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REGULAR ARTICLE

ESTIMATION OF UNCERTAINTY AND VALIDATION OF ANALYTICAL PROCEDURES AS A QUALITY CONTROL TOOL THE EVALUATION OF UNCERTAINTY FOR AMINO ACID ANALYSIS WITH ION-EXCHANGE CHROMATOGRAPHY – CASE STUDY

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ABSTRACT

The aim of this study was to assess the importance of validation and uncertainty estimation related to the results of amino acid analysis using the ion-exchange chromatography with post-column derivatization technique. The method was validated and the components of standard uncertainty were identified and quantified to recognize the major contributions to uncertainty of analysis. Estimated relative extended uncertainty (k=2, P=95%) varied in range from 9.03% to 12.68%. Quantification of the uncertainty components indicates that the contribution of the calibration concentration uncertainty is the largest and it plays the most important role in the overall uncertainty in amino acid analysis. It is followed by uncertainty of area of chromatographic peaks and weighing procedure of samples. The uncertainty of sample volume and calibration peak area may be negligible. The comparison of CV% with estimated relative uncertainty indicates that interpretation of research results can be misled without uncertainty estimation.

Keywords: amino acid analysis, combined standard uncertainty, expanded uncertainty

INTRODUCTION

Amino acid analysis is used in various areas of research, among others for analysis of products and components of foodstuffs and also of biotechnological or biological products (protein quality and quantity).

Even relying on the standarized and proven analytical methods does not guarantee that obtained results are reliable. A way to have required confidence in measurements is quality control: controlling all steps of performance, method validation and uncertainty estimation **(Konieczka and Namieśnik, 2009; Dobecki, 2004).** The uncertainty of measurement characterises the dispersion of the values that may be assigned to a measured value. There is a necessity to determine uncertainty as it enhances the confidence in the reliability of measurements results **(EURACHEM, 2012; ILAC, 2002; APLAC, 2010; ISO, 1993)**.

The aim of this study was to discuss and estimate the uncertainty related to the results of amino acid analysis using the ion-exchange chromatography with post-column derivatization method. The study intended to identify the uncertainty sources, evaluation of extended uncertainty and finally, to derive uncertainty budget with the aim of recognizing the major contributions to combined uncertainties associated with simultaneous determination of 17 amino acids of the acid hydrolysates of proteins.

MATERIAL AND METHODS

Amino acid analysis and validation

The hydrolysis of model protein (Bovine serum albumin) was classical liquid-phase hydrolysis in HCl solution (6 $mol.l^{-1}$) at 110^0C for 24 h **(Davidson, 2003)**. The hydrolysates were lyophilized, dissolved in an appropriate volume of dilution buffer and filtered through a 0.45 μm syringe filter before applying to the amino acid analyzer. Sulphur-containing amino acids were analysed as oxidation products (cysteic acid and methionine sulfon) obtained by performic acid oxidation followed by standard hydrolysis procedure with HCl. Amino acid analysis was done by ion-exchange chromatography with post-column derivatization with ninhydrin using an automatic amino acid analyser AAA400 [Ingos, Czech Republic] **(Ingos, 2007)**. For calibration of amino acid analyser the amino acid standard solution was used (Sigma, USA). All other analytical grade chemicals were from Sigma (USA), Fluka (Switzerland) or Applichem (Germany).

Method validation was performed according to standard validation protocols that are commonly known and can be find elsewhere **(EURACHEM, 1998; IUPAC, 2002; Reason, 2003)**.

Uncertainty sources identification

Identified uncertainty sources for examined amino acid analysis method are presented graphically on Ishikawa's cause-effect diagram.

Mathematical equations for determination of measured values and uncertainty calculation

Amino acid content in protein is defined by the equation:

$$Z_s = C_{std} \frac{Area_s \cdot V}{Area_{std} \cdot m} M_w \cdot F$$

where:

Z_S – content of amino acid in sample [%w/w],

C_{std} – amino acid concentration in analytical standard [nmol.ml^{-1}],

$Area_{std}$, $Area_S$– chromatographic peak area for analytical standard and sample respectively,

m – sample weight [mg],

V – sample volume [ml],

F – correction factor, equal to 0.0001 (value used for amino acid content expressed in % w/w),

M_w – molecular weight of amino acid.

Quantification of the uncertainty

Equation for estimation of expanded uncertainty for protein hydrolysates:

$$u_z = k \cdot Z_s \cdot \sqrt{\left(\frac{u_{C\,std}}{C_{std}}\right)^2 + \left(\frac{u_{Area\,s}}{Area_s}\right)^2 + \left(\frac{u_{Area\,std}}{Area_{std}}\right)^2 + \left(\frac{u_m}{m}\right)^2 + \left(\frac{u_V}{V}\right)^2}$$

where:

u_z – expanded uncertainty,

k – coverage factor (k=2 for level of confidence P=0.95),

Z_S – content of amino acid in sample [g per 100 g],

C_{std}, C_S – amino acid concentration in analytical standard and sample respectively [nmol.ml^{-1}],

$Area_{std}$, $Area_S$ – chromatographic peak area for analytical standard and sample respectively

m – sample weight [mg],

V – sample volume [mL].

Other abbreviations are explained in the text below.

Uncertainty components of combined standard uncertainty for amino acid analysis

- $u_{AREA\ std}$ – uncertainty of chromatographic peak area for analytical standard depends on accuracy of estimation of peak area, (which is estimated using rectangular distribution, which means that the indicated value is divided by √3) and standard deviation of peak area (in case of single-point calibration it is equal 0 for analytical standard)
- $u_{AREA\ s}$ – uncertainty of chromatographic peak area for amino acids in protein hydrolysate depends on accuracy of estimation of peak area (which is estimated using rectangular distribution, which means that the indicated value is divided by √3) and standard deviation of peak area
- $u_{C\ std}$ – uncertainty of analytical standard concentration - commercially available amino acid standard solution is usually diluted before application to chromatographic column, so its uncertainty depends on initial concentration and dilution volumes uncertainties
- u_m – weighing uncertainty depends on accuracy of weighing (which is estimated using rectangular distribution, which means that the indicated value is divided by √3) and standard deviation of sample weight
- u_V – sample volume uncertainty depends on volumetric flask volume uncertainty (estimated on the basis of accuracy specification given by the manufacturer (x) and using rectangular distribution which means that the indicated value is divided by √3), and volume uncertainty of adjustable piston pipettes *P1, P2 ... Pn,* estimated using rectangular distribution and manufacturer accuracy data (x).

Final equation for calculation of expanded uncertainty of amino acid analysis of protein hydrolysates is:

$$u_z = k \cdot Z_s \cdot \sqrt{\left(\frac{\sqrt{\left(\frac{u_{C\,0\,std}}{C_{0\,std}}\right)^2 + \left(\frac{u_{V\,0}}{V_0}\right)^2 + \left(\frac{u_{Vk}}{V_k}\right)^2}}{C_{std}}\right)^2 + \left(\frac{\sqrt{\left(\frac{Acc_{area}}{\sqrt{3}}\right)^2 + SD^2_{Area_s}}}{Area_s}\right)^2 + \left(\frac{\left(\frac{Acc_{area}}{\sqrt{3}}\right)}{Area_{std}}\right)^2 + \left(\frac{\sqrt{\left(\frac{Acc_w}{\sqrt{3}}\right)^2 + SD^2_m}}{m}\right)^2 + \left(\frac{\sqrt{\left(\frac{x_{Vf}}{\sqrt{3}}\right)^2 + \left(\frac{x_{Vp1}}{\sqrt{3}}\right)^2 + \left(\frac{x_{Vp2}}{\sqrt{3}}\right)^2 + \left(\frac{x_{Vp3}}{\sqrt{3}}\right)^2}}{V}\right)^2}$$

Uncertainty budget

On the basis of quantification of the combined uncertainty components and their contributions in combined standard uncertainty, the uncertainty budget was estimated to indicate major uncertainty components.

RESULTS AND DISCUSSION

Method validation

Calibration curves and linear range, limits of detection and quantification

Linear regression equations were determined for concentration range 0.025 – 1.25 nmol.ml^{-1}. The values of correlation coefficients for the calibration curves were ≥ 0.999 for all calibration curves. The average repeatability for each independent experimental point expressed as coefficient of variation (CV%) was 1.95 %, only incidentally exceeded 5% and was never worse than 10%. Results of the limit of detection varied in range 1.25 – 6.68 nmol.ml^{-1} (for methionine and tyrosine respectively), and limit of quanification 3.74 – 17.79 nmol.ml^{-1} (for methionine and proline respectively).

Repeatability and intermediate precision

Coefficients of variation for the repeatability of measurements among amino acids ranged from 0.99% to 5.37% with an average value of 2.78%. In case of intermediate precision the CV% ranged from 1.37% to 2.88% with an average value of 1.70%.

Uncertainty sources identification

The main components of uncertainty were identified as follows: (1) the uncertainty of commercially available analytical standard given by the supplier; (2) uncertainty of sample

preparation, which is associated with uncertainty of weighing and volumetric measurements; (3) uncertainty associated with the calibration of measuring equipment; (4) uncertainty of the measured signal related to repeatability of chromatographic peak area. Ishikava's cause-effect diagram is presented in Fig. 1.

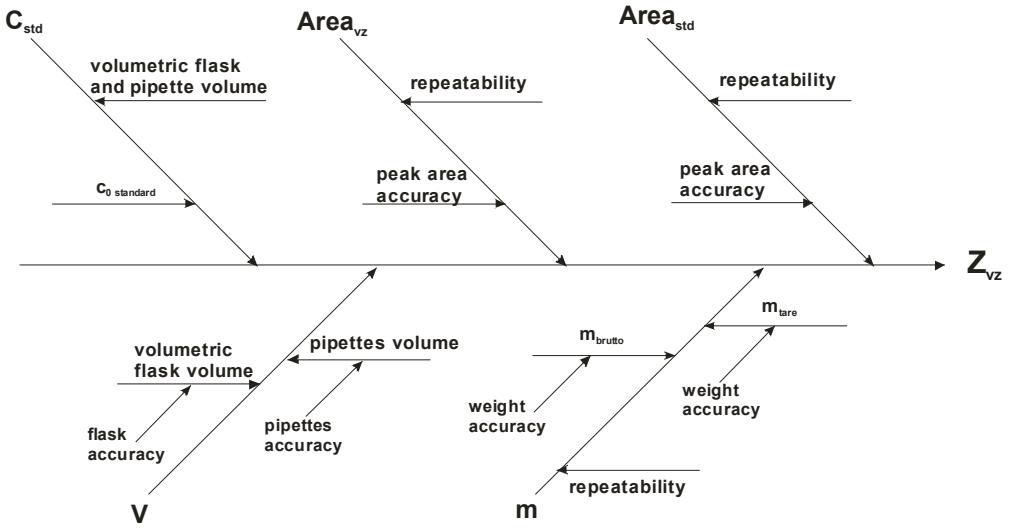

Figure 1 Ishikava's diagram for amino acid analysis

Uncertainty estimation

To estimate combined standard uncertainty, detailed evaluation of all uncertainty components associated with individual uncertainty sources during each step of analysis was performed. An expanded uncertainty was calculated at the 95% confidence level and corresponding coverage factor of 2 for each amino acid of model protein hydrolysate. Relative expanded uncertainties ranged from 9.03% to 12.68% and average relative expanded uncertainty was 9.82%. Results are presented in Fig. 2.

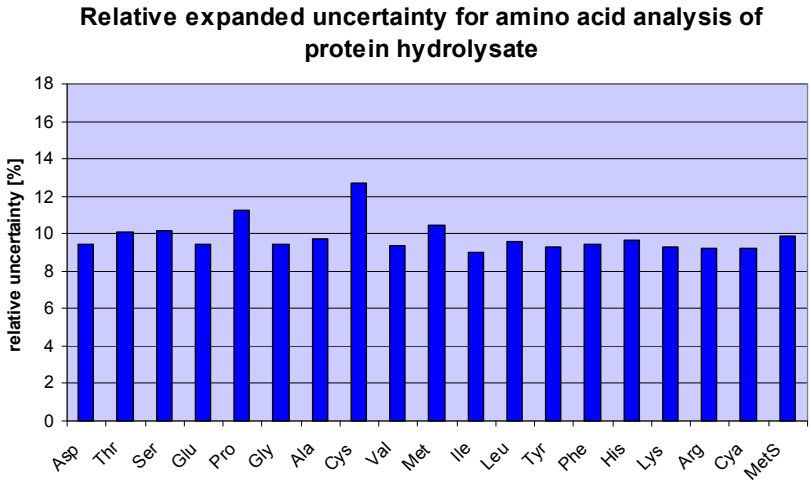

Figure 2 Relative expanded uncertainty in amino acid analysis

Analysis of uncertainty budget

Quantification of the combined uncertainty components indicates that the contribution of the uncertainty of the calibration concentration is the largest (51.5-74.4%), followed by area of chromatographic peaks and weighing procedure of samples (approx. 12 - 40% and 8.4 - 13% respectively). Alternatively, the uncertainty of sample volume and calibrator peak area may be negligible. Fig. 3 shows the graphic presentation of uncertainty budget.

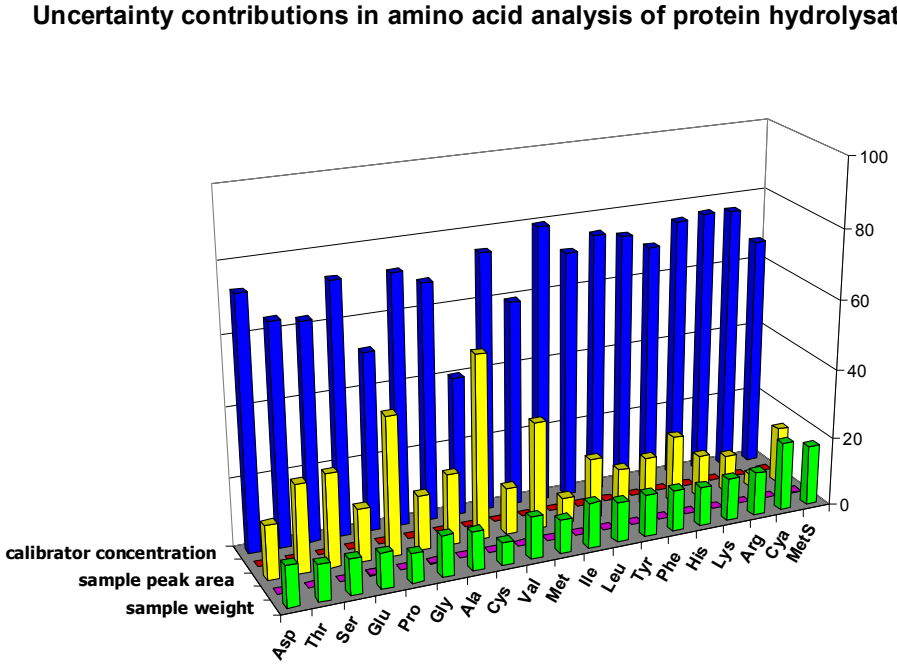

Figure 3 Uncertainty budget for amino acid analysis

CONCLUSIONS

Usually in most of the research works, the results of amino acid analysis are presented as value of amino acid content with standard deviation of final result and occasionally coefficient of variation. Satisfactory values of these parameters, expressing precision and repeatability, can suggest good reliability and quality of obtained results. In our study, the average coefficient of variation (CV%) for the repeatability of measurements was 2.78%. The achieved results of CV% compared with estimated relative uncertainty of approximately 9% suggest that in some cases concerning only standard deviation can be misleading during interpretation of research results. That can not be negligible especially in situation, when confidence in reliability of results is important.

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