Research Square

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Measurement uncertainty for the quantification of serum CysC by ID-LC-MS/MS method

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Abstract

Objective

Taking Cystatin-C (CysC) measurement uncertainty (MU) evaluation as an example to help researches to better understand the accuracy and limitations of results obtained by ID-LC-MS/MS method.

Methods

According to GUM principle, we summarized the MU evaluation process in the following four steps:1) Specification of the measurand, which is the mass concentration of CysC in serum. Then set a model equation between the measurand and everything important that affects it; 2)Identification of the uncertainty sources, we took pre-analytical (high-abundance protein depletion, trypsin digestion and solid-phase extraction) and analytical (calibration curve establishment and measurement imprecision) phases uncertainty sources into account; 3) Quantification and standardization of the uncertainty components, the detailed evaluation of the uncertainty components is either based on statistical methods (type A) or other methods (type B) and requires that the analyst makes assumptions regarding the underlying distribution (rectangular, triangular, normal, etc.); 4) Calculation of the combined standard uncertainty (u_c) and expanded uncertainty (U, k = 2) .

Results

The relative uncertainty of $u_{RE\text{protein}}$%, $u_{\text{calibration-curve}}$%, $u_{RE\text{hydrolysis}}$%, $u_{RE\text{SPE}}$%, u_{imp}% are 0.64%, 4.16%, 2.04%, 0.30% and 0.73% for y^9, and 0.64%, 1.23%, 2.24%, 0.27% and 0.77% for y^6. The u_c are 4.77% and 2.79% for y^9 and y^6, respectively. And the U are 9.54% and 5.58% (k = 2) for y^9 and y^6, respectively.

Conclusion

The two major contributions to total uncertainty arise from trypsin digestion and calibration curve, which account for 78.9% and 67.9% for y^9 and y^6, respectively. So we should strictly control the two processes when quantification of serum CysC by ID-LC-MS/MS.

1.Introduction

With the development and standardization of clinical medicine, high-quality test results are expected, which is a challenge to the medical laboratory. Although the method validation demonstrates the reliability of the results, it's not sufficient to correctly interpret and compare the results[1]. Measurement Uncertainty (MU) provides a quantitative assessment of the quality of a test result. The current international standard ISO 15189 has a tighter requirement, instructing us to consider "measurement

uncertainty of measured quantity values”[2]. The International Vocabulary of Metrology (VIM) defines “measurement uncertainty” as a “non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used”[3]. A number of parameters can contribute to making measurements uncertain. Causes of measurement uncertainty can occur at any stage of the laboratory process, such as pre-analytical, analytical or post-analytical. In 2008, the Working Group 1 of the JCGM (JCGM/WG1) published the latest version of the Guide to the Expression of Uncertainty in Measurement (GUM)[4], which is considered by the metrology global organizations as the seminal guide to evaluate measurement uncertainty. However, the concept of MU is still relatively new in the field of quantity measurement, particularly in medical laboratory. Therefore, practical examples should be highlighted the evaluation and expression of uncertainty. Some examples, evaluation of MU of calcium[5] and glucose[1, 5] in serum based on the GUM have been published.

Nowadays, ID-LC-MS/MS based methods become more and more popular for proteins quantification, because they do not require specific antibody and can provide reliable and robust results[6]. But these kinds of methods contain complex sample pretreatment process, high-abundance protein depletion[7, 8], trypsin digestion of target protein [9, 10]and solid phase extraction[11, 12], then followed by quantification of selected surrogate peptides[6, 13]. All of these processes are difficult to standardize and consistent, which makes quantification results obtained at different times or by different laboratories incomparable. So evaluation of related MU is essential when quantification of serum proteins by ID-LC-MS/MS method.

In this study, we chose the measurement of serum Cystatin C (CysC) to demonstrate the application of uncertainty in ID-LC-MS/MS tests. Following the evaluation procedures recommended by GUM (2008), we identified the sources of uncertainty, evaluated the components and combined them. We hope that this can help researches to better understand the accuracy of results and to fully reflect and appreciate the limitations of serum proteins quantification by ID-LC-MS/MS method.

2. Materials and methods

2.1 Reagents and materials

All reagents were ACS grade or higher, all solvents used, including water, were LC/MS grade. Urea, dithiothreitol (DTT), iodoacetamide (IAM), trifluoroacetic acid (TFA), ProteoPrep®Blue Albumin & IgG Depletion Kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-modified grade trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile and methanol were purchased from J. T. Baker®Chemicals (Center Valley, PA, USA). Ammonium bicarbonate (ABC) and formic acid (FA) were purchased from Fluke (St. Louis, MO, USA). Water was obtained from a Milli-Q system (Millipore Co., Bedford, USA). Sep-Pak C18 cartridges (200 mg, 3 mL) were purchased from Waters (Saint-Quentin, France). Recombinant Human Cystatin C 13.3-kDa was obtained from BBI Solutions (Cardiff, UK). The natural abundance peptide ALDFAVGEYNK (amino acids 26–36 of CysC) was purchased from ChinaPeptides Co., Ltd. (Shanghai, China). The isotopically enriched peptide labelled in

^{13}C and ^{15}N atoms A[$^{13}C_6{}^{15}N$]LDFAVGEYNK ($^{13}C^{15}N$-CysC peptide) was synthesized by using universally labelled leucine from ChinaPeptides Co., Ltd. (Shanghai, China).

2.2 Preparation of peptides solutions, CysC protein solution and calibration standards

Surrogate peptide (ALDFAVGEYNK) solution: used 1mL 30% ACN in H_2O to dissolve surrogate peptide powder, making the storage solution of surrogate peptide was 1mg/mL, then diluted to 200mg/L, 10mg/L and 1mg/L three kinds of working solutions.

Internal peptide (A[$^{13}C_6{}^{15}N$]LDFAVGEYNK) solution: used 1mL 30% ACN in H_2O to dissolve internal peptide powder, making the storage solution of internal peptide was 1mg/mL, then diluted to 1mg/L working solution.

CysC protein solution: 1 mg/mL CysC stock solution was prepared by dissolving the protein in water according to the manufacture's protocol. Then diluted to 5mg/L working solution. The solutions were stored at -70℃.

Calibration standards: CysC surrogate peptide solution was diluted to final concentrations of 0.0, 0.01, 0.025, 0.05, 0.1875, 0.3, 0.75, 1.0 and 1.5 mg/L in the presence of a fixed amount of internal standard (1mg/L, 100μL).

2.3 Established calibration curve

To determine the concentration of CysC surrogate peptide derived by trypsin digestion of serum samples, a calibration curve ($y = ax+b$) was generated. Calibration standards were generated where the pure CysC surrogate peptide solution was diluted to final concentrations of 0.0, 0.01, 0.025, 0.05, 0.1875, 0.3, 0.75, 1.0 and 1.5 mg/L in the presence of a fixed amount of internal standard (1mg/L, 100μL). The ID-LC-MS/MS analysis was performed for each data point under the same conditions described above. The statistical analysis of the calibration curve was processed by MedCalc 15.2 software. Then the concentration of CysC in human serum was deduced by using the peak area ratio between the endogenous CysC surrogate peptide derived by trypsin digestion and the internal standard based on the calibration curve.

2.4 Serum depletion and Urea Denaturation Protocol

Albumin and IgG were depleted from the samples (100μL) by using the ProteoPrep®Blue Albumin and IgG Depletion Kit according to the manufacturer's protocol. Then 180μL of each depleted sample was mixed with 70μL 25mM Ammonium bicarbonate solution (ABC). For Recombinant Human CysC protein, each 100μL CysC protein solution (5mg/L) was mixed with 150μL 25mM ABC. When carbamylate primary amines exposed to heat, the serum samples were heat-denatured prior to urea denaturation to prevent degradation of the urea into cyanate ions. This sample was allowed to cool on ice for 5min,

followed by chemical denaturation with about 96.1mg urea (8M). Approximately 5min later, the sample was diluted with 250μL 25mM ABC.

2.5 Reduction, Alkylation, and Digestion

All the denatured samples (serum samples and Recombinant Human CysC protein samples)were reduced by adding 100mM DTT (to a final concentration of 10mM) and incubated at 60℃ for 30min. Then the sample was alkylated by adding 550mM IAM (to a final concentration of 50mM) and incubated at room temperature for 30min in dark. To quench the alkylation, 185μL 100mM DTT was added, and this was allowed to react for 30min at room temperature. The modified sequencing grade trypsin was added to reduce and alkylate samples to give a 20:1 mass ratio of protein and enzyme, then samples were digested at 37℃ for 4h. The reaction was stopped by adding 35μL 5% TFA (to a final concentration of 0.1%).

2.6 Solid phase extraction by Sep-pak C18

The sorbent was conditioned with 3mL of MeOH followed by equilibration with 3mL of H_2O. 1mL surrogate peptide solution (1mg/L) or 1mL 30% ACN in H_2O were loaded onto the sorbent. The sorbent was washed with 3mL H_2O. The final elution with 1mL of 75% MeOH was carried out. The eluate was dried down and reconstituted in 1mL of 0.1%FA in H_2O, then 5μL 30% ACN in H_2O and 5μL surrogate peptide solution (200mg/L) were spiked into extracted sample (1mg/L surrogate peptide solution) and matrix (1mL 30% ACN in H_2O) after the extraction procedure, respectively.

Trypsin digestion serum samples: The sorbent was conditioned with 3mL of MeOH followed by equilibration with 3mL of H_2O. Then the trypsin digestion serum samples were loaded onto the sorbent. The sorbent was washed with 3mL H_2O. The final elution with 1mL of 75% MeOH was carried out. The eluate was dried down and reconstituted in 0.4mL of 0.1%FA in H_2O.

2.7 LC-MS/MS conditions

Tandem mass spectrometry was performed using a AB SCIEX TRIPLE QUAD™ 5500 tandem quadrupole MS system (AB Sciex Pte. Ltd, USA) with positive ion electrospray ionization (ESI). AB Sciex MultiQuant Software (version 3.0.2) was used for data acquisition. The LC system was a Shimadzu Nexera X2 UPLC system (Shimadzu Corporation, Japan). Columns used a 3.5μm, 2.1×150mm SymmetryShieldTM RP C18 column (from Waters Corp., Milford, MA) thermostated at 40℃. Flow rates was 0.2 mL/min. Solvent A was 0.1% FA in water, solvent B was 0.1% FA in ACN. A solvent gradient (A/B) was applied, with 5% B for 1 min, from 5 to 35% B for 17 min, from 35 to 80% B for 4 min, 80% B for 5 min, from 80 to 5% B for 0.1 min, and 5% B for 2.9 min. Injection volume was 5μL, depending on the sample loop installed. The LC flow was directly introduced into the MS source without flow splitting. The mass spectrometer was run under multiple reaction monitoring (MRM) mode with quadrupole mass filters Q1 and Q3 at unit resolution. Fragmentation was induced by using nitrogen under a high-pressure setting with collision energies of 25 eV and declustering potential (DP) of 80 V. The ESI source was operated under 5.5 kV ionsprayer voltage and 550℃ dry gas temperature. Other parameters were optimized to obtain

maximum signal intensities for two peptides. The data were collected and processed by using the AB Sciex MultiQuant Software (version 3.0.2).

3. Results

In this paper, we took into account pre-analytical (sample pretreatment) and analytical uncertainty sources for serum CysC quantification by ID-LC-MS/MS method. And we evaluated uncertainty following the GUM Guide, which summarizes the evaluation process in the following four steps[4, 5]:1) Specification of the measurand; 2)Identification of the uncertainty sources; 3) Quantification and standardization of the uncertainty components; 4) Calculation of the combined standard uncertainty (u_c) and expanded uncertainty (U) .

Step 1-Specification of the measurand

The measurand is specified as the mass concentration (mg/L) of CysC in human serum. The uncertainty concept also requires a functional relationship (model equation) between the measurand and everything important that affects it. Serum CysC quantification is based on calibration curve method[14], so the values are calculated by the following model equation.

$$C_{CysC} = \left(a \times \frac{S_{SP}}{S_{IS}} + b \right)$$

Where C_{CysC} is the mass concentration of CysC in human serum, mg/L. a and b are the slope and intercept of the calibration curve, respectively. S_{SP} and S_{IS} are the peak areas of CysC surrogate peptide and internal peptide, respectively.

Step 2-Identification of the uncertainty sources

In this step, we identify and list all important uncertainty sources. The main sources of uncertainty are analyzed in Fig. 1 and are shown to come from pre-analysis and analysis. Every input quantity in the model equation above, i.e. a, b, S_{SP}, S_{IS}, has an uncertainty associated with its value and contributes to the total uncertainty. Input quantities a, b, S_{SP} and S_{IS} refer to the analytical phase. ID-LC-MS/MS based method to quantify the concentration of serum CysC also requires complex sample pretreatment process, which includes high-abundance depletion, trypsin digestion of CysC protein and solid phase extraction of CysC surrogate peptide, then followed by quantification of selected surrogate peptide. So we should also include the uncertainties arising from the sample pretreatment process. Then the model can be modified as follow:

$$C'_{CysC} = \left(a \times \frac{S_{SP}}{S_{IS}} + b \right) / RE_{protein}\% / RE_{hydrolysis}\% / RE_{SPE}\%$$

Where $RE_{protein}$% is the recovery rate of CysC protein in serum after high-abundance depletion. $RE_{hydrolysis}$% is the trypsin digestion efficiency of CysC protein. RE_{SPE}% is the recovery rate of surrogate

peptide extracted by solid phase extraction.

Step 3-Quantification and standardization of the uncertainty components

The information about the uncertainty sources and their contribution comes from within the laboratory (method development, validation and new and previous measurements), from manufacturers of instruments and equipment (specifications and test reports), from certificates (e.g. these for the calibrators and reagents), and/or from the scientific literature. Before the calculation (Step 4), all uncertainty contributions must be expressed as relative standard uncertainties (relative standard deviations). The detailed evaluation of the uncertainty components is either based on statistical methods (type A) or other methods (type B) and requires that the analyst makes assumptions regarding the underlying distribution (rectangular, triangular, normal, etc.)[5].

1)Uncertainty of recovery rate of CysC protein

High abundance proteins Albumin and IgG were depleted from the serum samples (100μL) by using the ProteoPrep®Blue Albumin and IgG Depletion Kit according to the manufacturer's protocol. 100μL serum was sampling by pipettor (10–100μL), which expanded uncertainty *U* is 0.5% (*k* = 2) provided by calibration certificate. So the relative standard uncertainty of sampling u_{sampling}% is 0.25%. For each serum sample, we conducted high-abundance protein depletion experiment for three times, then we detected the CysC concentration after high abundance protein depletion by ADVIA 2400, 3 times per depletion sample. The average recovery rate is 52.00%, and its related impression is 0.59%. So the uncertainty of recovery rate of CysC protein can be calculated as follow:

$u_{RE_{protein}}\% = \sqrt{\left(u_{sampling}\%\right)^2 + CV^2_{RE_{protein}}}$, then the %$u_{RE\text{protein}}$ is 0.64%. The bias caused by high-abundance protein depletion should be corrected.

2)Uncertainty of calibration curve

To determine the concentration of CysC surrogate peptide derived by trypsin digestion of serum samples, a calibration curve ($y = ax+b$) was generated. Calibration standards were generated where the pure CysC surrogate peptide solution was diluted to final concentrations of 0.0, 0.01, 0.025, 0.05, 0.1875, 0.3, 0.75, 1.0 and 1.5 mg/L in the presence of a fixed amount of internal standard (100μL, 1mg/L). The ID-LC-MS/MS analysis was performed for each data point under the same conditions described above. The area ratios (y) of CysC surrogate peptide and internal peptide was plotted against the concentration of CysC surrogate peptide (x), and a linear response between 0.0 and 1.5 mg/L was observed, which showed $y = 5.08x + 0.018$ ($R^2 = 0.999$) for y^9, $y = 5.61x-0.047$ ($R^2 = 0.999$) for y^6. The uncertainty of linearity %u_{L} can be calculated as follow:

$$u_L\% = \sqrt{\left(s_a\%\right)^2 + \left(s_b\%\right)^2 + 2r_{a,b} \times s_a\% \times s_b\%}$$

Where S_a% and S_b% are the uncertainty of slope and intercept of the linearity equation, respectively. $r_{a,b}$ is the correlation coefficient of the slope and intercept. S_a%, S_b% and $r_{a,b}$ can be calculated as follows[15, 16]:

$$s_{y/x} = \sqrt{\sum_{i=1}^{n} e_i^2 / (n-2)}\,,\ s_a\% = s_{y/x}\sqrt{\frac{\sum_{i=1}^{n} x_i^2}{n\sum_{i=1}^{n}(x_i-\bar{x})^2}}/a \times 100\%,$$

$$s_b\% = \frac{s_{y/x}}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2}}/b \times 100\%\,,\ r_{a,b} = -\bar{x}\frac{s_b\%}{s_a\%}$$

Where $S_{y/x}$ is the residual standard deviation; e_i is the residual; n is the numbers of CysC surrogate peptide concentration, which is 10; a is the slope; b is the intercept; $\bar{x}$ is the average concentration of CysC surrogate peptide.

Then the uncertainties of u_L% are 4.15% and 1.18% for y^9 and y^6, respectively.

The purity of CysC surrogate peptide and internal peptide are 99.67% and 99.23%, respectively. So the bias are 0.33% and 0.77%, respectively, which considering follow uniform distribution, so the uncertainty of surrogate peptide purity is equal to 0.33%/$\sqrt{6}$=0.13%, and the uncertainty of internal peptide purity is equal to 0.77%/$\sqrt{6}$=0.31%. Then the combined uncertainty of purity is 0.34%.

So the uncertainty of the calibration curve can be calculated as follow:

$u_{calibration-curve}\% = \sqrt{(u_L\%)^2 + (u_{purity}\%)^2}$, then the $u_{\text{calibration-curve}}$% are 4.16% and 1.23% for y^9 and y^6, respectively.

3) Uncertainty of trypsin digestion of CysC protein

We used Recombinant Human CysC pure protein to study the trypsin digestion efficiency. The enzymatic digestion conditions were optimized, it was found that the digestion efficiency remained stable after 4 h of digestion when the mass ratio of enzyme and substrate was great than 1:50 at 37°C. We conducted trypsin digestion for three times, then we detected the peak areas of CysC surrogate peptide and internal peptide, and calculated the actual concentration of CysC according to the calibration curve. $RE_{\text{hydrolysis}}$% can be calculated as follow: $\%RE_{hydrolysis} = \frac{C_{CysC-actual}}{C_{CysC-theoretical}} \times 100\%$, the average $RE_{\text{hydrolysis}}$% are 82.5% and 84.7% for y^9 and y^6, respectively, and their related impression are 1.23% and 1.54%, respectively. The purity of Recombinant Human CysC is greater than 96% according to certificate, so the biggest bias is 4%, which considering follows uniform distribution, so the uncertainty of the purity is equal to 4%/$\sqrt{6}$=1.63%. So the uncertainty of trypsin digestion of CysC protein can be calculated as

follow: $u_{RE_{hydrolysis}}\% = \sqrt{(u_{purity}\%)^2 + CV^2_{RE_{hydrolysis}}}$, then the $u_{RE\text{hydrolysis}}$% are 2.04% and 2.24% for y^9 and y^6, respectively. The bias caused by trypsin digestion should be corrected.

4)Uncertainty of CysC surrogate peptide extracted by SPE

In order to know the recovery rate and its related measurement uncertainty of CysC surrogate peptide extracted by Sep-Pak C18 cartridge, we set up extracted samples and post-extracted spiked sample, respectively, each of them were three, and every sample were measured three times. Then Sep-Pak C18 cartridges were used to extract these samples according to section 2.6. Then a full scan LC-MS/MS analysis was performed with the surrogate peptide. The recovery rate were calculated as follow:

$$RE_{SPE}\% = \frac{\text{Re}\,sponse_{extracted\ sample}}{\text{Re}\,sponse_{post-extrated\ spiked\ sample}} \times 100\%$$

Where $\text{Response}_{\text{extracted-sample}}$ is the average peak area count for the CysC surrogate peptide in solution (1mL surrogate peptide solution, 1mg/L), which has been through the extraction process. $\text{Response}_{\text{post-extracted spiked sample}}$ is the average peak area count for the same quantity of surrogate peptide (5μL surrogate peptide solution, 200mg/L) spiked into extracted matrix (1mL 30% ACN in H_2O) after the extraction procedure.

Results showed that the maximum RE_{SPE}% were 89.61% and 87.72% for y^9 and y^6, respectively. And the imprecision were 0.27% and 0.24% for y^9 and y^6, respectively. The purity of the surrogate peptide is 99.67%, so the bias is 0.33%, which considering follow uniform distribution, so the uncertainty of the bias is equal to 0.33%/$\sqrt{6}$=0.13%. So the uncertainty of SPE can be calculated as follow: $u_{RE_{SPE}}\% = \sqrt{(u_{purity}\%)^2 + CV^2_{RE_{SPE}}}$, then the $u_{RE\text{SPE}}$% are 0.30% and 0.27% for y^9 and y^6, respectively. The bias caused by SPE should be corrected.

5) Measurement impression

The imprecision of the assay were assessed by observing the response of human serum samples with the concentrations of CysC at 3.5mg/L. The intra- and inter-day imprecision were expressed as coefficient of variation (*CV*). We detected 3 times per day and lasted for 3 days, the intra-day imprecision were 0.71% and 0.73% for y^9 and y^6, respectively. The inter-day imprecision were 0.16% and 0.26% for y^9 and y^6, respectively. So the measurement impression can be calculated as follow:

$$u_{imp}\% = \sqrt{(CV_{\text{int}\,ra-day})^2 + (CV_{\text{int}\,er-day})^2}$$

And the u_{imp}% were 0.73% and 0.77% for y^9 and y^6, respectively.

Step 4 - Calculation of the combined standard uncertainty and expanded uncertainty

In the last step of the evaluation, the value of the measurand and the combined standard uncertainty (u_c) are calculated from the model. The value for u_c is obtained by propagating the individual uncertainty contributions according to mathematical rules [4, 17]. Expanded uncertainty (U) was obtained by multiplying u_c by a coverage factor k (k = 2), which yields a confidence level of approximately 95%. The values of u_c and U are shown in Table 1. And the major contribution to uncertainty arises from the trypsin digestion and the calibration curve.

Table 1
Summary of uncertainty

Sources of component	**Quantities**	**Relative uncertainty (%)**	
		y^9	y^6
High-abundance peotein depletion	$u_{RE\text{protein}}$%	0.64	0.64
Calibration curve	$u_{\text{calibration-curve}}$%	4.16	1.23
Trypsin digestion	$u_{RE\text{hydrolysis}}$%	2.04	2.24
SPE	$u_{RE\text{SPE}}$%	0.30	0.27
Measurement impression	u_{imp}%	0.73	0.77
Combined uncertainty (u_c)		4.77	2.79
Expanded uncertainty U(k = 2)		9.54	5.58

4.Discussion

All measurements are imperfect and have many potential sources of variation. An estimate of measurement uncertainty (MU) provides an interval of values within which the true value is believed to lie with a stated probability, and is therefore a quantitative indication of the reliability of a measurement. MU estimates are essential for assessing whether methods are suitable for clinical use and for comparison of results of a similar type. MU estimates can help identify method limitations and opportunities for improvement[18]. So the current ISO 15189 edition (2012) requires its determination and also recommends that laboratories define the performance requirements for measurement uncertainty and regularly review their estimates of measurement uncertainty[2]. For many years the error concept has been widely used to quantify measurement unreliability and to set quality goals for methods. A limitation of the approach is that by adding bias and imprecision total error calculates the worst case error for the procedure, and does not recognise that individual results may have less error. In the 1990s it was recognised that measurement comparability between laboratories and methods required an internationally agreed approach to estimating and expressing measurement uncertainty,

which is described in the “Guide to the Expression of Uncertainty in Measurement” (GUM). Although GUM is primarily for measurements in physics, the principles are applicable to biological and chemical measurements. In practice, bias correction and replicate measurements can reduce, but not completely eliminate systematic and random errors, and therefore total error cannot be exactly known. It follows that the true value of a measured quantity cannot be exactly known either. This assumption is fundamental to the MU approach. The MU concept also assumes that if the bias of a procedure is known, then steps are taken to minimise it, e.g. by re-calibration. However, because the bias value cannot be known exactly, an uncertainty will be associated with such a correction. Thus the MU approach considers a single measurement result to be the best estimate of the measured quantity, and centred on the combined standard uncertainty provides an interval of values within which the true value of the measured quantity is believed to lie, with a stated coverage probability[20]. The GUM describes a bottom-up approach to estimating MU, by which an uncertainty budget for a given measurement procedure is assembled by identifying all potential sources of uncertainty (e.g. calibration, weighing, pipettings, temperature and instrument fluctuations) and attributing to each an uncertainty estimate as an SD obtained by experiment or from available information. The contributing uncertainties are combined in a mathematical model that best represents their interactions in the measurement process. The combining calculation yields the estimated combined standard measurement uncertainty for the whole procedure (u_c)[20].

The global metrology organizations, such as the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), as well as the ISO, recommend that MU shall be determined with GUM methods. Also in the medical laboratory field, The Australian National Pathology Accreditation Advisory Council (NPAAC) guideline (2007) recommends a set of GUM empirical approaches [19], and the Clinical and Laboratory Standards Institute (CLSI) C51-A guideline (2012) features GUM modular and empirical approaches[20].

To fully reflect and appreciate the limitations of serum CysC quantification by ID-LC-MS/MS method, it’s essential to determine the MU associated with the assays. Knowledge of MU will also facilitate the comparison and interpretation of results generated from different laboratories. In this study, we took pre-analytical (sample pretreatment) and analytical uncertainty sources into account for evaluation of MU of serum CysC by ID-LC-MS/MS method. Sources of high-abundance protein depletion, trypsin digestion and solid-phase extraction refer to the pre-analytical phase, calibration curve establishment and measurement imprecision refer to analytical phase. According to GUM (2008) principle, We quantified every uncertainty component, and calculated of the combined standard uncertainty and expanded uncertainty. Results shown that the major contribution to total combined uncertainty arises from the trypsin digestion and the calibration curve, which account for 78.9% and 67.9% for y^9 and y^6, respectively. Since the two processes have significant effect on the accurate quantification of serum CysC by ID-LC-MS/MS, we should strictly control the two processes. The bias mainly caused by pre-analytical phase, which is 48.0% arises from high-abundance protein depletion, and are 17.5% and 15.3% arises from the trypsin digestion for y^9 and y^6, respectively. And are 10.39% and 12.28% arises from the SPE for y^9 and

y^6, respectively. The measurement uncertainty principle is that bias should be corrected, and calculated its related uncertainty.

This paper gives a model for evaluation MU of serum proteins quantification by ID-LC-MS/MS method. We hope this can help researches to better understand the accuracy of results obtained by ID-LC-MS/MS method.

Declarations

Author Contribution

Ziyi Zhang and Yiwen Huang performed the measurement uncertainty calculations and wrote the first draft of the manuscript. Huoyan Ji conducted the experiments and analyzed the data. Xinlei Wang and Chen Qian conceived and designed the study. Chen Qian supervised the study and critically revised the manuscript for important intellectual content. Ziyi Zhang and Yiwen Huang contributed equally to this work. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

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Figures

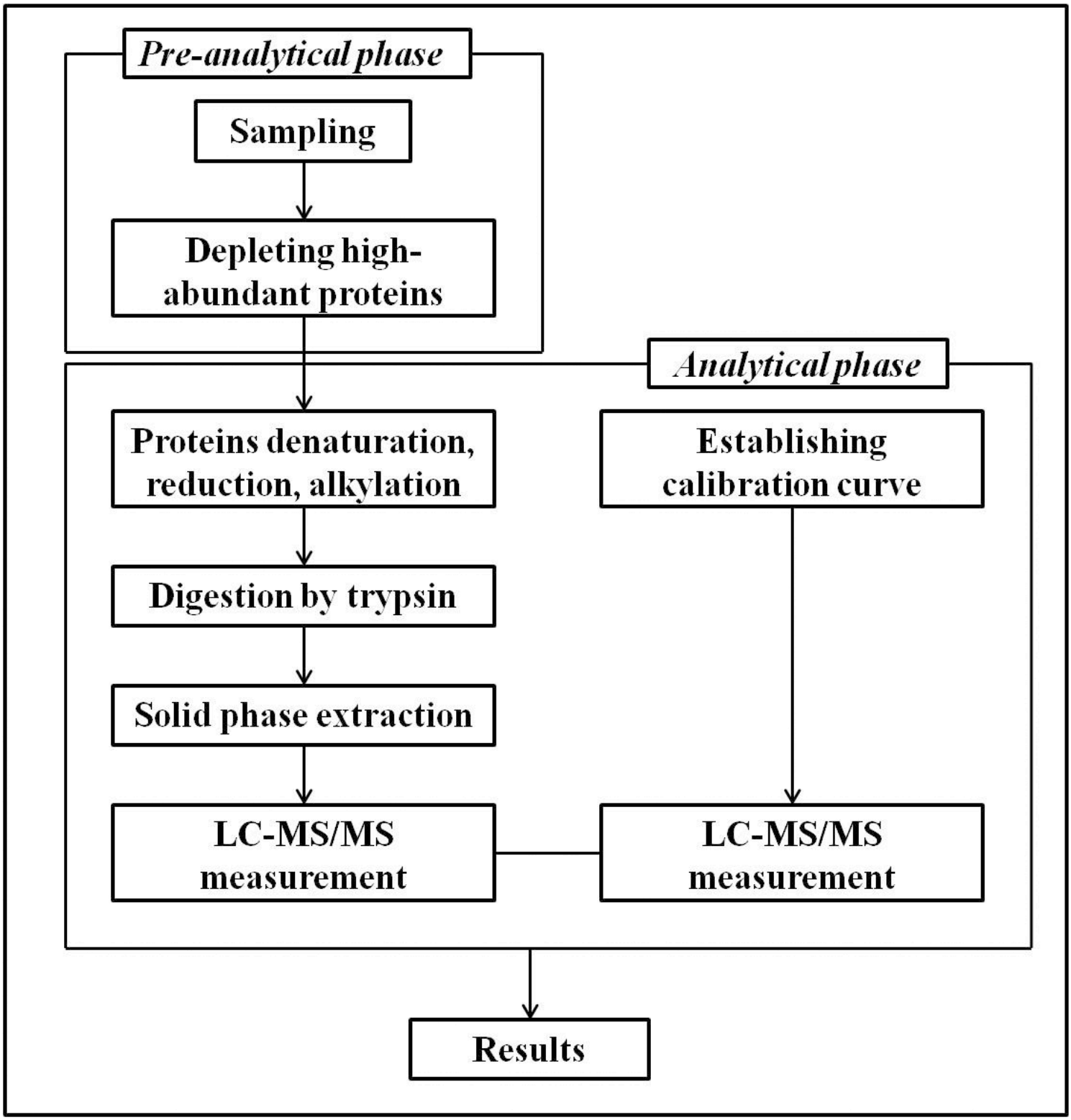


Figure 1

Overview of the measurement procedure.

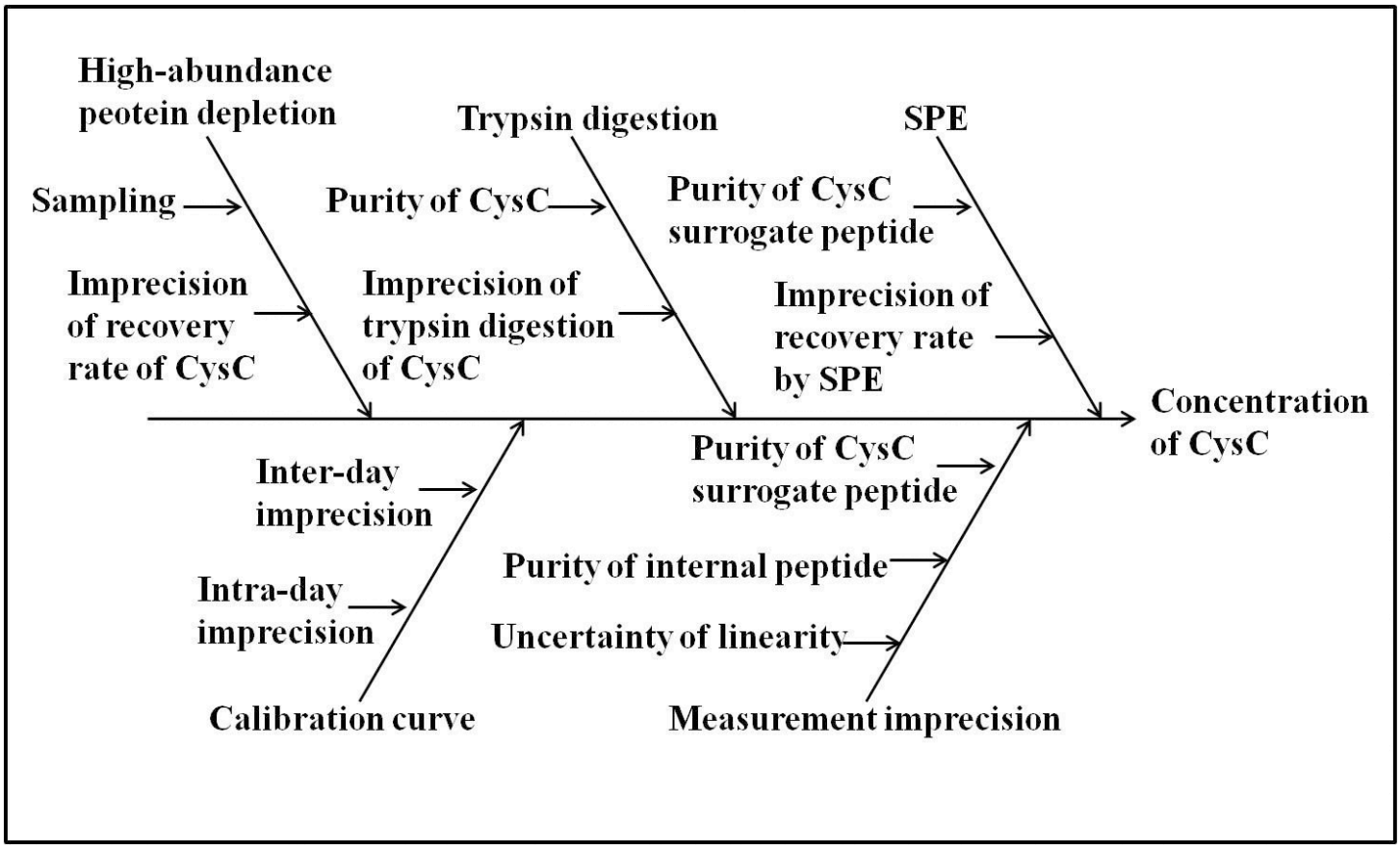


Figure 2

Cause and effect diagram for uncertainty of serum CysC measurement.

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Figure 3

Gives an overview of the contribution of each of the individual uncertainty sources.