Research Article

The Simultaneous Voltammetric Determination of Aflatoxins B\textsubscript{1} and M\textsubscript{1} on a Glassy-Carbon Electrode

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1. Introduction

Aflatoxin M\textsubscript{1} is a metabolite of aflatoxin B\textsubscript{1}, a product of life activity of \textit{Aspergillus} microscopic fungi. In natural conditions, aflatoxin B\textsubscript{1} contaminates cereals, legumes, various nuts, oil seeds, cocoa and coffee, animal feed, and other food products. It can be converted into aflatoxin M\textsubscript{1} in the body of animals and is present in meat [1]. In [2], the information on admissible levels of contents of the specified kinds of mycotoxins in flour obtained from various kinds of grains used for cooking human food, as well as in the composition of feed of various animal species, is presented. With contaminated feed, aflatoxins enter the body of animals, and their residual quantities are found in meat, milk, and eggs. Aflatoxins are the only mycotoxins that are strictly regulated in markets such as the EU and US [3]. The information on tolerable levels of mycotoxins taken in Australia, China, Guatemala, India, Ireland, Kenya, and Taiwan is reported [4]. Aflatoxin M\textsubscript{1} is a hydroxylated metabolite present in human milk and animals exposed to aflatoxin B\textsubscript{1}. Like its precursor, aflatoxin B\textsubscript{1}, aflatoxin M\textsubscript{1} already in low concentrations poses a serious threat to the health of animals and humans. Aflatoxin B\textsubscript{1} is found not only in whole milk (including reconstituted milk) but also in cottage cheese, cheese, and yoghurt. Dairy products contaminated with aflatoxin M\textsubscript{1} are environmentally hazardous to humans. In adult food and baby food, aflatoxins are not allowed. Figure 1 shows the structural formula of aflatoxins B\textsubscript{1} and M\textsubscript{1}.

Aflatoxin B\textsubscript{1} is (6aR-cis)(2,3,6a,9a)-tetrahydro-4-methoxycyclopenta[c]furo[2,3-h][1]benzopyran-1,11-dione with a molecular weight of 312. Aflatoxin M\textsubscript{1} is (2,3,6a,9a)-tetrahydro-9a-hydroxy-4-methoxycyclopenta[c]furo[2,3-h][1]benzopyran-1,11-dione with a molecular weight of 328.

There are known methods of simultaneous quantitative determination of aflatoxins B\textsubscript{1} and M\textsubscript{1}, aflatoxins B\textsubscript{2}, G\textsubscript{1}, G\textsubscript{2}, and M\textsubscript{1}, and ochratoxin A by the method of high-performance liquid chromatography with fluorescent detection in breast milk [5–8] with detection limits between 0.5 and 0.25 µg/L [7], of 5 ng/L [7], and from 0.005 to 0.03 ng/mL [8].
The method of high-performance liquid chromatography was used to determine aflatoxin M₁ in eggs [9], and together with tandem mass-spectrometry, it was used to determine aflatoxins B₁ and M₁ in fresh and dry milk after ultrasonic extraction; the detection limit was 0.05 μg/kg, and the limit of quantification was 0.1 μg/kg [10]. The detection limits of aflatoxins B₁, B₂, G₁, G₂, and M₁ and ochratoxin A in food products of animal origin were in the range of 0.07–0.59 μg/kg [11]. In case of simultaneous determination of six aflatoxins (B₁, B₂, G₁, G₂, M₁, and M₂) by this method in peanut, the detection limits are in the range of 0.03 to 0.26 ng/g and 0.1 to 0.88 ng/g [12]. The use of chromatography methods is complicated by the duration and the need to use expensive equipment and highly toxic solvents as a mobile phase.

The possibility of indirect competitive enzyme-linked immunosorbent assay for the determination of aflatoxin M₁ in various objects was demonstrated in [13]. At present, highly sensitive, inexpensive, and easy-to-use electrochemical methods, in particular voltammetry, are becoming increasingly popular for the determination of a number of organic substances including aflatoxins. In literature, there is a rather large number of works on the individual determination of aflatoxins B₁ [14–19] and M₁ [20–23] using amperometric and voltammetric immunosensors. The use of enzymes and nanomaterials to design sensors provides high sensitivity and selectivity for detection. At the same time, the analysis of numerous publications in the databases of Science Direct, Scopus, Web of Science, and so on shows that, at the moment, there is no research work on the simultaneous determination of aflatoxins B₁ and M₁ by the method of voltammetry.

The purpose of the work consists of studying the possibility of simultaneous voltammetric determination of aflatoxins B₁ and M₁ on a glassy-carbon electrode (GCE), selecting working conditions for measurements and developing a method for their determination in whole milk.

2. Methods and Materials

In this study, the voltammetric analyzer “STA” (Russia) consisting of electronic and measuring units and an IBM-compatible personal computer with the installed software package “STA” was used. As the indicator electrode, a glassy-carbon electrode (GCE) was used, and the conventional silver chloride electrode (CSE) served as an auxiliary and reference electrode.

The measurements were carried out in a constant-current sweep mode with the speed w = 30 mV/s in the potential range from 0.0 to +1.1 V. To mix the analyzed solution, vibration of the electrodes without removal of dissolved oxygen was used.

The working solutions of aflatoxins B₁ and M₁ were prepared from standard samples of aflatoxin B₁ (GSO 7936-2001) with a concentration of 10.0 mg/dm³ and aflatoxin M₁ (GSO 7935-2001) with a concentration of 1.0 mg/dm³ in the volume of 1.0 dm³ mixture of benzene and acetonitrile (in the 9:1 ratio), followed by diluting 10 times in ethanol alcohol. As background electrolytes, solutions with different pH: 0.1 M Na₃HPO₄, 0.1 M C₆H₅O₂(NH₄)₃, 0.1 M (NH₄)₂SO₄, 0.1 M Na₃PO₄, 0.1 M K₂HPO₄, 0.1 M Li₂CO₃, and 0.1 M ZnSO₄ were used.

3. Preparation of the Sample of Whole Milk

When preparing for analysis, a sample of whole milk 25.00 g is taken in a conical flask with the capacity of 100 cm³, and 1.0 cm³ of hydrochloric acid with a concentration of 6–7 mol/dm³ is added in portions of 0.2 cm³. The mixture is slightly stirred and left for 15 minutes, poured into centrifuge tubes, and then centrifuged at 15,000 rpm within 15 minutes.

The centrifugate is poured into a conical flask, and 5–6 g of ammonium sulfate ((NH₄)₂SO₄) is added in portions of 2 to 3 grams, each time stirring the contents of the flask with a glass rod until the salt dissolves. The flask is left for 20 minutes, after which the contents of the flask are poured into centrifuge tubes and centrifuged within 15 minutes at the speed of 6000 rpm. The centrifugate is filtered into a clean cup with the capacity of 30 cm³ through the double-layered filter paper (blue tape). The resulting filtrate is a prepared sample. For analysis, an aliquot of the prepared sample of 5.0 cm³ is taken.

4. Results and Discussion

Studies on the effect of the background electrolyte composition on the analytical signals of aflatoxins B₁ and M₁ on a glassy-carbon electrode under working conditions previously developed for the determination of aflatoxin B₁ were conducted [24]. Experiments on the choice of the background electrolyte showed that the value of the analytical signal aflatoxin M₁ on background electrolytes: 0.1 M Na₃PO₄, 0.1 M Na₃HPO₄, 0.1 M K₂HPO₄, and 0.1 M ZnSO₄, was found to be low, and it was high on background electrolytes: 0.1 M (NH₄)₂SO₄ and 0.1 M Li₂CO₃; the maximum current of its electric oxidation was obtained against the background of 0.1 M C₆H₅O₂(NH₄)₃. Changing the cation-anion composition and pH of the background electrolyte may negatively shift the peak potential of the electric oxidation peak of aflatoxin M₁ in the range (0.6 ± 0.08) V. The effect of the background electrolyte pH on the analytical signals of these aflatoxins was studied, and it was shown that it was preferable to use neutral or weak acidic solutions as background ones, since mycotoxins decompose into nontoxic or low-toxic compounds in the alkaline medium, and the use of background electrolytes with pH > 6.5 is impractical. In Figure 2, calibration curves of the peak current of electric oxidation of aflatoxins B₁ and M₁ in
Figure 2: Calibration dependences of electric oxidation of aflatoxins M₁ (1 and 3) and B₁ (2 and 4) on various background electrolytes: (1, 2) 0.1 M CaH₂O₇(NH₄)₃; (3, 4) 0.1 M (NH₄)₂SO₄.

Figure 3: Voltammograms of aflatoxins B₁ and M₁ electric oxidation with the joint presence on the GCE: (1) background electrolyte 0.1 M CaH₂O₇(NH₄)₃; (2) CₐfB₁ = 2 × 10⁻³ mg/dm³ and CₐfM₁ = 0; (3) CₐfB₁ = 2 × 10⁻³ mg/dm³ and CₐfM₁ = 2 × 10⁻₄ mg/dm³.

Figure 4 shows the dependences of the current derivatives of the peak of aflatoxins B₁ (curve 1) and M₁ (curve 2) on the accumulation potential of the GCE in the selected background electrolyte. It can be seen in Figure 4 that the maximum values of the electric oxidation currents of aflatoxins are observed at the potential of 0.0 V that was selected as the accumulation potential for further studies.

The mutual influence of aflatoxins B₁ and M₁ in their simultaneous determination on a glassy-carbon electrode was studied. For this purpose, the current derivative of the peak of aflatoxin B₁ electric oxidation was obtained as a function of the concentration of aflatoxin M₁ in the solution (Figure 5) and the calibration curves of aflatoxin M₁ in the presence of aflatoxin B₁ (Figure 6).

In Figure 5, it can be seen that, in the concentration range studied, the effect of aflatoxin M₁ on the aflatoxin B₁ current is practically negligible at the ratio CₐfB₁ : CₐfM₁ = 1 : 1 in the presence of a two- or threefold excess of aflatoxin M₁, the peak current of aflatoxin B₁ decreases by 10–15% (curve 1), and the potential of the peak remains unchanged. It is shown that the systematic error in determination of aflatoxin B₁ in the presence of aflatoxin M₁ at the ratio CₐfB₁/CₐfM₁ ≤ 1 : 40 does not exceed 20%.

In Figure 6, it is seen that, in the presence of aflatoxin B₁, the derivative of the peak current of aflatoxin M₁ decreases almost 1.5 times and the peak potential shifts to the anode region from +0.85 V to +0.95 V, but the linearity of the calibration dependence remains in a wide range which proves the possibility of their simultaneous determination.

Based on the conducted studies, the working conditions of the simultaneous voltammetric determination of aflatoxins B₁ and M₁ were proposed (Table 1).

On the basis of the obtained data of the electrochemical behavior of aflatoxins, an algorithm for quantifying these toxic substances in order to effectively control the detection of their minimum acceptable amounts in whole milk was developed. The algorithm for quantification of mycotoxins in whole milk includes the following steps:

1. Taking a sample
2. Acid hydrolysis with concentrated HCl and centrifugation
3. Precipitation of proteins with ammonium salts of sulfate (NH₄)₂SO₄ and centrifugation
4. Filtration of the obtained precipitate
5. Quantitative determination of the aflatoxins content by the method of differential voltammetry

Verification of the correctness of the proposed procedure was carried out by the "introduced-found" method (Table 2).

The data in Table 2 show that the voltammetric joint determination of quantities of aflatoxins B₁ and M₁ is possible with the measurement error of 15–20% in the concentration ranges 2 × 10⁻³ – 2 × 10⁻¹ mg/dm³ and 2 × 10⁻⁴ – 2 × 10⁻² mg/dm³, respectively.

The proposed method is simple, and it does not require a lot of reagents and labor. The range of detectable...
concentrations is from 0.001 to 0.12 mg/dm³. The relative standard deviation (Sr) is not more than 30%.

### 5. Conclusion

Thus, the possibility of the simultaneous voltammetric determination of aflatoxins B₁ and M₁ on the GCE in the background electrolyte 0.1 M C₆H₅O₇(NH₄)₃ has been shown.
When determining aflatoxins, the method of “soft” sample preparation has been used for separating the matrix by hydrolysis and salting out proteins followed by their separation by centrifugation or filtration which reduces the analysis time to less than one hour as compared with thin-layer and high-performance chromatography (GOST 30711-2001). The developed technique has a number of advantages in comparison with the already known methods of analysis. The algorithm of the technique is characterized by the express analysis (the analysis time does not exceed 1 hour), sensitivity (the range of the determined contents is not inferior, and in the case of aflatoxin M1, it exceeds the capabilities of chromatographic methods), and the equipment cheapness. The technique is characterized by simplicity of execution, minimal consumption of reagents, and improved metrological characteristics.

Conflicts of Interest

The authors declare that they have no conflicts of interest for this research.

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