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# Cathodic adsorptive stripping voltammetry of abscisic acid using pencil-lead bismuth-film electrode

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## Abstract

In this study, electrochemical behavior of phytohormone abscisic acid (ABA) at the bismuth-film electrode (BiFE) has been investigated using square-wave cathodic stripping voltammetry. BiFE was prepared *ex situ* on a supporting pencil-lead graphite electrode. ABA yielded a well-defined voltammetric response in phosphate buffer, pH 3.0 at -1.08 V (vs. Ag/AgCl) (a pre-concentration step being carried out at a fixed potential of -0.80 V for 120 s). The process could be used to determine ABA concentrations in the range 0.756–15.08 μM, with a detection limit of 0.209 μM (55.24 ng/ml). As an example, the practical applicability of pencil-lead BiFE was tested with the measurement of ABA in seed samples of maize plant (*Zea mays* L.).

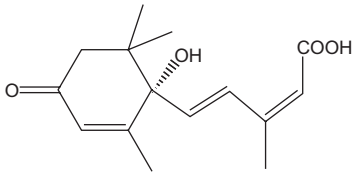
**Keywords:** abscisic acid; bismuth-film electrode; pencil-lead graphite; plant hormones; square-wave cathodic adsorptive stripping voltammetry.

## Introduction

Plant hormones or phytohormones, a group of naturally occurring substances, control the physiological processes at low concentrations. As minor components of the metabolome, phytohormones are of particular significance given their role in the regulation of germination, growth, reproduction, and the protective responses of plants against environmental stresses such as drought, cold, high temperatures, or salt stresses (Davies 2004). Since the discovery of the first plant hormones, auxins, early in the twentieth century, scientists have reported up to several types of phytohormones, mainly including auxins, cytokinins, gibberellins, and inhibitors (Rivier and Crozier 1987). Abscisic acid (ABA, see Scheme 1), a monocyclic sesquiterpenoid, belongs to the latter, which inhibits or delays the physiological or biochemical process of plants, including response to abiotic stress (abnormal temperature, ultraviolet, drought, etc.), regulation of seed dormancy and germination, and control of stomatal movement (Bray 2002). The levels of concentration of ABA in plant cells are dynamically maintained by continual synthesis, transport, and degradation. Metabolism can occur by several routes involving oxidation, reduction, and conjugation (Cutler and Krochko 1999). The study of the variation of ABA concentration in different plant tissues can therefore provide knowledge on the ABA biosynthesis and degradation pathways, and the correlation with different physiological and environmental parameters (Xiong and Zhu 2003, Koussa et al. 2004, Soar et al. 2004). Using biotechnology approaches to control ABA levels is seen as a powerful strategy for improving plant performance and productivity, and there is currently much research into the molecular mechanism of ABA action (Rock 2000).

Over the past few years, different sensitive and selective methods have been developed to detect and quantify ABA in plant tissues. The use of mass spectrometry (MS) coupled with an appropriate separation technique such as high-performance liquid chromatography (HPLC) (Ross et al. 2004, Vilaro et al. 2006, Hou et al. 2008, López-Carbonell et al. 2009) or gas chromatography (GC) (Cvikrová et al. 1998, Kamboj et al. 1999, Müller et al. 2002, Okamoto et al. 2009) allows the determination of trace amounts of ABA down to nanograms/gram fresh weight (ng/g fw). However, most of them require very expensive instruments and complicated sample treating. The other approach to free and conjugated ABA analysis is the use of immunoassays such as radioimmunoassay (RIA) (Weiler 1980), enzyme-linked immunosorbent assay (ELISA) (Daie and Wyse 1982, Montero et al. 1994), or immunoaffinity chromatography (IAC) (Hradecká et al. 2007). Although immunoassays show good sensitivity, selectivity, and accuracy for ABA, the analytical process is very complex, which can consume many hours of tedious work. It is, therefore, important to exploit a simpler and more rapid analytical method for the determination of ABA. Compared with the above-mentioned techniques, electrochemical methods, particularly the voltammetric ones, could meet these requirements because they are relatively simple to apply, fast, and reasonably cheap, providing good sensitivity and selectivity to electroactive analytes (Wang 2006). We have not, however, found any literature data on the electrochemical behaviors of ABA, except in a study by Hernández et al. who deals with its cathodic adsorptive stripping voltammetric determination using a hanging mercury drop electrode (Hernández et al. 1997). The hormone, which is not electroactive in basic medium, undergoes an electrochemical reduction process in acidic pH values, with one step involving four electrons.

Cathodic electrochemical detection of inorganic as well as organic compounds has been studied essentially at different types of mercury electrodes. Recently, much more care has been needed for the analytical use of mercury owing to toxicity and environmental pollution. In the past few years, a novel bismuth-film electrode (BiFE) has been recognized as




**Scheme 1** Chemical structure of abscisic acid (ABA).

an alternative to mercury electrodes for voltammetric stripping analysis (Wang et al. 2000). The favorable electroanalytical characteristics of BiFE, such as simple preparation, high sensitivity, excellent peak resolution, and insensitivity to dissolved oxygen in aqueous solutions, high hydrogen overpotentials or negligible toxicity, have encouraged many scientists to study for environmental and clinical applications in trace metal analysis (Hocevar et al. 2002, Kefala et al. 2003, 2004, Królicka and Bobrowski 2004, Wang 2005, Hutton et al. 2006). By contrast, to date, only a few articles have been published investigating the possibility of utilizing BiFEs for organic compounds (Hutton et al. 2001, 2004, Bučková et al. 2005, Guzsvány et al. 2006, Sánchez Arribas et al. 2006, Yang et al. 2006, Claux and Vittori 2007, Sattayasamitsathit et al. 2007, Du et al. 2008, Moreno et al. 2009, Nigovič et al. 2009). Therefore, there is an increasing need to investigate the practical application of BiFEs in the area of organic compound analysis.

To date, different materials have been used as substrates for BiFEs, including glassy carbon, carbon paste, carbon fiber, impregnated graphite, screen-printed inks, and noble metals (Wang 2005). More recently, pencil-based graphite substrate has been reported for the stripping analysis of trace metals (Demetriades et al. 2004, Rehacek et al. 2008). The main attractions of this material are high electrical conductivity, good mechanical rigidity, low cost, wide availability, and ease of modification.

The aim of this report was to study the possibility of applying BiFE consisting of a thin bismuth-film deposited onto pencil-lead graphite for a novel analytical quantification of ABA. The utility of the method developed was also demonstrated in real samples.

## Materials and methods

### Chemicals

Standard ABA was purchased from Sigma (Germany). Its stock solution ($5\times10^{-3}$ M) was prepared in methanol owing to its low solubility in water. On the day of the experiment working solutions were prepared by diluting the stock solution with a selected supporting electrolyte. Four different supporting electrolytes, namely perchloric acid (0.1 M), nitric acid (0.1 M), Britton-Robinson buffer (0.1 M, pH 2), and phosphate buffer (0.1 M, pH 3, 4, and 7.4) solutions containing 0.02 M NaCl were used. The Bi(III) ion stock solution (1000 mg/l) was prepared by dissolving bismuth nitrate (Merck, Germany) in 10 wt% nitric acid and diluted as required. An acetate buffer (0.1 M, pH 4.8 containing 0.02 M NaCl) served as the electrolyte solution for plating of the bismuth onto the substrate electrode material. All stock solutions were preserved at 4°C when not in use and protected from daylight during use in the laboratory. All other chemicals were of analytical reagent grade and used as received. Aqueous solutions were prepared with deionized water further purified via a Milli-Q unit (Millipore, France).

### Apparatus

Cyclic and square-wave voltammetric (CV, SWV) measurements were carried out using a μAutolab type III potensiostat (EcoChemie, The Netherlands) controlled by GPES 4.9 software. The raw square-wave voltammograms were treated by using the Savitzky and Golay filter (level 2) of the GPES software (EcoChemie, The Netherlands) followed by the moving average baseline correction with a peak width of 0.01 V.

In all measurements, the reference electrode was an Ag/AgCl (3 M NaCl) (Model RE-1, BAS, USA) and the auxiliary electrode was a platinum wire. A bare pencil-lead graphite electrode (PGE) or bismuth-coated PGE (BiFE) served as the working electrodes. For PGE, a mechanical pencil Model T 0.5 (Rotring, Germany) was used as a holder for pencil-lead (Tombo, 0.5 mm, 2B, Japan), which were purchased from a local bookstore. The details of the preparation of PGE have been described in our previous study (Levent et al. 2009). Before use, pencil-lead graphite did not require any electrode pretreatment protocol as a bare or substrate electrode. The measurements were carried out in a standard home-made 5-ml glass voltammetric cell at a laboratory temperature (20–25°C). The convective transport was provided with a magnetic stirring of 300 rpm.

### Preparation of BiFE

The *ex situ* electroplating of the bismuth film on the PGE was performed in air saturated acetate buffer solution (pH 4.8) containing 5 mg/l of Bi(III) by applying a constant potential of -1.0 V for a fixed time (150 s), while stirring the plating solution. The electrode was then gently rinsed with acetate buffer solution (pH 4.8) and transferred into the fresh analyzed solution.

### Electrochemical measurements

Electrochemical measurements were carried out in non-deaerated solutions containing the selected supporting electrolyte, which was spiked with the required volume of the stock ABA solution. For stripping studies, the working electrode was kept in the cell, and the solution was stirred at a chosen accumulation potential (-0.8 V) throughout the selected accumulation period (120 s). After a resting period of 10 s, a cathodic SW scan was applied. The common instrumental parameters for SWV were as follows: pulse amplitude, 30 mV; frequency, 100 Hz; scan increment, 8 mV. Successive measurements were carried out by repeating the above assay protocol on a new surface of working electrode.

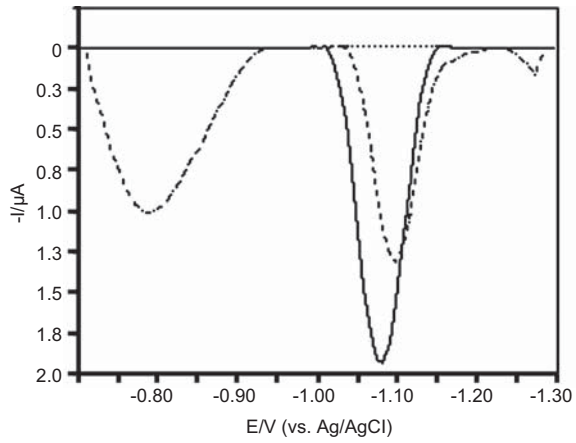



**Figure 1** SW voltammograms of 40 μM ABA in 0.10 M phosphate buffer pH 3.0 (with 0.02 M NaCl) at PGE (dashed line) and at pencil-lead BiFE (solid line). SWV parameters: frequency, 25 Hz; scan increment, 8 mV; pulse amplitude, 20 mV. The blank solution is represented (…) at pencil-lead BiFE.

## Results and discussion

For a comparison between a bare PGE and BiFE plated *ex situ* on pencil-lead, in a solution containing 40 μM ABA in phosphate buffer (pH 3.0, with 0.02 M NaCl), the SWV detection mode was initially employed in the cathodic potential range of -0.7 to -1.3 V without prior accumulation time (Figure 1). ABA undergoes electrochemical reaction in the negative potential window and can be easily detected at the BiFE with the cathodic peak at a potential of -1.08 V. As clearly evident from Figure 1, the ABA reduction behavior at the BiFE is advantageous over that observed at the bare PGE under identical conditions, as the peak potential is approximately 16 mV less negative and the peak current is approximately 45% higher, indicating that the electron transfer rate is favored in BiFE. Moreover, BiFE showed lower background and no adverse influence of the oxygen reduction was observed in the opposite to bare PGE. As expected, the presence of dissolved oxygen affected the SWV current performance at the PGE, causing a strong and broad cathodic peak in the potential range of -0.7 V to -0.9 V. We performed the same measurement at BiFE without and with the nitrogen bubbling procedure. The electrode produced identical baseline characteristics in both cases, which corresponds with literature information (Wang et al. 2000, Bučková et al. 2005), where removal of oxygen does not play any role for the analysis and hence deaeration was not necessary in the case of BiFE.

Experiments performed in media which are more acidic (pH≤2), such as 0.1 M perchloric acid, 0.1 M nitric acid, 0.1 M Britton-Robinson buffer pH 2 solutions containing 0.02 M NaCl, showed the dramatic increase of background current in the more negative potential region. The cathodic limit is given by the reduction of protons and therefore it shifted with increasing pH to the more negative values of potential up to -0.85 V for 0.1 mol/l HCl (pH 1.0). The studies reported earlier (Hutton et al. 2001) have concluded that the negative cathodic limit of the BiFE is shifted with decreasing pH to the less negative values of potential (e.g., -0.85 V for a 0.1 M strong acid) owing to the background discharge (commencement of hydrogen evolution). For this reason, in such solutions the ABA peak was either completely obscured or was superimposed on a sloping baseline that made difficult the precise measurement of the peak height. It was observed that hormone reduction peak almost disappeared at pH values higher than approximately 3.0. This observation confirms the results at hanging mercury drop electrode for ABA (Hernández et al. 1997). Therefore, the 0.1 M phosphate buffer (pH 3.0, containing 0.02 M NaCl) was selected using BiFE for further experiments.

Next, the optimization experiments for the quality of bismuth coating using SWV signal were conducted by varying the plating solution composition, Bi concentration, plating potential and time. Different acidic media, including 0.1 M $HNO_3$ (with 0.02 M NaCl), 0.1 M $HClO_4$ (with 0.02 M NaCl) and 0.1 M acetate buffer (pH 4.8, with 0.02 M NaCl) were tested for 5 mg/l of Bi(III). Although Bi(III) hydrolysis at relatively low pH values is known, the Bi film formation using acetate buffer (pH 4.8, with 0.02 M NaCl) was not only successfully accomplished but showed its optimum cathodic detection behavior. The concentration of bismuth was varied in the range 2–20 mg/l by keeping both the film deposition time (150 s) and potential (-1.0 V) constant. The response increased slightly with the level of bismuth at first up to 5 mg/l, then more slowly and nearly leveled off above 10 mg/l bismuth. It was further noted that when the bismuth concentration was higher than 20 mg/l, the low solubility of Bi(III) was observed in the plating solution. Using the concentration at 5 mg/l, the plating potential and time were investigated in the range of -0.2 to -1.2 V and 30–250 s, respectively. By using the same criteria, -1.0 V and 150 s were found as optimum selected conditions.

Further research was dedicated towards studying the electrochemical behavior of ABA at BiFE in phosphate buffer (pH

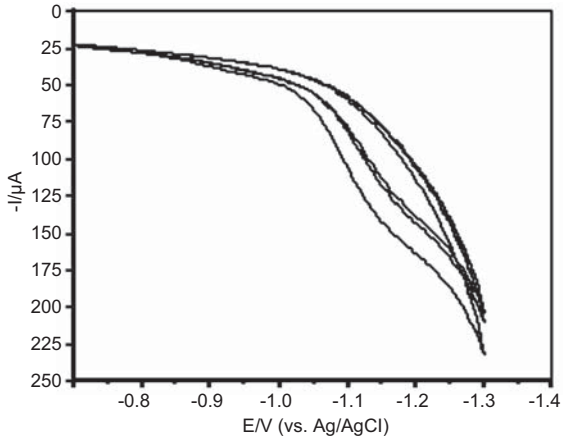



**Figure 2** The repetitive cyclic voltammograms of 0.5 mM ABA solutions in phosphate buffer, pH 3.0 (with 0.02 M NaCl) for pencil-lead BiFE. Scan rate, 200 mV/s.

3.0, with 0.02 M NaCl) upon the CV response, without performing an accumulation step. The potential range from -0.7 to -1.3 V was used for CV analysis. ABA showed a single irreversible reduction wave as depicted in Figure 2. Multi-scan CV recordings revealed that the waves decreased upon the second and subsequent scans at the same bismuth film, pointing to certain adsorption activity at the electrode surface.

Scan rate examination between 25 and 300 mV/s on the reduction of ABA at BiFE was also carried out in phosphate buffer (pH 3.0, with 0.02 M NaCl). The peaks became deformed at scan rates above 300 mV/s, which poses problems for the use of high scan rates. Plotting the logarithm of peak current (log $i_p$) vs. logarithm of scan rate (log $v$) yielded a straight line with a slope of 0.78. The slope denotes that the process is predominantly controlled by adsorption. The equation obtained is:

$$\log i_p\ (\mu A) = 0.78 \log v\ (mV/s) - 0.59,\ r = 0.996$$

The observed responses suggested the possibility of setting up a method to determine ABA by cathodic stripping voltammetry. Among the stripping waveforms, the square-wave modulation combines good sensitivity with high speed, and insensitivity to dissolved oxygen which allows the analysis to be carried out in the presence of oxygen and avoided the time-consuming deoxygenation step. As a consequence, with the aim of obtaining the highest electroanalytical performance of the BiFE for measuring ABA, pre-concentration/stripping conditions, such as accumulation potential and time, and the key operational parameters for SWV mode were optimized.

The effect of the accumulation time between 0 and 240 s on the ABA stripping peak height at open circuit potential in a stirring solution containing 40 μM ABA is illustrated in Figure 3A. The signal increased considerably up to 120 s and at longer accumulation times it started to diminish. The accumulation potential was examined either at open circuit condition or in the potential window between -0.1 V and -1.0 V in combination with an accumulation time of 120 s. The value for the stripping current obtained at -0.1 V was nearly equal to the value obtained at open circuit voltage. As can be seen in Figure 3B, the response increased while decreasing the accumulation potential from -0.1 to -0.8 V, whereas at more negative accumulation potentials the response attenuated. For the rest of the study, an accumulation time and potential of 120 s and -0.8 V were used, respectively.

The SWV parameter optimization was carried out in solution of 40 μM ABA (data not shown). The ranges studied were 5–125 Hz for frequency, 4–14 mV for scan increment, and 10–40 mV for pulse amplitude. The scan rate in SWV is the result of the product of the frequency and scan increment. Therefore, the peak heights increased with the increase in the frequency and scan increment. At higher values than 100 Hz and 8 mV, a broadening and a distortion in the voltammograms were observed. The analytical signal is also dependent on the pulse amplitude even if this parameter seems to be less important than the frequency. Peak heights increased linearly up to 30 mV. However, with higher pulse amplitudes a broadening of the peak occurred. For all subsequent studies the

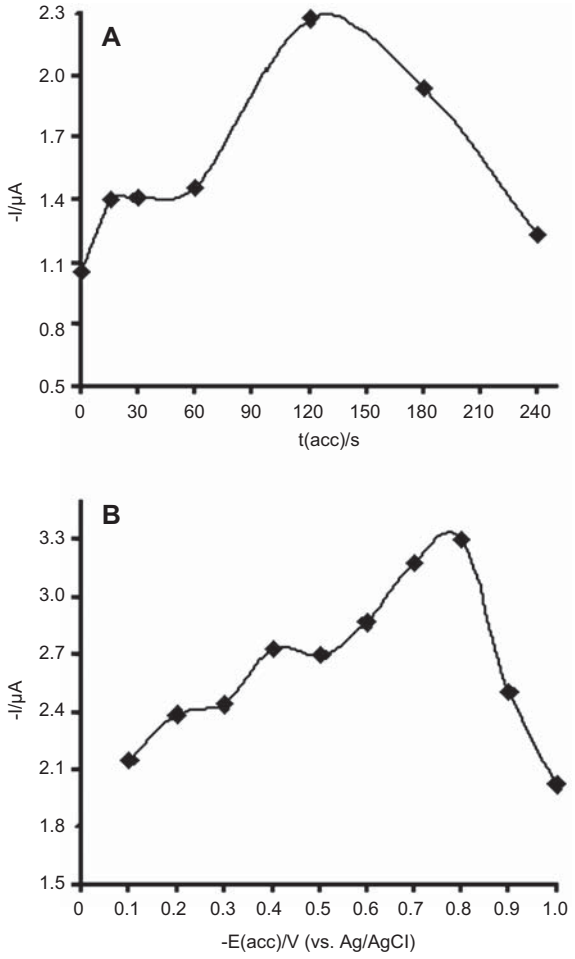



**Figure 3** The effect of accumulation variables on the stripping signals for 40 μM ABA solution in phosphate buffer, pH 3.0 (with 0.02 M NaCl). Electrode, pencil-lead BiFE; SWV parameters as indicated in Figure 1.

selected conditions were: frequency, 100 Hz; scan increment, 8 mV; and pulse amplitude, 30 mV.

The previously optimized adsorptive stripping SWV parameters were employed to record the analytical curve for ABA in phosphate buffer (pH 3.0) using the BiFE. The electrode revealed a linear behavior for stripping signals in the concentration range 0.756–15.08 μM (Figure 4), which fits the expression

$$i_p\ (\mu A) = 0.1433\ C\ (\mu M) + 0.0777,\ r = 0.997$$

The sensitivity of the proposed method was evaluated both by the limit of detection (LOD) and limit of quantification (LOQ) values. The LOD and LOQ values were calculated using the following equations:

$$LOD = 3\ s/m;\ LOQ = 10\ s/m,$$

where $s$ is the standard deviation of the peak current (three runs) of the lowest concentration of the linearity range (0.756 μM) and $m$ is the slope of the related calibration

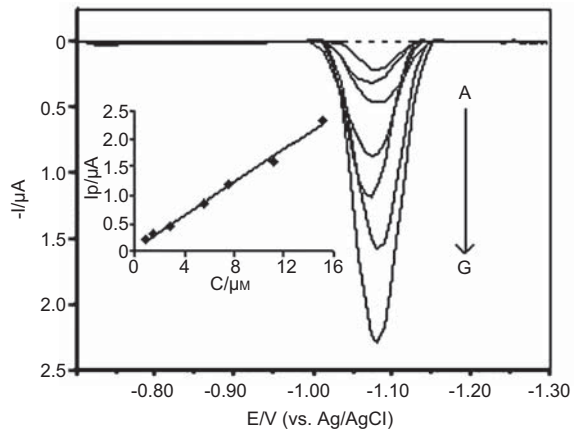



**Figure 4** The stripping voltammograms for increasing levels of ABA concentrations 0.756–15.08 μM (A–G) in phosphate buffer, pH 3.0 (with 0.02 M NaCl) and dashed line represents the blank solution at pencil-lead BiFE. Inset depicts a corresponding calibration plot for the quantitation of ABA. SWV parameters: frequency, 100 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

equation. LOD and LOQ values were calculated as 0.209 μM (55.24 ng/ml) and 0.696 μM (183.95 ng/ml), respectively. The repeatability on a new electrode surface was tested for six measurements of the current peak for solutions of 2.82 μM ABA. The relative standard deviation was found to be 7.84% and under optimized conditions. Table 1 compares the analytical performance of ABA at the proposed methods. To evaluate the selectivity of the proposed method, increasing concentrations of the possible interfering agents such as some metal ions which are bound up with growth of plants and phytohormone compounds which are usually present in the plants were added to a solution with a fixed amount of ABA (5.64 μM), and the corresponding voltammograms were recorded. The tolerance limit was defined as the maximum concentration of potential interfering substance that causes a relative error less than ±5% for determination of 5.64 μM ABA. At approximately 100-fold excess, $Fe^{3+}$, $Mg^{2+}$, $Zn^{2+}$, gibberellic acid, indole-3-acetic acid, methyl jasmonat did not significantly influence the height of the peak currents, whereas at approximately 10-fold excess $Cu^{2+}$ seriously interfered with ABA assay at BiFE electrode. It could be supposed that a procedure could be used including chelation of $Cu^{2+}$ followed by a simple solvent extraction or precipitation to decrease interference remarkably.

It is clear that this new voltammetric method can be used to determine ABA quantitatively in the ideal laboratory samples. To testify its practical application, this voltammetric method was applied to the analysis of the seed samples of maize plant (*Zea mays* L.). The samples were provided by the Department of Field Crops, Yüzüncü Yıl University, Van, Turkey. Thecollected materials were weighted, powdered by pestle and mortar in liquid nitrogen, and kept in a deep freezer until analysis. Extraction of plant material was carried out as described previously (Battal et al. 2008). Appropriate volume (100 μl) of the extract of maize seeds was placed into a cell containing the phosphate buffer pH 3.0 (with 0.02 M NaCl) and the voltammetric procedure was followed. Quantifications were performed by means of the calibration curve method. No signal for ABA was observed when the samples were analyzed by using the method described above, as its concentration in this sample could be lower than the detection limit of the proposed method. In the reported study (Hernández et al. 1997), a direct voltammetric measure at mercury electrode has been applied to an extract of pears. ABA signal has not been observed because the nature of the sample produces adsorption in the electrode. Thus, samples were spiked with known amounts of ABA standard solutions. ABA peaks were clearly displayed for re-analyzed spiked plant extracts. Recovery of ABA was calculated by comparing the concentration obtained from the spiked mixtures with those of the pure ABA. The results are summarized in Table 2. Recovery of the spiked ABA was also observed to be good in samples.

**Table 2** Recoveries of ABA added to the seed extracts of maize by square-wave adsorptive stripping voltammetry at pencil-lead BiFE[a].

| ABA added (μM) | ABA found (μM) | Recovery (%)±RSD (%) |
|---|---|---|
| 4.7 | 4.35 | 92.55±11.94 |
| 8.8 | 9.31 | 105.79±3.93 |

[a]Values reported are the average of three independent analyses of each spiked sample.

## Conclusion

The BiFE prepared *ex situ* on a supporting PGE was recognized as a suitable electrochemical sensor for its application in voltammetric measurements of the biologically significant phytohormone, ABA, by square-wave cathodic stripping voltammetry in acetate buffer solution (0.1 M, pH 4.8 containing 0.02 M NaCl). Bi(III) is a much softer oxidant than both oxygen and mercury(II) and, moreover, in media containing halide ions, the corresponding values of its formal redox potentials are even lower owing to formation of chloro- and/or bromobismuthates. In acetate buffers, the value of formal redox potential is also lower because of formation of hydroxocomplexes (Vytřoeas et al. 2002). Combined with

**Table 1** Comparison of the efficiency of the electrodes in the determination of ABA.

| Electrode | pH | LOD (ng/ml) | r | Was the $O_2$ eliminated? | References |
|---|---|---|---|---|---|
| HDME | 1 | 30.00 | 0.999 | Yes | (Hernández et al. 1997) |
| BiFE | 3 | 55.24 | 0.996 | No | This work |

being inexpensive and disposable of PGE, pencil-lead BiFE offers the advantage of avoiding the toxicity and pollution of mercury electrodes. Although the new proposed method is less sensitive than the previously published voltammetric method (detection limit, 30 ng/ml), ABA signal has also not been observed at mercury electrode by applying the direct electrochemical measurement in extract of pears (Hernández et al. 1997). More research is currently being carried out in our laboratory to investigate the Nafion coating on the pencil-lead BiFE, by coupling HPLC as the preparatory technique as reported by Hernández et al. (1997), in order to improve the sensitivity and selectivity of the presented voltammetric method.

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