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ESTIMATION OF UNCERTAINTY OF TRYPSIN INHIBITOR ACTIVITY MEASUREMENT IN LEGUME CROPS

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Abstract. *Irradiation of legume seeds has emerged as an attractive alternative compared to conventional chemical treatments in seed production. Irradiation is also used for the decontamination of food and feed in order to increase the shelf-life of fresh and dry food materials. The effects of irradiation on nutritive and anti-nutritive factors such as trypsin inhibitors are usually reported together with the measurements obtained by using the quantitative analytical methods. The objective of this study was to measure trypsin inhibitor activity (TIA) of common bean cultivar Oplenac using the microtiter plate method and to identify factors that contribute to the uncertainty of TIA measurement according to the current Guide to the Expression of uncertainty in measurement (GUM). Dominant sources of uncertainty of TIA measurement were: absorbance measurements of sample and positive control reaction mixtures and preparation of the final sample solution using a graduated cylinder (V4). Absorbance measurement of sample reaction mixtures took 37.8 % of the overall measurement uncertainty. Preparation of the final sample solution using a graduated cylinder (V4) and absorbance measurement of positive control reaction mixture contributed to the overall uncertainty with 35.1 % and 15.8 %, respectively. Acquired insight into factors that contribute to the uncertainty of TIA measurement gives directions for the improvement of TIA testing methods and TIA results management.*

Key words: *Measurement uncertainty, trypsin inhibitor activity, common bean*

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1. INTRODUCTION

Irradiation of legume seeds has emerged as an attractive alternative when compared to conventional chemical treatments used to minimize losses that occur during seed storage and production. In addition, ionizing radiation has been used as another technique for decontamination of food and feed in order to increase the shelf-life of fresh and dry food materials [1], [2], [3]. As a consequence, the effects of an irradiation on nutritional characteristics of food and feed materials were investigated in many studies.

Legume crops are of great concern to food and feed industry as an important source of proteins, carbohydrates, fats, fibers, essential vitamins, and minerals. The presence of anti-nutritional factors, such as protease inhibitors, could decrease nutritional value of legume crops and limit their utilization in human and animal nutrition, but could also be beneficial to human health by preventing diseases such as cancer [4], [5], [6]. Depending on time and dose of consumption, protease inhibitor will have beneficial or anti nutritional effects [7]. As a consequence, exploration of protease inhibitors, especially of trypsin inhibitors is of great concern, and in some countries allowed trypsin inhibitor activity (*TIA*) of new legume cultivars is statutorily prescribed. Since it was reported that irradiation could have effects on nutritional characteristics of food and feed, a number of investigations were conducted in order to estimate the irradiation effects on anti-nutritional factors such as tryspin inhibitors.

Considerable increase in protein values, and decrease in *TIA* during germination of irradiated green grams was reported by [8]. Significant linear relationships have been reported in chick pea between the loss of *TIA* and increasing radiation dose (0.25–1.00 kGy) with little or no effect on protein content [9]. The loss of *TIA* was found to be 54.5 % when soybeans were subjected to 10 kGy [10]. According to Serbian national legislation [11] and Directive 1999/2/EC of the European Parliament concerning foods and food ingredients treated with ionising radiation [12], legume crops may be treated with ionising radiation with doses less than 1 kGy. The effects of irradiation on nutritive and anti-nutritive components of legumes are usually reported together with measurements obtained by using the quantitative analytical methods.

Methods for *TIA* measurement are based on the hydrolysis of Nα-Benzoyl-,L-arginine 4-nitroanilide hydrochloride (L-BAPNA) by trypsin and includes spectrophotometric measurement of the reaction

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products. Measurement results of *TIA* are usually accompanied with standard deviation, however, analysis of uncertainty sources of *TIA* measurement were not previously analyzed. In order to demonstrate the quality of measurement results it is important to estimate measurement uncertainty. Results accompanied with statement of measurement uncertainty increase confidence in the validity of a measurement results and enable comparisons between results obtained by different techniques or compliance with regulatory levels. The aim of this study was to estimate and to analyze uncertainty of *TIA* measurements using the microtiter plate method according to the concept of measurement uncertainty described in the current Guide to the Expression of uncertainty in measurement (GUM) [13].

2. MATERIALS AND METHODS

2.1. Preparation of sample solution

Starting material for *TIA* measurement was seed of common bean *(Phaseolus vulgars),* variety Oplenac, originating from the collections of the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Extraction of trypsin inhibitors was performed using method reported by [14]. Trypsin inhibitors were extracted from the grounded raw seeds (0.2 g) using 20 mL of distilled water (V_1). Obtained sample suspension were completed by adding 20 mL (V_2) of assay buffer (50 mM Tris buffer, pH 8.2, containing 10 mM $CaCl_2$), and after shaking for 2 -3 minutes it was filtered through a Whatman No. 2 paper (Sigma Aldrich, USA). After filtration, 1 mL of filtrate (initial sample solution, V_3) was additionally diluted with 6.5 mL of distilled water (V_4) in order to obtain final sample solution which would give 30 % - 70 % of trypsin inhibition.

2.2. TIA test

TIA testing was carried out using microtiter plate method with assay conditions described by [14]. Reaction mixtures of sample, positive and negative controls were set up in each microtiter plate row. Reaction mixtures were set up by mixing: 45-µl of the final sample solution or distilled water (for control reaction mixtures), 22.5 µl of trypsin solution and 90 µl of L-BAPNA solution. Pre-incubation of final sample solution or distilled water with trypsin solution was performed at 37 °C for 10 minutes, and after adding 90 µl of solution of L-BAPNA incubation was carried out for 30 minutes at 37 °C. Positive control reaction mixture gave non-inhibited reaction of enzyme (trypsin) and substrate (L-BAPNA), while negative control reaction mixture was used as reagent blank, since 45 µl of 30 % acetic acid was added immediately after trypsin solution in order to stop reaction. The absorbances of reaction mixtures were measured by using *Multiskan Ascent* microtiter plate photometer (Thermo Fisher Scientific, California, USA).

TIA was calculated according to Equation 1 provided by [14]. The trypsin inhibitor activity was expresed in number of trypsin units inhibited (TIU) per miligram of seed sample, taking into account the fact that one trypsin unit is defined as an increase of 0.01 absorbance units at 405 nm.

$$TIA = \frac{\left(A_{pc} - A_s\right)\times 100}{m_s} \qquad (1)$$

where:

m_s - is mass (mg) of original material (seed) contained in 1 mL of final sample solution

A_{pc} - absorbance measurement of the positive control reaction mixture

A_s - absorbance measurement of the sample reaction mixture

2.3. Uncertainty of TIA measurement

A large number of experiments were conducted and uncertainty analysis was obtained using a procedure reported by [15].

The main sources of uncertainty of a *TIA* measurement are identified from measurement function (Equation 1) and they include: absorbance measurement of the sample and positive control reaction mixture and concentration of the original material in the final sample solution. As a result combined standard uncertainty of *TIA* was calculated as follows:

$$u(TIA) = \sqrt{\left(\frac{\partial TIA}{\partial A_{pc}}u(A_{pc})\right)^2 + \left(\frac{\partial TIA}{\partial A_s}u(A_s)\right)^2 + \left(\frac{\partial TIA}{\partial m_s}u(m_s)\right)^2} \qquad (2)$$

Factors that influence the uncertainty of the absorbance measurement of positive control reaction mixture (*A*pc) includes: correction due to the dispersion of absorbance measurement results of the positive control reaction mixture and the related standard uncertainty; correction due to the calibration of the photometer and the related standard uncertainty; correction due to the finite indication resolution of absorbance measurement of the positive control reaction mixture and related standard uncertainty; volume of trypsin solution; volume of L-BAPNA solution; correction due to the variation of individually delivered volumes of trypsin solution using an automatic pipette and related standard uncertainty; correction due to the variation of individually delivered volumes of L-BAPNA solution using an automatic pipette and related standard uncertainty.

Similar to the previous estimation of the combined standard uncertainty of *A*pc, the combined standard uncertainty of absorbance measurement of the sample reaction mixture *A*s includes: correction due to the dispersion of absorbance measurement results of the sample reaction mixture and related standard uncertainty; correction due to the calibration of the photometer and the related standard uncertainty; correction due to the finite indication resolution of absorbance measurement of the sample reaction mixture and related standard uncertainty; correction due to the variation of individually delivered volumes of final sample solution using an automatic pipette and related standard uncertainty; correction due to the calibration of the automatic pipette used for the volume delivery of the final sample solution and correction due to the volume delivery variation of the final sample solution caused by temperature variation.

Uncertainty sources related to the quantity m_s are: correction due to the calibration of the analytical balance used for weighing of ground seed and related standard uncertainty; sensitivity coefficients, defined as the partial derivatives of function m_s of input quantities x_i (x_i are m, V_1, V_2, V_3 and V_4); combined standard uncertainty of delivered volumes; correction due to the calibration of the graduated cylinder used for delivery of the volume V_i and associated standard uncertainty; correction due to the volume delivery V_i variation caused by temperature variation and associated standard uncertainty; correction due to the variation of individually delivered volume V_i and associated standard uncertainty.

3. Results and discussion

Measured *TIA* of common bean variety Oplenac was 56.2 TIU/mg and expanded measurement uncertainty (with coverage factor k=2) was aproxitemately 5.2 TIU/mg (9 %).

Dominant sources of uncertainty of *TIA* measurements were: absorbance measurements of the sample and the positive control reaction mixture, and preparation of the final sample solution using graduated cylinder (V_4) (Fig. 1, Fig. 2).

Absorbance measurement of the sample reaction mixture (*A*s) took 37.8 % of the overall uncertainty of *TIA* measurement with repeatability of absorbance measurement (*A*s,m) contributing dominantly to the uncertainty with 37 %. Volume delivery of the final sample solution (*V*s), trypsin solution (*V*s,T) and L-BAPNA solution (*V*s,B) using automated pipette, when the sample reaction mixtures were prepared, had smaller contribution to uncertainty with 4.6 %, 0.3 % and 1.6 %, respectively. Absorbance measurement and preparation of sample reaction mixtures took the largest percent (44 %) of overall uncertainty of *TIA* value.

Absorbance measurement of positive control reaction mixture (*A*pc) took 15.8 % of *TIA* measurement uncertainty with repeatability of absorbance measurement (*A*pc, m) contributing to uncertainty with 15.06 %. Volume delivery of the trypsin solution (*V*pc,T) and L-BAPNA solution (*V*pc,B) using automated pipette had contribution to uncertainty of *TIA* measurement with only 0.3 % and 1.6 %, respectively. Absorbance measurement and preparation of positive control reaction mixtures had the smallest contribution to uncertainty of *TIA* measurement (18 %)

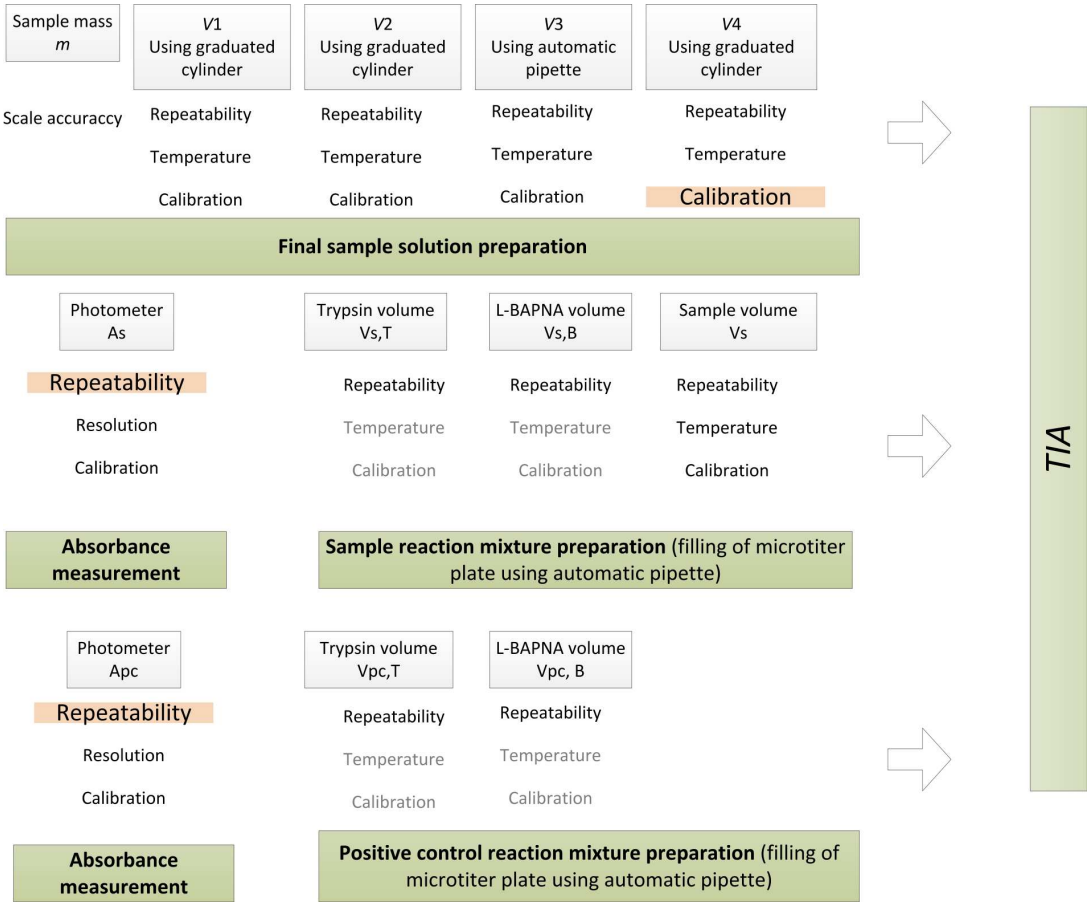

Figure 1. Sources of uncertainty of *TIA* measurement

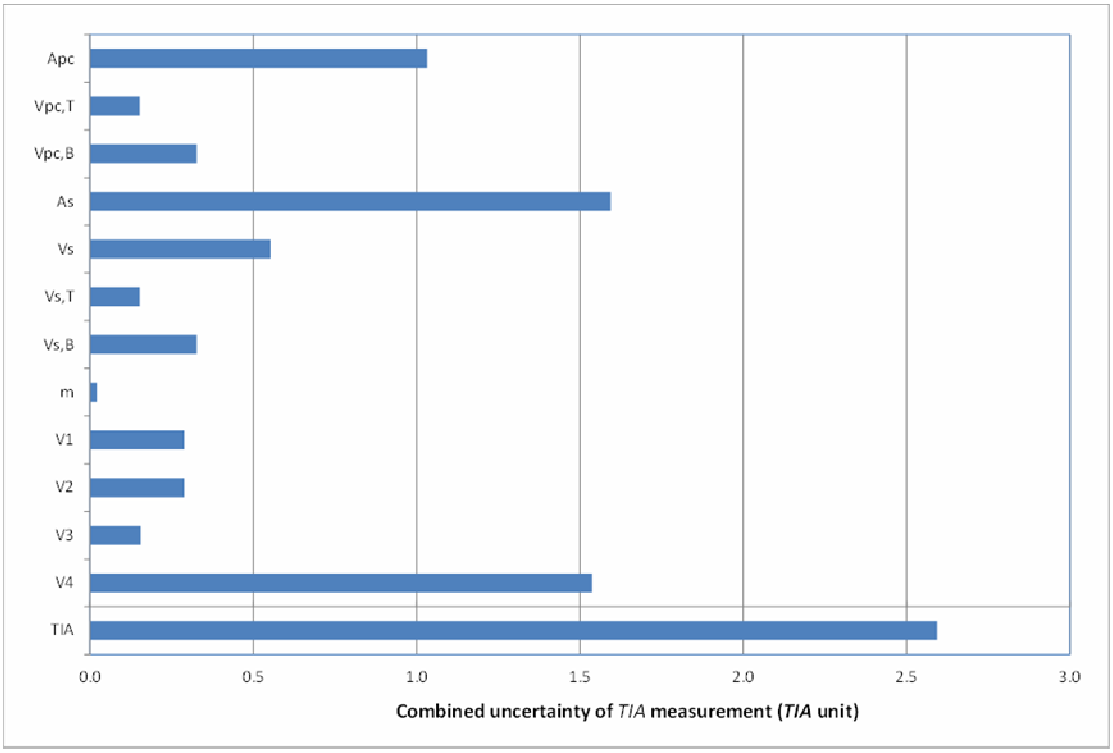

Figure 2. Contribution of uncertainty sources to combined uncertainty of *TIA* measurement

Preparation of the final sample solution considered using graduated cylinders ($V1$, $V2$ and $V4$) and automatic pipette ($V3$). Preparation of the final sample solution took 38 % of overall uncertainty of *TIA* measurement. The dominant influence (35.1 %) had preparation of the final sample solution using graduated cylinder ($V4$) with correction due to calibration contributing to uncertainty with 34.8 %. Smaller contribution showed volume delivery of $V1$, $V2$ and $V3$ with 1.2 %, 1.2 % and 0.4 %, respectively. Contribution of the preparation of the final sample solution to uncertainty of *TIA* measurement was almost equal to contribution of repeatability of absorbance measurement of sample reaction mixture (37 %) indicating that the preparation of the final sample solution had considerable impact on uncertainty and that it should be performed by using more accurate volumetric instruments than graduated cylinders are, or graduated cylinders should have low systematic error.

The higher contribution of repeated absorbance measurement of sample reaction to uncertainty of *TIA* measurement compared to repeated absorbance measurement of positive control reaction mixture was expected. Sample reaction mixture is more complex medium than the positive control reaction mixture having non- specific components which interact with coloration of L-BAPNA. According to [16] protein extraction using alkaline buffer is less specific resulting in extraction of non- specific components. However, repeated absorbance measurement of positive control reaction mixture had also considerable contribution to uncertainty of *TIA* measurement, indicating that part of uncertainty is derived from experimental conditions.

According to [17] divergence of absorbance measurements is in correlation with volume of reaction mixture and error of *TIA* measurement could be minimized if assaying is performed using 4 mL volume reaction mixtures or greater. Aliquot of the final sample solution used for preparation of the sample reaction mixture in microtiter plate method is only 45 µl, however, [16] showed consistency of microtiter plate and AFNOR reference methods. According to [14] the smaller amounts of trypsin inhibitors can be measured by decreasing the volume of the reaction mixture while the concentration of reagents is kept unchanged. This was confirmed by [15] who compared *TIA* measurements of soybean variety Vojvodjanka obtained by microtiter plate method and modified AOCS method. AOCS method was performed with reaction mixture of 4 mL [14], [18] Although it was shown that reference and microtiter plate method give consistent *TIA* measurements, estimation of uncertainty factors that contribute to uncertainty of *TIA* measurement indicates that experimental conditions as well as the way of the preparation of the final sample solution could have a great impact on that uncertainty.

4. Conclusion

This study provides advancement in the processing of *TIA* results by revealing the sources that influence uncertainty of *TIA* measurement using a microtiter plate method. Estimated uncertainty of *TIA* measurement provided insight into the range of *TIA* values that should be expected. In addition, identified sources of measurement uncertainty also gave ability to compare results obtained by different methods. This could be of crucial importance considering the fact that there are three standards and many other similar methods for *TIA* measurement. Providing information

on uncertainty sources that influence *TIA* measurements gives directions for improvement of methods used for *TIA* testing and contributes to improvement of *TIA* results management.

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