Account of the uncertainty of measurements of the degree of free radical oxidation in wheat germ by the method of chemiluminescent analysis

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Abstract. Uncertainty is a more objective concept for evaluating the accuracy of measuring product or process indicators than error. The article studies the adequacy of the assessment of the process of free radical oxidation in wheat germ by chemiluminescent analysis (CLA). This process in the product is due to the conjugated action of enzymes: lipase, lipoxygenase and catalase. To confirm the close relationship between their activity and the CLA parameters (the maximum value of the intensity and the slope of the kinetic curve), a linear correlation coefficient was calculated. As a result, a direct close relationship was established between the studied parameters, which confirms the possibility of assessing the degree of oxidation in the product by the CLA method. An algorithm for obtaining the uncertainty value is formed, which includes four main stages: a description of the measured values (the intensity of the peroxide processes, the inhibitory ability in the product); identification of sources of uncertainty (related to instrumental, methodological and subjective reasons, as well as to a random component); simplification of components to eliminate mutually compensating effects and calculation of uncertainties. The calculation of standard and expanded uncertainties was performed. Their assessment led to the conclusion about a high degree of accuracy of the obtained results with a reliability of 95%.

1. Introduction

Currently, the terms «error» and «uncertainty» of measurements are used to express the concept of measurement accuracy [1]. Initially, in Russia, error was used to assess the reliability of the measurement. Abroad initially there was a concept «error of measurement». It is now increasingly necessary to assess the accuracy of measurements (for example, this requirement is imposed in laboratory accreditation) in terms of «uncertainty». In connection with Russia's accession to the WTO, it was decided to translate the rules for carrying out and evaluating the quality of works (including metrological ones) into accordance with ISO international standards. All measurement laboratories of WTO member countries should assess measurement accuracy in terms of uncertainty.
According to RMG 91-2009 «Joint use of the concepts «measurement error» and «measurement uncertainty»» [2] these concepts are interpreted as follows:

- measurement error measurement result (measured value of magnitude) minus the reference value of the magnitude;
- measurement uncertainty is a non-negative parameter characterizing the scatter of the values of the quantity attributed to the measured quantity based on the information used.

It should be noted the fundamental difference between the concepts of «uncertainty» and «error». The error in accordance with RMG 29-2013 [3] is defined as the difference between an individual result and the true value of the measured quantity, i.e. this is the only clearly defined value that can be taken into account as a correction to the measurement result. However, due to the fact that we cannot know the concept of the true value of the measured quantity, the error is an idealized concept. The estimation of uncertainty forms a certain range of values within which the investigated quantity is located with a given probability.

Thus, if it is necessary to establish the accuracy of a single measurement, it is advisable to use the concept of error. In the case when it comes to the generalized result of a series of experiments or the application of the analysis method, it is necessary to calculate the measurement uncertainty.

There are studies on the possibility of assessing the degree of free radical oxidation in products by the CLA method [4, 5, 6].

Chemiluminescence is the phenomenon of radiation arising due to the formation of chemical reaction products in the excited state. The excitation energy is emitted as photons during the quantum transition to the ground state of the molecule. The brightness of the chemiluminescence is usually proportional to the quantum output—the ratio of the number of photons emitted by the chemical system to the number of reacted particles, which is practically defined as the ratio of the luminescence intensity to the rate of the chemical reaction [4].

The method of inducing chemiluminescence by hydrogen peroxide with iron sulfate is based on the catalytic decomposition of peroxide by metal ions with variable valence—divalent iron by the Fenton reaction. The resulting free radicals (R', OH', RO', RO_2', O_2') enter into the process of initiating free radical oxidation in the studied biological substrate. Recombination of RO_2' radicals leads to the formation of an unstable tetroxide that decays with the release of a quantum of light. The free-radical process is registered within 40 seconds. The intensity of this process was determined by the value of the maximum signal intensity and the chemiluminescence light sum during this time [4].

Wheat germ has become widespread in the food industry due to its unique nutrient composition: vitamins E and group B, proteins, which include essential amino acids (lysine, leucine, valine, phenylalanine and others), as well as macro and micronutrients, including potassium, phosphorus, magnesium [6, 7, 8]. However, this product, which is valuable from a biochemical point of view, quickly loses its valuable properties under the influence of a conjugated system of enzymes: lipase, lipoxygenase, and catalase. Under the action of lipase of wheat germ hydrolysis of lipids occurs with the formation of free fatty acids and further intensive oxidation of the latter. With the participation of lipoxygenase, a deep decomposition of hydroperoxides of fatty acids into products of an acidic nature takes place in parallel. The action of catalase leads to the release of oxygen, which intensifies the oxidative processes in the product [6, 9]. The CLA method was used to assess the degree of free radical oxidation [5], but there is no information about the degree of accuracy and adequacy of the assessment.

2. The purpose of the study
In connection with the prospects of using CLA to assess the degree of free radical oxidation in wheat germ [10], the purpose of the study was to study the measurement uncertainty using this technique.

3. Materials and methods
As the object of the study, wheat germ selected for varietal grinding of wheat of the 4th grade of technical degree of maturity at JSC «Voronezh flour mill» was selected.
3.1 CLA method
When determining the intensity of free-radical processes in wheat embryos during storage, an extract prepared by grinding to a degree of dispersion of 0.1 mm of the embryos and extracting them with 0.05 M Tris/NaCl buffer (pH = 8.0) for 30 minutes at 4 °C. The resulting mixture was subjected to centrifugation at 5000 g for 15 minutes. The determination was carried out in a measuring cuvette of the biohemiluminometer BCL-06, into which 0.4 ml of supernatant, 0.2 ml of phosphate buffer (pH 7.5), 0.2 ml of iron sulfate (0.05 mm), then 0.2 ml of 2% \( \text{H}_2\text{O}_2 \) solution were added [7]. The values of the maximum chemiluminescence intensity and the tangent of the angle of inclination of the kinetic curve were recorded on the device screen [5].

3.2 Methodology for conducting correlation analysis
To establish the probability of the existence of a linear relationship between random variables \( X \) (enzyme activity) and \( Y \) (CLA parameters), a linear correlation coefficient \( r_{xy} \) is used, which takes values in the range of \(-1 < r_{xy} < 1 \) [11]. This indicator characterizes the tightness of the connection and is calculated according to the following relationships:

\[
 r_{xy} = \frac{S(xy)}{S(xx)S(yy)} ;
\]

\[
 S(xy) = \sum_{i=1}^{n} y_i x_i - \frac{\left( \sum_{i=1}^{n} x_i \right) \left( \sum_{i=1}^{n} y_i \right)}{n} ;
\]

\[
 S(xx) = \sum_{i=1}^{n} x_i^2 - \frac{\left( \sum_{i=1}^{n} x_i \right)^2}{n} ;
\]

\[
 S(yy) = \sum_{i=1}^{n} y_i^2 - \frac{\left( \sum_{i=1}^{n} y_i \right)^2}{n} .
\]

The conclusion about the tightness of linear correlation was made on the basis of the obtained results as follows: if random variables \( X \) and \( Y \) are connected by an exact linear functional dependence, then the value \( r_{xy} \) tends to \( |1| \); if the random variables are independent, then the \( r_{xy} \) value is close to 0. The sign before the numerical value \( r_{xy} \) characterizes the form of the dependence: with the «+» sign - a direct dependence; with a «-» sign - otherwise.

To test the null hypothesis \( H_0: r_{xy} = 0 \), a random variable, \( z \), is used, which is calculated by the formula (1):

\[
 T = \frac{r_{xy} \sqrt{n-2}}{\sqrt{1-r_{xy}^2}} .
\]

If the null hypothesis is valid, there is a Student distribution with \((k = n – 2)\) degrees of freedom. The competing hypothesis is \( H_1: r_{xy} \neq 0 \), in this connection, a two-sided critical region is constructed using the formulas (2), based on the requirement that the probability of the criterion \( T \) falling into this region, assuming the validity of the null hypothesis, is equal to the accepted level of significance \( \alpha \).
\[ P(T < t_{\text{left critical}}) = \frac{\alpha}{2}, \quad P(T > t_{\text{right critical}}) = \frac{\alpha}{2}. \] (2)

Since the value of \( T \) has a Student distribution, and it is symmetric with respect to zero, critical points are also symmetric with respect to zero. It is enough to find the right boundary of the bilateral critical region to find the critical region itself:

\[ T < -t_{\text{two-sided critical}}(\alpha; k), \quad T > t_{\text{two-sided critical}}(\alpha; k). \]

If the calculated observed value of the criterion \( T_{\text{obs}} < t_{\text{two-sided critical}}(\alpha; k) \), there is no reason to reject the null hypothesis. If \( T_{\text{obs}} > t_{\text{two-sided critical}}(\alpha; k) \) the null hypothesis is rejected, then the variables \( X \) and \( Y \) are correlated, i.e. connected by a linear relationship.

### 3.3 Uncertainty calculation

In the course of the study, the uncertainty was calculated \([12, 13]\) according to type A \((u_A(x))\), if the assessment was based on a statistical series of observations) according to formula (3), according to type B \((u_B(x))\) otherwise according to formulas (4):

\[ u_A(x) = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}, \]

\[ u_B(x) = \frac{b}{\sqrt{3}}, \quad u_B(x) = \frac{b}{\sqrt{6}}, \quad u_B(x) = \frac{b}{2}. \] (4)

The choice of formula (4) is carried out depending on the type of distribution of the possible values of these quantities (respectively, uniform, triangular or normal).

The standard uncertainty, \( U_c \), was calculated by the formula (5):

\[ U_c = \sqrt{\sum_{i=1}^{n} \left( \frac{\partial f}{\partial x_i} \right)^2 \left( \frac{\partial f}{\partial x_j} \right)^2}. \] (5)

The expanded uncertainty, \( U_e \), which determines the interval around the measurement result, within which a large part of the distribution of values is contained, which with a given reliability can be attributed to the measured result was calculated by the formula (6):

\[ U_p = k \cdot U_c. \] (6)

where \( k \) – the coverage coefficient, which depends on the level of confidence (in the studies, the confidence level was 95 %, with \( k = 2 \)).

### 4. Discussion of the results

To establish the nature and closeness of the relationship between chemiluminescence parameters (maximum intensity value and the tangent of the kinetic curve angle) and the enzymatic activity of lipase, lipoxygenase, and catalase, the action of which leads to the intensification of free radical oxidation, a correlation analysis was performed.

To study the correlation between chemiluminescence indicators and the enzymatic activity of wheat germ, data were used during storage for 2 months. The measurements were performed from one of the joint samples at the same time once a week. As a result, graphs of the relationship between lipase, lipoxygenase, and catalase activity with the maximum chemiluminescence intensity (figure 1) and the tangent of the kinetic curve angle were obtained (figure 2).
Figure 1. Diagram of the relationship between the activity of lipase (1), lipoxygenase (2) and catalase (3) and the maximum intensity of chemiluminescence.

Figure 2. Diagram of the relationship between the activity of lipase (1), lipoxygenase (2) and catalase (3) and the tangent of the angle of inclination of the kinetic curve.

Analysis of the results calculate the coefficients of linear correlation (table 1) showed that the activity of lipase, lipoxygenase and catalase have a close direct relationship with the maximum intensity of chemiluminescence and the back tangent of the slope of the kinetic curve. A lower value of coefficient of linear correlation for the tangent of the slope of the kinetic curve CLA probably due to the fact that the inhibitory ability of the product itself is not so much the activity of enzymes, as with the biochemical composition of wheat germ and a content of antioxidants.

Table 1. The coefficients of linear correlation between enzymatic activity and indicators of CLA

| Enzyme       | \( r_{sy} \) value for the indicator | \( I_{max} \) | \( \text{tg} \ \alpha \) |
|--------------|--------------------------------------|--------------|-----------------|
| Lipase       | 0.954                                | -0.932       |
| Lipoxygenase | 0.982                                | -0.946       |
| Catalase     | 0.977                                | -0.872       |

The hypothesis about the significance of linear correlation coefficients was tested at the level of \( \alpha = 0.05 \) based on the Student's criterion. As a result, the significance of \( r_{sy} \) is confirmed. In this regard, it is possible to estimate the uncertainty of CLA for studying the degree of free-radical oxidation of wheat germ.

The algorithm for estimating extended measurement uncertainty includes 4 stages:
1. study and description of the measured value;
2. identifying sources of uncertainty;
3. calculation of standard uncertainties;
4. calculation of the expanded uncertainty.

At the first stage of the uncertainty assessment, both CLA indicators were selected. The maximum intensity of chemiluminescence is the highest point of the kinetic curve of the reaction occurring between the peroxides and hydroperoxides contained in the product with the introduced reagents with the release of a quantum of light that is registered by the device. The tangent of the angle of inclination of the kinetic curve characterizes the inhibitory ability of the product, that is, its antioxidant activity. The higher the value of this indicator, the higher the antioxidant potential of the test sample.
The second stage is to identify sources of uncertainty. To do this, we selected all possible sources of uncertainty [14] and conditionally divided them into the following groups:

- instrumental effects (the error of the analytical balance, the error of biochemiluminimeter, the accuracy of volumetric glassware);
- the purity of the reagents (due to the fact that the concentration of solutions cannot be determined with absolute accuracy, because there is uncertainty associated with the method of this test; in addition, the reagents may contain impurities);
- measurement conditions (temperature and relative humidity may deviate from the normal values of the instrument application);
- methodological errors (different time of sample preparation, time of using freshly prepared samples and reagents, working with the device);
- computational effects (errors in instrument settings, incorrect rounding in calculations);
- influence of the operator (skills of working with the device, ability to prepare reagents, visual ability when using measuring utensils);
- random effects (this factor is always included in the default uncertainty calculation).

At the next stage, it is necessary to make some simplifications in order to avoid duplication of reasons: remove mutually compensating effects, factors that affect equally and at the same time combine, etc. For example, the ability to work with the device is inherent in both the methodological and subjective group of reasons. An increase in the ambient temperature, on the one hand, leads to disruption of the analytical balance in the direction of reducing the mass value, and on the other hand, forms the optimal mode of enzyme activity.

At the third stage, standard uncertainties were calculated separately by type A (random effects, methodological errors, influence of measurement conditions) and by type B (hardware effects, purity of reagents).

At the fourth stage, the total uncertainty was calculated according to the formula (10), and then the extended uncertainty was estimated at the 95% confidence level.

To perform the calculation, a series of 10 experiments were performed to measure the CLA indicators in wheat embryos. The work was carried out by the same operator under the same conditions (temperature and relative humidity fluctuated within 3%). The results of the calculations are shown in table 2.

### Table 2. Estimation of the uncertainty of measuring the level of free radical oxidation by the CLA method

| Uncertainty                        | Value for the indicator |
|------------------------------------|-------------------------|
|                                    | I_{max}, mV | tg α |
| The parameter value                | 2.362        | 0.954 |
| Standard uncertainty for           |              |     |
| type A                             | 0.018        | 0.003 |
| type B                             | 0.011        | 0.004 |
| Total uncertainty                  | 0.021        | 0.005 |
| The expanded uncertainty           | 0.042        | 0.010 |

5. Conclusion

A correlation analysis was performed between the value of wheat germ enzyme activity and CLA indicators, which resulted in a close direct relationship - in the case of maximum intensity, and a close inverse relationship - in the case of the tangent of the kinetic curve inclination angle.

Based on the method's uncertainty estimation algorithm, the causes of uncertainty were identified. At the 95% confidence level, the parameters were evaluated and the result was obtained: the maximum chemiluminescence intensity was (2.362 ± 0.042); the tangent of the angle of inclination of the kinetic curve was (0.954 ± 0.010).
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