Physikalisch-Technische Bundesanstalt

Braunschweig und Berlin

TC-MC 1351

HLT05 "Transferrin in Human Serum"

Final Report

1 Introduction

Metalloproteins are particularly important in medical diagnosis as they represent around 30 % of the whole proteome. Transferrin (Tf) is an acute-phase protein and the iron transporter in the human body. Its blood concentration indicates inflammation and the composition of the sugar residues are used to prove alcohol abuse.

A reference method or matrix matched reference material is not available for Tf, even though the directive 98/79/EC on *in vitro* diagnostic medical devices clearly states that "the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order". Furthermore, the standard EN ISO 17511:2003 also demands reference measurement systems including reference measurement procedures. Up to now, the applied methods in intercomparisons for clinical laboratories are turbidimetry and nephelometry with only

method specific consensus values (determined as the median for the various methods) and method specific limits instead of a SI traceable reference value.
This TC-MC 1351 comparison is the first step to establish traceability of Tf quantification, which might lead to a CCQM comparison for Tf in human serum in the future.

2 Participants

Three National Metrology Institutes (NMIs) and one industrial laboratory registered to participate in the TC-MC 1351 comparison. For more details refer to table 1.

Table 1: Participants of TC-MC 1351 in alphabetical order of their acronyms.

Institute	Country	Contact
IL- Industry Laboratory	-	not to be disclosed
LNE - Laboratoire national de métrologie et d'essais	France	M. Estela Del Castillo Busto
PTB – Physikalisch-Technische Bundesanstalt	Germany	Claudia Swart Christine Brauckmann
TÜBITAK UME - TÜBITAK National Metrology Institute	Turkey	Gonca Coskun Alper Isleyen

3 Samples

3.1 Sample preparation

The basic material for the samples was the reference material BCR®-637 charge numbers: 797, 810, and 811 from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). These three aliquots were homogenised and then divided into 13 aliquots of about 1 g in 2 mL Protein LoBind tubes from Eppendorf. Two samples, each containing approximately 1 g pooled BCR®-637 human serum, were provided by PTB to each participant. All aliquots had the same Tf mass fraction of about 2 mg/g and the same serum matrix. The BCR®-637 serum was sterile filtered prior to filling and no preservatives were added by the manufacture [1]. BCR®-637 serum material was produced from blood from healthy blood donors. Each portion of BCR®-637 serum was tested negative for Anti-HIV-1&2, Anti-HCV and Anti-HTLV-I&II. The samples were shipped cooled with dry ice and all

participants stored the serum at -70 °C or below. Before opening the serum, it was left to warm to room temperature under continuously gently shaking. All participants made sure that the sample was well homogenised before use. The minimum amount of sample to be used was 50 µL.

3.2 Stability and Homogeneity

In accordance with ISO Guide 35 the samples were checked for homogeneity and stability issues [2]. The homogeneity of Tf in the BCR®-637 serum was tested in four different aliquots (HLT05-TRF-S2, HLT05-TRF-S6, HLT05-TRF-S9 and HLT05-TRF-S12). For the analysis three samples were prepared from each aliquot and measured three times, respectively. 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):

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

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

$$u_{\text{bb}} = s_{\text{bb}} = \frac{s_{\text{among}}^2 - s_{\text{within}}^2}{n_0} \quad (3)$$

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

The stability of Tf in BCR®-637 serum was monitored from September 29th 2014 to January 13th 2015. From n = 32 samples the stability related uncertainty u_{lts} was calculated with a linear approach to describe changes of the Tf mass fraction over the time period t_Δ = 106 d.

$$w = a_0 + a_1 t \quad (5)$$

The measurements were repeated in 35 d - 36 d intervals. For the calculation a linear ordinary least square (OLS) regression model was applied and evaluated by means of Excel [3]. The stability related uncertainty u_{lts} was estimated using the following equations:

$$u_{lts} = t_\Delta \cdot s(a_1) \quad (6)$$

$$u(k_{\text{homstab}}) = \sqrt{u_{\text{bb}}^2 + u_{\text{lts}}^2} \quad (7)$$

Table 2 summarises the uncertainty contributions due to homogeneity and stability.

Table 2: Uncertainty contributions due to homogeneity u_{bb} and stability u_{lts} along with their combined contribution $u(k_{homstab})$. The relative uncertainty contributions were calculated with the arithmetic mean of the stability /homogeneity measurements.

	in µg/g	in %
u_{bb}	0.03	1.5
u_{lts}	0.14	7.0
$u(k_{homstab})$	0.15	7.1

The uncertainty of the homogeneity reflects the limits of the HPLC-ICP-MS analysis rather than an inhomogeneity of the material. The uncertainty of the stability u_{lts} amounts to 7.0 % which is caused by high variations between the measurements, even though no degradation of the protein can be observed as shown in figure 1.

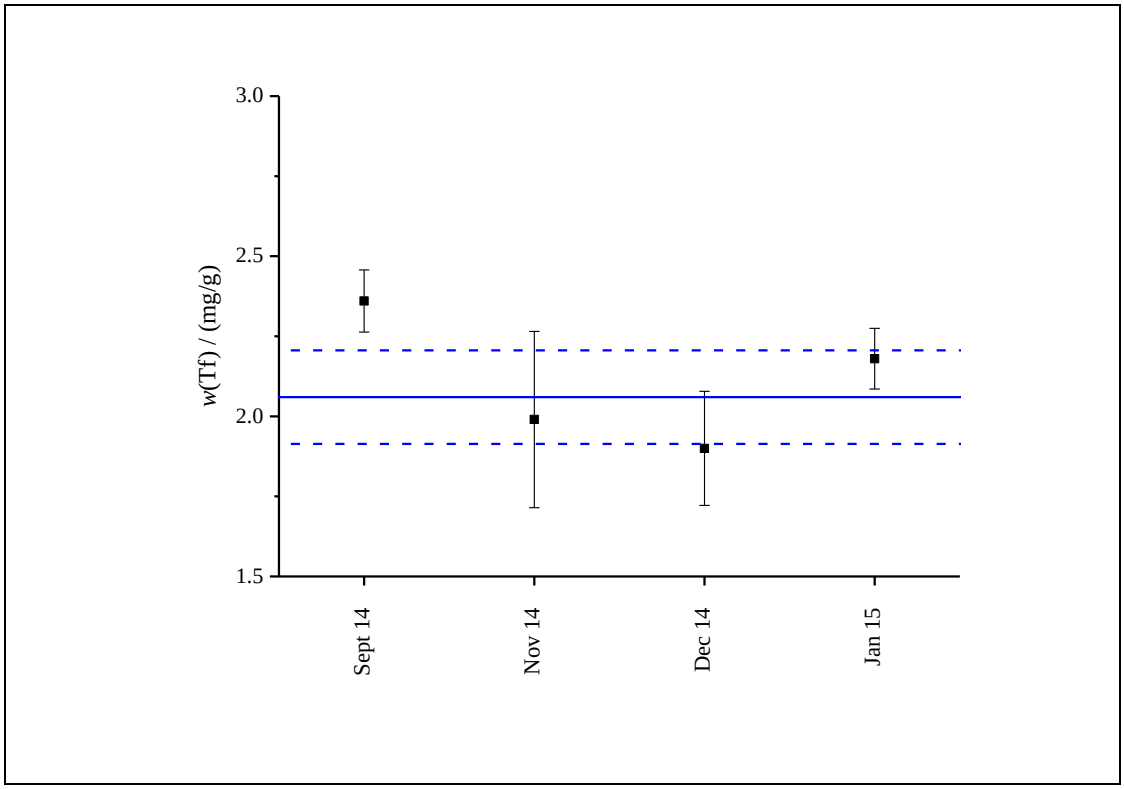

Figure 1: Tf mass fraction *w*(Tf) as measured in the stability tests. Error bars denote the expanded uncertainty *U*(*w*(Tf)) for a coverage factor of *k* = 2. The blue line shows the arithmetic mean of all values. The dashed blue lines indicate the range of the combined uncertainty $u(k_{homstab})$.

Furthermore, the student *t*-factor is below the absolute value of the slope a_1:

$$|a_1| < t(p = 0.95, n-2) \cdot s(a_1)$$

This result indicates that the changes of stability are lower than the uncertainty of the measurement.

4 Instructions to the participants

Together with the samples, a technical protocol was sent to all participants of the interlaboratory comparison, providing information about the properties of the samples, the sample handling and the recommended procedure to check for losses and correct for evaporation effects during storage. The sample bottles were accompanied by an individual table (Excel-file) compiling the masses of the empty bottles and of the respective solutions needed to carry out the loss checking/evaporation correction procedure.
The appendix shows the technical protocol of TC-MC1351.

5 Reference materials, methods, and instrumentation

The participants were asked to apply their most accurate methods of measurement, preferably primary methods. The relative expanded measurement uncertainty U_{rel} associated with the result should not exceed 10 %. In table 3 the instrumentation, methods, and analytes are listed which were applied by the participants.

Table 3: Instrumentation/methods and calibration strategies used by the participants as reported.

Institute	Instrumentation	Method	Heteroatom
IL	HPLC/ICP-SF-MS	standard addition	S
LNE	HPLC/ICP-SF-MS	double SS-IDMS	Fe
PTB	HPLC/ICP-MS	triple SS-IDMS	Fe
TÜBITAK	HPLC/ICP-QQQ-MS	double SS-IDMS	Fe

6 Results

6.1 Tf mass fractions

The selected sample material for the interlaboratory comparison was the certified reference material BCR®-637, which is only certified for Al, Se and Zn, but not for Tf or Fe [1]. Therefore, no reference values for the applied serum were available. Even though, this material was selected because it shows a high homogeneity and stability (section 3.2), but has the same characteristic as a real human serum sample. These conditions cannot be observed for Tf certified reference materials such as the ERM-470kDa/IFCC reference material. Within this interlaboratory comparison the ability to quantify real samples was requested. Following quantities had to be determined:

- Mass fraction w(Tf) of Tf in human serum (w_i).

The results of the comparison are listed in table 4 and the graphical evaluation is shown in figure 2.

Table 4: Tf mass fraction w(Tf) of all participants as reported. The uncertainty are given using a coverage factor of k = 1.

Participant	(w (Tf) ± u(w(Tf))/ mg/g	u_{rel}
PTB 1	2.14 ± 0.05	2.5 %
PTB 2	2.23 ± 0.05	2.3 %
LNE 1	2.26 ± 0.05	2.2 %
LNE 2	2.27 ± 0.05	2.2 %
TÜBITAK UME 1	2.43 ± 0.06	2.5 %
TÜBITAK UME 2	2.49 ± 0.08	3.2 %
IL 1	3.91 ± 0.17	4.4 %
IL 2	3.93 ± 0.18	4.7 %

Since no reference value was available for the BCR®-637 serum, consensus values were evaluated based on the participants' data. Therefore, the arithmetic mean $\overline{w}_A$ (eq. (8)), the median $\overline{w}_M$ (eq. (10) +(11)) and the uncertainty weighted mean $\overline{w}_U$ (eq. (13)) were calculated along with their associated uncertainties $u(\overline{w}_A)$ (eq. (9)), $u(\overline{w}_M)$ (eq. (12)) and $u(\overline{w}_U)$ (eq. (15)) for all participants and for the NMI results.

$$\overline{w}_A = \frac{1}{N}\sum_{i=1}^{N} w_i \tag{8}$$

$$u(\overline{w}_A) = \sqrt{\frac{1}{N\cdot(N\text{-}1)}\sum_{i=1}^{N}(w_i - \overline{w}_A)^2} \tag{9}$$

Please note that when carrying out eq. (10) and (11), respectively, the participants' results w_i have to be arranged in the order of increasing values, while when carrying out equation (19) the absolute deviations of the participants' results from the median $(|w_i - \overline{w}_M|)$ have to be arranged in the order of increasing values.

$$\overline{w}_M = \frac{1}{2}(w_{N/2} + w_{N/2+1}) \quad N \text{ even} \tag{10}$$

$$\overline{w}_M = w_{(N+1)/2} \quad N \text{ odd} \tag{11}$$

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

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

As proposed in [4] the data set was checked for consistency by means of the chi-squared test. χ^2_{obs} was calculated according to eq. (14).

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

In table 5 are the results listed for all participants and for the NMIs. In both cases the chi-squared test was not passed. For comparison a chi-squared distribution with a 95 percentile and (N-1) = degrees of freedom $\chi^2_{0.05,N-1}$ was used [5].

Table 5: 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	8	190	14	no
NMIs	6	27	11	no

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

$$u(\overline{w}_{\mathrm{U}}) = \sqrt{\frac{\chi^2_{\mathrm{obs}}}{N\text{-}1} \left(\sum_{i=1}^{N} \frac{1}{u^2(w_i)} \right)^{-1}} \tag{15}$$

In figure 2 to 7 all calculated consensus values with their associated uncertainties and the results of TC-MS 1351 are illustrated. The consensus values were calculated from all participants' and also separately from the NMIs' results, respectively.

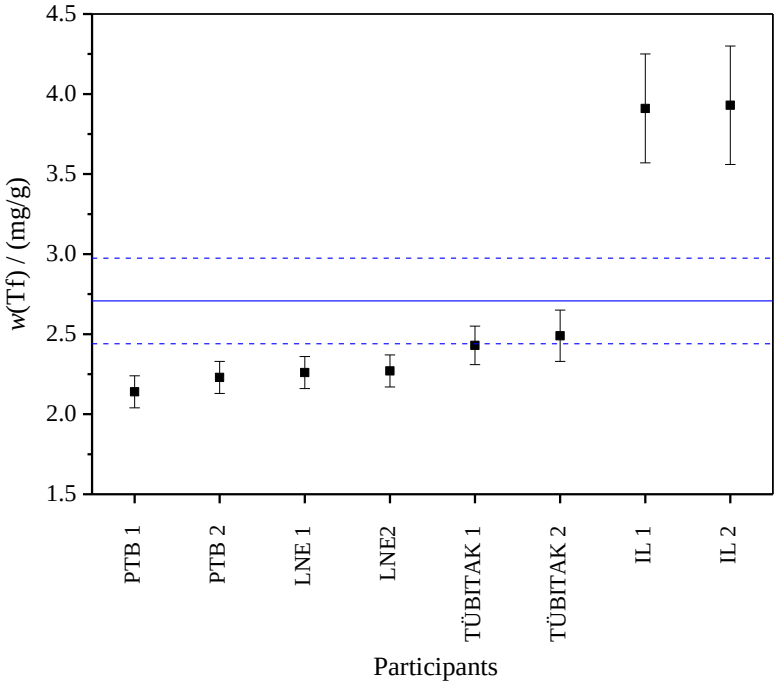

Figure 2: Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The blue line shows the arithmetic mean of all participants: $\overline{w}_{\mathrm{A}}$ = 2.71 mg/g. The dashed blue lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{A}})$ associated with the arithmetic mean.

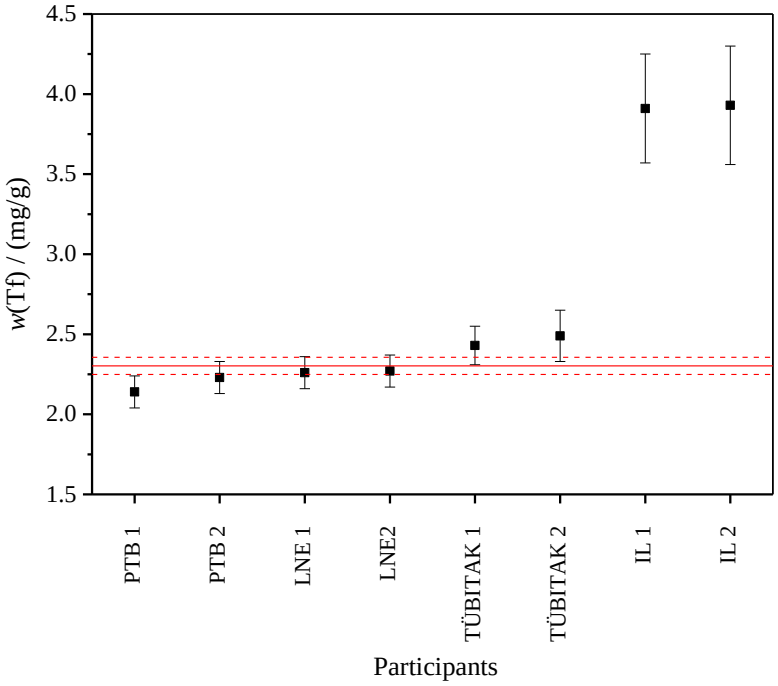

Figure 3: Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The red line shows the arithmetic mean of the NMIs: $\overline{w}_{\mathrm{A\,NMI}}$ = 2.30 mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{A\,NMI}})$ associated with the arithmetic mean.

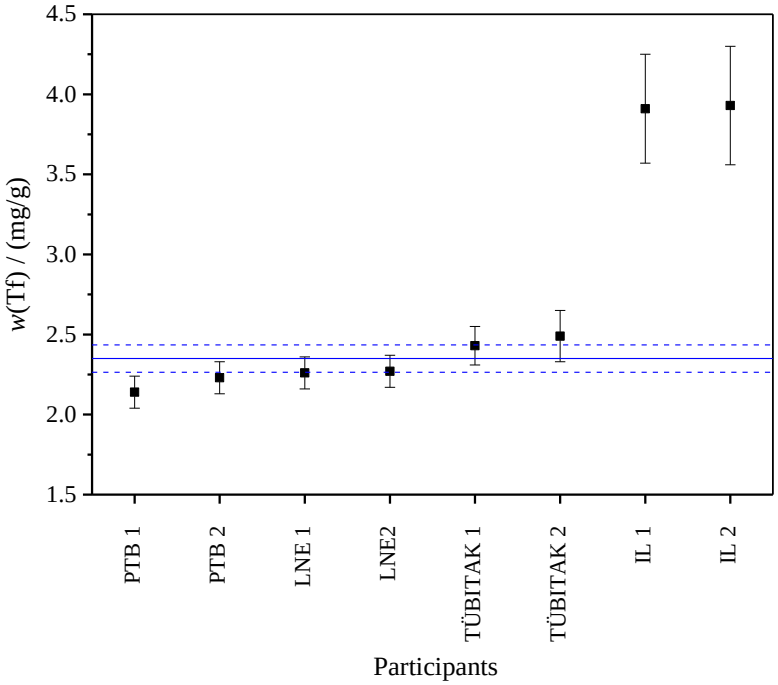

Figure 4: Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The blue line shows the median of all participants: $\overline{w}_{\mathrm{M}}$ = 2.35 mg/g. The dashed blue lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{M}})$ associated with the median.

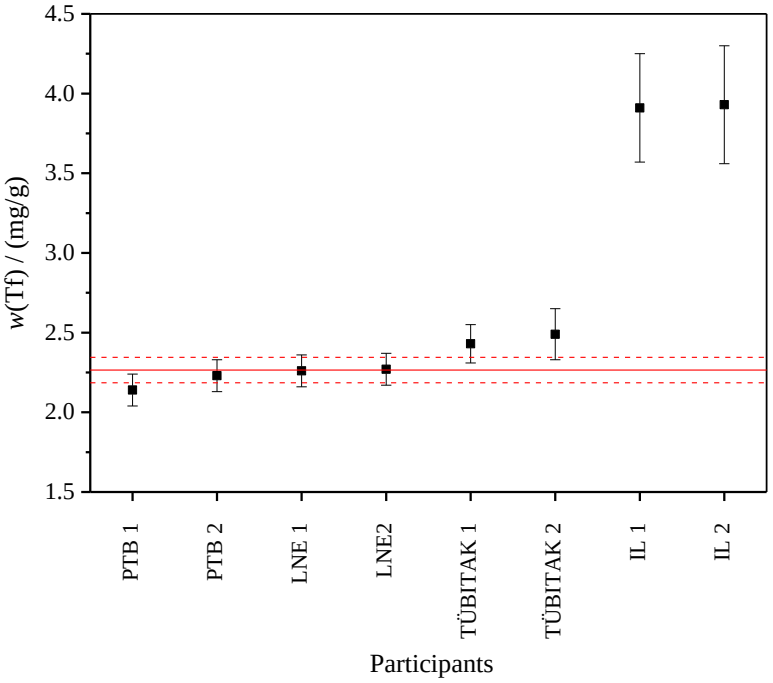

Figure 5 Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The red line shows the median of the NMIs: $\overline{w}_{\mathrm{M\,NMI}}$ = 2.27 mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{M\,NMI}})$ associated with the median.

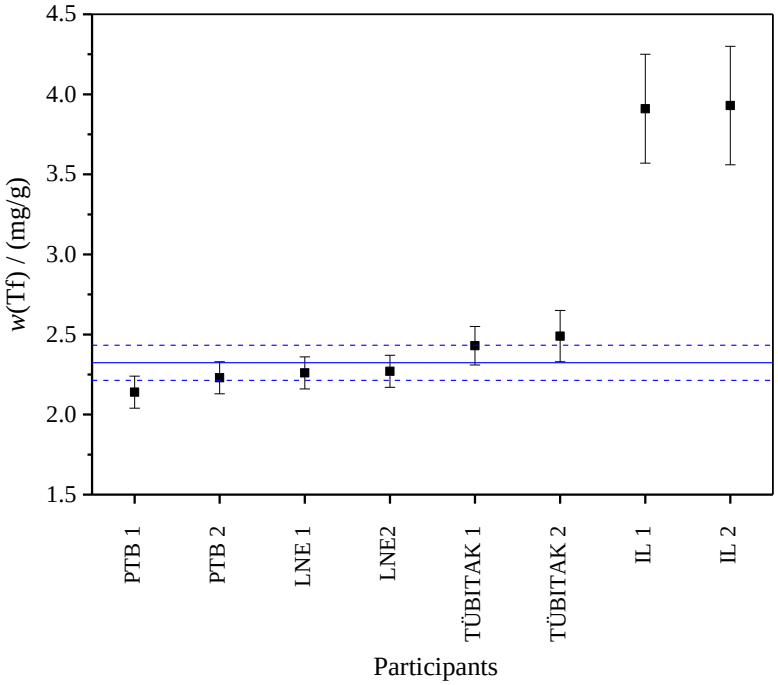

Figure 6 Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The blue line shows the uncertainty weighted mean of all participants: $\overline{w}_{\mathrm{U}}$ = 2.32 mg/g. The dashed blue lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{U}})$ associated with the uncertainty weighted mean.

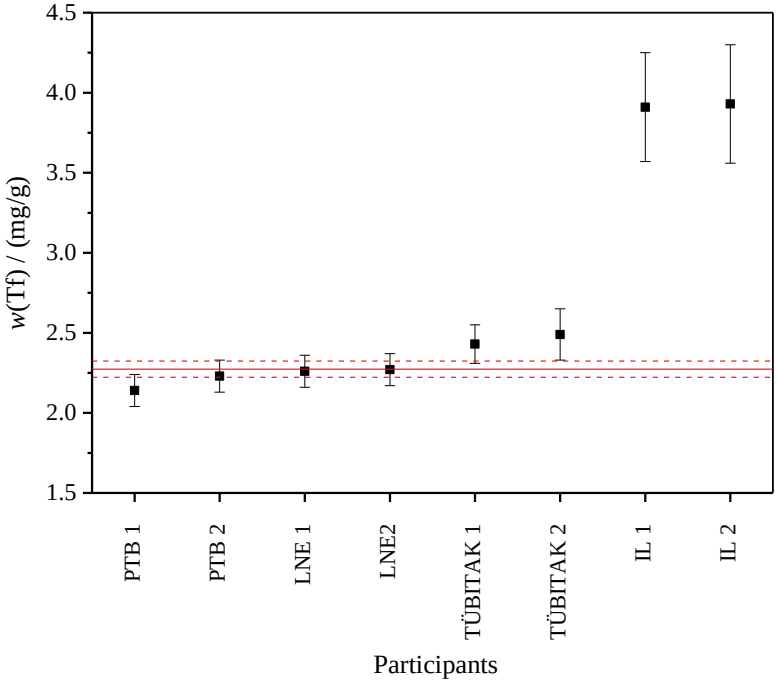

Figure 7: Tf mass fraction *w*(Tf) as reported by the participants. Error bars denote the uncertainty *u*(*w*(Tf)) for a coverage factor of *k* = 1 as reported. The red line shows the uncertainty weighted mean of the NMIs: $\overline{w}_{\mathrm{U\,NMI}}$ = 2.27 mg/g. The dashed red lines indicate the range of the combined uncertainty $u(\overline{w}_{\mathrm{U\,NMI}})$ associated with the uncertainty weighted mean.

6.2 Degrees of equivalence

The degree of equivalence (DoE) (d_i) of a result w_i equals its deviation from the consensus value, which are in our case six different possible values. For all these values the d_i with the corresponding uncertainties $u(d_i)$ as well as the normalized error (E_n) were calculated using eq. (16) – (18) [3, 6].

$$d_i = w_i - w_{ref} \tag{16}$$

$$u(d_i) = \sqrt{u^2(w_i) + u^2(w_{ref})} \tag{17}$$

$$E_n = \frac{d_i}{U(d_i)} \tag{18}$$

The results are summarised in table 6 – 11 and plotted in figure 8 – 13. When E_n is between 0 and 1, the results and consensus value are in agreement within their estimated uncertainties.

Table 6: Mass fractions *w*(Tf) and their associated combined and relative expanded uncertainties *u*(*w*(Tf)) and u_{rel}, resp., using a coverage factor *k* = 1 as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of *k* = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the arithmetic mean of all participants $\overline{w}_A$ was applied.

Consensus value: $\overline{w}_A$ = (2.71 ± 0.27) mg/g					
Participant	(*w* (Tf) ± *u*(*w*(Tf))/ mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.5675	0.5442	-1.0428
PTB 2	2.23 ± 0.05	2.3 %	-0.4775	0.5442	-0.8774
LNE 1	2.26 ± 0.05	2.2 %	-0.4475	0.5442	-0.8223
LNE 2	2.27 ± 0.05	2.2 %	-0.4375	0.5442	-0.8039
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	-0.2775	0.5483	-0.5062
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	-0.2175	0.5584	-0.3895
IL 1	3.91 ± 0.17	4.4 %	1.2025	0.6339	1.8971
IL 2	3.93 ± 0.18	4.7 %	1.2225	0.6504	1.8795

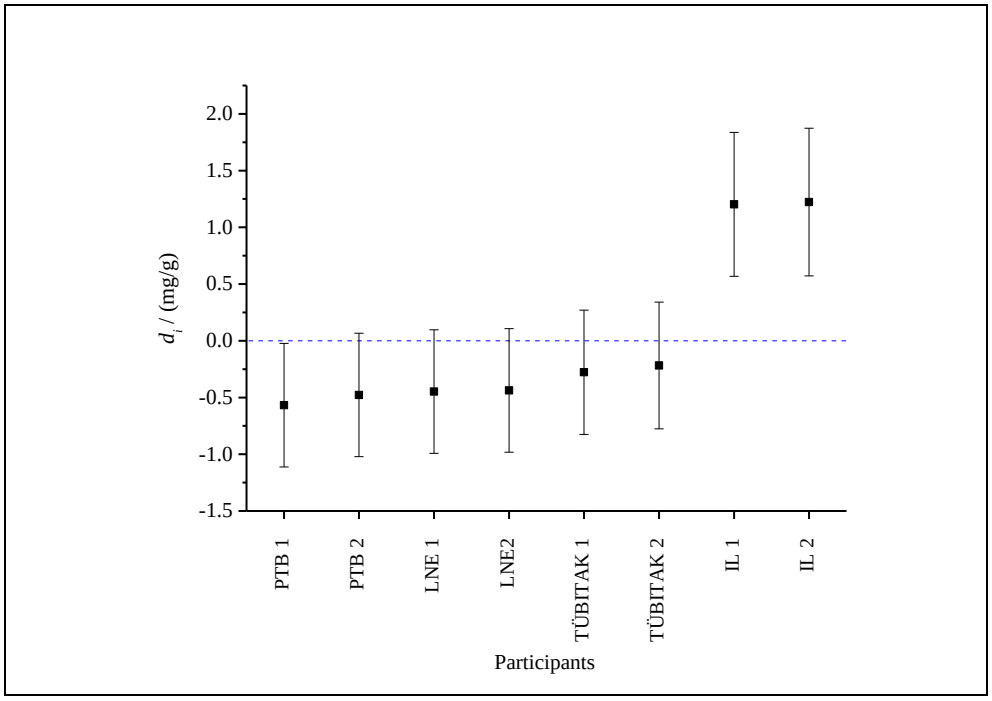

Figure 8: Graphical representation of the equivalence statements related to the arithmetic mean of all participants $\overline{w}_A$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_A$.

Table 7: Mass fractions w(Tf) and their associated combined and relative uncertainties $u(w(\mathrm{Tf}))$ and u_{rel}, resp., using a coverage factor $k = 1$ as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of $k = 2$ was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the arithmetic mean of the NMIs $\overline{w}_{ANMI}$ was applied.

Consensus value: $\overline{w}_{ANMI} = (2.30 \pm 0.05)$ mg/g

Participant	(w (Tf) ± $u(w(\mathrm{Tf}))$/ mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.1633	0.1465	-1.1150
PTB 2	2.23 ± 0.05	2.3 %	-0.0733	0.1465	-0.5006
LNE 1	2.26 ± 0.05	2.2 %	-0.0433	0.1465	-0.2958
LNE 2	2.27 ± 0.05	2.2 %	-0.0333	0.1465	-0.2276
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	0.1267	0.1608	0.7877
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	0.1867	0.1925	0.9697
IL 1	3.91 ± 0.17	4.4 %	1.6067	0.3565	4.5074
IL 2	3.93 ± 0.18	4.7 %	1.6267	0.3852	4.2232

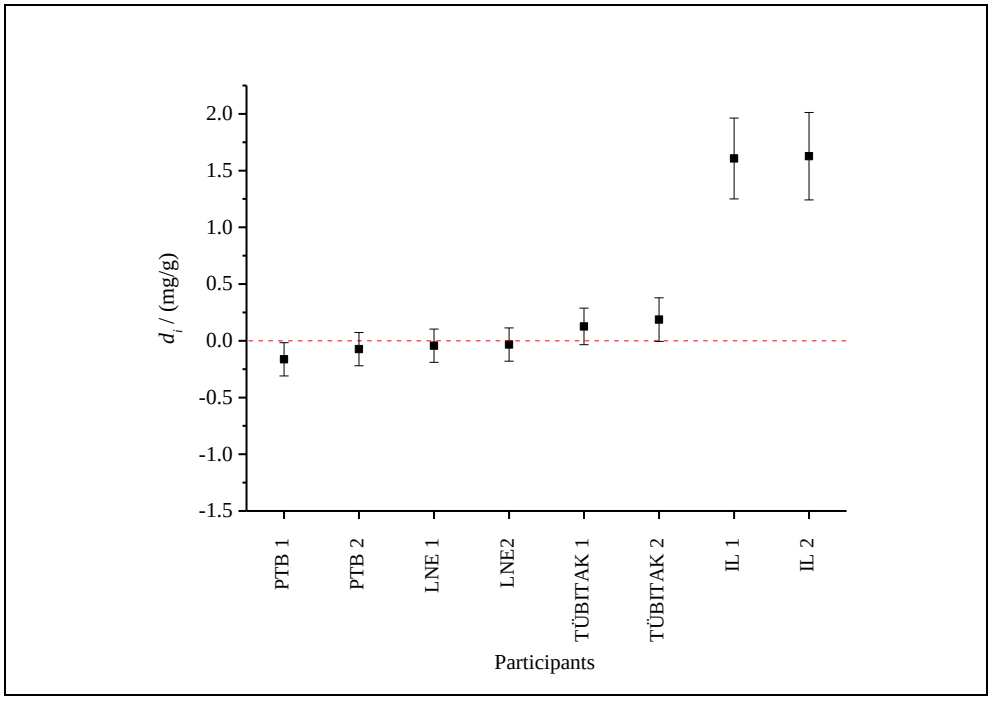

Figure 9: Graphical representation of the equivalence statements related to the arithmetic mean of the NMIs $\overline{w}_{ANMI}$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_{ANMI}$.

Table 8: Mass fractions *w*(Tf) and their associated combined and relative expanded uncertainties *u*(*w*(Tf)) and u_{rel}, resp., using a coverage factor *k* = 1 as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of *k* = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the median of all participants $\overline{w}_M$ was applied.

Consensus value: $\overline{w}_M$ = (2.35 ± 0.09) mg/g

Participant	(*w* (Tf) ± *u*(*w*(Tf)) / mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.2100	0.1980	-1.0608
PTB 2	2.23 ± 0.05	2.3 %	-0.1200	0.1980	-0.6062
LNE 1	2.26 ± 0.05	2.2 %	-0.0900	0.1980	-0.4546
LNE 2	2.27 ± 0.05	2.2 %	-0.0800	0.1980	-0.4041
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	0.0800	0.2088	0.3832
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	0.1400	0.2341	0.5981
IL 1	3.91 ± 0.17	4.4 %	1.5600	0.3805	4.0997
IL 2	3.93 ± 0.18	4.7 %	1.5800	0.4075	3.8769

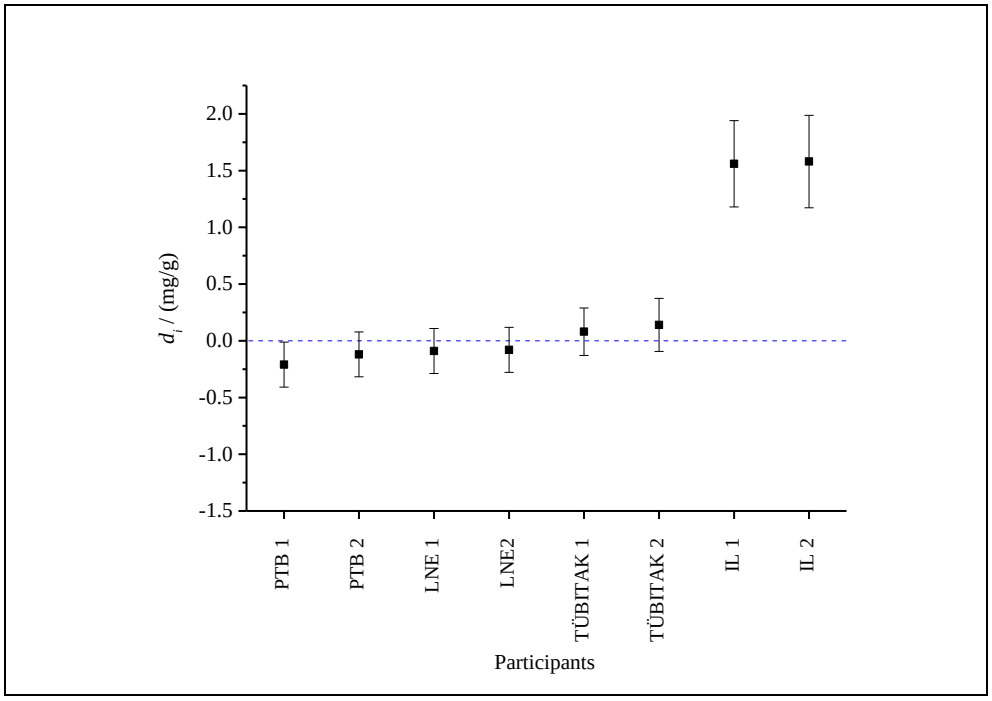

Figure 10: Graphical representation of the equivalence statements related to the median of all participants $\overline{w}_M$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_M$.

Table 9: Mass fractions *w*(Tf) and their associated combined and relative expanded uncertainties *u*(*w*(Tf)) and u_{rel}, resp., using a coverage factor of *k* = 1 as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of *k* = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the median of the NMIs $\overline{w}_{MNMI}$ was applied.

Consensus value: $\overline{w}_{MNMI}$ = (2.27 ± 0.08) mg/g

Participant	(*w* (Tf) ± *u*(*w*(Tf))/ mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.1250	0.1879	-0.6654
PTB 2	2.23 ± 0.05	2.3 %	-0.0350	0.1879	-0.1863
LNE 1	2.26 ± 0.05	2.2 %	-0.0050	0.1879	-0.0266
LNE 2	2.27 ± 0.05	2.2 %	0.0050	0.1879	0.0266
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	0.1650	0.1992	0.8282
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	0.2250	0.2256	0.9974
IL 1	3.91 ± 0.17	4.4 %	1.6450	0.3754	4.3826
IL 2	3.93 ± 0.18	4.7 %	1.6650	0.4027	4.1343

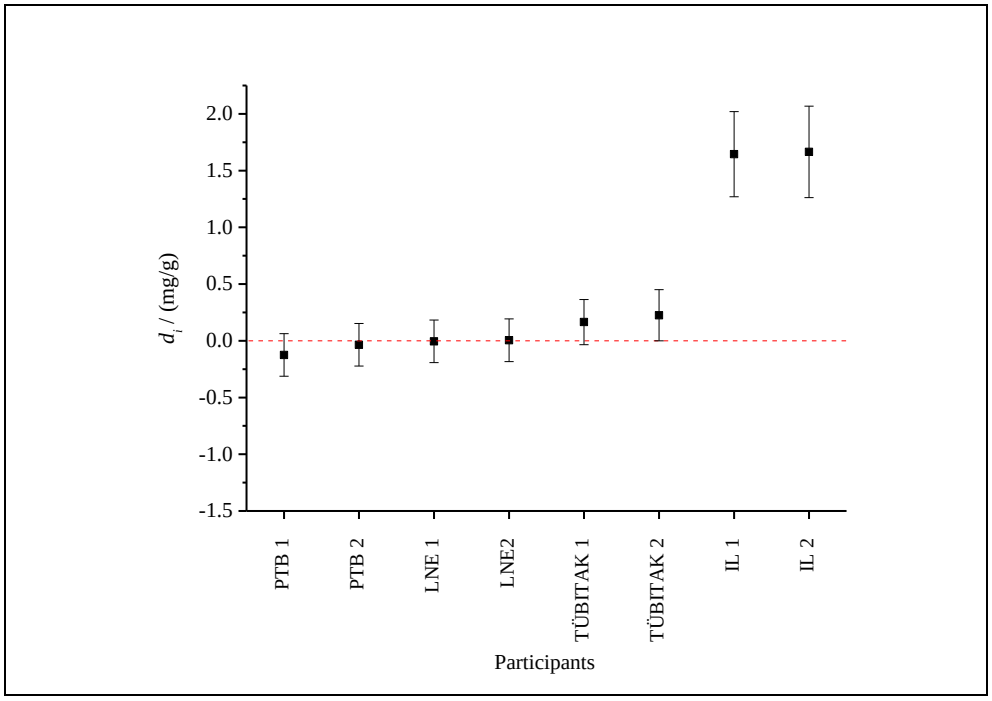

Figure 11: Graphical representation of the equivalence statements related to the median of the NMIs $\overline{w}_{MNMI}$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_{MNMI}$.

Table 10: Mass fractions w(Tf) and their associated combined and relative expanded uncertainties $u(w(\mathrm{Tf}))$ and u_{rel}, resp., using a coverage factor of k = 1 as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of k = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the uncertainty weighted mean of all participants $\overline{w}_U$ was applied.

Consensus value: $\overline{w}_U$ = (2.32 ± 0.11) mg/g

Participant	(w (Tf) ± $u(w(\mathrm{Tf}))$/ mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.1834	0.2483	-0.7386
PTB 2	2.23 ± 0.05	2.3 %	-0.0934	0.2483	-0.3762
LNE 1	2.26 ± 0.05	2.2 %	-0.0634	0.2483	-0.2554
LNE 2	2.27 ± 0.05	2.2 %	-0.0534	0.2483	-0.2151
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	0.1066	0.2570	0.4146
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	0.1666	0.2780	0.5992
IL 1	3.91 ± 0.17	4.4 %	1.5866	0.4090	3.8792
IL 2	3.93 ± 0.18	4.7 %	1.6066	0.4343	3.6997

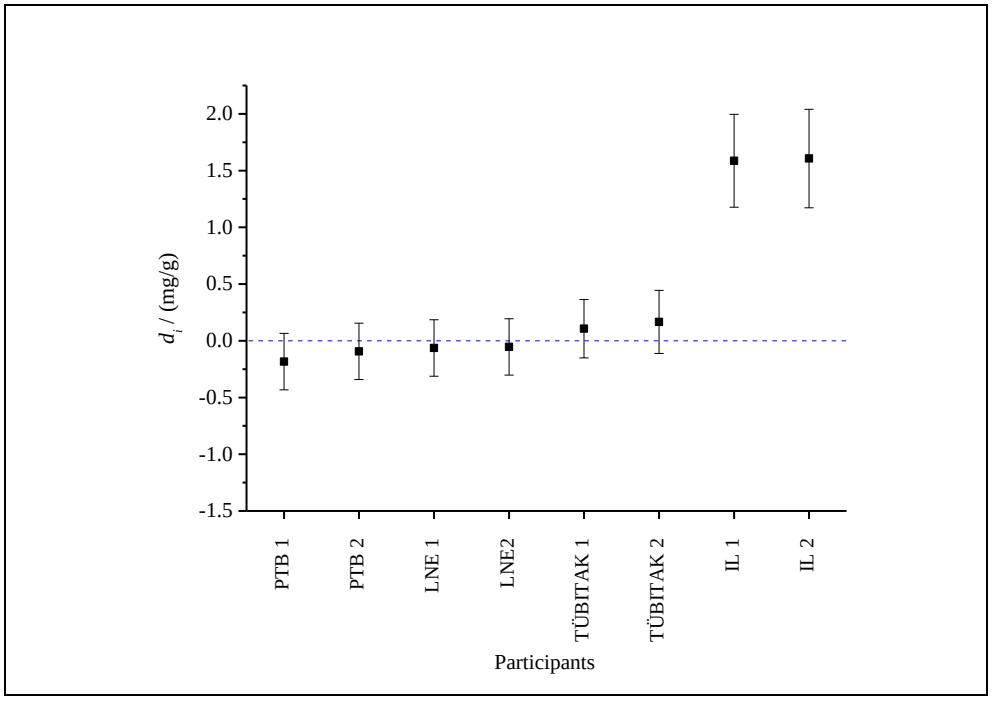

Figure 12: Graphical representation of the equivalence statements related to uncertainty weighted mean of all participants $\overline{w}_U$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_U$.

Table 11: Mass fractions w(Tf) and their associated combined and relative expanded uncertainties $u(w(\mathrm{Tf}))$ and u_{rel}, resp., using a coverage factor k = 1 as reported by the participants in the order of increasing mass fraction values. Degrees of equivalence d_i and their associated expanded uncertainty $U(d_i)$ were calculated according to equation (16) and (17). A coverage factor of k = 2 was used to calculate $U(d_i) = k \cdot u(d_i)$. As consensus the uncertainty weighted mean of the NMIs $\overline{w}_{\mathrm{UNMI}}$ was applied.

Consensus value: $\overline{w}_{UNMI}$ = (2.27 ± 0.05) mg/g

Participant	(w (Tf) ± $u(w(\mathrm{Tf}))$/ mg/g	u_{rel}	d_i	$U(d_i)$	E_n
PTB 1	2.14 ± 0.05	2.5 %	-0.1334	0.1427	-0.9344
PTB 2	2.23 ± 0.05	2.3 %	-0.0434	0.1427	-0.3038
LNE 1	2.26 ± 0.05	2.2 %	-0.0134	0.1427	-0.0936
LNE 2	2.27 ± 0.05	2.2 %	-0.0034	0.1427	-0.0235
TÜBITAK UME 1	2.43 ± 0.06	2.5 %	0.1566	0.1574	0.9953
TÜBITAK UME 2	2.49 ± 0.08	3.2 %	0.2166	0.1897	1.1423
IL 1	3.91 ± 0.17	4.4 %	1.6366	0.3549	4.6113
IL 2	3.93 ± 0.18	4.7 %	1.6566	0.3838	4.3169

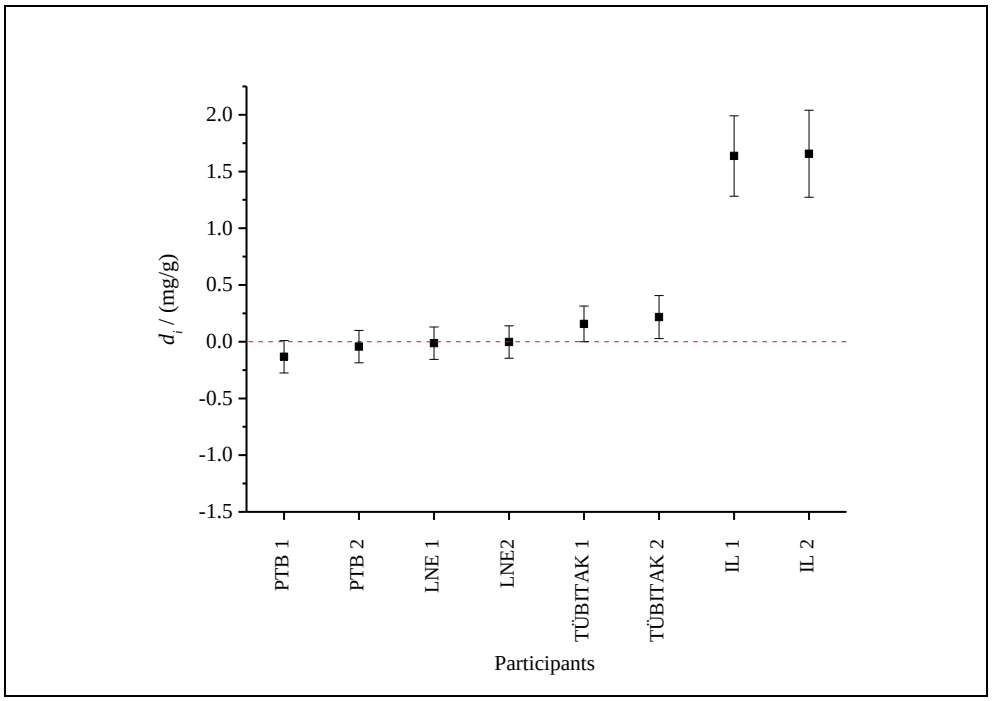

Figure 13: Graphical representation of the equivalence statements related to the uncertainty weighted mean of all participants $\overline{w}_{\mathrm{UNMI}}$ – DoE-plot of the data reported by the TC-MC 1352 participants according to table 4. The black dots show the DoE d_i, while the error bars denote the expanded uncertainty associated with the degree of equivalence $U(d_i)$ according to eq. (17), calculated 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 $\overline{w}_{\mathrm{UNMI}}$.

7 Discussion

The NMI results are in good agreement for all applied consensus values since no or only one value has an $E_n > 1$. E_n should be in a range of 0 to 1 to ensure that the determined results are within the uncertainty of the target value. The E_n values of the IL exceed 1 in case of all calculated consensus values. A gravimetric reference value is not available in this interlaboratory comparison, for this reason it cannot be concluded that the values of IL are out of the range and the NMIs passed the comparison. A further detailed evaluation has to be performed because the measurement procedures of the NMIs and the IL differ significantly. The NMIs quantified Tf via the Fe content whereas the IL focused on S. Both strategies have advantages and disadvantages that have to be evaluated. S detection is more unspecific than Fe because almost all human serum proteins contain S. Therefore, in case of S detection a very good separation from the complex serum matrix has to be guaranteed. The disadvantage of Fe in case of Tf is that the Fe saturation is not 100 % under physiological conditions whereas the S content in Tf is constant (47 S per Tf). For this reason a complete saturation of the protein with Fe (2 Fe per Tf) has to be ensured by adding Fe to the samples and removing the excess metal.

For a further evaluation of the variations observed in TC-MC 1351, further investigations and comparisons are necessary including quantification of Tf via Fe and S by all participants.

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10 Appendix

Physikalisch-Technische Bundesanstalt

Braunschweig und Berlin

HLT05

"Transferrin in Human Serum"

Technical Protocol

1. Introduction

Metalloproteins are particularly important in medical diagnosis as they represent around 30 % of the whole proteome. Many of them such as haemoglobin (Hb), transferrin (Tf), superoxide dismutase (SOD) or ceruloplasmin (Cp) are important markers for diseases such as Down's syndrome in the prenatal diagnostic (e.g. SOD), inflammation (acute-phase proteins like Tf or C-reactive protein (CRP)) or deficiency diseases (e.g. Hb, Tf, and Cp). Moreover, they are used for the control of treatment efficiency e.g. total Hb as the most important marker for anaemia treatment.

However, for proteins such as Tf, SOD or Cp, which are important markers for deficiency diseases or ischemic myocardium, reference measurement procedures are not yet available.

As a consequence most inter-laboratory comparisons have only a method specific consensus value (determined as the median for this method) with method specific limits instead of a reference value which is traceable to the SI.
The comparison is the first step to establish traceability of metalloproteins which might lead to a CCQM comparison for Tf in human serum in the future.

2. Samples

Two samples containing approximately 1 g pooled human serum each are provided by PTB. Both samples have the same Tf concentration and the same matrix. The serum was sterile filtered prior to filling and no preservatives were added. This serum material was produced from blood from healthy blood donors. Each portion of serum was tested negative for Anti-HIV-1&2, Anti-HCV and Anti-HTLV-I&II. However, the material is of human origin and should be handled with adequate care. For in vitro analysis only.

Table 12: mass of the empty container and mass of the serum.

Sample	mass	Air buoyancy correction
Bottle 1	1.14275 g	68.9 %, 22.3 °C, 1001 hPa
Serum (HLT05-TRF-S5)	2.15583 g	68.9 %, 22.3 °C, 1001 hPa
Bottle 2	1.15637 g	68.4 %, 22.5 °C, 1001 hPa
Serum (HLT05-TRF-S11)	2.16527 g	68.3 %, 22.6 °C, 1001 hPa

3. Sample handling

The samples should be stored at -70 °C or below. Before opening the serum should be thawed to room temperature under continuously gently shaking. Please make sure that the sample is well homogenised before use. The minimum amount of sample to be used is 50 µL.

4. Analysis

Please apply your most accurate methods of measurement, preferably primary methods. Note that the relative expanded measurement uncertainty U_{rel} associated with your result must not exceed 10 %. You are asked to determine the following quantities:

- Mass fraction *w* (Tf) of transferrin in human serum.

5. Reporting

Note that the reporting deadline has been changed. The new **deadline** for the submission of results is **30.01.2015**. Please send your report via E-mail.
Please report all your results 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 bottles for the first time. Please refer to section 6 to do this.
Please calculate uncertainties for all the results reported according to the GUM [1]. Please, report also your sources of traceability along with a short description of the method(s) you used.

If you need further assistance or encounter any kind of problem, please contact Claudia Swart

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6. Checking for losses / correcting evaporation effects

In addition to this "Technical Protocol" you should have received a table summarizing all bottles enclosed in your parcel together with the masses of the empty bottles m_{bottle} and the masses of the serum in these bottles m_{serum}.

These masses were determined from the apparent masses (weighing values) of the empty bottle m_1 and the bottle containing the according serum 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 (PE in case of the bottle, ρ_{bottle}, and the different serum, ρ_{serum}) were calculated to convert the apparent masses m_1 and m_2 into the masses m_{bottle} and m_{serum}.

$$m_{\mathrm{bottle}} = K_{\mathrm{bottle},1} \cdot m_1$$

$$K_{\mathrm{bottle},1} = \frac{1 - \dfrac{\rho_{\mathrm{air},1}}{\rho_{\mathrm{cal}}}}{1 - \dfrac{\rho_{\mathrm{air},1}}{\rho_{\mathrm{bottle}}}}$$

$$\rho_{\mathrm{air},1} = \frac{0.348444\,\dfrac{\mathrm{kg/m^3}}{\mathrm{hPa}} \cdot p_1 - \varphi_1 \cdot \left(0.252\,\dfrac{\mathrm{kg/m^3}}{^{\circ}\mathrm{C}} \cdot \vartheta_1 - 2.0582\,\dfrac{\mathrm{kg}}{\mathrm{m^3}}\right)}{273.15 + \dfrac{1}{^{\circ}\mathrm{C}} \cdot \vartheta_1}$$

$$m_{\mathrm{serum}} = K_{\mathrm{serum},2} \cdot \left(m_2 - \frac{m_{\mathrm{bottle}}}{K_{\mathrm{bottle},2}}\right)$$

$$K_{\mathrm{bottle},2} = \frac{1 - \dfrac{\rho_{\mathrm{air},2}}{\rho_{\mathrm{cal}}}}{1 - \dfrac{\rho_{\mathrm{air},2}}{\rho_{\mathrm{bottle}}}} \quad \text{and} \quad K_{\mathrm{serum},2} = \frac{1 - \dfrac{\rho_{\mathrm{air},2}}{\rho_{\mathrm{cal}}}}{1 - \dfrac{\rho_{\mathrm{air},2}}{\rho_{\mathrm{serum}}}}$$

$$\rho_{\mathrm{air},2} = \frac{0.348444\,\dfrac{\mathrm{kg/m^3}}{\mathrm{hPa}} \cdot p_2 - \varphi_2 \cdot \left(0.252\,\dfrac{\mathrm{kg/m^3}}{^{\circ}\mathrm{C}} \cdot \vartheta_2 - 2.0582\,\dfrac{\mathrm{kg}}{\mathrm{m^3}}\right)}{273.15 + \dfrac{1}{^{\circ}\mathrm{C}} \cdot \vartheta_2}$$

The following parameters were used to perform the calculations above: $\rho_{\mathrm{bottle}} = 2150\ \mathrm{kg/m^3}$, $\rho_{\mathrm{serum,Cr}} = 1030\ \mathrm{kg/m^3}$ in case of serum type 1 and 2, respectively, as well as $\rho_{\mathrm{cal}} = 8000\ \mathrm{kg/m^3}$.

Before sampling the first aliquot from a bottle, you are asked to weigh the bottle (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 bottle and tighten it immediately afterwards to equilibrate the pressure inside and outside the bottle. To calculate the correction, please follow the step-by-step recipe:

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

$$\rho_{air,3} = \frac{0.348444\,\frac{\mathrm{kg/m^3}}{\mathrm{hPa}} \cdot p_3 - \varphi_3 \cdot \left(0.252\,\frac{\mathrm{kg/m^3}}{^\circ\mathrm{C}} \cdot \vartheta_3 - 2.0582\,\frac{\mathrm{kg}}{\mathrm{m^3}}\right)}{273.15 + \frac{1}{^\circ\mathrm{C}} \cdot \vartheta_3}$$

Step 2: Calculate the air buoyancy correction factor of the bottle $K_{bottle,3}$

$$K_{bottle,3} = \frac{1 - \frac{\rho_{air,3}}{\rho_{cal,3}}}{1 - \frac{\rho_{air,3}}{\rho_{bottle}}}$$

Step 3: Calculate the air buoyancy correction factor of the serum $K_{serum,3}$

$$K_{serum,3} = \frac{1 - \frac{\rho_{air,3}}{\rho_{cal,3}}}{1 - \frac{\rho_{air,3}}{\rho_{serum}}}$$

Step 4: Calculate the mass $m_{serum,3}$ of the serum at the time t_3 before sampling the first aliquot from the bottle

$$m_{serum,3} = K_{serum,3} \cdot \left(m_3 - \frac{m_{bottle}}{K_{bottle,3}}\right)$$

Step 5: Calculate the loss Δm

$$\Delta m = m_{\text{serum},3} - m_{\text{serum}}$$

Step 6: In case it is reasonably small (-10 mg < Δm < 0 mg) this loss can be attributed to evaporation effects. In this case calculate an according evaporation losses correction factor f_{evap} (assuming the element content is still present completely in the bottle, causing a slightly elevated mass fraction of the element in question) and apply this to the mass fraction w_3 you have determined in the particular serum in order to retrieve the original mass fraction of the element at the time t_2 immediately after bottling the serum. Please report this corrected mass fraction w_2.

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

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 bottle m_{bottle} and with the mass of the serum m_{serum}, respectively: $u(m_{\text{bottle}})$ = 0.0005 g and $u(m_{\text{serum}})$ = 0.0007 g.

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

Symbol	**Unit**	**Quantity**	**Comment**
m_{bottle}	g	Mass of the empty bottle (corrected for air buoyancy)	Individually listed for every bottle no. in the table sent to each participant
m_{serum}	g	Mass of the serum (corrected for air buoyancy)	Individually listed for every bottle no. in the table sent to each participant; determined immediately after bottling in the pilot laboratory (PTB)

$m_{serum,3}$	g	Mass of the serum (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 serum (corrected for air buoyancy)	Difference between m_{serum} and $m_{serum,3}$; determined prior to sampling in the participant's laboratory
m_1	g	Apparent mass (reading of the balance) of the empty bottle	Determined in the pilot laboratory (PTB); used to calculate m_{bottle}
m_2	g	Apparent mass (reading of the balance) of the sum of the empty bottle and the serum	Determined in the pilot laboratory (PTB) immediately after bottling; used to calculate m_{serum}
m_3	g	Apparent mass (reading of the balance) of the sum of the empty bottle and the sample/calibration serum	Determined in the participant's laboratory prior to sampling; used to calculate $m_{serum,3}$
w_2	g/kg	Mass fraction of the particular element	Value corrected for evaporation losses; calculated from w_3
w_3	g/kg	Mass fraction of the particular element	Value actually measured in the participant's laboratory
f_{evap}	1	Factor to correct the measured mass fraction for evaporation losses	To be calculated by the participant
$K_{bottle,1}$	g/g	Air buoyancy correction factor	Valid for the bottle material (PE) at the time of the determination of m_1
$K_{bottle,2}$	g/g	Air buoyancy correction factor	Valid for the bottle material (PE) at the time of the determination of m_2
$K_{bottle,3}$	g/g	Air buoyancy correction factor	Valid for the bottle material (PE) at the time of the determination of m_3

$K_{serum,2}$	g/g	Air buoyancy correction factor	Valid for the serum A, and B, respectively, at the time of the determination of m_2
$K_{serum,3}$	g/g	Air buoyancy correction factor	Valid for the serum A, and B, respectively, 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 (PTB)
$\rho_{air,2}$	kg/m³	Air density	At the time of the determination of m_2 in the pilot laboratory (PTB)
$\rho_{air,3}$	kg/m³	Air density	At the time of the determination of m_3 in the participant's laboratory
ρ_{bottle}	kg/m³	Density of the bottle material (PE)	Assumed to be sufficiently constant throughout the temperature range in question; ρ_{bottle} = 2150 kg/m³
ρ_{serum}	kg/m³	Density of the particular sample/calibration serum	Determined in the pilot laboratory (PTB); listed in the text above; assumed to be sufficiently constant throughout the temperature range in question
p_1	hPa	Air pressure	At the time of the determination of m_1 in the pilot laboratory (PTB)
p_2	hPa	Air pressure	At the time of the determination of m_2 in the pilot laboratory (PTB)
φ_1	1	Relative air humidity	At the time of the determination of m_1 in the pilot laboratory (PTB)
φ_2	1	Relative air humidity	At the time of the determination of m_2 in the pilot laboratory (PTB)
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 (PTB)
ϑ_2	°C	Air temperature	At the time of the determination of m_2 in the pilot laboratory (PTB)
ϑ_3	°C	Air temperature	At the time of the determination of m_3 in the participant's laboratory

7. References

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