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Analytical Methods

Determination of aflatoxins B1 and B2 by adsorptive cathodic stripping voltammetry in groundnut

R. Hajian [a,*], A.A. Ensafi [b]

[a] Department of Chemistry, Islamic Azad University Branch of Gachsaran, Gachsaran 75818-63876, Iran
[b] College of Chemistry, Isfahan University of Technology, Isfahan, 84156-83111, Iran

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ABSTRACT

In this study, adsorptive stripping voltammetry was proposed for determination of aflatoxins B1 (AFB1) and B2 (AFB2) using hanging mercury drop electrode (HMDE) as the working electrode. Both aflatoxins were found to adsorb and undergo irreversible reduction reaction at the working mercury electrode. The experimental conditions were optimised by one-at-a time and experimental design to obtain the best characterised peak in terms of peak height with analytical validation of the method for each aflatoxin. The calibration curves for aflatoxins AFB1 and AFB2 were linear in the ranges of 0.4–40 ng ml^{-1} and 0.2–70 ng ml^{-1} with the limit of detections (LOD) 0.15 and 0.10 ng ml^{-1}, respectively. The proposed method was applied for the analysis of aflatoxins in groundnut samples and the results were compared with those obtained by the HPLC technique.

1. Introduction

Aflatoxins are toxic compounds which are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* growing on a variety of food products and are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. Among 18 different types of aflatoxins identified, the major ones are aflatoxin B1 (AFB1) and B2 (AFB2) (Scheme 1). The International Agency for Research on Cancer (IARC) has classified all four aflatoxins as Group 1 carcinogens (in order of toxicity, AFB1 > AFG1 > AFB2 > AFG2) (Chiavaro et al., 2001).

The European Committee Regulations (ECR) establish the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits either for direct human consumption, or as an ingredient in foods: 4 ppb for total aflatoxins (AFB1, AFG1, AFB2 and AFG2) and 2 ppb for AFB1 alone (Moss, 2002; Stroka & Anklam, 2002).

Several methods for aflatoxin determination in various samples have been developed and reported in the literature. Methods based on thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with UV-absorption, fluorescence, mass spectrometry or amperometric detection, have been reported (Gilbert & Vargas, 2003; Kok, 1994; Panalaks & Scott, 1976; Taguchi, Fukushima, Summoto, Yoshida, & Nishimune, 1995). However, these methods require well equipped laboratories, trained personnel, harmful solvents and several hours to complete an assay. Novel methods for the detection of aflatoxins such as the application of surface plasmon resonance biosensors, flow injection monitoring, fibre optic sensors, capillary electrokinetics and electrochemical transduction have been proposed (Gilbert & Vargas, 2003). Several approaches have been made in the past to elaborate and validate means for the fluorescence determination on TLC plates with commercially available or simple prototype apparatus (Dikens, Mcclure, & Whitaker, 1980; Peterson, Ciegler, & Hall, 1967; Pons, Robertson, & Goldblatt, 1968; Robertson & Pons, 1968; Stroka & Anklam, 2000; Whitaker, Dickens, & Slate, 1980). Impressive results by simple means have been made with so-called "spotmeter" prototypes (Dikens et al., 1980; Peterson et al., 1967). These devices were dedicated to determine the fluorescence with a probe that was positioned over the aflatoxin spot. Spots as low as 1 ng were recorded. However, both devices measured the fluorescence transmission on the TLC plate.

In this work, we prompted to extend the adsorptive stripping voltammetry for total determination of aflatoxins B1 (AFB1) and aflatoxin B2 (AFB2). This paper presents a method based on accumulation and reduction of AFB1 and AFB2 species on the surface of hanging mercury drop electrode (HMDE), which offers both sensitivity and selectivity. To the best of our knowledge it is the first reported paper for the determination of aflatoxins by stripping voltammetry.

2. Experimental

2.1. Reagents

All the chemicals were of analytical grade and were purchased from Aldrich or fluka. Double distilled water was used throughout.

* Corresponding author. Tel.: +98 742 3334751; fax: +98 742 3334750.
E-mail address: hajian@iaug.ac.ir (R. Hajian).

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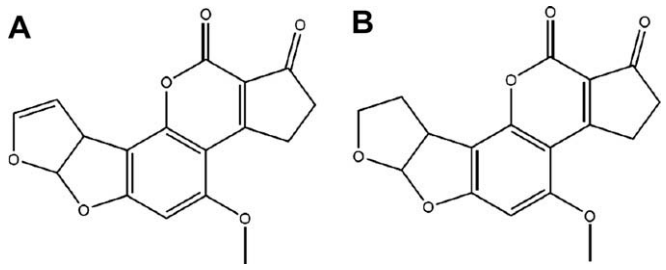

Scheme 1. Molecular structures of (a) AFB1 and (b) AFB2.

Aflatoxin B1 and aflatoxin B2 were supplied by Khatam Co. with a stock concentration of 1 mg l^{-1}. The solutions were kept in a refrigerator at 4 °C in dark. More dilute solutions were prepared by serial dilution with double distilled water.

Britton–Robinson (B–R) buffers (0.1 mol l^{-1} in phosphate, acetate and borate) in the pH range of 2.0–11.0 were used, throughout.

2.2. Apparatus

Voltammetric measurements were carried out using a Metrohm instrument, Model 797 VA, computrace with stand three-electrodes containing hanging mercury drop electrode as a working electrode, a carbon rod as an auxiliary electrode and an Ag/AgCl (3.0 M KCl) reference electrode.

The HPLC system used was a Waters model comprising WATERS 600 controller, WATERS 717 auto sampler, WATERS temperature control module and MILLENIUM chromatography manager, equipped with a model WATERS 420-AC fluorescence detector filled with standard optical filters with 338 nm bandpass excitation filter and 425 nm longpass emission filter. For chromatographic separation, a Nova-Pak C-18 steel column (150 × 3.9 mm, 4 um particle size) was employed. The separation was carried out at room temperature using, as the mobile phase of 75% water, 20% methanol and 5% acetonitrile with a flow rate of 1.4 ml min^{-1} at column temperature of 28 °C. The injection volume was 40 μl with 15 min for running time.

A pH-metre (Corning, Model 140) with a double junction glass electrode was used to check the pH of the solutions.

A Pentium IV (2.53 MHz) computer controlled all of the setting and data processing with the use of MATLAB (version 6.02).

2.3. Preparation of real samples

Groundnut samples were obtained from different parts of Gachsaran city, which is located in the Province of Kohgilooye and Boyerahmad, Iran.

Groundnut samples were first ground in a household blender at high speed for 3 min. For extraction, 10 ml of CH_3OH-H_2O (80:20) was added to 5 g of sample, followed by 5 ml hexane. The suspension was hand shaken for 3 min and then passed through Whatman No. 4 filter paper. The aqueous layer was diluted 1 in 10 for the assays to avoid any interference. The extraction took approximately 15 min to perform (Garden & Strachan, 2001).

2.4. Recommended procedure

For determination of AFB1 and AFB2, 5.0 ml of the supporting electrolyte solution (B–R buffer at pH 9.0) was transferred into the electrochemical cell. The solution was purged with nitrogen gas for 4 min. The adsorption potential at −0.40 V vs. Ag/AgCl was applied to a fresh HMDE and the adsorption were carried out in a stirred solution (2000 rpm) for a period of 60 s. Then a square wave stripping voltamogram was recorded from −0.9 to −1.5 V with a potential scan rate of 0.6 V s^{-1}, pulse amplitude of 0.02 V, voltage step of 0.012 s and frequency of 50 Hz. After the background voltamogram was obtained (Ip_b), aliquots of the sample solution containing a certain concentration of aflatoxins (B1 or B2) were introduced into the cell whilst maintaining a nitrogen atmosphere above the solution. A square wave stripping voltamogram was recorded as described above to obtain the sample peak currents (Ip_s). The difference currents ($Ip_s - Ip_b$) were considered as a net signal (ΔIp). Calibration graphs were prepared by plotting the net peak currents versus aflatoxins (B1 or B2) concentration.

3. Results and discussion

Fig. 1A and B displays square wave voltammograms of aflatoxins B1 and B2 for a solution of Britton–Robinson buffer (a) at pH 9.0 and in the presence of 20 ng ml^{-1} aflatoxins without accumulation time (b) and with accumulation time of 60 s (c) at pH of 9.0 in the potential range of −0.9 to −1.5 V versus Ag/AgCl. According to the voltammograms b and c, the reduction currents for the aflatoxins B1 and B2 system are depending on accumulation time. Addition of 10 μg ml^{-1} Triton X-100 causes reducing the peak currents to about 30% of the initial value. These phenomena show the adsorptive characteristic of the system.

3.1. Effect of operational parameters

In order to find the optimum conditions with highest sensitivity for determination of aflatoxin B1 and B2, the influence of various parameters including pH, accumulation potential and accumulation time on the peak current were studied by traditional (one-at-a time) and also by experimental design, and the results were compared with each other.

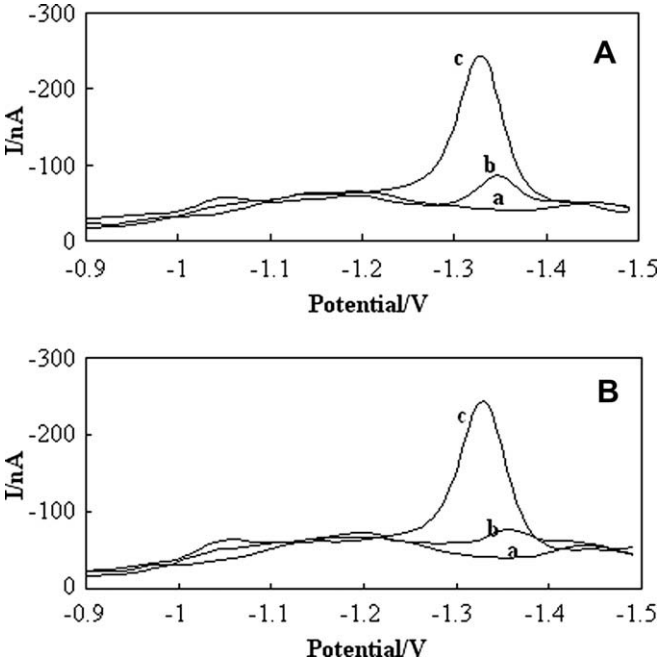

Fig. 1. Square wave voltammogram of AFB1 system (A): (a) Britton–Robinson buffer (pH 9.0) with 60 s accumulation time at −0.50 V, (b) 20.0 ng ml^{-1} AFB1 in Britton–Robinson buffer (pH 9.0) without accumulation time and (c) b with accumulation time of 60 s. (B): Square wave voltammogram of AFB2 system. (a) Britton–Robinson buffer (pH 9.0) with 60 s accumulation time at −0.50 V, (b) 20.0 ng ml^{-1} AFB1 in Britton–Robinson buffer (pH 9.0) without accumulation time and (c) b with accumulation time of 60 s.

3.1.1. One-at-a time optimisation

The dependence of the pH of solution on the peak currents for aflatoxins B1 and B2 was investigated using Britton–Robinson buffer. The results show that the peak currents were independent to pH in the ranges of 2.0–4.0 and 2.0–5.0 for aflatoxins B1 and B2, respectively. However, by increasing pH from 5.0 to 6.0 the peak current of AFB1 increased. For the higher pH values, the peak current leveled off. Similarly, by increasing pH from 4.0 to 7.0 the peak current of AFB2 increased and then leveled off. So pH of 9.0 was selected for further optimisation steps.

The effect of accumulation potential on the stripping peak currents of aflatoxins was examined over the potential range of +0.10 to −0.80 V. By changing in the potential range from +0.10 to −0.10 V the peak currents increased for both AFB1 and AFB2 and at more negative accumulation potentials, currents were leveled off. Therefore an accumulation potential of −0.40 V was selected as an optimum accumulation potential for determination of AFB1 and AFB2.

The effect of accumulation time on the reduction peak currents of AFB1 and AFB2 was studied under the optimised conditions described above. As it is expected in adsorption process, by increasing accumulation time the peak currents for both of the aflatoxins were increased and then leveled off because of the saturation of electrode surface. Therefore for taking higher sensitivity and time consuming of analysis, accumulation time of 60 s was elected for further studies.

3.1.2. Factorial design optimisation

In order to investigate the interactions between influencing parameters on the current we used experimental design optimisation.

According to the above results, it can be noticed that by increasing the accumulation time up to 60 s, the peak currents of AFB1 and AFB2 increased due to increasing the concentration of their compounds on the surface of the electrode. In addition, the peak currents were reached to their maximum values with scan rate of 0.40 V s^{-1}. Our results showed that those two variables were independent of the other variables. Thus, for simplicity (to reduce the variables in our model), in all experiments, accumulation time and scan rate were chosen as 60 s and 0.4 V s^{-1}, respectively, for further study. Therefore, we have optimised the effect of two parameters including pH and accumulation potential on the peak currents using full factorial design optimisation. The level for each factor was chosen as Table 1.

In making the model, the response (the peak current) was written as a function of pH, accumulation potential and all possible interactions. The coefficients of these parameters were obtained by multiple least squares regression. For each parameter and interaction, the parameter coefficient, the standard error and the *t* value for the null hypothesis (H_0) were calculated (Tables 2 and 3). A program written in MATLAB and was used to perform the calculations. The null hypothesis states that the value of the parameter coefficient is zero. The *t* value is the probability that a parameter coefficient can be zero. If the *t* value for each parameter is smaller than the critical value ($t_{crit.}$), the parameter has no significant effect in the model (confidence limit, 95%), and can be eliminated (the value of the parameter coefficient is taken to be zero).

Table 1
Selected levels for each factor.

Level	pH	E_{acc} (V)
−2	2	−0.50
−1	4	−0.30
0	7	0.00
+2	9	+0.1

Table 2
Full factorial design parameters for aflatoxin B1.

Parameter	Parameter value	Standard error	*t* for H_0
Intercept	−3.09	0.58	−5.31
pH	1.72	0.10	17.25
E_{acc}	31.81	3.28	9.70
pH × E_{acc}	−21.98	2.09	−10.52
$pH^2 \times E_{acc}$	1.3	0.23	5.65
pH × E_{acc}^2	−8.40	3.57	−2.35
$pH^2 \times E_{acc}^2$	−2.36	0.27	−0.2

Table 3
Full factorial design parameters for aflatoxin B2.

Parameter	Parameter value	Standard error	*t* for H_0
Intercept	−4.15	0.54	−7.73
pH	2.48	0.09	27.61
E_{acc}	46.58	3.33	13.99
pH × E_{acc}	−19.10	2.13	−8.95
$pH^2 \times E_{acc}$	−0.54	0.23	−2.34
pH × E_{acc}^2	5.46	3.72	1.47
$pH^2 \times E_{acc}^2$	−2.56	0.47	−5.49

The analyses of variance (ANOVA) of the models showed that there wasn't any lack of fit in the two models for aflatoxins B1 and B2. The *F* value of the model (regression) describes that the regression at the confidence limit of 95% is significant. The models have correlation coefficients, adjusted R^2, 0.9921 and 0.9619, explaining 99.21% and 96.19% of variances in the response values for AFB1 and AFB2 models, respectively. There is no lack of fit in the confidence limit 95% (calculated $F_{lack\ of\ fit}$ is less than the critical value). From the model, a program in MATLAB was written to calculate the optimum values for the each parameter in two models. Within the confidence limit of 95%, the models show one significant second-order interaction and two significant third-order interactions.

The current calculated from the models was plotted versus the measured currents for AFB1 and AFB2, respectively. The correlation coefficients for the plots are 0.9921 and 0.9619, which indicates good performance for the models. By plotting the residuals versus the number of experiments for AFB1 and AFB2, it was shown that hemoscedastic error was propagated between the experiments.

The optimum values for pH and accumulation potential calculated with MATLAB programming are 9.2 and −0.37, respectively, for both AFB1 and AFB2. The results showed that the optimisation results obtained by one-at-a time and full factorial design are more similar to each other. Therefore, we used pH of 9.0, accumulation potential of −0.40 V, an accumulation time of 60 s and a scan rate of 0.40 V s^{-1} for both determination of AFB1 and AFB2.

3.2. Figures of merit

Under the optimised conditions, two calibration graphs for separate determination of AFB1 and AFB2 were obtained. The calibration plot for AFB1 was linear over the range of 0.4–40.0 ng ml^{-1} with regression equation of $\Delta I = 6.22(\pm0.61)C - 2.68(\pm0.42)$ ($r^2 = 0.9923$ ($n = 15$)). Similar calibration curve for AFB2 was plotted with a linear dynamic range of 0.2–70.0 ng ml^{-1} with regression equation of $\Delta I = 6.30(\pm1.62)C + 6.38(\pm2.40)$ ($r^2 = 0.9928$ ($n = 26$)), where ΔI and C are net current (nA) and concentration of aflatoxins (ng ml^{-1}), respectively.

The limit of detections ($3S_B$), were 0.15 and 0.10 ng ml^{-1} for AFB1 and AFB2, respectively. The relative standard deviations ($n = 6$) for 15.0 ng ml^{-1} AFB1 and 40.0 ng ml^{-1} AFB2 were 2.5% and 1.4%, respectively.

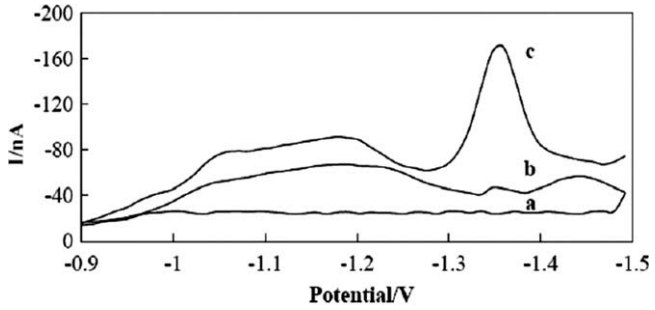

Fig. 2. Square wave voltammograms of Britton–Robinson buffer (pH 9.0) with 60 s accumulation time at −0.40 V (a), injection of 100 μl real sample (b) and with the addition of 10 ng ml^{-1} AFB1 (c).

3.3. Interference study

The effects of some species on the determinations of 5.0 ng ml^{-1} of AFB1 and 10.0 ng ml^{-1} for aflatoxin B2 were studied under the optimised conditions described above. The tolerance limit was defined as the foreign-species concentration causing an error smaller than 5.0% for the determining aflatoxins. The results showed that Methanol, Ethanol, Urea, Glucose, Saccarose, Lactose, Fructose and Benzoic acid were not interference in 1000 times more that the analytes. Also, Alanine, Tyrosine, Glycine, Asparagine, Phenylalanine, Cystine, Serine, Leucine and Asparatic acid were not any significant error in concentrations until 400 times. Ascorbic acid was the effective interference in concentrations more than 100 times. The results indicate that many of species did not interfere. Only AFB2 interfered at level equal to AFB1 and vice versa due to the similar chemical structures. So, this method can be used for total determination of AFB1 and AFB2.

3.4. Determinations of AFB1 and AFB2 in real samples

The proposed method under the optimum condition was applied to the determination of total aflatoxins in 10 groundnut real samples. The extraction and clean-up method for aflatoxins in real samples which have been previously applied by other researchers (Garden and Strachan, 2001) were tried in order to obtain the highest yield of aflatoxins with the minimum matrix effect.

For each sample, 100 μl of the solution was spiked into 5 ml supporting electrolyte followed with general voltammetric determination procedure. Voltammograms of each samples were recorded and any peak appeared was observed. For determination of aflatoxin in real sample, single standard addition method was applied by spiking 10 ng ml^{-1} of aflatoxin standard followed with general procedure for voltammetric analysis (Fig. 2). Because of the equal sensitivities for AFB1 and AFB2, the amount of total aflatoxins in groundnut samples were calculated according to the formula as stated below:

Table 4
Total aflatoxins contents in four types of groundnuts.

Samples	Total aflatoxins/ng g^{-1}	
	Proposed method	HPLC
1	2.75 (±0.18)	2.54 (±0.21)
2	1.12 (±0.09)	1.23 (±0.12)
3	1.20 (±0.87)	1.35 (±0.91)
4	3.47 (±0.31)	3.28 (±0.37)
5	1.75 (±0.15)	1.50 (±0.22)
6	1.85 (±0.12)	1.70 (±0.25)
7	2.80 (±0.27)	2.96 (±0.58)
8	3.12 (±0.34)	3.08 (±0.42)
9	1.20 (±0.26)	1.42 (±0.35)
10	3.22 (±0.35)	3.39 (±0.39)

$$\text{Aflatoxin}(\text{ng ml}^{-1}) = \frac{P'}{P} \times C \times 20 \quad (1)$$

where;

P' is peak current of sample (nA), P is peak current of standard aflatoxin (after subtract from P') (nA), C is the concentration of aflatoxin spiked in the cell (ng ml^{-1}) and 20 is a Factor value after the sample weight, volume of methanol/water used in the extraction and preparation of injection sample have been considered. The detailed information on how this formula was formulated is shown in supplementary data.

The results were compared with HPLC as a reliable standard method (Table 4). The good comparably between results indicate the successful applicability of proposed method for determination of total aflatoxins.

4. Conclusions

The present study demonstrates that Adsorptive Stripping voltammetry is a suitable method for determination of total aflatoxins (B1 and B2) in real samples. This method has some advantages such as high sensitivity, extended linear dynamic range, simplicity and speed, a combination very better than previously reported systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2008.12.009.

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