for the blood at the time of the determination of m_3
$\rho_{air.1}$	kg/m³	Air density	At the time of the determination of m_1 in the pilot laboratory (Lab 1)

$\rho_{air.2}$	kg/m³	Air density	At the time of the determination of m_2 in the pilot laboratory (Lab 1)
$\rho_{air.3}$	kg/m³	Air density	At the time of the determination of m_3 in the participant's laboratory
ρ_{tube}	kg/m³	Density of the tube material (PP)	Assumed to be sufficiently constant throughout the temperature range in question; ρ_{tube} = 946 kg/m³
$\rho_{blood.2}$	kg/m³	Density of the blood before lyophilisation	Determined in the pilot laboratory (Lab 1); listed in the text above; assumed to be sufficiently constant throughout the temperature range in question
$\rho_{blood.3}$	kg/m³	Density of the blood after reconstruction	Determined in the participants laboratory.
ρ_{cal}	kg/m³	Density of the calibration masses of the balance	Value for the balance used in the pilot laboratory (Lab 1) to determine m_1 and m_2
$\rho_{cal.3}$	kg/m³	Density of the calibration masses of the balance	Value for the balance used in the participant's laboratory to determine m_3.
p_1	hPa	Air pressure	At the time of the determination of m_1 in the pilot laboratory (Lab 1)
p_2	hPa	Air pressure	At the time of the determination of m_2 in the pilot laboratory (Lab 1)
p_3	hPa	Air pressure	At the time of the determination of m_3 in the participant's laboratory
Φ_1	1	Relative air humidity	At the time of the determination of m_1 in the pilot laboratory (Lab 1)
Φ_2	1	Relative air humidity	At the time of the determination of m_2 in the pilot laboratory (Lab 1)
Φ_3	1	Relative air humidity	At the time of the determination of m_3 in the participant's laboratory; please use numerical values $0 \leq \varphi_3 \leq 1$
ϑ_1	°C	Air temperature	At the time of the determination of m_1 in the pilot laboratory (Lab 1)
ϑ_2	°C	Air temperature	At the time of the determination of m_2 in the pilot laboratory (Lab 1)
ϑ_3	°C	Air temperature	At the time of the determination of m_3 in the participant's laboratory

Annex II

Participants' Reports

Participating Laboratory

Institute: Lab code 1_1

Reporting date: 18.12.2019

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
120.1	1.2	2.0	2.5

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
38	LC-MS/MS	119.1	118.6	119.2	
61	LC-MS/MS	118.6	118.8	120.1	
89	LC-MS/MS	123.1	121.8	121.3	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	3*80 mg per blood sample

Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	Samples were reconstituted as recommended**.** Correction factor "f_{blood}" for sample reconstitution was 0.925 (vial-38), 0.899 (vial-61) and 0.952 (vial-89) Aliquots (80 mg) were taken from each reconstituted blood-sample. Each aliquot was further diluted with 20000 mg of 50 mM TRIS-buffer (pH: 7.6). Each aliquot (30 mg) of diluted blood was spiked with a solution (22 mg) of U-^{15}N-HbA0. The concentration of U-^{15}N-HbA0 was 11.148 pmol/mg in this spike-solution. For calibration of the mass spectrometer, mixtures of defined amounts of HbA0 reference material and U-^{15}N-HbA0 were prepared and treated in the same way as the aliquots of diluted blood. Pure crystalline HbA0 was obtained from Lab1_2 and value-assigned by amino acid analysis (AAA). Purity of this material was found to be 44.4% by weight (AAA by R. Ohlendorf). Solutions of HbA0 reference material were prepared in 50 mM TRIS-buffer (pH: 7.6) and used for preparation of calibration mixtures as described above. Spiked samples of diluted blood and calibration mixtures were digested over night using trypsin. Raw digestion products were desalted using solid phase extraction (SPE). Extracts were submitted to LC-MS/MS analysis. Three signature peptides of the α-globin VGAHAGEYGAEALER (T4), MFLSFPTTK (T5) and TYFPHFDLSHGSAQVK (T6) were analyzed by LC-MS/MS. Mean-values of results for all 3 signature peptides were calculated for each aliquot of reconstituted blood. All single results for T4, T5 and T6 are shown in the table at the end of this report.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	Instrumentation and important MS-acquisition parameters are described in Chim Acta 487(2018)318, doi: 10.1016/j.cca.2018.10.024
Calibration method/design used	One-point calibration using a calibrator as described above. Labeled Hb (U-^{15}N HbA0) was used as internal standard.

Separation Conditions (column, eluents, gradient and flow rate)	see Instrumentation
Equation used to calculate the mass fraction of total Hb in blood	**CM ref M spike(sample) R ref - R M(ref) R spike - R M(sample)** C_{Hb} **=** ---------- **x** ---------- **x** ---------- **x** ---------- **M sample M spike(ref) R M(sef) - R spike R M(sample) - R sample** **C_{Hb}** : concentration of Hb in sample (mol/g) **C** ref : concentration of Hb-working solution (mol/g) **M** ref : weight of Hb-working solution (g) **CM r**ef : amount of substance of Hb (mol), (**C** ref x **M** ref) **M** spike(sample): weight of working solution of U-^{15}N-HbA0, added to sample (g) **M** spike(ref): weight of working solution of U-^{15}N-hbA0, added to calibrator (g) **M** sample: weight of sample (g) **R** M(sample): isotope ratio of mixture "sample + U-^{15}N-HbA0", with **R** M(sample)= $FL_{labeled\ T4}/FL_{T4}$, $FL_{labeled\ T5}/FL_{T5}$ or $FL_{labeled\ T6}/FL_{T6}$, FL= peak area in XIC **R** M(ref): isotope ratio of mixture "calibrator + U-^{15}N-HbA0", with **R** M(ref)= $FL_{labeled\ T4}/FL_{T4}$, $FL_{labeled\ T5}/FL_{T5}$ or $FL_{labeled\ T6}/FL_{T6}$ **R** sample : isotope ratio(natural) of Hb in sample (R sample = 0) **R** ref : isotope ratio (natural) of reference material (HbA0), R ref = 0 **R** spike : isotope ratio of U-^{15}N-HbA0 **C** spike : concentration of U-^{15}N-HbA0-working solution (mol/g)
Air buoyancy corrected (ambient conditions, density after reconstitution)	Reconstitution of blood samples and dilution of blood aliquots was corrected for air buoyancy. Details for this correction are provided upon request.

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	- standard uncertainty of determination of the mass fraction (w_{Hb}) : 0.37 g/kg - standard uncertainty contribution of value-assignment of HbA0-reference solution used for preparation of HbA0-solution in Tris buffer: 1.0 g/kg - standard uncertainty of weighing: 0.00072 g/kg (reconstitution of blood by addition of water) - standard uncertainty of weighing: 0.025 g/kg (reference mat.) - standard uncertainty of weighing: 0.0006 g/kg (dilution of reference mat. by addition of 2g Tris-buffer) - standard uncertainty of weighing: 0.092 g/kg (80 mg - aliquot of blood) - standard uncertainty of weighing: 0.0086 g/kg (dilution of blood by addition of 20 g Tris-buffer) - unknown factor for systematic unidentified discrepancies including sample preparation and LC-MS/MS interferences, estimated value: 0.6 g/kg combined standard uncertainty: $u_c = \sqrt{0{,}37^2 + 1{,}0^2 + 0.00072^2 + 0.025^2 + 0.0006^2 + 0{,}092^2 + 0.0086^2 + 0{,}6^2}$ $U_c = 2.5\ g/kg$ (Urel = 2.1 %) with k = 2.0
Additional Comments or Observations	

Single results obtained by determination of signature peptides T4, T5 and T6 of α-globin

		total Hb (g/kg)		
sample	**aliquot**	**T4**	**T5**	**T6**
38	1	119.1	119.1	119.1
38	2	120.2	117.7	117.9
38	3	120.6	118.9	118.1
61	1	119.5	118.0	118.3
61	2	120.4	117.5	118.4
61	3	121.5	119.9	119.0

89	1	121.3	124.4	123.7
89	2	123.0	122.4	119.9
89	3	122.2	121.7	120.1

T4: VGAHAGEYGAEALER

T5: MFLSFPTTK

T6: TYFPHFDLSHGSAQVK

Participating Laboratory

Institute: Lab code 1_2

Reporting date: 07.11.2019

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
117.4	0.55	2.0	1.1

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
04	LC-ICP-MS	118.752	114.687	117.755	
41	LC-ICP-MS	118.355	115.760	117.932	
81	LC-ICP-MS	115.68	118.477	116.540	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	3*ca 90 mg per blood sample

Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	Samples were reconstituted as recommended**.** Correction factor for sample reconstitution vs. original whole blood sample was 0.952 (vial-04), 0.956 (vial-41) and 0.947 (vial-81) Aliquots (88 mg) were taken from each reconstituted blood-sample. Each aliquot was further diluted 1:100 with 12.5 mM TRIS-buffer (pH: 7.8). 30 mg of each aliquot of diluted blood was spiked with 30 mg ^{54}Fe-HbA_0 solution (0.96 mg/g). To perform double ID-MS, mixtures of defined amounts of HbA_0 reference material and ^{54}Fe-HbA_0 were prepared and treated in the same way as the aliquots of diluted blood. Pure crystalline HbA_0 was obtained from Sigma-Aldrich and value-assigned by amino acid analysis (AAA) by Lab 1_1. Purity of this material was found to be 44.4% by weight (AAA by R. Ohlendorf). Solutions of HbA_0 reference material were prepared in 12.5 mM TRIS-buffer (pH: 7.8) and used for preparation of calibration mixtures as described above. 10 µL KCN solution (50 mM) was added to all blends and all solutions were allowed to homogenise on a vortexer at 4 °C for up to 30 min. The samples were then centrifuged for 15 min and the supernatants were transferred to a HPLC vial.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	HPLC (Agilent 1200) coupled to ICP-MS (Agilent 7700) ICP-MS conditions:	
	RF power	1500 W
	nebulizer	PFA-ST 700 µL MicroFlow
	Spray chamber temp	2 °C
	Plasmagas	Ar, 15 L/min
	Reactionsgas	H_2, 6 mL/min
	m/z detected	54, 56, 57, 58
	m/z used for calculation	54, 56
	Detection mode	TRA
	Integration time	0.1 s
Calibration method/design	ID-MS using ^{54}Fe-labelled haemoglobin as spike material.	

used	
Separation Conditions (column, eluents, gradient and flow rate)	Column: Zenix-C® SEC-300, 30 cm × 4,6 mm, 3 µm, PEEK, Supelco (Sigma-Aldrich, St Louis, USA) Eluent: 12,5 mmol/kg Tris + 125 mmol/kg NH4Ac, pH = 7.8 Gradient: isocratic Column temp: 30 °C Flowrate: 0.35 mL/min Injection volume: 5 µL Autosampler temp: 4 °C
Equation used to calculate the mass fraction of total Hb in blood	$w_x = w_z * \frac{m_{yx}}{m_x} * \frac{m_z}{m_{yz}} * \frac{R_y - R_{bx}}{R_{bx} - R_x} * \frac{R_{bz} - R_z}{R_y - R_{bz}}$ w_x mass fraction in sample solution x w_z mass fraction in calibration solution z m_{yx} mass of spike y in sample solution x m_x mass of sample solution x m_z mass of calibration solution z m_{yz} mass of spike y in calibration solution z R_y isotope ratio $^{54}Fe/^{56}Fe$ in spike solution y R_{bx} isotope ratio $^{54}Fe/^{56}Fe$ in sample-spike blend bz R_x isotope ratio $^{54}Fe/^{56}Fe$ in sample solution x R_{bz} isotope ratio $^{54}Fe/^{56}Fe$ in calibration-spike blend bz R_z isotope ratio $^{54}Fe/^{56}Fe$ in calibration solution z
Air buoyancy corrected (ambient conditions, density after reconstitution)	Reconstitution of blood samples and dilution of blood aliquots was corrected for air buoyancy according to study protocol.

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	R_{bz} 52.7 % w_{pur} 31.9 % (purity of calibration material) R_{bx} 11.6 m_{yx} 0.6 % m_{x} 0.6 % m_{z} 0.6 % m_{yz} 0.6 % m_{HbA0} 0.6 % (mass of calibration material in stock solution) m_{sol} 0.6 % (mass of calibration stock solution)
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 2_1

Reporting date: 28/02/2020

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
124,9	1,6	2	3,2

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB B3 25	UV/Vis	123,5 (80 mg)	123,4 (60 mg)	124,4 (40 mg)	129,9 (20 mg)
WB B3 47	UV/Vis	122,5 (100 mg)	122,5 (80 mg)	122,4 (60 mg)	125,8 (40 mg)
WB B3 64	UV/Vis	124,9 (100 mg)	125,0 (80 mg)	126,1 (60 mg)	128,3 (40 mg)

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	20, 40, 60 and 80 mg (WB B3 25); 40, 60, 80 and 100 mg (WB B3 47, 64).

Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	Samples were kept at RT for 10 min, briefly opened to equilibrate with atmospheric pressure before weighting, gravimetrically ressuspended in type I water, homogenized at RT for 1h under vortexing with occasional tube inversion. Temperature, relative humidity, and atmospheric pressure were recorded. Measurement was performed according to DIN 58931:2010, method AHD574. Sample aliquots (20-100 mg) were gravimetrically suspended in conversion solution, vortexed for 1 min and then kept in the dark for at least 2h before reading at 574nm (UV 2700 Shimadzu spectrophotometer; slit width 2nm; absorbance mode; equipped with a thermal controller TCC 240A). Temperature was kept at 21°C. Chlorohemin (Sigma H9039 lot SLCC3454) and JCCRM 912-3 (H and M levels) were employed as QCMs.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	UV 2700 Spectrophotometer operating on absorbance mode, slit width 2 nm equipped with a thermal controller TCC 240A (Shimadzu Inc.); Spectrasil S cuvettes 10 mm optical path.
Calibration method/design used	Transmittance values calibrated against standard filters traceable to Lab 2 national standards
Separation Conditions (column, eluents, gradient and flow rate)	Not applicable
Equation used to calculate the mass fraction of total Hb in blood	According to DIN 58931:2010, applying a correction factor for reconstitution mass of dried samples
Air buoyancy corrected (ambient conditions, density after reconstitution)	Corrected as stated in the reconstitution process. (atm p; RH%, T; ρ) WB B3 25 (1005,8 ; 49,5; 19,6; 1,05) WB B3 47 (1004,3 ; 46,0; 20,3; 1,05) WB B3 64 (1004,3 ; 46,0; 20,3; 1,05)
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	-optical path (2%); reconstitution (negligible); dilution in conversion solution (18%); absorbance measurement (80%, including repeatability according to DIN 58931:2010)
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 2_2

Reporting date: 28/02/2020

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
126,8	5,3	2	10,6

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB B3 25	LC-MS/MS	124,0	127,3	126,8	
WB B3 47	LC-MS/MS	119,6	118,3	126,2	
WB B3 64	LC-MS/MS	127,2	130,1	127,8	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	40 mg

Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	Samples were kept at RT for 10 min, briefly opened to equilibrate with atmospheric pressure before weighting, gravimetrically resuspended in type I water, homogenized at RT for 1h under vortexing with occasional tube inversion. Temperature, relative humidity, and atmospheric pressure were recorded. 40 mg of sample were diluted in 50 mM Ammonium Bicarbonate buffer (2g final mass) and, after homogenization, 40mg of were further diluted in buffer (200 mg final mass). From this diluted solution, 10mg were taken for tryptic digestion (using promega´s V5111 trypsin). The Hb peptides / transitions measured were: T4 (510 > 209); (510 > 488); T5 (536 > 349); (536 > 446); (536 > 593); T6 (611 > 363); (611> 589);

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	H Class Acquity UPLC (Waters) Xevo TQS Triple Quadrupole (Waters); ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm (Waters)
Calibration method/design used	A 5 point calibration curve (45, 90, 130, 175, 220 mg/g) was prepared using a reference material (912-3H; JCCRM) and analyzed in triplicate. Previously three calibration curves were performed using the standard addition method (reference material addition to the sample) and the slope of these curves was equivalent (Equal Slope Test) to the slope of a calibration curve prepared in buffer.
Separation Conditions (column, eluents, gradient and flow rate)	ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm (Waters). The following gradient of water and methanol (both with 0,1% Formic Acid) was used: Time (min) Water (%) Methanol (%) 2 95 5 24 10 90

Equation used to calculate the mass fraction of total Hb in blood	$c_a = \frac{c_0}{m_{sol}}$ $\quad c_0 = \left(\frac{A - b_0}{b_1}\right)$ Where: c_a = concentration of the analyte (measurand) A = Area of the analyte in the sample b_0 = Linear coefficient of the calibration curve m_{sol} = Final mass of the injected dilute solution b_1 = Angular coefficient of the calibration curve c_0 = mass fraction in the injected dilute solution
Air buoyancy corrected (ambient conditions, density after reconstitution)	Corrected as stated in the reconstitution process. (atm p; RH%, T; ρ) WB B3 25 (1005,8 ; 49,5; 19,6; 1,05) WB B3 47 (1004,3 ; 46,0; 20,3; 1,05) WB B3 64 (1004,3 ; 46,0; 20,3; 1,05)
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	- Reconstitution and dilutions (negligible); Repeatability (0,3%); standard curve interpolation (96,7%); Reference material JCCRM 912-3H uncertainty (3%)
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 3

Reporting date: 2019-09-30

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
124.36	0.85	2.14	1.82

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)	Mass fraction of total Hb in aliquot 5 (opt) (g/kg)
22	**UV/Vis**	**125.1**	**124.8**	**124.7**	**124.1**	**124.4**
58	**UV/Vis**	**124.5**	**123.3**	**124.6**	**124.9**	**124.6**
82	**UV/Vis**	**124.2**	**124.1**	**124.0**	**123.9**	**124.2**

Analytical conditions

Sample treatment

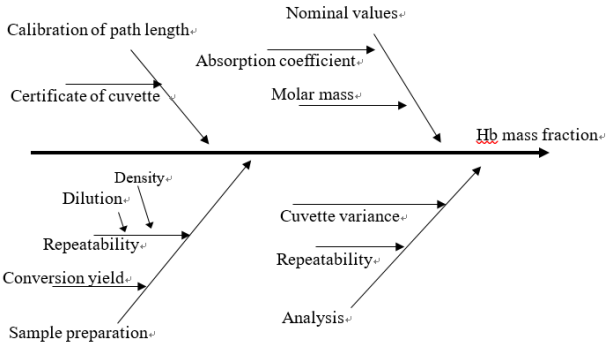
Sample amount used for analysis (mg)	**40**
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	After reconstitution, approx. 40 mg of sample and AHD-conversion reagent (~5 g) were mixed in PP tube by gravimetric preparation. Gently mix the diluted samples, and transfer to a quartz cuvette for UV analysis.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical**	UV/vis spectrophotometer (Lambda 25 UV/VIS Spectrophotometer, PerkinElmer), Alkaline Hematin Detergent 575 (AHD) method was used for analysis.

technique used	
Calibration method/design used	UV filter CRM (NIST 931h, 2034) for UV calibration
Separation Conditions (column, eluents, gradient and flow rate)	
Equation used to calculate the mass fraction of total Hb in blood	$\mathrm{C}(Hb_m) = \frac{A_{AHD} \cdot M(Hb_m)}{d \cdot \varepsilon_{AHD} \cdot \Phi}$ *C(Hb$_m$)*: the mass fraction of the monomeric hemoglobin in the sample *A$_{AHD}$:* the spectral absorbance of the test sample *M(Hb$_m$):* molecular weight of the monomeric hemoglobin (16114.5 g/mol) *d*: layer thickness of the rectangular spectrophotometric cuvette ε_{AHD}: molar decadic absorption coefficient of the AHD complex ϕ: volume fraction of the sample in the test sample
Air buoyancy corrected (ambient conditions, density after reconstitution)	Ambient conditions are inserted into template from Lab 1 (excel file). No further measurement for sample density by Lab 3 due to lack of sample amount (at least 1 mL is required for density measurement)

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)



Factor	Type	U (%)	contribution
sampling	Type A	0.37%	25.0%
density	type A	0.07%	4.7%
cuvette	type A	0.10%	6.8%
absorption coefficient	type B	0.27%	18.7%
UV absorbance	Type B	0.33%	22.7%
measurement repeatability	type A	0.00%	0.0%
method repeatability	type A	0.36%	24.8%
Combined uncertainty		0.68%	0.85
Coverage factor *(k)*			2.14
Expanded uncertainty		1.47%	1.82

Descriptions of uncertainty factors

Sampling: weighing for sample and conversion reagent, and air buoyancy correction via pressure, temp, humid

Density: repeatability of densitometer

Cuvette: difference of the spectral absorbance between

	cuvettes Absorption coefficient: value from literature [1] UV absorbance: standard uncertainty from certificate of UV filter CRM (NIST SRM 931h) Measurement repeatability: standard uncertainty of repeated measurement/within sample Method repeatability: standard deviation of reproduced sample measurement/between samples Coverage factor: coverage factor with degree of freedom in 95% confidence [1] H.UweWolf, H. Link, W. Lang; Biol. Chem. Hoppe-Seyler, 373,305-313, 1992.
Additional Comments or Observations	Although there are several scientific literatures about adopted measurement method (AHD method) for hemoglobin, there is no complete metrological traceability to SI in this method yet. Due to the lack of purity-assessed certified reference material of chlorohaemin for absorption coefficient by Lab 3, this result cannot be clearly declared as SI-traceable result. Except absorption coefficient, the results have SI-traceability to the absorbance by UV filter CRM, length, and mass.

Participating Laboratory

Institute: Lab code 4_1

Reporting date: 2019-10-31

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
119.89	1.02	2	2.04

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB-B3-53	**HiCN**	119.76	119.96	119.79	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	After reconstitution, the weights of the aliquots were 12mg for analysis.
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	100 µl of blood is added to 25 mL reagent, mixed and left for 5 minutes in darkness. Absorbance is read at 540 nm against a reagent blank. The absorbance of HiCN standard is measured in the same way. The reaction reagent including: 200 mg Potassium ferricyanide (K3Fe(CN)6) + 50 mg Potassium cyanide (KCN) + 140 mg Dihydrogen potassium phosphate (KH2 PO4) + 1 mL Non-ionic detergent (e.g. Triton X-100), and above diluted to 1000 mL in distilled water.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	Absorbance is read at 540 nm against a reagent blank. The absorbance of HiCN standard is measured in the same way. Worked on the PerkinElmer Lambda 950 spectrometer.
Calibration method/design used	
Separation Conditions (column, eluents, gradient and flow rate)	None.
Equation used to calculate the mass fraction of total Hb in blood	1. The mass fraction of signature peptide in peptide working solution was first calculated by the following formula: $$w2 = \frac{\mathrm{A} \times 16114.5 \times \mathrm{F}}{11.0 \times d \times 1000 \times \rho} \times f_{blood}$$ **Where:** $w2$: The mass fraction of Hb in original reference standard solution. A: Absorbance at 540 nm. 16114.5: The molecular weight of monomer average of Hb. F: Dilution multiple of Hb. 11.0: extinction coefficient. d: optical paths, 1.000 cm. 1000: Convert 1000 milligram to gram. ρ: Density of the blood. f_{blood}: The correction factor
Air buoyancy corrected (ambient conditions, density after reconstitution)	After the sample was reconstituted, ambient condition, apparent mass of the tube and the density of blood were measured immediately. Apparent mass $m_3 = 1.68910$g Relative humidity of the air $\varphi_3 = 45.8\%$ Air pressure $p_3 = 1018$ Air temperature $\vartheta_3 = 26.3$℃ Density $\rho_{blood,3} = 1.043$ Correction factor $f_{blood} = 0.9595$

Uncertainty budget (please list all components

and their contribution to the combined standard uncertainty)

The following uncertainty sources were taken into consideration:

$$C = \frac{\mathrm{A} \times 16114.5 \times \mathrm{F}}{11.0 \times d \times 1000}$$

$$U_{A1} = \frac{\mathrm{U_{spectrometer}} \times \ \bar{\mathrm{A}}}{\sqrt{3}} = \frac{0.003 \times \ \bar{0}.3544}{\sqrt{3}} = 6.10 \times 10^{-4}$$

$$U_{A2} = \frac{\mathrm{SD}_{A540}}{\sqrt{6}} = \frac{4.15 \times 10^{-4}}{\sqrt{6}} = 1.69 \times 10^{-4}$$

$$U_A = \sqrt{UA1^2 + UA2^2} = 6.37 \times 10^{-4}$$

$$F = \frac{m_{reagent} + m_{blood}}{m_{blood}}$$

$$U_{m\mathrm{reagent}} = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$

$$U_{mblood} = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$

$$U_F = \sqrt{\left(\frac{1}{m_{blood_{mean}}} \times Um_{reagent}\right)^2 + \left(\frac{m_{reagent_{mean}}}{mblood_{mean}^{\ 2}} \times Um_{blood}\right)^2} = 1.4 \times 10^{-2}$$

$$U_d = \frac{U_{cuvette}}{2} = 1.00 \times 10^{-4}$$

$$U_C = C_{mean} \times \sqrt{\left(\frac{\mathrm{U}_A}{A_{mean}}\right)^2 + \left(\frac{\mathrm{U}_F}{F_{mean}}\right)^2 + \left(\frac{\mathrm{U}_d}{d}\right)^2} = 0.22\ g/L$$

$$w_3 = \frac{\mathrm{C}}{\rho_{blood}};\ \ \rho_{blood} = \frac{m_{blood}}{\mathrm{v}};\ \ \mathrm{v} = \frac{m_{H2O}}{\rho_{H2O}};$$

$$U_v = \sqrt{\left(\frac{1}{\rho_{H2O}} \times U_{m_{H2O}}\right)^2 + \left(\frac{\mathrm{SD}_{m_{H2O}}}{\sqrt{6}}\right)^2} = 4.10 \times 10^{-4}$$

	$$U_\rho = \sqrt{\left(\frac{1}{v} \times U_{m_{blood}}\right)^2 + \left(\frac{m_{blood}}{v^2} \times U_v\right)^2} = 9.09 \times 10^{-3}$$ $$U_{w_3} = \sqrt{\left(\frac{1}{\rho} \times U_{C_{mean}}\right)^2 + \left(\frac{C_{mean}}{\rho^2} \times U_\rho\right)^2} = 1.07$$ All the above uncertainty components were combined to calculate standard uncertainty: $$U_{w_2} = f_{blood} \times U_{w_3} = 1.02$$ The expended factor k was 2 corresponding to 95% confidence level and the expanded uncertainty can be calculated by: $$U = k \times u = 1.02 \times 2 = 2.04\ g/kg$$
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 4_2

Reporting date: 2019-10-30

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
120.69	1.75	2	3.50

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB-B3-16	LC-MS/MS	120.65	121.09	120.34	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	After reconstitution, three aliquots were taken from the sample tube. The weights of the aliquots were 93.51 mg, 93.09 mg and 88.94 mg.
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	**1. Sample preparation** After reconstitution, aliquots were taken from the sample tube. The weight of each aliquot was about 90 mg. The aliquots were diluted with solvent by a mass ratio of about 120. The solvent used for the dilution was acetonitrile (ACN)/water (3/7, v/v) with 0.1% formic acid (FA) (v/v). After dilution, the mass concentration of Hb in the samples was about 1 mg/g. The obtained solutions were used for subsequent tryptic hydrolysis. **2. Tryptic hydrolysis**

	Specific peptides: T4 and T5 from the α-chain of Hb was chosen as the specific peptides. The amino acid sequences of T4 and T5 were VGAHAGEYGAEALER and MFLSFPTTK, respectively. Isotopically labeled T4* (VGAHAGEYGAEAL*ER) and T5* (MFL*SFPTTK) were also synthesized and used as the internal standanrds. Stock solutions of T4, T4*, T5 and T5* were prepared in ultrapure water. **Buffer solution:** 350 mg Tris, 460 mg Tris•HCl, and 130 mg $CaCl_2$•$6H_2O$ were dissolved in 10 mL ultrapure water. The obtained solution was used as the buffer for the tryptic hydrolysis experiments. **Tryptic hydrolysis:** Each time, 100 µL diluted sample solution was used for the tryptic hydrolysis. 100 µL T4* and 100 µL T5* solutions were added to the sample. The mole ratio of T4*/T5* to Hb in the sample was 2:1. Then, 100 µL buffer solution was added to the sample. After a slight shaking of the tube, 20 µL trypsin solution was added to the sample. The obtained solution was incubated at 37℃ for 3 h. **3. Desalting** After hydrolysis, the samples were desalted before mass spectrometry (MS) analysis. The desalting was performed using Sep-Pak® Vac 1cc C18 (200 mg, Waters, Ireland) cartridges. The elution buffers were (A) H_2O with 0.1% trifluoroacetate (TFA) and (B) ACN with 0.1% TFA. The activation of the column was achieved by washing the column with 100% B, 50% B and 0% B, sequentially. This process was repeated for three times. Afterwards, the sample was loaded onto the column. The column was washed with 0% B for two times. Then, the sample was eluted with 3 × 0.6 mL 80% B into 2 mL Eppendorf tube (FisherScientific). The obtained sample solution was dried at 40 °C (< 1 mbar) for 4 h. Finally, the sample was re-dissolved in 600 µL ACN/water (7:93, v/v, 0.8% FA) for subsequent MS analysis. **4. source of traceability** The value of Hb's concentration was traced to the reference material CRM-IRMM/IFCC-467. The mass fraction of Hb in the reference material was 119.7 mg/g, with an uncertainty of 3.7 mg/g (k = 2). The reference material was used to determine the concentration of T4 and T5 in their standard solutions via isotope dilution mass spectrometry (ID-MS). The standard solutions of T4 and T5 were then used to determine the concentration of Hb in the comparison sample via ID-MS.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important	**Mass spectrometer**: Triple quadrupole mass spectrometer (TSQ Altis, Thermo Scientific) was used for the measurement. **MS Parameters:** The mass spectrometer was run in a multiple reaction

instrument parameters) **and analytical technique used**	monitoring (MRM) mode with quadrupole mass filter Q1 and Q3 at unit resolution. The transitions monitored were as follows: m/z 510.69→488.25 and m/z 512.95→495.29 for T4 and T4*, corresponding to $[M+3H]^{3+}$→y4; m/z 536.11→793.49 and m/z 539.79→800.56 for T5 and T5*, corresponding to $[M+2H]^{2+}$→y7. Fragmentation was induced using nitrogen at a high-pressure setting and at collision energies of 16 eV for T4 and T4* and 20 eV for T5 and T5*. The ESI source was operated at a 3500 V sprayer voltage and a 325 °C dry gas temperature. All other parameters were optimized to obtain maximum signal intensities for T4,T4* and T5,T5*. Under these conditions, the retention time of T4,T4* was 6.02 min and was 11.29 min for T5,T5*.
Calibration method/design used	Single point calibration mode was used for both sample measurement.
Separation Conditions (column, eluents, gradient and flow rate)	**High performance liquid chromatography (HPLC):** UHPLC+ Vanquish (Thermo Scientific) **Column:** ZORBAX Eclipse Plus C18, 2.1×150 mm, 1.8 μm. **Eluents:** A: H_2O (0.8% FA), B: Acetonitrile (0.8%FA). **Flow rate:** 0.2 mL/min. **Oven temperature:** 40℃. **Gradient:** 0−14 min: A:B = 92:8→64:36; 14−17 min: A:B = 64:36→0:100; 17−24 min: A:B = 0:100→92:8.
Equation used to calculate the mass fraction of total Hb in blood	1. The mass fraction of signature peptide in peptide working solution was first calculated by the following formula: $$\omega_{pep} = \frac{\omega_{ref} m_1 m_3 A_2 m_6}{(m_1 + m_2) m_4 A_1 m_5} \cdot \frac{M_{r\,pep}}{M_{r\,Hb}}$$ **Where:** ω_{ref}: The mass fraction of Hb in original reference standard solution. m_1: Weight of Hb standard solution added to prepare Hb reference working solution. m_2: Weight of buffer added to prepare Hb reference working solution. m_3: Weight of Hb reference working solution added to prepare peptide caribrator solution. m_4: Weight of peptide* working solution added to prepare peptide caribrator solution. m_5: Weight of the peptide sample. m_6: Weight of peptide* working solution in the spiked sample.

	A_1: Peak area ratio of the peptide to its isotopic one in the calibrator solution. A_2: Peak area ratio of the peptide to its isotopic one in the sample. $M_{r\,pep}$: Molecular weight of peptide. $M_{r\,Hb}$: Molecular weight of Hb. 2. The Hb mass fraction in blood was then calculated by the following formula: $$\omega_2 = f_{blood} \cdot \frac{\omega_{pep} m_9 A_4 m_{12}(m_7 + m_8)}{m_{10} A_3 m_{11} m_7} \cdot \frac{M_{r\,Hb}}{M_{r\,pep}}$$ **Where:** f_{blood}: The correction factor. ω_{pep}: The mass fraction of peptide in peptide working solution. m_7: Weight of blood sample added to prepare sample working solution. m_8: Weight of buffer added to prepare sample working solution. m_9: Weight of peptide working solution added to prepare caribrator solution. m_{10}: Weight of peptide* working solution added to prepare caribrator solution. m_{11}: Weight of the blood sample working solution. m_{12}: Weight of peptide* working solution in the spiked sample. A_3: Peak area ratio of the peptide to its isotopic one in the calibrator solution. A_4: Peak area ratio of the peptide to its isotopic one in the sample. $M_{r\,pep}$: Molecular weight of peptide. $M_{r\,Hb}$: Molecular weight of Hb.
Air buoyancy corrected (ambient conditions, density after reconstitution)	After the sample was reconstituted, ambient condition, apparent mass of the tube and the density of blood were measured immediately. Apparent mass $m_3 = 1.67782$ Relative humidity of the air $\varphi_3 = 34.4$ Air pressure $p_3 = 1018$ Air temperature $\vartheta_3 = 20.1$ Density $\rho_{blood,3} = 1.053$ Correction factor $f_{blood} = 0.9439$.

Uncertainty budget (please list all components

and their contribution to the combined standard uncertainty)

1. Uncertainty budget for mass fraction of peptide in standard solution

The following uncertainty sources were taken into consideration:

(1) Uncertainty of reference Hb standard solution

According to the certificate Hb standard solution, its mean is 119.7 mg/g, uncertainty is 3.7 mg/g, and expanded factor is 2. Therefor its relative uncertainty is

$$u_1 = \frac{3.7}{119.7 \times 2} = 1.5455 \times 10^{-2}$$

(2) Balance

Weighing was performed on an ME235S balance. The imprecisions of the balance was 0.01 mg. Uncerainty components during weighing were calculated according to rectangle distribution. The uncertainty component is as follows:

$$u_2 = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$

(3) Method reproducibility: Type A uncertainty evalution method was used to calculate this uncertainty source. Relative standard deviations were calculated based on 12 times measurement.

$$u_A = RSD$$

Based on the preparation process for working solution and samples, and the result calculation formula of,

$$\omega_{pep} = \frac{\omega_{ref} m_1 m_3 A_2 m_6}{(m_1 + m_2) m_4 A_1 m_5} \cdot \frac{M_{r\,pep}}{M_{r\,Hb}}$$

Sensitivity factors were calculated for every uncertainty components:

$$c_i = \frac{\partial f}{\partial x_i}$$

All the above uncertainty components were combined to calculate standard uncertainty:

$$u_c = \sqrt{\sum_{i=1}^{n} (\frac{\partial f}{\partial x_i} \cdot u_i)^2}$$

Table 1. Uncertainty budget for mass fraction of signiature peptide T4 in working solution

Uncertainty source	Uncertainty component	Relative uncertainty	Type	Data source

ω_{ref}		1.5455×10^{-2}	B	Certificate of standard solution
m_1	5.77×10^{-6}	1.9245×10^{-4}	B	Balance manufacture
m_2	5.77×10^{-6}	2.8868×10^{-6}	B	Balance manufacture
m_3	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_4	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_5	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_6	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
Method reproducibility		6.8895×10^{-3}	A	
Combined relative uncertainty		1.6923%		

Table 2. Uncertainty budget for mass fraction of signiature peptide T5 in working solution

Uncertainty source	Uncertainty component	Relative uncertainty	Type	Data source
ω_{ref}		1.5455×10^{-2}	B	Certificate of standard solution
m_1	5.77×10^{-6}	1.9245×10^{-4}	B	Balance manufacture
m_2	5.77×10^{-6}	2.8868×10^{-6}	B	Balance manufacture

m_3	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_4	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_5	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_6	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
Method reproducibility		4.2839×10^{-3}	A	
Combined relative uncertainty		1.6040%		

2. Uncertainty budget for mass fraction of Hb in blood

The following uncertainty sources were taken into consideration:

(1) Uncertainty of peptide working solution

The uncertainty of peptide working solutions were calculated above.

$$u_{T4} = 1.6923 \times 10^{-2}$$

$$u_{T5} = 1.6040 \times 10^{-2}$$

(2) Balance

Weighing was performed on an ME235S balance. The imprecisions of the balance was 0.01 mg. Uncerainty components during weighing were calculated according to rectangle distribution. The uncertainty component is as follows:

$$u_3 = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$

(3) Method reproducibility: Type A uncertainty evalution method was used to calculate this uncertainty source. Relative standard deviations were calculated based on 14 times measurement.

$$u_A = RSD$$

Based on the preparation process for working solution and samples, and the result calculation formula of,

$$\omega_2 = f_{blood} \cdot \frac{\omega_{pep} m_9 A_4 m_{12}(m_7 + m_8)}{m_{10} A_3 m_{11} m_7} \cdot \frac{M_{r\,Hb}}{M_{r\,pep}}$$

where,

$$f_{blood} = \left(1 + \frac{\Delta m}{m_{blood,2}}\right) = \frac{m_{blood,3}}{m_{blood,2}}$$

Sensitivity factors were calculated for every uncertainty components:

$$c_i = \frac{\partial f}{\partial x_i}$$

All the above uncertainty components were combined to calculate standard uncertainty:

$$u_c = \sqrt{\sum_{i=1}^{n} (\frac{\partial f}{\partial x_i} \cdot u_i)^2}$$

The uncertainty of mass fraction of Hb in blood base on both signature peptide T4 and T5 was combinded according to the following formula:

$$u_{c(Hb)} = \frac{\sqrt{u_{(T4)}^2 + u_{T5}^2}}{2}$$

The expended factor k was 2 corresponding to 95% confidence level and the expanded uncertainty can be calculated by :

$$U = k \times u$$

Table 3. Uncertainty budget for mass fraction of Hb in blood based on T4

Uncertainty source	Uncertainty component	Relative uncertainty	Type	Data source
ω_{pep}		1.6923×10^{-2}	B	Certificate of standard solution
m_7	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_8	5.77×10^{-6}	5.7735×10^{-7}	B	Balance manufacture
m_9	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_{10}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_{11}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture

m_{12}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
$m_{blood,2}$	1×10^{-4}	1.8968×10^{-4}	B	P201 technical protocol
$m_{blood,3}$	1×10^{-4}	2.0094×10^{-4}	B	P201 technical protocol
Method reproducibility		1.2803×10^{-2}	A	
Combined relative uncertainty		2.1221%		

Table 4. Uncertainty budget for mass fraction of Hb in blood based on T5

Uncertainty source	Uncertainty component	Relative uncertainty	Type	Data source
ω_{pep}		1.6040×10^{-2}	B	Certificate of standard solution
m_7	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_8	5.77×10^{-6}	5.7735×10^{-7}	B	Balance manufacture
m_9	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_{10}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_{11}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
m_{12}	5.77×10^{-6}	5.7735×10^{-5}	B	Balance manufacture
$m_{blood,2}$	1×10^{-4}	1.8968×10^{-4}	B	P201 technical protocol

$m_{blood,3}$	1×10^{-4}	2.0094×10^{-4}	B	P201 technical protocol
Method reproducibility		1.1483×10^{-2}	A	
Combined relative uncertainty		1.9727%		

Table 5. Uncertainty budget for mass fraction of Hb in blood

Uncertainty source	Mass fraction of Hb (g/kg)	Relative uncertainty	Uncertainty (g/kg)
T_4	120.72	2.12%	2.56
T_5	120.67	1.97%	2.38
Measured value	120.69		
Combined uncertainty		1.45%	1.75
Combined relative uncertainty		2.90%	3.50

Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 4_3

Reporting date: 2019-10-31

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
121.17	1.91	2	3.82

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB-B3-48	LC-MS/MS	118.06	120.95	124.49	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	After reconstitution, the weights of the aliquot were 0.20 mg.
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	**Sample preparation** For in-solution tryptic digestion, the two protein samples [Hb +Label-Hb, and references protein + Label-Hb], 200 µg) were denatured with 8 M urea and 10 mM DTT, and incubated at 37℃ for 2 h; the samples were alkylated by adding 100 mM iodoacetamide and storing them in the dark for 1 h. Trypsin and 50 mM NH4HCO3 (pH 8.2) were added at an enzyme: protein ratio of 1:50. The samples were then incubated at 37 ℃ for overnight. The samples were desalted before mass spectrometry (MS) analysis by SPE C18 columns.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	**Mass spectrometer**: Triple quadrupole mass spectrometer (AB QTRAP 5500) was used for the measurement. **MS Parameters:** The mass spectrometer was run in a multiple reaction monitoring (MRM) mode with quadrupole mass filter Q1 and Q3 at unit resolution.
Calibration method/design used	Single point calibration mode was used for both sample measurement.
Separation Conditions (column, eluents, gradient and flow rate)	**High performance liquid chromatography (HPLC):** **Column:** Kinetex C18, 2.1×150 mm, 2.6 µm **Eluents:** A: 10% H_2O+ 90% ACN (0.1 % TFA), B: Acetonitrile (0.1% TFA). The samples were analyzed using a flow rate of 0.2 mL/min and the following gradient: 5% B from 0 to 1 min, 60% B at 12 min, 95% B at 14 min, and 5% B during the last 2 min from 14 to 16 min.
Equation used to calculate the mass fraction of total Hb in blood	1. The mass fraction of signature peptide in peptide working solution was first calculated by the following formula: $$C_{blood} = \frac{\frac{A_{blood}}{A_{label}} \times m_{ref.} \times m_{label'}}{\frac{A_{ref.}}{A_{label}} \times m_{label} \times V_{blood}} \times f_{blood}$$ **Where:** C_{blood}: The mass fraction of Hb in original reference standard solution. $\frac{A_{blood}}{A_{label}}$: Peak area ratio of the peptide to its isotopic one in the Hb. $\frac{A_{ref.}}{A_{label}}$: Peak area ratio of the peptide to its isotopic one in the calibrator solution. $m_{ref.}$: Weight of Hb standard solution added to prepare Hb reference working solution. $m_{label'}$: Weight of Label-Hb working solution in the spiked sample. m_{label}: Weight of Label-Hb working solution added to prepare calibrator solution. V_{blood}: Weight of the Hb sample. f_{blood}: The correction factor
Air buoyancy corrected	After the sample was reconstituted, ambient condition, apparent mass

(ambient conditions, density after reconstitution)	of the tube and the density of blood were measured immediately. Apparent mass $m_3 = 1.68444\ g$ Relative humidity of the air $\varphi_3 = 44.9\%$ Air pressure $p_3 = 1013.5$ Air temperature $\vartheta_3 = 20.2\ ℃$ Density $\rho_{blood,3} = 1.043$ Correction factor $f_{blood} = 0.9552$
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	The following uncertainty sources were taken into consideration: $$C_{blood} = \frac{\frac{A_{blood}}{A_{label}} \times m_{ref.} \times m_{label\prime}}{\frac{A_{ref.}}{A_{label}} \times m_{label}}$$ $$U_{\frac{A_{blood}}{A_{label}}} = \frac{ratio_{max} - ratio_{min}}{1.69} = 3.14$$ $$U_{\frac{A_{ref.}}{A_{label}}} = \frac{ratio_{max} - ratio_{min}}{1.69} = 1.28$$ $$U_{m_{label}} = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$ $$U_{m_{label\prime}} = \frac{0.00001}{\sqrt{3}} = 5.77 \times 10^{-6}$$ $$U_{m_{ref.}} = \sqrt{\left(m_{ref.} \times U_{m_{blood}}\right)^2 + \left(\frac{m_{blood}}{v^2} \times U_v\right)^2} = 0.69$$ $$U_{m_{blood.}} = \sqrt{\left(\frac{U_{\frac{A_{blood}}{A_{label}}}}{\frac{A_{blood}}{A_{label}}}\right)^2 + \left(\frac{U_{\frac{A_{ref.}}{A_{label}}}}{\frac{A_{ref.}}{A_{label}}}\right)^2 + \left(\frac{U_{m_{ref.}}}{m_{ref.}}\right)^2 + \left(\frac{U_{m_{label}}}{m_{label}}\right)^2 + \left(\frac{U_{m_{label\prime}}}{m_{label\prime}}\right)^2} = 3.41$$ $$U_{c_{blood}} = \sqrt{\left(\frac{1}{v_{blood}} \times U_{m_{blood}}\right)^2 + \left(\frac{m_{blood}}{v^2} \times U_v\right)^2} = 2.00$$

	All the above uncertainty components were combined to calculate standard uncertainty: $$U_{cblood} = f_{blood} \times U_{c_{blood}} = 1.91$$ The expended factor k was 2 corresponding to 95% confidence level and the expanded uncertainty can be calculated by: $$U = k \times u = 1.91 \times 2 = 3.82\ g/kg$$
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 4_4

Reporting date: 2019-09-30

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
115.5	1.0	2.0	2.1

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB-B3-84	HPLC-ID-ICP-MS	112.1	117.2	114.7	118.0

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	WB-B3-84

Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	➢ **Reconstitution** The reconstitution process was according to the protoc ol. 0.39236 g purified water was added to the tube. ➢ **Digestion** Approximate 20 µL sample was accurately weighted, and 1.0 g ^{54}Fe spike with the concentration of 7.8337 µ g g^{-1} was added. 2 mL HNO3 was used for digestion at 185℃ for 4 h. Four parallel samples were prepared. ➢ **Separation by HPLC** Take 50 µL the reconstitution sample solution and dilute to 2 mL. Take a certain amount of sample into HPLC for separation.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	**HPLC.** The HPLC system consisted of a LC-30AD pump, a SIL-20A automatic sampler, and a SPD-20A UV-detector (Shimadzu, Japan). **HR-ICP-MS:** Sector field ICP mass spectrometer (Elemen t 2) operated at medium resolution (m/Δm=4000). ^{54}Fe an d ^{56}Fe were measured.
Calibration method/design used	Isotope dilution was used for total Fe determination. Isoto pe ^{54}Fe CRMs (GBW04462) from NIM China was used for calibration.
Separation Conditions (column, eluents, gradient and flow rate)	**Column:** TSK-gel G3000SWxl column (7.8 mm x 300 mm) **Mobile phase:** 12.5 mmol/L Tris + 125 mmol/L NH_4Ac at pH = 7.8 **Gradient:** isocratic **Flow rate:** 0.35 mL/min
Equation used to calculate the mass fraction of total Hb in blood	The total Fe was measured by isotope dilution HR-ICP-M S after sample digestion. And the amount of Hb was calcul ated by the following equation. $C_{Hb}=(C_{Total\ Fe}-C_{Fe\ in\ Tf}) \times (m_{water}+m_{dry\ blood})/m_{blood} / M_{Fe}/4 \times M_{Hb}$
Air buoyancy corrected (ambient conditions, density after reconstitution)	Pressure: 1008.1 hPa; humidity: 51.3%; temperature: 25. 7 ℃

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	In the determination of total Fe by IDMS, the uncertainty b udget include concentration of ^{54}Fe spike, procedure blank subtraction, isotope ratio of blend b R56/54, isotope ratio i n the primary assay standard R56/54, isotope ratio in the spike R56/54, mass fraction ofsample in blend, mass fracti on of spike in blend, measured result of total Fe.
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 4_5

Reporting date: 2019-09-24.

Overall Results

Mass fraction total Hb in blood (mean of single results from replicate determinations in g/kg)	Combined Standard Uncertainty (g/kg)	Coverage Factor (*k*)	Expanded Uncertainty (g/kg)
115.3	1.2	2	2.4

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	Instrumentation (e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)	Mass fraction of total Hb in aliquot 1 (g/kg)	Mass fraction of total Hb in aliquot 2 (g/kg)	Mass fraction of total Hb in aliquot 3 (g/kg)	Mass fraction of total Hb in aliquot 4 (opt) (g/kg)
WB-B3-84	HPLC-ID-ICP-MS	116.2	114.1	115.7	

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	**WB-B3-84**
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	At first, sample preparation according to technical protocol. And then take 50 µL sample solution and dilute to 2 mL. Take a certain amount of sample into HPLC for separation and purification.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) **and analytical technique used**	**HPLC.** The HPLC system consisted of a LC-30AD pump, a SIL-20A automatic sampler, and a SPD-20A UV-detector (Shimadzu, Japan).

	HR-ICP-MS. The sector field ICP-MS was an Element 2 from Thermo Fisher Scientific (Bremen, Germany), which was operated at medium resolution (m/Δm = 4000) through all the experiments. In this study, we developed a method based on high performance liquid chromatography (HPLC) coupled to ICP-MS via post-column isotope dilution for the quantification of total haemoglobin in Blood using enriched ^{54}Fe isotopic solutions.
Calibration method/design used	Species-unspecific isotope dilution analysis
Separation Conditions (column, eluents, gradient and flow rate)	**Column:** TSK-gel G3000SWxl column (7.8 mm x 300 mm) **Mobile phase:** 12.5 mmol/L Tris + 125 mmol/L NH_4Ac at pH = 7.8 **Gradient:** isocratic **Flow rate:** 0.35 mL/min
Equation used to calculate the mass fraction of total Hb in blood	$$MF_s = c_{sp} d_{sp} f_{sp} \frac{M_s}{M_{sp}} \frac{A_{sp}^b}{A_s^b} \frac{R_m - R_{sp}}{R_s - R_m}$$ **MF_s:** the mass flow of iron in the sample eluting from the column. **$c_{sp}d_{sp}f_{sp}$:** the mass flow of the spike solution cali brated by reversed IDA using a standard with na tural isotopic abundance. **M_s, M_{sp}:** are the atomic weights of the ele-ment i ron in the spike and the sample, resp-ectively. **A_s, A_{sp}:** are the abundance of the isotope “b” (^{54} Fe) in the spike and the sample. **R_m, R_{sp}, and R_s**: are the isotope ratios (isotope a /isotope b, ^{56}Fe/^{54}Fe) in the mixture, spike, and t he sample, respectively.
Air buoyancy corrected (ambient conditions, density after reconstitution)	**air pressure**：1008.1 hPa; **air humidity**：55. 3%**; air temperature**：25.7°C **material density after reconstitution:** 1.02 g/c m^3

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	The uncertainty budget include the measurement of uncertainty, enriched ^{54}Fe isotopic solution, mass of sample in sample blend, mass of enriched spike in sample blend, mass of enriched spike in calibration blend, mass flow of the spike solution calibrated, mass of standard in calibration blend, Dilution factor of sample and standard, procedure blank subtraction, isotope ratio of blend b R56/54, isotope ratio in the primary assay standard R 56/54, isotope ratio in the spike R56/54.
Additional Comments or Observations	

Participating Laboratory

Institute: Lab code 5

Reporting date: 9/18/2019

Overall Results

Mass fraction **total Hb in blood (mean of single results from replicate determinations in g/kg)**	**Combined Standard Uncertainty (g/kg)**	**Coverage Factor (*k*)**	**Expanded Uncertainty (g/kg)**
127.8	**3.0**	**2.31**	**6.9**

[1]The molar mass of Hb is 64458 g/mol.

Single results

Sample number	**Instrumentation** **(e.g. LC-ICP-MS, LC-MS/MS, Raman, UV/Vis)**	**Mass fraction** **of total Hb in aliquot 1** **(g/kg)**	**Mass fraction** **of total Hb in aliquot 2** **(g/kg)**	**Mass fraction** **of total Hb in aliquot 3** **(g/kg)**	**Mass fraction** **of total Hb in aliquot 4 (opt)** **(g/kg)**
WB3 B3-43	**ICP-MS**	**136.9**	**118.2**	**129.1**	
WB3 B3-78	**ICP-MS**	**112.6**	**125.3**	**125.7**	
WB3 B3-91	**ICP-MS**	**131.4**	**130.4**	**141.1**	

<u>Analytical conditions</u>

Sample treatment

Sample amount used for analysis (mg)	50 mg.
Brief description of the sample treatment (e.g. sample preparation, dilution steps, purification and separation, derivatization, source of traceability etc.)	Approximately 0.05 g CCQM sample was weighed into a 2 mL polypropylene vial. The sample was quantitatively transferred into a microwave vessel and digested in a mixture of nitric acid and hydrogen peroxide at temperatures up to 205 °C. Iron in the digested sample was quantified by the method of standard addition using ICP-MS. The iron values are traceable to the calibration standard SRM 3126a Iron (Fe) Standard Solution.

Measurement method and evaluation

Instrumentation (e.g., type of liquid-chromatograph, mass spectrometer or spectrometer, manufacturer, important instrument parameters) and analytical technique used	Agilent 7500cs ICP-MS operated in collision/reaction mode using H_2 as the collision/reaction gas. The analyte Fe and the internal standard Y were measured at 56 u and 89 u.	
Calibration method/design used	Calibration by the method of standard addition.	
Separation Conditions **(column, eluents, gradient and flow rate)**	None.	
Equation used to calculate the mass fraction of total Hb in blood	$w_2 = \frac{usp}{sp-usp} * \frac{w_{sp}}{w_{sa}} * c * dil * \frac{M_{Hb}}{4*M_{Fe}} * f_{blood}$ where w_2 is the mass fraction of Hb in the pre-lyophilized sample; *sp* and *usp* are the internal-standard-corrected count rate of the spiked and the unspiked samples; w_{sa} and w_{sp} are the mass of the reconstituted sample and the mass of the spike solution; *c* and *dil* are the mass fraction of Fe in the spike solution and the dilution factor of the sample; M_{Hb}, and M_{Fe} are the molar mass of Hb and Fe, respectively; f_{blood} is the reconstitution correction factor.	
Air buoyancy corrected (ambient conditions, density after reconstitution)	Yes (998 hPa, 0.543 RH, 21.0 °C, ρ=1100 kg/m^3)	
Uncertainty budget (please list all components **and their contribution to the combined standard uncertainty)**	All values are in g/kg units	
	$_{eps}$ *(Sample replication)*	2.9
	$_{epb}$ *(Blank replication)*	0.62
	(Calibrant)	0.15
	(Weighing)	0.054
	(Reconstitution)	0.051
Additional Comments or Observations	Gross mass (g) of as-received CCQM P-201 sample when the package was opened the first time	
	WB-B2-43 1.28602	WB-B3-78 1.28897	WB-B3-91 1.30083