

# “Goldberg Validations”: Refractometric Protein Concentration Calibration of Pooled Source Plasma and Validation of Clinical Serum Protein Refractometers

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## Abstract

Digital protein refractometry is a convenient and resource-saving (“green”) method for measuring protein concentrations of biological fluids with complex composition. Conflicting historical data for the specific refractive index increment  $dn/dc$  of human plasma/serum protein have warranted an experimental re-calibration for citrated source plasma for fractionation. Measurements by *Dumas* combustion and digital refractometry applied the pharmacopeial protein-nitrogen ratio of 6.25. The non-protein matrix was separated by centrifugal ultrafiltration. Refractometric data agreed excellently with *Dumas*-based protein concentrations. Validation of a digital and a manual clinical refractometer demonstrated a good agreement between a benchtop analytical and the portable clinical refractometers in differential (sample-matrix) or a temperature-uncompensated direct “serum protein” mode, respectively. The found  $dn/dc$  value for plasma protein of 0.000194 mL/mg agreed well with results obtained for human serum in the 1920’s. The average temperature coefficient of both the sample and the matrix refractive index of  $-0.00011/^\circ\text{C}$  suggested that thermostating consistency may appear as more critical than temperature accuracy. Both the digital and the Goldberg TS refractometer gave a reasonable accuracy in the specified range of 2.5 to 15 mg “serum protein”/mL. The  $dn/dc$  values obtained for both refractometers indicate that the protein-nitrogen ratio of 6.25 still provides the calibration base for the determination of plasma/serum protein in the dedicated clinical instruments. Digital refractometry for protein concentration measurement thus presents a viable option especially when data have to be obtained at real-time.

## Highlights

- Protein refractometry for plasma protein concentration determination is applied.
- Untransparent calibration data traceability hampers the use of the method.
- Calibration data of commercially available refractometers are evaluated.
- An accurate refractometric method for the plasma protein measurement is provided.

## Keywords

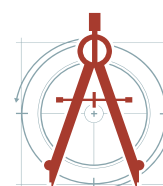
Digital refractometry, Protein determination, Plasma/serum, Validation, Real time data

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## Introduction

Previously, we have described the refractometric calibration of albumin and of purified immunoglobulin G based on nitrogen-based protein concentration values [1]. A comprehensive list of the specific refractive index (RI)<sup>a</sup> increment ( $dn/dc$ ) values has been provided in the [Supplementary Material](#). There are only few data for human blood plasma [2], but many in part conflicting values for human serum protein (see [Table 1](#) [2-15]).

In the first 50 years of refractometry, defibrinated blood serum had been a much more common sample material than blood plasma. Analytical attention towards the latter has arisen only with the introduction of pooled plasma for transfusion [16], and the establishment of the cold-ethanol plasma fractionation [17]. Before, blood plasma was regarded only as a clinical sample material ([Supplemental Material](#)). Although several theses at European hematological departments had focused on the application of refractometry on plasma and serum samples, these apparently did not find wider distribution and attention ([Supplemental Material](#)).

<sup>a</sup>The following abbreviations were used: RI: Refractive Index; RSD: Relative Standard Deviation; RT: Room Temperature; SP: Serum Protein; VP: Veronal Buffer.

Temperature-compensated manual refractometers were introduced in the 1960s and licensed for *in vitro* diagnostic use, such as the Goldberg TS refractometer [18]. This hand-held, telescope-shaped device does not contain any moving parts and uses a fixed scale from 2.5 to 15% serum protein, calculated with a protein-nitrogen ratio of 6.54. Upon re-calculation from this scale and the RI versus concentration table in the manual results in  $dn/dc$  values of 0.000195 and 0.000193 mL/mg, respectively, which are very close to the value for serum protein ( $dn/dc = 0.000194$  mL/mg) as provided in [4].

Two additional aspects may emphasize the reemerging importance of a valid  $dn/dc$  value for plasma protein: First, starting plasma pools for fractionation must contain not less than 50 g protein/L [19] and are usually analyzed by *Kjeldahl* digestion [20] or *Dumas* combustion [21]. While *Kjeldahl* digestion is time consuming and uses hazardous reagents, *Dumas* combustion requires a fixed instrument installation and a sample pre-drying step. Both methods cannot deliver real-time results. Second, single plasma donations are still checked for their protein content with hand-held or benchtop refractometers to reject single donations with a “total protein concentration” below 6.0 g/100 mL (US 21 CFR 640.63 [22]). State-of-the art requirements for such analytics include not only a valid  $dn/dc$  value, but also instrument validation.

**Table 1:** Historical RI increment values for human plasma/serum protein.

Sample	Remarks (solvent, conditions, calibration)	$dn/dc$ [mL/mg]	Reference
Plasma	19 plasma pools for fractionation	0.0001833	[2]
Serum	0.9% NaCl	0.0001784	[3]
Serum	15 normal sera, UF permeate-subtracted, Kjeldahl	0.000194	[4]
Serum	69 sera, gravimetric	0.000196	[5]
Serum	N/D	0.000195	[6]
Serum	20 pathological sera	0.000210	[7]
Serum	32 pathological sera	0.000197	[8]
Serum	10 pathological sera	0.000200	[9]
Serum	47 pathological sera	0.000201	[9]
Serum	39 sera, gravimetric	0.0001906	[10]
Serum	4 h after venipuncture (VP)	0.000189	[11]
Serum	Veronal buffer (VP), pH = 8.6 (dialyzed, 4 h after VP)	0.000193	[12]
Serum	VP, pH = 8.6 (dialyzed, 24 h at room temperature)	0.000204	[12]
Serum	Personal communication to authors	0.000179	[13]
Serum	Recalculated using a protein to N ratio of 6.54	0.000187	[14]

**Remarks:** Robertson (1912) determined a  $dn/dc$  value of 0.000195 mL/mg for bovine serum protein [15].

Altogether, this warrants an investigation of single-donor plasma samples after a valid  $dn/dc$  value has been established. Furthermore, validation of an exemplary temperature-compensated clinical instrument as compared to a high-resolution, high precision, thermostated instrument seemed to be appropriate.

Since its first description for refractometry [7], the centrifugally accelerated separation of a protein-free matrix appears to have found occasional application in clinical analysis only after the availability of mechanically durable devices [23-25]. The duration of the centrifugation step may bias the refractometric permeate value [26] as a “Donnan” shift may result in an imbalance of the low-molecular matrix [27,28]. Upon crowding, protein-associated matrix components may

become increasingly displaced and forced into the permeate fraction. Therefore, the sample preparation procedure, especially that applied for single donor plasma samples, may need to be optimized for both permeate yield and Donnan bias minimization.

Here, we present an evaluation of the calibration data of commercially available refractometers intended to increase the transparency of the data traceability and present and furthermore provide an accurate method for the plasma protein measurement.

## Materials and Methods

### Instruments

A manual Reichert Goldberg TS temperature-compensated clinical refractometer [5,29] and two

**Table 2:** Parameter comparison of the digital refractometers used for this study.

Instrument	Rudolph Research J357CC	Reichert TS D (1 <sup>st</sup> generation) <sup>a)</sup>
Type	Analytical refractometer	Clinical refractometer
Applications	RI, Brix, custom scale	RI, Brix, %serum protein, urine SG, % CuSO <sub>4</sub>
Serum protein (% = g/100 mL)	Not implemented	0.0-15.0 ( $dn/dc$ not stated)
Installation/operation	Bench-top	Bench-top and handheld
Light source	589.3 nm LED	589.3 nm LED
Prism	Sapphire ( $n_D = 1.7681$ )	Heavy crown glass ( $n_D = 1.60994$ ) <sup>a)</sup>
Sample well	Stainless steel	Stainless steel
Detector	CCD pixel array <sup>b)</sup>	CCD pixel array, 3648 pixels <sup>a)</sup>
RI range	1.26 - 1.70	1.33-1.56
RI resolution	0.00001 $n_D$	0.0001 $n_D$
Temperature resolution	0.01 °C	0.1 °C
Temperature accuracy	± 0.01 °C (thermostatization)	± 0.2 °C (measurement)
Temperature compensation	4 - 95 °C (sucrose Brix)	15-40 °C (sucrose Brix)
Precision	0.00002 $n_D$	0.0001 $n_D$
Accuracy (for protein)	± 0.00002 $n_D$ (~ 0.1 mg/mL)	± 0.0001 $n_D$ (~ 0.5 mg/mL)
Zero calibration	Water, air	Water
Dimensions (L × W × H, cm)	43.5 × 30.5 × 33 (lid open)	18 × 9 × 3.5 (no lid)
Weight (kg)	10.4	0.41
User interface	Touchscreen, USB keyboard	Three keys, double-line alphanumeric display
Communication ports	USB, RS-232, ethernet	IR to optional IR receiver connected to PC
21 CFR 11 compliance	Optional	Not stated
Power	AC mains	6 V DC (4 × AAA batteries)
Cost (approximate USD)	~ 15,000	1,385 (MSRP, current version)

**Remarks:** <sup>a)</sup> 1<sup>st</sup> instrument generation according to patent description; the current version is equipped with a sapphire prism; <sup>b)</sup> The overall number of sensor pixels is less relevant than the accuracy of the shadow-line location algorithm.

digital refractometers were used (Supplemental Figure 2). The manual instrument is delivered with calibration data from 2.5 to 14.6 g protein/100 mL. The digital refractometers are compared in Table 2 [30,31]. The analytical, digital high precision refractometer is equipped with a stable and accurate thermostatisation. The clinical instruments, also a digital one, belongs to the first model generation with a high-refractivity glass prism; the current version is equipped with a scratch-resistant sapphire prism. Any documentation about the serum protein calibrations traceability has not been provided in the manual.

### Instrument validation and verification

The manual refractometer was verified with water only, while the digital refractometers were initially validated with water and sucrose solutions and then checked daily with water as system suitability test. 4 M guanidine hydrochloride solution was used as a cleaning agent. Precision and linearity of the clinical refractometer were checked with neat and diluted albumin solutions.

### Plasma samples

Citrate-anticoagulated single donor plasma samples were purchased as a kit of 50 healthy US donors (George King Bio Medical Inc., Overland Park, Kansas, USA). In addition, 39 frozen source plasma pool samples from US donors (BioLife, Baxter) were used. After thawing at 37 °C, ultrafiltration was done at room temperature (RT) in a fixed-angle-rotor centrifuge at 6,000 rpm for at least 15 min or in a swinging bucket centrifuge at 4,500 rpm for at least 5 min through pre-conditioned [1] Sartorius Vivaspin 2 5kDa (Sartorius, Göttingen, Germany) or Amicon Ultra 4 10 kDa (Merck, Vienna, Austria) centrifugal filters, respectively, to obtain at least 200 µL of a protein-free permeate.

### Plasma centrifugation time for *Donnan* effect evaluation

Four mL pooled plasma were incrementally centrifuged through the 10 kDa membrane at 4,500 rpm for a cumulative duration of 5, 12, 22, 37, and 60 min at RT, with weighing and RI measurement of the obtained permeate fraction. A total RI index was calculated for each cumulative centrifugation time.

### Plasma protein concentration accuracy and linearity

An approximately double-concentrated pooled

plasma, obtained by prolonged centrifugation of 2 × 10.4 mL plasma in Amicon Ultra 15 10 kDa centrifugal ultrafiltration tubes (60 min at 4,500 rpm and 37 °C), was mixed with its respective permeate in a pre-defined ratio and measured on all instruments in all modes, i. e., with and without temperature compensation in the clinical digital refractometer.

### Refractometric measurements and $dn/dc$ calibration

The RI of samples and their respective permeates was measured with the thermostated (20.00 °C) Rudolph refractometer for all sample types or with the Reichert refractometer for the single donor plasma samples without using the programmed temperature compensation. As verified by a temperature series between 17.5 and 30 °C (in 2.5 °C increments),  $dn/dc$ -values for plasma and corresponding permeate samples were shown to be essentially constant in this temperature range. The net RI (= difference between RIs of sample and permeate) was then divided by the protein concentration as obtained by the *Dumas* method (pooled source plasma) using a factor of 6.25. The  $dn/dc$  value of pooled plasma protein was used as the divisor for single donor samples.

### Results

For total plasma protein, a specific RI increment  $dn/dc = 0.0001942$  was determined with a relative standard deviation (RSD) of 1.09% (Supplemental Table 2). The average protein concentration of all 39 plasma pools was 55.7 mg/mL with RSDs of 1.06% and 0.83% for the *Dumas* and the refractometry method, respectively. Paired t- test, calculated by using GraphPad Prism 8.0, did not detect any significant differences between the results obtained with both methods ( $p = 0.9793$ ). The mean difference was 0.0025 mg/mL with a 95% confidence interval ranging from -0.2012 to 0.1961 mg/mL. The average permeate value was as 1.33535 with single data in the narrow range of 1.33525-1.33542 demonstrating consistent composition of plasma samples in terms of low molecular weight compounds. A *Donnan* shift of ultrafilterable non-protein-solids was observed upon prolonged centrifugation time, with a significant relative protein concentration error of about -1.7% after 60 min of cumulative centrifugation (Table 3).

For the linearity check, a permeate obtained by

**Table 3:** Effect of centrifugation time on the non-protein permeate refractive index of plasma.

Time (min)	Permeate (g) <sup>a)</sup>	$n_D^{20}$ permeate <sup>a)</sup>	Permeate total (g)	$n_D^{20}$ total (calculated)	net $n_D^{20}$ <sup>b)</sup>	Protein (mg/mL) <sup>c)</sup>	Relative error <sup>d)</sup>
5	0.415	1.33483	0.415	1.33483	0.01132	58.4	0.00%
12	0.511	1.33488	0.926	1.33486	0.01129	58.2	-0.24%
22	0.554	1.33498	1.480	1.33490	0.01125	58.0	-0.65%
37	0.536	1.33511	2.016	1.33496	0.01119	57.7	-1.13%
60	0.475	1.33528	2.491	1.33502	0.01113	57.4	-1.68%

**Remark:** <sup>a)</sup> The column “Permeate (g)” shows the incremental weight of permeate obtained at the given ultrafiltration times, while the column “ $n_D^{20}$  permeate” gives the corresponding RIs determined; <sup>b)</sup> The net  $n_D^{20}$  value was calculated as the difference between the values determined for the plasma sample before starting the ultrafiltration ( $n_D^{20} = 1.3461$ ) and at the given ultrafiltration times; <sup>c)</sup> The protein concentration of the plasma was calculated for the different ultrafiltration times; <sup>d)</sup> The relative error was calculated as the percent deviation from the plasma protein concentration determined after 5 min ultrafiltration.

**Table 4:** Dilution linearity of plasma protein.

Sample/ Dilution	J-357 digital	TS-D digital			Goldberg TS
	mg/mL <sup>a)</sup>	mg/mL <sup>a)</sup>	mg/mL SP (direct) <sup>b)</sup>	mg/mL SP (20 °C) <sup>c)</sup>	mg/mL SP <sup>d)</sup>
Permeate	0.0	0	7	9 (23.7 °C)	ND
0.10	16.8	17	21	22 (22.6 °C)	~ 20
0.15	25.2	26	26	28 (23.0 °C)	26
0.20	34.0	34	35	37 (23.3 °C)	35
0.30	49.6	49	51	53 (23.4 °C)	51
0.40	66.5	66	67	70 (23.8 °C)	68
0.50	82.9	82	84	86 (23.8 °C)	85
0.60	99.3	98	100	102 (23.9 °C)	102
0.70	115.9	115	117	119 (24.1 °C)	118
0.80	132.4	131	133	136 (24.1 °C)	136
0.90	150.4	150	152	155 (24.2 °C)	~ 154
Concentrate	168.3	169	171	173 (24.3)	ND

**Remarks:** ND stands for not determined, SP for serum protein: <sup>a)</sup> Calculated protein concentrations using the  $dn/dc$  value of 0.000192 for the permeate-corrected RI values; <sup>b)</sup> Serum protein concentrations as calculated by the instrument without temperature and permeate correction; <sup>c)</sup> Serum protein concentrations as obtained after temperature compensation automatically done by the instrument. Numbers in brackets show the temperature actually measured; <sup>d)</sup> Serum protein concentrations as obtained by the Goldberg TS device.

prolonged ultrafiltration provided an essentially constant matrix composition. Values for the mixtures from the double-concentrate pooled plasma with its respective permeate agreed very well between the data obtained with either the J-357 or the clinical instrument TS-D-Digital and the latter's direct serum protein (SP) mode for concentrations within the tabulated range of 2.5 to 14.6 g/100 mL (Table 4). Upon correlation of the RI versus the uncompensated SP results, a  $dn/dc$  value of 0.000192 mL/mg was re-calculated.

Permeates alone gave however falsely positive protein concentrations of about 7 to 9 mg/mL. The agreement between both digital and the manual refractometer was rather moderate - the latter gave a bias of about 0.2 g/100 mL higher values.

The temperature effect proved negligible (Table 5) since the RI changes were parallel for plasma and the corresponding permeate. The (mean) temperature coefficient  $\Delta n_D/\Delta T$  of -0.000113/°C for a temperature range from 17.5 °C



**Table 5:** Effect of the temperature on the apparent protein concentration.

T (°C)	$n_D$		Net $n_D$ <sup>a)</sup>	Protein (mg/mL) <sup>b)</sup>
	Plasma	10 kDa permeate		
17.5	1.34617	1.33506	0.01111	57.3
20.0	1.34592	1.33484	0.01108	57.1
22.5	1.34566	1.33459	0.01107	57.1
25.0	1.34538	1.33432	0.01106	57.0
27.5	1.34507	1.33403	0.01104	56.9
30.0	1.34476	1.33372	0.01104	56.9

**Remarks:** <sup>a)</sup> Net  $n_D$  is calculated as the difference between the RIs determined for plasma and its corresponding 10 kDa permeate; <sup>b)</sup> A  $dn/dc$  value of 0.0001942 was used for the calculation of the protein concentration.

to 30 °C confirmed the value obtained at the Zeiss instrument factory [32]. The protein-containing solution showed a slightly stronger temperature-dependence than the protein-free permeate ( $\Delta n_D/\Delta T = -0.000108/^\circ\text{C}$ ), as shown previously for carp serum and distilled water [33]. As the measured concentration decreases by about 0.5% from 20 to 30 °C, the actual contribution of the  $dn/dc$  value to the relative error can be estimated as about 50%, while the rest will most likely be caused simply by thermal volume expansion.

For the single-donor plasma samples, a particularly good agreement between both digital refractometers was found for the permeate subtraction method, while the SP values in the clinical instrument were again in general slightly higher (Supplemental Table 3) with an average bias of +2.39% as calculated by the Bland-Altman technique [34] (95% limits of agreement: 0.10%-4.69%, Supplemental Figure 3). The permeate RI varied only moderately (1.33514-1.33548) confirming the stated healthy donor population [35] as required for qualified plasma donors.

## Discussion

The present work demonstrated the practical benefits of digital refractometry. The  $dn/dc$  value obtained for pooled plasma (0.0001942 mg/mL) is however considerably higher than the single literature value as given at the beginning of commercial plasma fractionation [2], but practically the same as the value stated for plasma first in 1928 (0.000195 mL/mg) [10], as the experimental result determined by the permeate subtraction technique in 1923 for human serum [7], and as the value of dialyzed serum given in 1952 (0.000193 mL/mg) [12]. With about 55 mg/mL, the measured source

plasma pools tested fulfill the pharmacopeial requirement for plasma for fractionation. The data also agree well with findings from a study [36], where a lower total protein content (about 55 mg/mL) was measured for US source plasma than for recovered and European plasma (about 60 mg/mL). Of note, not all single donor plasma samples would however have met the minimum protein content of 6 g/100 mL for source plasma donations.

The comparison between plasma protein concentrations obtained by refractometry and the *Dumas* method confirmed the (expected) good agreement between the both methods as the means determined for 39 plasma pools did not show significant differences. A clear benefit of the refractometric method, however, can be seen in the immediate availability of results supporting real-time decisions when needed associated with low energy resources required for the analysis, which might become of even more importance for the construction of carbon-neutral facilities. Clearly, the *Dumas* (or *Kjeldahl*) analysis of neat pooled source or single donor plasma can never discriminate between protein and non-protein nitrogen. A base value of non-protein N (0.214 mg/mL in the present calibration) must thus be subtracted for source plasma pools. Protein-rich diet or renal insufficiency are known to markedly increase the non-protein N content. The total and the permeate RI would however not correlate only with the total or the residual N content, respectively: Elevated glucose and high lipid levels may add up as well [24]. Elevated cholesterol levels caused a high bias in a direct refractometric measurement as compared to micro-*Kjeldahl* results [37]. With the present permeate subtraction technique, only an excess of non-ultrafilterable lipoproteins might

**Table 6:** Specific refractive index increments re-calculated from “serum protein” scales.

Manufacturer/brand	Model	ATC	Low RI	High RI	dn/dc (calc.)
Atago [38]	Master SUR/Na	Yes	1.3365 (0%) <sup>a)</sup>	1.3575 (12%)	0.000175
Reichert [39]	Goldberg TS	Yes	1.3400 (3%)	1.3634 (15%)	0.000195
Reichert [40]	TS-400	Yes	1.3357 (0%)	1.3564 (11.5%)	0.000180
Sper Scientific [41]	300005	No	1.3349 (0%)	1.3581 (12%)	0.000193
Bellingham & Stanley [42]	E-line veterinary <sup>b)</sup>	Yes	1.3367 (0%)	1.3540 (9%)	0.000192
Teckoplus [43]	RETK-70	Yes	1.3350 (0%)	1.3582 (12%)	0.000193
Refractometer. Eu	RUR-2	Yes	1.3353 (0%)	1.3588 (12%)	0.000196
Dr. Richter	N/A <sup>b)</sup>	Yes	1.3366 (0%)	1.3571 (12%)	0.000171
Arcarda	REF312 <sup>b)</sup>	Yes	1.3366 (0%)	1.3584 (12%)	0.000190
TC	THC-200 <sup>b)</sup>	Yes	1.3365 (0%)	1.3570 (12%)	0.000171

**Remarks:** The abbreviation ATC and N/A stand for automatic temperature compensation and not available, respectively: <sup>a)</sup> Numbers in brackets represent the approximate representative serum protein concentrations; <sup>b)</sup> Denotes a veterinary instrument.

lead to a too high result biased by protein-bound lipids. Other than patients, therapeutic plasma donors are not required to donate fasted, so that glucose, lipid, and non-protein N may be elevated in the single donation; in the source plasma pool, such outliers would however be averaged out. In addition, source plasma becomes always “thinned down” by the anticoagulant mixture, usually 4% trisodium citrate solution in a ratio of about 1/16 or 1/25, depending on the plasmapheresis machine type. The data presented here apply primarily to such a citrate-anticoagulated source plasma and in particular to its permeate; other plasma types such as plasma recovered from whole-blood donations with ACD or CDP anticoagulants in a higher volume ratio may give a different permeate RI and a different non-protein N content.

As the temperature effect on the specific RI increment turned out to be practically negligible, its earlier identification as the main cause of inaccuracy in analytical refractometry cannot be substantiated at least at 0.0001 nD resolution. The obvious reluctance to accept and establish refractometry as a standard technique might thus be attributed rather to the conflicting *dn/dc* values, the not yet standardized “de-proteination” procedures, and practically to the delicate handling with a considerable workload of cleaning and maintenance for serial analyses. The question of the calibration of “serum protein” refractometer scales arises as the following examples illustrate conflicting data shown in Table 6 [38-43].

Atago traces back its scale to “2000 measurements done at the Tokyo’s Women Medical College” and claims it to be “the most reliable one” [38]; Reichert does not state any origins of its scale value for the Goldberg refractometer [39,40]. The value, however, agrees almost perfectly with the tabulated plasma/serum value [10,15], and the experimental data for ultrafiltered [4] or dialyzed serum [12], and our data for plasma presented here. Similarly, no source is provided for Reichert’s TS-400 instrument transformations [40]. The built-in “serum protein” scale in the Reichert TS-D refractometer, at least, has not been documented or specified anywhere, but by back-calculation from data within the specified concentration range, the *dn/dc* value of ~0.000192 mL/mg agrees surprisingly well both with the present results for plasma and the literature increments for serum (Noteworthy, the Atago Type 3 high-resolution (0.0001 nD) Abbe refractometer’s German manual refers to the Zeiss tables [32] with the *Reiss-Alder* *dn/dc*-value of 0.000195 mL/mg).

This discrepant data may raise again a question on the validity of established nitrogen-determined reference values and of their complementation by refractometry. Even after the sophisticated experimental works for albumin and immunoglobulin G [44] and for plasma [45] had shown the actual agreement, the validity of the assumption of 16% nitrogen in the total plasma protein and in albumin was still been doubted later, as seen from extensive data compilations [46,47]. The aforementioned experimental studies however would still set the

reference due to the methodological precautions: A micro-*Dumas* combustion analysis eliminates any effect of the *Kjeldahl* digestion catalyst on the nitrogen recovery by a complete conversion of all organic nitrogen; the denaturing ethanol-ether precipitation with delipidation and desalting of the precipitate together with a final drying step rules out any bias from such non-volatile non-protein matrix components on the gravimetric calibration. While the Sunderman *Kjeldahl* factor of 6.54 for serum protein is referenced in both the Reichert Goldberg TS and the TS-400 refractometer scales, the  $dn/dc$  values as re-calculated from the table in the respective manuals however agree with our experimental data based on the conventional - and compendial - conversion factor of 6.25. In the aforementioned studies, conversion factors of 6.24 and 6.27 were obtained for total plasma protein and for albumin, respectively by the micro-*Dumas* technique. The compendial standard value of 6.25, as mandatory in the corresponding Ph. Eur. Chapter, can therefore still be utilized with sufficient accuracy.

Even as the present work succeeded in establishing a valid  $dn/dc$  value for plasma protein in pooled plasma, it emphasizes again a desperate need for standardization of reference values and instruments. Collaborative trials, as ideally organized jointly by regulatory authorities, compendial boards, and industry organizations, would only require a relatively simple experimental layout, an access to the now common *Dumas* analyzers, and an agreement on the N content and the respective "*Kjeldahl* factor" of plasma protein. With such a standardization, any confusion by inconsistent historical data, which apparently has put off many potential users from refractometry, may be avoided bringing the method with long-lasting historical roots in compliance with state-of-the-art requirements for analytical methods.

## Remarks

The authors explicitly advise against any clinical or diagnostic use of refractometers without regulatory license for such applications in human medicine. Pooled plasma results were in part presented at the Plasma Product Biotechnology Meeting 2017.

## Author Contribution

HA lead the investigation, provided the methodology and wrote the original draft; KH and

SZ were involved in data curation and reviewed the original draft; AW supervised the study and reviewed and edited the original draft.

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## Conflicts of Interests

All authors were at the time of the study full-time employees at Baxalta Innovations GmbH or Baxter AG, now part of Takeda. AH and WA hold Takeda stocks. AH and AW are great admirers of keyboard music by Johann Sebastian Bach, in particular of the Goldberg Variations, reflected intentionally by the phrasing of the title.

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