Sensitivity and Specificity Improvement of an Ion Sensitive Field Effect Transistors-Based Biosensor for Potato Glycoalkaloids Detection

Yaroslav I. Korpan,*,† Frank M. Raushel,‡ Elena A. Nazarenko,†
Alexey P. Soldatkin,† Nicole Jaffrezic-Renault,§ and Claude Martelet§

Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine,
150 Zabolotnogo St., UA-03143, Kyiv-143, Ukraine, Department of Chemistry, Texas A&M
University, P.O. Box 30012, College Station, Texas 77842-3012, and Ecole Centrale de Lyon,
CEGELY, UMR CNRS 5005, 36 avenue Guy de Collongue, F-69134 Ecully Cedex, France

Butyryl cholinesterase of different origin along with variations of the time of enzyme immobilization on the potentiometric transducer surface is offered to control the ion sensitive field effect transistor (ISFET)-based biosensor sensitivity. Because butyryl cholinesterase has been already used to develop the sensors for heavy metals, organophosphorus/carbamate pesticides, and steroidal glycoalkaloids analysis, the present study has been focused on the investigation and adjustment of the ISFET-based biosensor specificity exclusively to the glycoalkaloids. Utilization of ethylendiaminetetracetate (a complexon of heavy metal ions) and phosphotriesterase (a highly efficient catalyst for the hydrolysis of organophosphorus compounds) enabled the highly specific determination of glycoalkaloids at the background of lead and mercury (up to 500 μM of ions concentration) and paraoxon (up to 100 μM of pesticide concentration). The developed biosensor has been validated for glycoalkaloids detection in potato varieties cultivated in Ukraine, and the results obtained are compared to those measured by the methods of HPLC and TLC.

INTRODUCTION

The cultivated potato (*Solanum tuberosum* L.), as one of the world's major agricultural crops, is consumed daily by millions of people from diverse cultural backgrounds. Potatoes are grown in ~80% of all countries, and worldwide production stands in access of 350 million tons per annum, a figure exceeded only by wheat, maize, and rice (*1*). Despite its status as a food of global importance, the potato tuber contains toxic steroidal glycoalkaloids (SGA) that cause sporadic outbreaks of poisoning in humans and many livestock deaths (*2*). Potatoes and individual alkaloids have been shown to be teratogenic and embryotoxic (*3, 4*). It has been also found that SGA specifically induce membrane disruptive effects on cholesterol containing membranes and are potent in permeabilizing the outer membrane of mitochondria. In vitro studies have revealed that α-solanine and α-chaconine inhibit substantially the activity of cholinesterase (*5*). α-Solanine noncompetitively inhibits calcium transport in vivo in rat duodenum. Either α-chaconine or α-solanine reduces the active transport of sodium ion in frog skin (*6, 7*). Their high concentration may cause acute poisoning, including gastrointestinal and neurological disturbances, with death being caused by depression of the central nervous system. Other studies also suggest that there is an increased risk for cancers of the brain, breast, endometrium, lung, and thyroid associated with the consumption of large quantities of potatoes (*8*). Available information suggests that the susceptibility of humans to SGA poisoning is both high and very variable: oral doses in the range of 1−5 mg kg^{-1} body weight are marginally to severely toxic to humans, whereas 3−6 mg kg^{-1} body weight can be lethal (*9*). Neither α-chaconine nor α-solanine levels are affected by food processing and preparation. On the contrary, baking and frying, resulting in low moisture-containing products, considerably concentrate SGA in products such as potato chips and fried peel, and the risk of possible toxicity increases greatly (*10*). The widely accepted safety limit for SGA in tubers remains 200 mg kg^{-1} fresh weight. However, because of the large and often unpredictable variations in the SGA levels, which can arise from differences in variety, locality, cultural practices, and stress factors (light, disease, mechanical injury, conditions of storage and pretreatment, insect damage, and chemicals), and the fact that so many aspects of the biochemistry and toxicity of these compounds remain poorly understood, the World Health Organization has proposed that the limit should be reduced to

* Author to whom correspondence should be addressed [telephone/fax +38-044-522-28-24; e-mail korpan@imbg.org.ua or ya_korpan@yahoo.com].
† National Academy of Sciences of Ukraine.
‡ Texas A&M University.
§ CEGELY.

20−100 mg per kg. According to Parnell et al. (*11*), "many authors have assumed without further evidence that levels below 200 mg/kg are safe. They ignore the fact that the 200 mg/kg [FW] level only relates to acute and/or sub-acute effects and not to possible chronic effects ..."

These considerations are sufficient to highlight the need for the control of SGA levels in agriculture, food analysis, and health care.

The methods in current use for the determination of SGA include colorimetry (*12*), thin-layer chromatography (*13*), gas chromatography (*14*), mass spectrometry (*15*), liquid chromatography (*16*), high performance liquid chromatography (*9, 17*), high performance thin-layer chromatography (*18*), and immunoassays (*19*). It must be pointed out that each method has its relative disadvantages. SGA in potatoes are usually determined by liquid chromatography but includes time-consuming sample preparation by solid-phase extraction and nonspecific UV detection at 202 nm. Moreover, the relative standard deviation (RSD), obtained for the detection of alkaloids by this method, ranges from 8% to 13% (*16*). In addition, SGA does not contain strong chromophores, and a derivatization step is generally needed to determine them. ELISA (*20*) is extensively used to analyze the total GA fraction. Extraction of the alkaloids by simple solubilization prior to the final assay makes the whole procedure much easier than alternative methods. It is carried out in the wells of plastic microtitration plates. The immunoassay procedure is often extremely specific, technically simple, and does not require the services of an experienced analyst. However, the main problem of immunoassay is the high price of the biologically active material, that is, monoclonal antibodies and enzyme-labeled antibodies. Biosensors are a very promising tool to overcome most of the problems described above. A basic idea for the ion sensitive field effect transistor (ISFET)-based monitoring of α-solanine, α-chaconine, and solanidine has been proposed by Korpan et al. (*21*), and recently optimized by Arkhypova et al. (*22*).

This paper describes novel approaches for adjusting the sensitivity of a potentiometric biosensor developed earlier (*21, 22*) and examines the possibility of selective potato SGA determination within a background of some heavy metals ions and pesticides. The content of SGA in several varieties of potatoes, which are being cultivated in Ukraine, was determined and compared using biosensor, HPLC, and TLC methods.

MATERIALS AND METHODS

Chemicals. Butyryl cholinesterase (BuChE, pseudocholinesterase EC 3.1.1.8) was from horse serum, with a specific activity of 13 U/mg solid; phosphotriesterase from *Pseudomonas putida* was prepared in the laboratory of Prof. F. Raushel with a specific activity of 2000 U/mL; butyrylcholine chloride (BuChCl), bovine serum albumin (BSA, fraction V, 98% purity), α-chaconine, α-solanine, and solanidine from potato sprouts were purchased from Sigma (France). Aqueous solution (25% w/v) of glutaraldehyde (GA) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade and were used without any further treatment.

The alkaloids (α-chaconine and α-solanine) were prepared as 1 mM stock solutions in 5 mM acetic acid.

Enzyme Immobilization. Biologically active membranes were formed by cross-linking butyryl cholinesterase with bovine albumin on the transducer surface in saturated GA vapors (*23*). The mixture containing 5% (w/v) enzyme, 5% (w/v) bovine albumin, and 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.5) was deposited on the sensitive surface of one transducer by the drop method, while the mixture of 10% (w/v) bovine albumin and 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.5) was placed on the surface of a reference transducer. The sensor chip was then placed in saturated GA vapors.

After a 30 min exposure in GA, the membranes were dried at room temperature for 15 min. In some cases, 0.2 % (w/v) phosphotriesterase was incorporated into the biologically active membrane containing BuChE to avoid the influence of organophosphorus pesticides on BuChE activity and consequently to achieve the desirable specificity.

Sensor Design. The ISFETs were fabricated at the Research Institute of Microdevices (Kiev, Ukraine). The potentiometric sensor chip contains two identical Si_3N_4-ISFETs; their design and operation mode has been previously described (*24*). The ISFETs were operated at a constant source current and drain-source voltage mode ($I_s = 200 \mu A$, $V_{ds} = 1$ V). Their pH-sensitivity was linear for pH values ranging from 2 to 12 with a slope of about 40 mV/pH unit.

Measurement of Enzyme Activity and Inhibition. Measurements were conducted in daylight at room temperature (20 °C) in a glass cell (2 mL volume) filled with K,Na-phosphate buffer, pH 7.5. The biosensor was immersed in a vigorously stirred sample solution. After baseline stabilization, BuChCl was added to the vessel. The differential output signal between the measuring and reference sensitive elements was registered with a "homemade" laboratory setup, and the steady state or kinetic response of the biosensor was plotted as a function of the BuChCl concentration.

The evaluation of the enzyme inhibition by SGA was carried out at an excess concentration of BuChCl and included the following steps:

The biosensor was soaked (approximately for 20 min) in 5 mM K,Na-phosphate buffer, pH 7.5, until a stable baseline output signal was reached.

BuChCl was added to the measurement cell. The kinetic (is reached within 10 s) or steady-state (is reached within 3 min) output signal of the biosensor was taken as an index of the maximal catalytic activity of the immobilized enzyme, and the value of the biosensor response was used for further evaluation of the inhibition effect of a specific SGA concentration.

After being washed twice (6 min), the sensor output was registered in the buffered solution containing target steroidal alkaloid/s and BuChCl as described in the previous step.

The level of inhibition due to the action of a specific concentration of SGA was evaluated by comparison of the biosensor response levels without and with inhibitor.

Analysis of SGA in Potato Tubers by TLC and HPLC. The analysis of SGA in potato varieties has been performed according to the procedures described by Bodart et al. (*25*) for TLC, and Hellenas et al. (*16*) and Arkhypova et al. (*26*) for HPLC technique.

RESULTS AND DISCUSSION

ISFET-Based Biosensor Sensitivity Improvement. The sensitivity of the ISFET-based biosensor developed earlier is sufficient for quantitative detection of potato SGA levels in foodstuffs because the measurements are reliable within the range 1−100 μM of target analyte concentration, which is lower than the SGA concentration in edible potato (25−250 μM). However, the SGA concentration in the blood serum is substantially lower: α-solanine, 0.2 μM; α-chaconine, 0.4 μM. Thus, a new approach is obviously necessary to improve the analytical characteristics of the biosensor developed, especially to lower the sensitivity threshold toward SGA.

The variation of the time of BuChE immobilization in the saturated GA vapors has been proposed to solve this problem. Increasing the immobilization time of BuChE on the transducer surface (**Figure 1**) results in a higher sensor sensitivity and slightly broader dynamic range for α-chaconine. It seems logical to suggest that this can be explained by the fact that the sensor response is mainly determined by the enzyme activity in the sensor membrane, that is, by the number of enzyme active sites able to bind the molecules of both inhibitor and substrate: the fewer number of enzyme active sites in the sensor biologically active membrane, the higher degree of inhibition at constant SGA concentration.
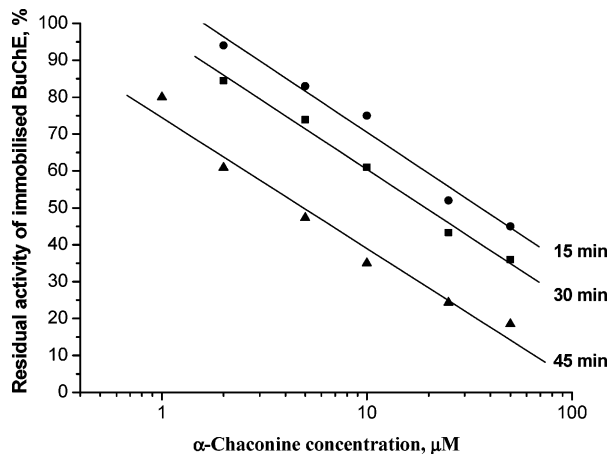
ISFET-Based Biosensor for Potato Glycoalkaloids Detection

J. Agric. Food Chem., Vol. 54, No. 3, 2006   **709**

Figure 1. Calibration curves for α-chaconine determination at different durations of BuChE immobilization (15, 30, and 45 min) in glutaraldehyde vapors, measured in 5 mM phosphate buffer, pH 7.3.
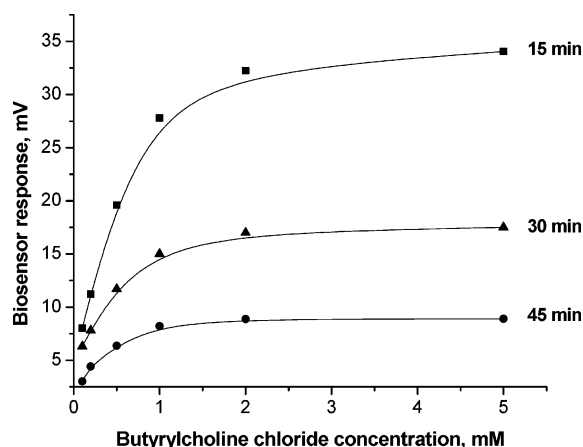

Figure 2. Calibration curves for butyrylcholine chloride determination at different durations of BuChE immobilization (15, 30, and 45 min) in glutaraldehyde vapors, measured in 5 mM phosphate buffer, pH 7.3.

Figure 3. Inhibition of human serum (**A**) and horse serum (**B**) butyryl cholinesterases by α-solanine, α-chaconine, and their mixture (at ratio 1:1), measured in 5 mM phosphate buffer, pH 7.3.

The results shown in **Figure 2** suggest that the number of enzyme active sites in the membrane decreases when the immobilization time is greater. Indeed, the sensor with 45-min immobilization gives the lowest response to a constant substrate concentration. The formation of more covalent binding between the GA molecules and enzyme amino groups decreases the number of enzyme active sites and results in lower sensor response.

Because the variations in the duration of BuChE immobilization onto the surface of potentiometric transducer did not improve the sensitivity of the ISFET-based biosensor, human serum BuChE was proposed as a more sensitive element instead of the horse serum BuChE. The biosensor with the human serum BuChE has been shown (**Figure 3**) to be substantially more sensitive toward potato SGA. The linear dynamic range was 0.03−5 μM as compared to 1−20 μM in case of the horse serum BuChE. The inhibition of BuChE in the experiments with the samples containing an equimolar mixture of α-solanine and α-chaconine was not additive and corresponds to the data (*27*) on the inhibition of human blood serum BuChE. This phenomenon, the antagonistic effect of alkaloids mixture on the immobilized BuChE activity, can be explained by a competition between α-solanine and α-chaconine toward the BuChE active site.

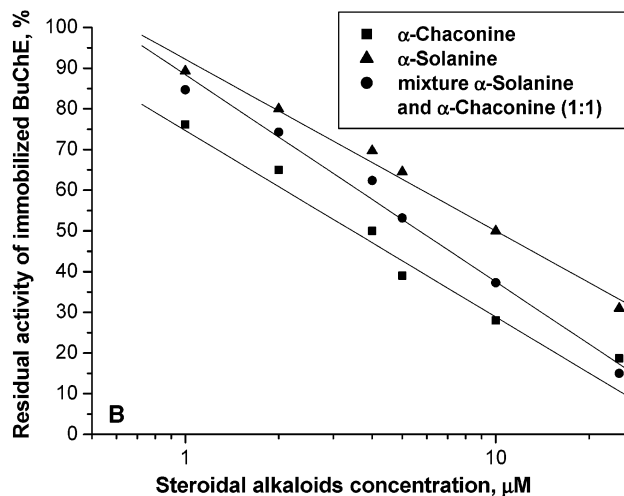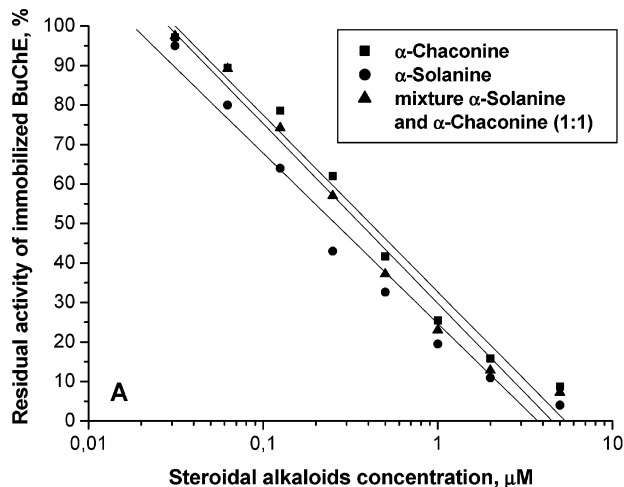**ISFET-Based Biosensor Selectivity/Specificity Improvement.** BuChE is known for a comparatively low specificity toward different types of inhibitors (*28, 29*), in particular, to heavy metal ions, organophosphorus (OP), organochlorine, and carbamate pesticides. This does not enable the specific determination of potato SGA by the potentiometric biosensor developed earlier in the presence of some heavy metal ions and pesticides.

It has been established that adding 1 mM ethylenediamine tetraacetate (EDTA) to the working buffer did not influence the ISFET-based biosensor sensitivity toward SGA. This observation enabled the actual value to be determined in the presence of mercury and lead up to a concentration of 50 μM (**Figure 4**). The further increase in the concentration of heavy metal ions in the measuring sample had no significant influence on the inhibition of SGA (**Figure 5**). Raising the ion concentration of either mercury or lead in the sample up to 500 μM does not change substantially the accuracy of SGA analysis (**Figure 6**).

The possibility of creating a biosensor insensitive to OP pesticides has been investigated. For this purpose, an enzyme phosphotriesterase was co-immobilized with BuChE in a bioselective membrane of the biosensor. Because phosphotriesterase is known to hydrolyze OP pesticides, it was proposed that in the presence of this enzyme in a bio-selective membrane the biosensor would be insensitive to OP pesticides. It has been shown that immobilization of phosphotriesterase in a bioselective membrane does not influence the ISFET-based biosensor response to SGA in the presence of the OP pesticide,
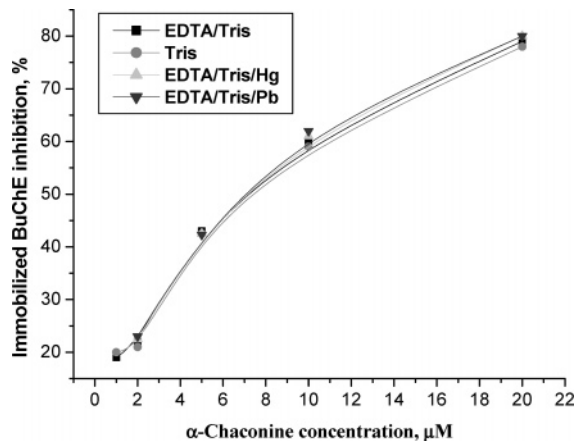
Figure 4. Calibration curves for α-chaconine detection by ISFET-based biosensor, measured in 10 mM tris-HNO$_3$ buffer (pH 8.1); 10 mM tris-NO$_3$ buffer (pH 8.1) containing 1 mM EDTA; 10 mM tris-NO$_3$ buffer (pH 8.1) containing 1 mM EDTA with 50 μM Hg^{2+} or Pb^{2+}.
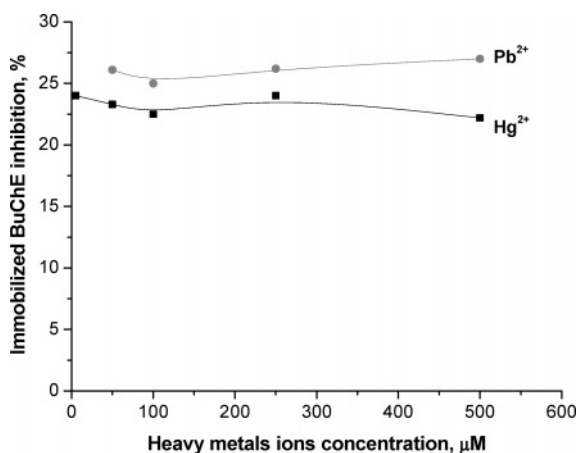

Figure 5. Dependence of the ISFET-based biosensor response on concentration of heavy metal ions (Pb^{2+} and Hg^{2+}) in the presence of 2 μM α-chaconine, measured in 10 mM tris-NO$_3$ (pH 8.1) buffer containing 1 mM EDTA.
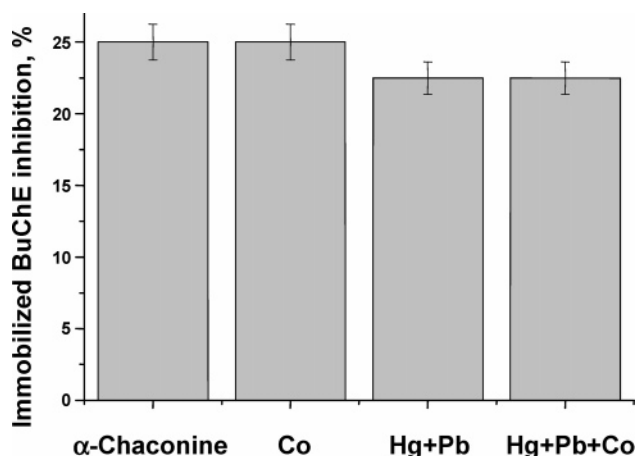

Figure 6. Effect of mercury and lead ions on the ISFET-based biosensor response, measured in 10 mM tris-NO$_3$ (pH 8.1) buffer containing 1 mM EDTA. [α-chaconine], 2 μM; [Co^{2+}], 500 μM; [Hg^{2+}], 250 μM; [Pb^{2+}], 250 μM.

paraoxon (**Figure 7**). Consequently, usage of 1 mM EDTA and phosphotriesterase resulted in a high specificity of SGA analysis in the presence of both interfering compounds (paraoxon, Pb^{2+}, and Hg^{2+}) at a concentration of 100 μM (**Figure 8**).
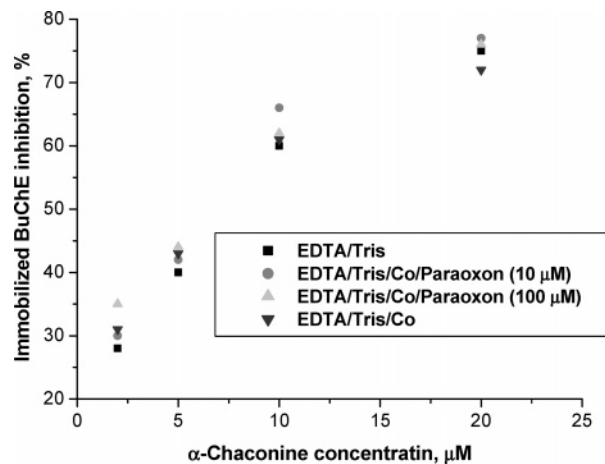

Figure 7. Calibration curves for α-chaconine determination by ISFET-based biosensor in the presence of paraoxon, measured in 10 mM tris-NO$_3$ (pH 8.1) buffer containing 1 mM EDTA and 500 μM cobalt ions. Bio-selective membrane contains BuChE and phosphotriesterase.

Figure 8. Effect of paraoxon and heavy metal ions (Hg^{2+} and Pb^{2+}) on ISFET-based biosensor response to 2 μM α-chaconine, measured in 10 mM tris-NO$_3$ (pH 8.1) buffer containing 1 mM EDTA and 500 μM cobalt ions. Bio-selective membrane contains BuChE and phosphotriesterase.
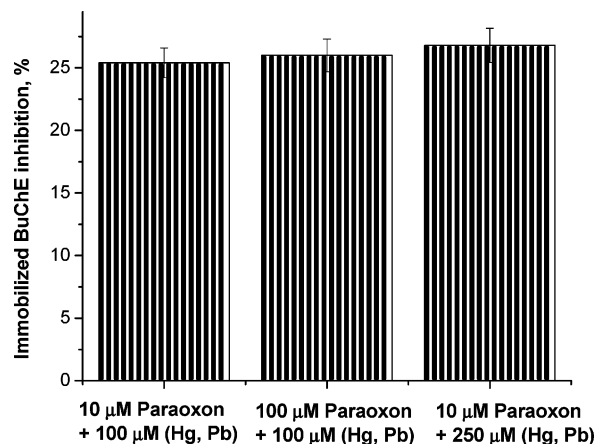
The results presented above demonstrate that the addition of EDTA and phosphotriesterase prevents the inhibition of BuChE immobilized onto the transducer surface by mercury, lead (up to the concentration 500 μM), and paraoxon (up to the concentration 100 μM). Moreover, the inhibitory potency of solanaceous alkaloids studied is not altered and the separate detection of the investigated classes of toxins, that is, SGA, OP, and heavy metals ions, may be possible.

ISFET-Based Biosensor Validation for SGA Detection in Potato Cultivated in Ukraine. Fourteen varieties of potatoes, bred by traditional selection methods at the Institute of Potato-Planting of Ukrainian Academy of Agricultural Sciences, among them early varieties (Povin', Serpanok, Borodyanska rozheva), middle-early (Svitanok Kyivskiy, Vodograj, Obriy, Cupava, Zabava), middle-ripe (Slov'yanka, Yavir, Lugovs'ka), and middle-late (Rakurs, Zarevo, Promin') 2003 harvest, were tested. The results obtained by ISFET-based biosensor were compared to those measured by HPLC and TLC.

According to the results (**Table 1**), there are only three varieties of potatoes (Serpanok, Obriy, and Zabava) that correspond to "generally accepted" International standards. Other varieties have a higher level of toxins; some of them are

ISFET-Based Biosensor for Potato Glycoalkaloids Detection

J. Agric. Food Chem., Vol. 54, No. 3, 2006 **711**

Table 1. Steroidal Glycoalkaloids Content in Potato Varieties Cultivated in Ukraine

		SGA content, mg/100 g of fresh weight		
	variety of potato	ISFET-based biosensor	HPLC	TLC
early	Povin'	15.6 ± 0.8	15.8 ± 0.5	13.8 ± 1.3
	Serpanok	8.2 ± 0.4	8.0 ± 0.6	6.0 ± 0.6
	Borodyanska rozheva	13.0 ± 0.6	12.8 ± 0.6	9.4 ± 0.9
middle-early	Svitanok kyivskiy	14.9 ± 0.7	14.3 ± 0.5	11.5 ± 1.1
	Vodograj	12.7 ± 0.6	12.9 ± 0.5	12.8 ± 1.3
	Obriy	5.4 ± 0.3	5.6 ± 0.5	7.0 ± 0.7
	Cupava	11.4 ± 0.6	11.7 ± 0.4	9.7 ± 0.9
	Zabava	6.1 ± 0.3	6.1 ± 0.2	6.1 ± 0.6
middle-ripe	Slov'yanka	11.9 ± 0.6	12.2 ± 0.3	13.7 ± 1.3
	Yavir	10.8 ± 0.5	10.3 ± 0.8	13.2 ± 1.3
	Lugovs'ka	15.8 ± 0.8	15.2 ± 0.4	14.2 ± 1.4
middle-late	Rakurs	17.9 ± 0.9	17.4 ± 0.9	29.4 ± 2.9
	Zarevo	19.3 ± 0.9	19.6 ± 1.0	19.9 ± 2.0
	Promin	9.7 ± 0.5	9.3 ± 0.4	17.1 ± 1.7
number of replicate measurements		20	22	22

on the edge of allowed levels. Concern is expressed toward the concentration in such varieties as Rakurs and Zarevo.

The connection between the duration of ripening and SGA content has also been studied. The lowest characteristics were indicated in early and middle-early varieties, although the cases of exceeded concentration content of solanine and chakonine (e.g., Povin', Svitanok Kyivskiy, Vodograj) have been observed. All middle-ripe varieties possessed considerable SGA content, which varied between 11 and 16 mg/100 g of fresh weight, and middle-late possessed higher values, from 17 to 30 mg/100 g of fresh weight. Thus, the increase in the concentration of SGA pro rata vegetation period is observed. This can be explained by a prolonged influence of different factors that stimulate SGA synthesis such as herbicides, insecticides, damage caused by plant pests, fungi, and other infections.

It is well known that TLC is generally considered to be a qualitative and not a quantitative procedure. To prove this statement, the correlation of the results between biosensor/TLC and biosensor/HPLC has been estimated. From the results represented above (**Table 1**), it is evident that the correlation coefficients constitute 0.76 and 0.97, for biosensor/TLC and biosensor/HPLC methodologies, respectively. Thus, TLC can be considered as a preselection tool for SGA measurements only.

ABBREVIATIONS USED

SGA, steroidal glycoalkaloids; ISFET, ion sensitive field effect transistors; GA, glutaraldehyde; BuChE, butyryl cholinesterase; BuChCl, butyrylcholine chloride; EDTA, ethylenediamine tetraacetate; OP, organophosphorus pesticides; TLC, thin-layer chromatography.

ACKNOWLEDGMENT

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Korpan et al.

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