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Research Article

The Simultaneous Voltammetric Determination of Aflatoxins B_1 and M_1 on a Glassy-Carbon Electrode

G. B. Slepchenko, T. M. Gindullina, M. A. Gavrilova, and A. Zh. Auelbekova

National Research Tomsk Polytechnic University, Tomsk 634050, Russia

Correspondence should be addressed to G. B. Slepchenko; slepchenkogb@mail.ru

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For the first time, the possibility of using stripping voltammetry for the simultaneous determination of aflatoxins B_1 and M_1 on a glassy-carbon electrode has been shown. The influence of various factors ($E_э$, $\tau_э$, w, and the nature of the background electrolyte) on the potential and magnitude of the oxidation current of mycotoxins has been estimated. Working conditions for voltammetric determination and reproducibility of analytical signals for two mycotoxins have been selected. The mutual influence of aflatoxins B_1 and M_1 on the value of analytical signals in their simultaneous presence has been studied. It has been found that, in the range of their detectable contents, the presence of aflatoxin B_1 reduces the analytical signal of aflatoxin M_1 by 45–50%, but the linearity of the calibration dependence is preserved. The content of aflatoxin M_1 in determination of aflatoxin B_1 does not exert a significant effect in the range of 10–15%. Based on the results obtained, a procedure has been proposed for determining the content of aflatoxins B_1 and M_1 in their joint presence in milk by voltammetry in the concentration ranges $2\times10^{-3} \div 2\times10^{1}$ mg/dm^3 and $2\times10^{-4} \div 2\times10^{2}$ mg/dm^3, respectively (Sr not more than 18%).

1. Introduction

Aflatoxin M_1 is a metabolite of aflatoxin B_1, a product of life activity of *Aspergillus* microscopic fungi. In natural conditions, aflatoxin B_1 contaminates cereals, legumes, various nuts, oil seeds, cocoa and coffee, animal feed, and other food products. It can be converted into aflatoxin M_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_1 is a hydroxylated metabolite present in human milk and animals exposed to aflatoxin B_1. Like its precursor, aflatoxin B_1, aflatoxin M_1 already in low concentrations poses a serious threat to the health of animals and humans. Aflatoxin B_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_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_1 and M_1.

Aflatoxin B_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_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_1 and M_1, aflatoxins B_1, B_2, G_1, G_2, and M_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].

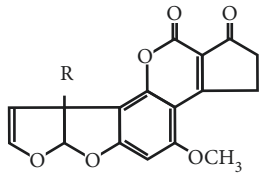

Figure 1: Structural formula of aflatoxins B_1 (R=H) and M_1 (R=OH).

The method of high-performance liquid chromatography was used to determine aflatoxin M_1 in eggs [9], and together with tandem mass-spectrometry, it was used to determine aflatoxins B_1 and M_1 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 B_1, B_2, G_1, G_2, and M_1 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 (B_1, B_2, G_1, G_2, M_1, and M_2) 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 M_1 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 B_1 [14–19] and M_1 [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 quantification of aflatoxins B_1 and M_1 by the method of voltammetry.

The purpose of the work consists of studying the possibility of simultaneous voltammetric determination of aflatoxins B_1 and M_1 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 B_1 and M_1 were prepared from standard samples of aflatoxin B_1 (GSO 7936-2001) with a concentration of 10.0 mg/dm^3 and aflatoxin M_1 (GSO 7935-2001) with a concentration of 1.0 mg/dm^3 in the volume of 1.0 dm^3 mixture of benzene and acetonitrile (in the 9 : 1 ratio), followed by diluting 10 times in ethyl alcohol. As background electrolytes, solutions with different pH: 0.1 M Na_2HPO_4, 0.1 M $C_6H_5O_7(NH_4)_3$, 0.1 M $(NH_4)_2SO_4$, 0.1 M Na_3PO_4, 0.1 M K_2HPO_4, 0.1 M Li_2CO_3, and 0.1 M $ZnSO_4$, 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 cm^3, and 1.0 cm^3 of hydrochloric acid with a concentration of 6–7 mol/dm^3 is added in portions of 0.2 cm^3. 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 ($(NH)_2SO_4$) 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 cm^3 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 cm^3 is taken.

4. Results and Discussion

Studies on the effect of the background electrolyte composition on the analytical signals of aflatoxins B_1 and M_1 on a glassy-carbon electrode under working conditions previously developed for the determination of aflatoxin B_1 were conducted [24]. Experiments on the choice of the background electrolyte showed that the value of the analytical signal aflatoxin M_1 on background electrolytes: 0.1 M Na_3PO_4, 0.1 M Na_2HPO_4, 0.1 M K_2HPO_4, and 0.1 M $ZnSO_4$, was found to be low, and it was high on background electrolytes: 0.1 M $(NH_4)_2SO_4$ and 0.1 M Li_2CO_3; the maximum current of its electric oxidation was obtained against the background of 0.1 M $C_6H_5O_7(NH_4)_3$. Changing the cation-anion composition and pH of the background electrolyte may negatively shift the peak potential of the electric oxidation peak of aflatoxin M_1 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 B_1 and M_1 in

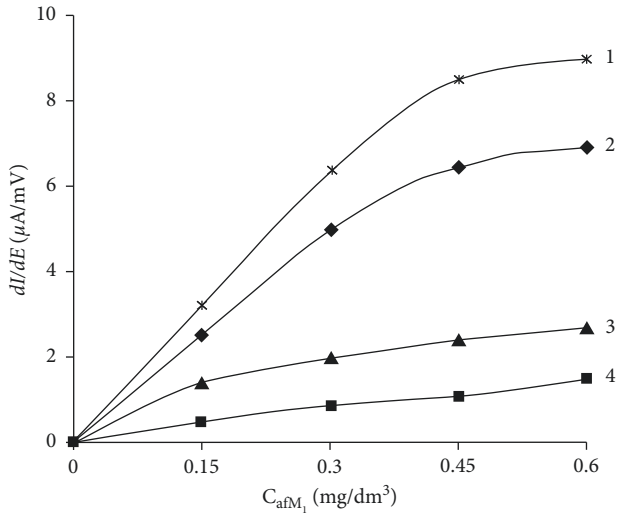

FIGURE 2: Calibration dependences of electric oxidation of aflatoxins M_1 (1 and 3) and B_1 (2 and 4) on various background electrolytes: (1, 2) 0.1 M $C_6H_5O_7(NH_4)_3$; (3, 4) 0.1 M $(NH_4)_2SO_4$.

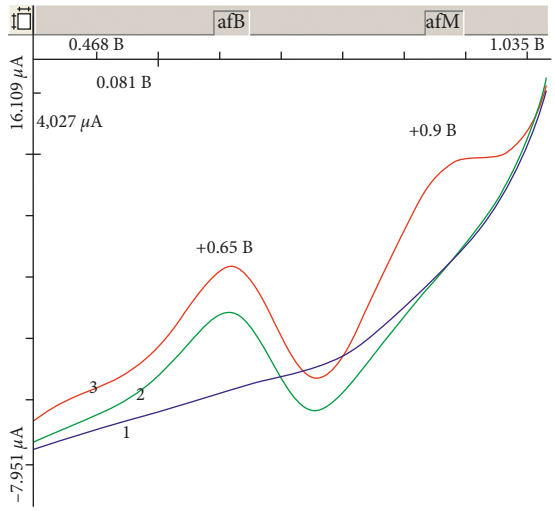

FIGURE 3: Voltammograms of aflatoxins B_1 and M_1 electric oxidation with the joint presence on the GCE: (1) background electrolyte 0.1 M $C_6H_5O_7(NH_4)_3$; (2) $C_{afB_1} = 2 \times 10^{-3}$ mg/dm^3 and $C_{afM_1} = 0$; (3) $C_{afB_1} = 2 \times 10^{-3}$ mg/dm^3 and $C_{afM_1} = 2 \times 10^{-4}$ mg/dm^3.

various background electrolytes are presented. According to the calibration curves, two background electrolytes were selected: 0.1 M 3-substituted ammonium citrate and 0.1 M ammonium sulfate solution, providing a high detection sensitivity coefficient in the range of determined contents $2 \times 10^{-4} \div 0.6$ mg/dm^3.

In Figure 3, voltammograms of the electric oxidation of aflatoxins B_1 and M_1 on the GCE in the selected background electrolyte are shown. Analytical signals are well separated and reproduced.

Both electrolytes can be used for the joint quantification of mycotoxins, but 0.1 M $C_6H_5O_7(NH_4)_3$ was selected as the working background electrolyte providing sufficient resolution and satisfactory reproducibility of the analytical signal.

Figure 4 shows the dependences of the current derivatives of the peak of aflatoxins B_1 (curve 1) and M_1 (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 B_1 and M_1 in their simultaneous determination on a glassy-carbon electrode was studied. For this purpose, the current derivative of the peak of aflatoxin B_1 electric oxidation was obtained as a function of the concentration of aflatoxin M_1 in the solution (Figure 5) and the calibration curves of aflatoxin M_1 in the presence of aflatoxin B_1 (Figure 6).

In Figure 5, it can be seen that, in the concentration range studied, the effect of aflatoxin M_1 on the aflatoxin B_1 current is practically negligible at the ratio $C_{a\Phi B_1} : C_{a\Phi M_1} = 1:1$ in the presence of a two- or threefold excess of aflatoxin M_1, the peak current of aflatoxin B_1 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 B_1 in the presence of aflatoxin M_1 at the ratio $C_{B_1}/C_{M_1} \leq 1 : 40$ does not exceed 20%.

In Figure 6, it is seen that, in the presence of aflatoxin B_1, the derivative of the peak current of aflatoxin M_1 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 B_1 and M_1 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 $(NH_4)_2SO_4$ 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 for determination of quantities of aflatoxins B_1 and M_1 is possible with the measurement error of 15–20% in the concentration ranges $2 \times 10^{-3} \div 2 \times 10^{-1}$ mg/dm^3 and $2 \times 10^{-4} \div 2 \times 10^{-2}$ mg/dm^3, respectively.

The proposed method is simple, and it does not require a lot of reagents and labor. The range of detectable

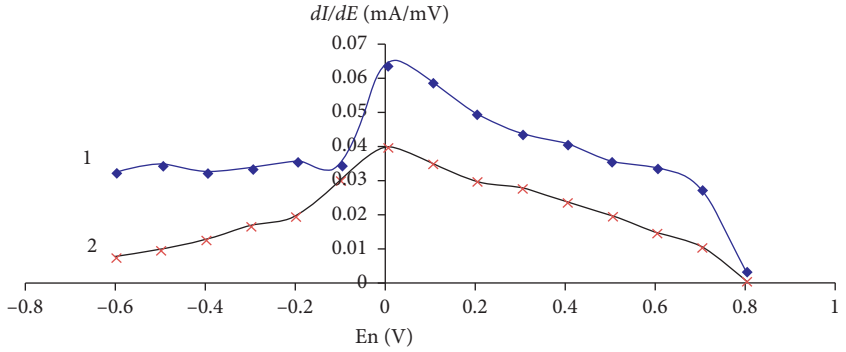

Figure 4: Dependences of the current derivatives of the aflatoxins B_1 and M_1 peak on the accumulation potential of the GCE. The background electrolyte is 0.1 M $C_6H_5O_7(NH_4)_3$; $\tau_э = 30$ s; $w = 30$ mV/s; (1) $C_{afM_1} = 2 \times 10^{-3}$ mg/dm^3; (2) $C_{afM_1} = 2 \times 10^{-4}$ mg/dm^3.

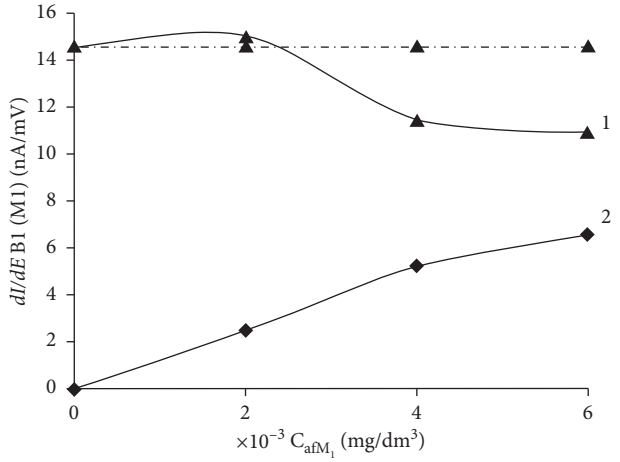

Figure 5: Dependences of the current derivatives of the peak of aflatoxins B_1 (1) and M_1 (2) on the aflatoxin M_1 content in the background electrolyte on the GCE: the background electrolyte is 0.1 M $C_6H_5O_7(NH_4)_3$; (1) $C_{afB_1} = 2 \times 10^{-3}$ mg/dm^3 and C_{afM_1} = var (on 2×10^{-3} mg/dm^3); (2) dependence of $I_{p\cdot afM_1}$ on C_{afM_1}.

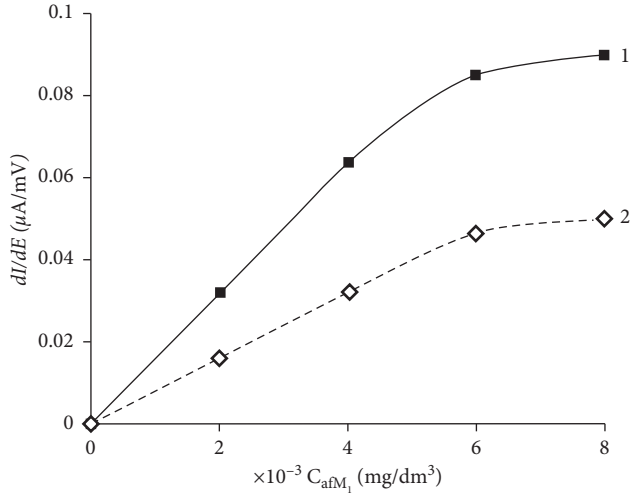

Figure 6: Calibration dependences of aflatoxin M_1 on the GCE: the background electrolyte is 0.1 M $C_6H_5O_7(NH_4)_3$; $E_e = 0{,}0$ B; $\tau_e = 30$ s; (1) $C_{afB_1} = 0$; (2) $C_{afB_1} = 2 \times 10^{-3}$ mg/dm^3.

Table 1: Working conditions of the simultaneous voltammetric determination of aflatoxins B_1 and M_1.

Parameters of votammetric determination of aflatoxins	Parameters values	
	B_1	M_1
The system used	3-electrode	
Electrodes		
(i) Indicator	GCE	
(ii) Comparison/auxiliary	CSE/CSE	
Background electrolyte	0.1 M $C_6H_5O_7(NH_4)_3$	
Electrolysis potential $E_э$ (V)	0,0	
Potential sweeping range (V)	0,0 ÷ +1,1	
Potential changing speed w (mV/s)	30	
Registration mode	Differential	
Peak potential $E_п$ (V)	+0.65 ± 0.05	+0.90 ± 0.05

Table 2: Verification of correctness of the voltammetric method for determination of the content of aflatoxins B_1 and M_1 in model samples of whole milk by the "introduced-found" method ($P = 0.95$ and $n = 5$).

Object	Component	Content of aflatoxins $B_1(10^3)$ and $M_1(10^4)$ (mg/dm^3)		
		In samples	Introduced	Found
Cow's milk Fat content: 3.8%	B_1	2.79 ± 0.41	2.00	4.81 ± 0.72
	M_1	3.01 ± 0.45	2.00	5.43 ± 0.81
Cow's milk Fat content: 1.5%	B_1	1.94 ± 0.35	2.00	3.95 ± 0.65
	M_1	2.12 ± 0.32	2.00	4.21 ± 0.98
Goat's milk Fat content: 4.4%	B_1	3.92 ± 0.58	4.00	7.85 ± 1.12
	M_1	2.08 ± 0.31	4.00	5.95 ± 0.91
Sour milk Fat content: 2.5%	B_1	13.5 ± 1.9	10.0	22.6 ± 3.1
	M_1	15.3 ± 2.1	10.0	26.1 ± 3.7

concentrations is from 0.001 to 0.12 mg/dm^3. The relative standard deviation (Sr) is not more than 30%.

5. Conclusion

Thus, the possibility of the simultaneous voltammetric determination of aflatoxins B_1 and M_1 on the GCE in the background electrolyte 0.1 M $C_6H_5O_7(NH_4)_3$ 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 M_1, 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.

Acknowledgments

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