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Simplified direct Kjeldahl method suitable as a primary reference procedure for the determination of total protein in reference materials used in clinical chemistry

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SUMMARY
Chromý et al. found in 2009, that albumin-calibrated analysis of serum total protein with biuret reagent according to a candidate reference method developed by Doumas working group in 1981, provide results with unacceptable positive bias, caused namely by endogeneous serum bilirubin and lipids. They also found, that a simplest way to rectify this defect, is the use of serum and/or plasma-based protein standards, commutable with patients samples, which are certified by the Kjeldahl method. In our foregoing two articles, (Chromý et al., 2015, and Vinklárková et al., 2015), we reviewed Kjeldahl methods adopted by laboratory medicine, selected a direct analysis on washed protein precipitates with trichloroacetic acid and verified its preliminary analytical performance parameters. In this article, we describe a simplified Kjeldahl analysis with modified digestion flask, now directly connectable to accordingly adapted Parnas and Wagner distillation unit, and present its re-validation. Simplified Kjeldahl method is suggested as a standard operating procedure for total protein in reference materials used in clinical chemistry
Keywords: total protein, the Kjeldahl method, serum-based reference materials.

SOUHRN
Vinklárková B., Chromý V., Bittová M., Šprongl L., Žaludová L.: Zjednodušená Kjeldahlova metoda vhodná jako primární referenční metoda pro stanovení celkové sérové bílkoviny v referenčních materiálech užívaných v klinické chemii.
Chromý a kol. v r. 2009 zjistili, že stanovení celkové sérové bílkoviny biuretovým činidlem za použití albuminových kalibrátorů, vypracované v r. 1981 Doumasovou pracovní skupinou jako kandidáta na referenční metodu, poskytuje výsledky s neakceptovatelnou systematickou pozitivní chybou, způsobenou zejména endogenním sérovým bilirubinem a lipidy. Zjistili též, že k nápravě stačí užít bíkovinné standardy na bázi séra nebo plasmy, které jsou komutabilní se vzorky pacientů, a jsou certifikovány Kjeldahlovou metodou. V našich dvou předchozích publikacích, (Chromý a kol., 2015 a Vinklárková a kol., 2015), jsme ověřili Kjeldahlovy postupy užívané v laboratorní medicíně. Zvolili jsme přímou analýzu ze sraženiny bílkovin promyté kyselinou trichloroctovou a prověřili jsme její analytické provozní parametry. V této práci popisujeme zjednodušenou analýzu s modifikovanou Kjeldahlovou digesční baňkou, kterou lze přímo připojit k tomu přizpůsobené Parnes-Wagnerově destilační aparatuře a uvádíme její validační parametry. Zjednodušenou Kjeldahlovu metodu navrhujeme jako standardní operační postup pro stanovení celkové bílkoviny v referenčních materiálech užívaných v klinické chemii.
Klíčová slova: celková bílkovina, Kjeldahlova metoda, referenční sérové materiály.

Introduction

Chromý et al. found in 2009 [1], that routine analyses of serum total protein (TP) based on albumin calibrators and biuret reagent developed by Doumas et al. in 1981 [2-4], and suggested as "A candidate reference method", provide results with 3-5 % unacceptable positive bias overlapping the total error ~3.4 % allowed and reserved now for analysis of serum TP in patients. The simplest way to remedy this fault was the use of human-based serum/plasma standards certified by the Kjeldahl method (KM). Such standards, commutable with patients samples, compensate for the offset caused namely by serum lipids and bilirubin in most normal and partly in pathological patients´ sera [1].

Unfortunately, there was not any KM applicable as a primary reference procedure for TP according to requirements of EN ISO 15193 now mandatory for reference laboratories [5]. Therefore, we reviewed KMs adopted for TP by laboratory medicine [6]. For our preliminary experiments, we chose, completed and verified KMs described originally by Alma Hiller et al. [8-10]. Based on hopeful preliminary performance parameters, we selected a direct KM on isolated and washed protein precipitates with trichloroacetic acid and manual visual acidimetric titration of steam-distilled ammonia as a base for primary reference procedure for both serum/plasma and urine TP [7]. Short description of selected Kjeldahl procedure presented in details in [7]: Using a vortex stirrer and centrifuge with test tubes, TP is precipitated and washed by trichloroacetic acid; TP precipitate is dissolved in alkali, transferred into a Kjeldahl flask, (comprising a 10-mL bulb with long, narrow neck), and digested in an aluminium block at ~385 °C in the excess of concentrated sulphuric acid with powdered catalyst, (copper and potassium sulfates 1+10), resulting in a digest containing ammonium sulphate; To prevent a solidification, still warm digest is diluted with water, transferred into distillation Parnas and Wagner apparatus, al-

kalized by NaOH and released ammonia is steam-distilled to a boric acid receiver; Receiver-trapped ammonium as ammonium dihydrogen borate is quantified manually/visually by direct acidimetric titration with standardized sulphuric acid ~0.005 mol/L, using mixed acid-base indicator, (bromocresol green and methyl red 10 + 1), which pH-colour change is between 4 and 6. Used indicator changes colour from a bright blue-green to pink with the first excess of titrant.

We further simplified preliminary selected KM [7] by the use of modified Kjeldahl digestion flask (MKF), now connectable directly through a ground-glass joint to accordingly converted Parnas and Wagner distillation unit, and usable for both digestion and distillation. However, such a modification necessitated to re-validate the whole procedure. In this paper, we present abridged form of simplified KM and its re-validation. The standard operating procedure (SOP), based on this article and on previously described procedures and reagents [7], will be used for the certification of TP in reference materials.

Material and methods

Equipment

We used most of laboratory equipment already described in our previous article [7]. To dose powdered Kjeldahl catalyst on the bottom of MKF, we used ~0.50-g glass spoon-doser and a funnel with long, wide stem reaching ~1 cm over the bottom of MKF. All special glassware was made by our glass blower. Samples and reagent solutions were tempered to (20±0.5) °C before their preparation and/or use in a 5-L water bath with a thermostat unit capable to keep bath temperature within ±0.2 °C. To compare an acidimetric manual visual versus automatic titration of ammonia, we used potentiometric titrator.

The principal changes between previously described KM [7] and this simplified procedure, consist in MKF, accordingly modified bottom part of originally one-piece Parnas and Wagner still and in the aluminium heating block with greater flask ports. MKF is now a straight-walled ~90-mL and ~17-cm long test tube with 24/40 interchangeable ground-glass joint, which fits to accordingly adapted bottom part of the still and serves for both digestion and steam distillation. The ground joint is fixed with a steel clip. Heating block has now seven greater circular ports 37x25 mm (diameter by depth) to fit with MKF placed in block-port vertically. Original sampling funnel of the still, (with a glass tube reaching to the bottom of MKS and serving for both dosing and as a steam supply), remained unchanged. To prevent a loss of boiling digest, we inserted into the neck of each MKF a glass funnel with short-wide-bent-stem, serving as a partial reflux and preventing digest to splash out.

Materials, samples and standards

We utilized pure chemical standards described and used already in [7]. All standards were dried over the silica gel before weighing and their producer-certified batch contents were included in calculations. Samples, standards, reagent solutions, titrant and water for their preparation were tempered before use to (20±0.5) °C and handled as described in [7].

Recovery and validation tests were performed with ammonium sulphate, (chemical standard recommended for steam distillation and titration of ammonia), with glycine, (chemical standard for the digestion and following steps), and with freeze-dried 5-mL Serum Control N (SCN), which we now used as our in-house reference material for the whole KM. TP in SCN we assessed by a direct KM as 64.05 g/L [7].

We used for calculations statistical methods described by Clinical laboratory standards institute, which are referred to in our foregoing paper [7].

Reagents for the Kjeldahl procedure

We used chemicals and reagents described in our foregoing paper [7]. Only 5.0 mL of 40 % NaOH, (used originally to alkalize digest before steam distillation of ammonia), was substituted for more convenient 10.0 mL of 20 % NaOH. To prevent the contamination by aerial ammonia, all reagents and solutions were kept in tightly closed bottles and dosed to the Kjeldahl reaction immediately before use.

Direct determination of TP

We accomplished a direct determination of TP on single-washed protein precipitates prepared from SCN [7]. To wash down scattered droplets of completed digest and prevent digest solidification, the neck of MKF and its reflux-funnel with still warm digest were rinsed out by six portions of water, (~1 mL each). MKF with water-diluted digest was either connected immediately to the adapted still, digest was alkalized and analysis continued, or MKF was tightly stoppered and stored for a later distillation. Seven days standing before distillation caused no statistically significant deviations in resulting TP.

Our digestion mixture contained uniformly: ~0.1 mL of glycine standard solution ~0.80 mol/L and/or washed protein precipitate prepared from ~0.1 mL of SCN, 1.0 mL of NaOH 0.6 mol/L (precipitate dissolution), 5.0 mL of water (quantitative transfer of alkali-dissolved TP into MKF), 0.50 g of powdered catalyst and 1.0 mL of concentrated sulphuric acid. Blanks contained 0.1 mL of water instead of a sample.

Results and Discussion

Manual visual titration and automatic potentiometry

We prepared and standardized manually and visually three sulphuric acid titrants ~0.005 mol/L. Found standard deviations of all titres were uniformly ~0.003 and blanks consumed ~0.05 mL of titrant. To compare precision of manual/visual versus automatic potentiometric titration, we prepared solution of ammonium dihydrogen borate ~0.01 mol/L and titrated its 5-mL aliquots ten times with the third titrant using both visual

and potentiometric indications. The standard deviation from potentiometry was only a half of those one found in the visual titration. However, the difference between both average consumptions of titrant was only negligible 0.002 mL, which testifies that the end-point colour change of used acid-base indicator is practically identical with the equivalent point in potentiometry. Nevertheless, an automatic titration could slightly improve the precision of the whole titrimetry, i.e. both the standardization of titrant and acidimetric quantification of the ammonia.

Digestion and distillation

Modified Kjeldahl flask and still unit now enables to connect MKF with complete and diluted digest directly to the still and omit delicate transfers of digests and/or ammonium sulphate standards into initially one-piece still through its sampling funnel. Because initially used steamer and steam input to the still remained unchanged [7], our modification of still bottom part did not change the speed of distillation and the final volume of distillate was the same ~10 mL as found previously [7].

Even though a preheating temperature and time ~150 °C and 10 min, digestion temperature ~(385±2) °C and the composition of digestion mixture remained the same [7], changed dimensions of MKF and aluminium block and vertical position of MKF in aluminium block affected the heat transfer to a digestion mixture and reduced the length of digestion time. We repeated digestion of both glycine and isolated protein precipitates. The digestion in a shorter and wider MKF without a reflux funnel was too violent, caused scattered results and accelerated the solidification of still warm digest. Originally used Kjeldahl flask [7], (a bulb with longer and narrower neck, which was placed in block port askew), slowed down digestion and its inclined position acted against a loss of boiling mixture.

The digestion in MKF with inserted reflux funnel also slowed down the digestion and functioned similarly/flawlessly as a longer flask used initially. The digestion of glycine is now completed ~15 min from the start at a temperature of about 380 °C. The digestion of TP needs a higher temperature ~385 °C and is completed ~25 min from the start. We determined a digestion time in MKF, (usable for both glycine and TP), as ~50 min from the start, i.e., when temperature was set from ~150 °C (preheating) to the final ~385 °C (digestion).

Verification of simplified Kjeldahl apparatus

We repeated quality tests described already in [7]. Verification of distillation and titration steps was realized with ammonium sulphate. The recovery of ammonium sulphate found previously was 99.8 %. In modified Kjeldahl equipment, we recovered (100.1±0.2) %, which satisfies the limit "better than 99.5 %" claimed for industrial KMs by Parsson et al. [11]. In the verification of digestion-distillation-titration steps with glycine, we now recovered (100.1±0.3) % of glycine. Previous glycine recovery was 100.0 % [7]. Both recoveries "lies within ±1 % relative" specified also by Parsson for glycine quantification by KM.

An overall verification of modified KM was made with SCN, which we now used as our in-house reference material. We recovered (64.2±0.2) g/L of TP, which corresponds closely with our previously Kjeldahl-found TP ~64.05 g/L [7]. Good concordance in recoveries of both chemical and protein standards is also an indirect proof of the correct standardization of used titrant.

Validation of simplified Kjeldahl method

We repeated and re-validated all basic performance parameters of modified /simplified KM. Their comparison with previously assessed analytical performance data shows Table 1. Previously assessed combined uncertainty (u_C) was calculated from eight individual uncertainties selected and summarized in the uncertainty budget in [7] as: $u_C = \sqrt{(u_1^2 + \ldots + u_8^2)} = 0.47$ %.

Combined uncertainty, presented in this study, was calculated using the coefficient of variation (CV) of 34 analyses of our in-house RM, which were performed during 34 weeks as: $u_C = 1.96$ CV = 0.52 %. Both combined uncertainties are practically the same.

We also repeated the calibration and TP-recovery with two protein solutions prepared from a 5-mL freeze-dried serum control N (SCN) dissolved either in originally prescribed 5 mL of water (High solution, marked as SCN) or in 25 mL of water (Low solution, marked as Solution A). We proceeded as described in [7]. Each calibrator was analyzed in triplicate (Table 2). Originally found [7] TP in SCN 64.05 g/L was used to calculate TP in all five calibrators. Newly assessed performance parameters are in good concordance with data found in [7].

The blanks reflect a titrant consumed by air-present ammonia absorbed in water and reagent solutions, by the ammonia from N-impurities in used chemicals and by the volume of titrant necessary to induce a colour change of visual acid-base indicator. Blanks found in individual Kjeldahl steps with 0.005 mol/L titrant differed form the least 0.05 mL (visually assessed titre) up to 0.20 mL (TP precipitation, dissolution, digestion, distillation and titration). A 0.10 mL blank corresponds to 0.14 mg N, which equals to serum TP ~0.9 g/L.

To facilitate a new analyst with presented simplified KM, we describe and summarize visual changes proceeding in a digestion mixture with isolated TP. MKF with inserted reflux funnel was put for 10 min into the block preheated to ~150 °C. Within ~6 min, water droplets condensed inside of MKF. After 10 min, block temperature was rised from ~150 (preheating) to the final ~385 °C, (the start of digestion), and digest started to boil at ~210 °C, see Table 3.

The essential difference in a digestion of pure glycine and a real TP consists in their different behaviour. While a slower and longer digestion of TP is always accompanied by charring carbohydrates and its finish is signalized by so-called point-of-clearing, glycine digests to the ammonia in a clear solution and its finish is accompanied only by fumes of SO3. However, prolonged overall digestion time ~50 min secures the complete digestion of both glycine standard and TP without any distinct differences in resulting TP.

Table 1 Validation parameters

Parameter	Found previously [7]	Newly assessed
LOD / LOQ (g/L) [a]	0.03 / 0.09	0.03 / 0.10
MR / IMP (%) [b]	0.65 / 0.65	0.58 / 0.60
Glycine bias (%)	0.16	0.12
Serum Control N (g/L)	64.05	64.18
Calibration curve [c]	y = 0.9952x + 0.37	y = 0.9979x + 0.42
TP bias (g/L)	0.37	0.42
Correlation, R^2	0.9999	0.9999
Combined uncertainty (%)	0.47	0.52

[a] Lower limit of detection / quantification; [b] Measurement-repeatability / intermediate-measurement-precision; [c] Calculated (y) vs. found TP (x), see Table 2.

Table 2 Recovery of total protein (g/L)

mL			TP (g/L)		
No	**SCN**	**Solution A**	**Calculated**	**Found [7]**	**Newly found**
1	2 x 0.100	-	128.4	128.0	127.9
2	1 x 0.100	2 x 0.100	89.9	89.9	89.5
3	1 x 0.100	-	64.2	63.9	64.2
4	-	3 x 0.100	38.5	37.9	37.7
5	-	1 x 0.100	12.8	12.7	12.7

Calibration curves are specified in Table 1.

Table 3 Visual changes in TP digests with increasing time and temperature

Visual change	Time (~min) /Temperature (~ºC)
Digest boils, water evaporates	6 / 210
Turbidity + slight SO3 fumes	14 / 317
Charring carbohydrates	18 / 340
Massive SO3 fumes	21 / 350
Digest starts to clear	35 / 380
Pale-green digest is clear	40 / 385
Digest stays clear and still	40-70 / 385 *

Temperature was measured in aluminium block; Temperature was after 10 min turned from 150 to 385 ºC – the start of digestion;
* Final digestion temperature fluctuates within ±2 ºC.

Conclusions

1. Chromý et al. proved [1], that the use of artificial albumin-based calibrators, together with a biuret reagent developed for TP by working group of Doumas et al. [2-4], provide TP with 3-5 % unacceptable positive bias overlapping the total error of 3.4 % reserved now for the TP analyses in serum. The simplest way to remedy this fault was the use of human-based serum/plasma standards certified by the Kjeldahl method.
2. We reviewed the Kjeldahl methods adopted for TP by laboratory medicine [6], selected and completed the direct KM on separated and washed protein precipitates and verified, that selected KM is suitable as a primary reference procedure for TP in reference materials [7].
3. In this study, we further simplified selected KM and re-validated its analytical performance parameters which are practically identical with data found previously [7]. This and foregoing article [7] were used to devise detailed SOP according to EN ISO 15193 now mandatory for reference laboratories [5].
4. We are prepared to apply for an accreditation of our university laboratory as a reference laboratory for TP in standards used in clinical chemistry. We are also prepared to assist to any laboratory, which would be interested in the implementation of the direct KM and to send them our SOP, which is now in the form of controlled document consisting of 21 pages, 20 paragraphs and three pictures.

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