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Short Communication

Quartz Crystal Microbalance Biosensor for Ergotamine Detection

Miroslav Pohanka

Faculty of Military Health Sciences, University of Defence, Trebesska 1575, CZ-500 01 Hradec Kralove, Czech Republic
E-mail: miroslav.pohanka@gmail.com

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Ergotamine is a toxin from ergot fungi. It interacts with various receptors in the organisms including the one for adrenaline, serotonin and dopamine. People can be poisoned by contaminated food but ergotamine is also marketed as a drug for migraine therapy. Because of survey on toxin presence in the food and making countermeasures against them, there are required simple and available methods for ergotamine assay. In this paper, quartz crystal microbalance (QCM) sensor with basic 10 MHz frequency served as a platform for immobilization of an antibody against ergotamine. The ergotamine was determined in a sandwich format using rabbit polyclonal antibody. The assay exerted limit of detection 12.6 μg/ml respectively 9.55 μmol/l, it was successfully validated to enzyme-linked immunosorbent assay (ELISA) method and it was not sensitive to interference by ergometrine, albumin or caffeine. The assay was also suitable for measurement of ergotamine in flour and milk that had no significant matrix effect on the results. In a conclusion, the biosensor is a simple and reliable tool for measurement of ergotamine in natural samples. No elaborative steps or techniques are necessary to finish the assay.

Keywords: alkaloid; antibody; biosensor; biorecognition; ergot; ergotamine; immunosensor; food; label free assay; piezoelectric; QCM

1. INTRODUCTION

Ergotamine is a secondary metabolite from a group of ergot alkaloids (ergopeptides) produced by family of ergot fungi with notable representative *Claviceps purpurea*. Considering its occurrence and practical importance, ergotamine is probably the most important ergot alkaloid but ergometrine, ergosine, ergocornine, ergocryptine and ergocristine are another relevant alkaloids produced by the ergot family [1]. Structural formula of ergotamine can be learned from figure 1. Poisoning by ergot alkaloids is called ergotism and it is typically started by consummation of contaminated cereals where ergot fungi grow. Ergotamine as well as the other ergot alkaloids are psychoactive and vasoconstricting agents [2]. Ergotamine as well as the most of ergot alkaloids exert affinity to adrenaline, serotonin and dopamine

receptors and acting on these receptors is the principle of ergotamine toxicodynamics [3-5]. Ergotamine and developed drugs structurally derived from ergotamine can be used for vasoactivity and vasoconstriction regulation [6,7]. In the current medicine, drugs containing ergotamine are indicated for migraine therapy [8-11]. Ergotamine is considered as a toxic compound though its toxicity is not high. Expected median lethal dose (LD_{50}) is between 60 and 100 mg/kg for small mammals (rats, mice, rabbits) and intravenous application. Thought the expected LD_{50} value is not low enough to entitled ergotamine as a highly toxic substance, it can represent a problem when food contaminated because ergotamine can be presented there in quite abundant amount.

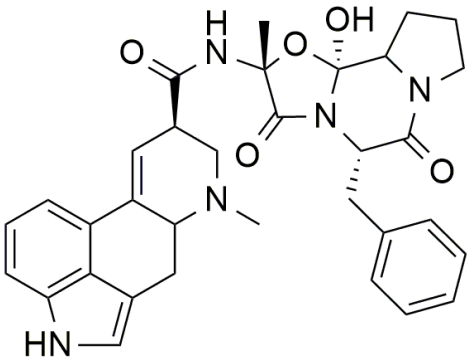

Figure 1. Chemical structure of ergotamine.

Presence of ergot alkaloids in food is controlled and legislatively regulated. For instance upper limit of ergot sclerotia contamination in the food is 0.05 % by an European Union regulation [1]. Ergotamine can be analyzed in real samples by standard chemical and immunochemical methods. Liquid chromatography (LC) and high-performance liquid chromatography (HPLC) with fluorescence detector or mass spectrometer on the output are convenient for ergotamine assay [12-15]. Immunoassays like enzyme-linked immunosorbent assay (ELISA) are another option for ergotamine determination [16-18].

In this paper, a cheap and easy to perform biosensor device is developed for determining of ergotamine presence in food and water samples. It is intended to prepare economically competitive method providing reliable data about ergotamine presence in samples where the toxin can be expected. Piezoelectric assay based on quartz crystal microbalance (QCM) sensor was chosen for the assay purpose because expected simplicity of measurement process and low demands on material and reagents. The choice of platform is an outcome from previous reviews on the issue [19,20].

2. MATERIALS AND METHODS

2.1. Biosensor construction

The biosensors were constructed on the platform of 10 MHz QCM sensors purchased from manufacturer Krystaly (Hradec Kralove, Czech Republic). The sensors were circle shaped with quartz

disc having diameter 20 mm and thickness 0.166 mm. Gold electrodes were also circle shaped and they were placed on the opposite sites and they had diameter 7 mm. New sensors were washed in 96 % v/v ethanol before further modifications and then dried. The biosensor preparation started by application of cysteamine solution 50 mg/ml that activated the gold surface. In a total 50 µl of the solution was given per one electrode and let to incubate in a wet box for at least five hours and then the electrodes were rinsed with deionized water and dried in an open air. In the second step, 50 µl of glutardialdehyde 5 % w/w was spread over one electrode and let to react in a wet box for another five hours. Rinsing by deionized water and drying followed. Protein A (Sigma-Aldrich St. Louis, MO, USA) was in amount 50 µl per one electrode and concentration 1 mg/ml was applied, let to incubate in a wet box for 12 hours and then rinsed with deionized water. Finally, anti-ergotamine antibody of IgG class (rabbit origin, polyclonal purified antibody) achieved from Argisera (Vannas, Sweden) was diluted in saline solution up to concentration 1 mg/ml and applied on electrode in a volume 50 µl and let to incubate in a wet box for 12 hours. The prepared biosensors were rinsed with saline solution, let dry under laboratory conditions and kept in a fridge at 4 °C until use.

2.2. Biosensor performance

In the beginning of assay, equilibrium frequency of each biosensor (f_1) was measured using ICM Level Oscillator 10.000 MHz (ICM, Oklahoma City, OK, USA) with frequency counter UZ 2400 (Grundig, Nuremberg, Germany) on the output. After that, application of a sample sized 50 µl followed and it was let to react for 30 minutes.

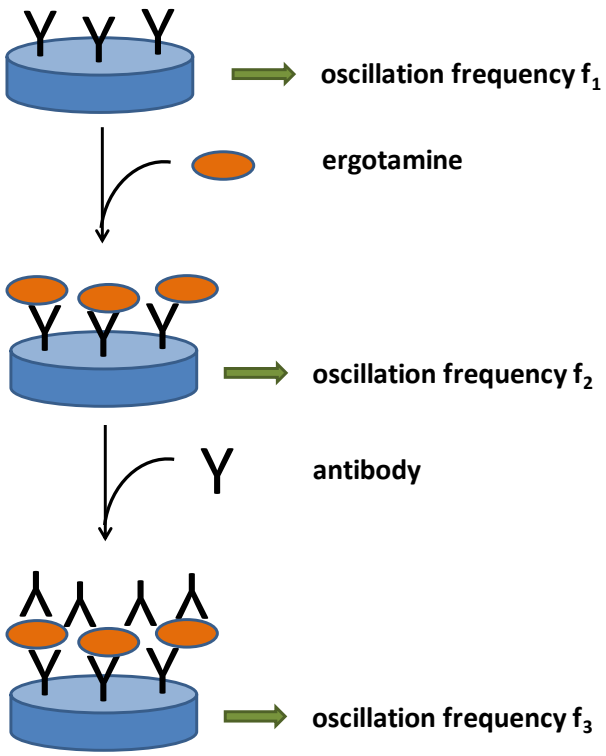

Figure 2. General principle of assay by biosensor.

Ergotamine tartrate (Sigma-Aldrich) in analytical purity served as a standard for the assay purpose. Following the incubation, the biosensor was washed by distilled water and let to dry. Oscillation frequency (f_2) was measured again. In the final step, solution of polyclonal antibody against ergotamine (the same antibody as used for biosensor construction) was given in a concentration 1 mg/ml and amount 50 µl per one electrode and let to incubate for 30 minutes, then the biosensor was washed by distilled water again, dried under laboratory conditions and oscillation frequency (f_3) was measured. One sample was independently measured by five biosensors. General principle of assay by the biosensor is explained in figure 2.

2.3. ELISA for samples measurement

ELISA was done on standard microplates with 96 wells and flat bottom (MaxiSorp, Nunc, Roskilde, Denmark). The wells were coated with 100 µl of sample (ergotamine, tested interferent or blank) and let to incubate in a wet box for 12 hours. After the incubation, the solution was sucked out and 100 µl of 0.1 % w/w gelatin was given per one well and let to interact in wet box for another 12 hours. Excess of gelatin solution was sucked out and the wells were washed by phosphate buffered saline pH 7.4. In the next step, application of 100 µl anti-ergotamine antibody (Argisera) solved in saline solution up to concentration 0.5 mg/ml followed. After 12 hours of incubation and washing by phosphate buffered saline pH 7.4 with 0.1 % (w/w) Tween 20, anti-rabbit IgG antibody labelled with horse radish peroxidase (Sigma-Aldrich) solved in phosphate buffered saline 1:8000 as recommended by manufacturer was applied in an amount 100 µl, let to incubate 12 hours, and washed by phosphate buffered saline pH 7.4 with 0.1 % (w/w) Tween 20. Finally, o-phenylenediamine in a concentration 0.5 mg/ml in a mixture containing also 5 mmol/l hydrogen peroxide was given in a well. In a total 100 µl of the mixture was applied and incubation of five minutes was ended by immediate injection of 100 µl sulfuric acid 2 mol/l. Optical density of the mixture was measured in a microplate reader at 490 nm. Wells with albumin 10 mg/ml instead of sample with ergotamine served for blank purpose. One sample was measured in five wells.

3. RESULTS AND DISCUSSION

In the beginning of experiments, detection of ergotamine was performed by the constructed biosensors. Concentration range of ergotamine tartrate 0 (pure water), 0.00763, 0.0153, 0.0305, 0.0610, 0,122, 0.244, 0.488, 0.977, 1.95, 3.91, 7.81, 15.6, 31,3, 62,5, 125, 250 and 500 mg/ml in water (two-fold dilution scale from 500 mg/ml) served for calibration purposes. When calculated change of oscillation frequency $\Delta f_e = f_1 - f_2$ due to direct interaction between ergotamine and immobilized antibody, no significant signal was achieved and the oscillation frequency change was less than 1 Hz even for sample of ergotamine with concentration 500 mg/ml. However, the situation was different when a secondary antibody used and change of oscillation frequency $\Delta f_a = f_1 - f_3$ taken as an outputting signal. Calibration curve for this assay is depicted as figure 3. It is not a surprise that the direct interaction between biosensor surfaces and ergotamine was not recordable while use of secondary antibody helped the assay.

Ergotamine is quite small molecule with molecular weight 581.11 g/mol, piezoelectric assay is more sensitive to molecules with higher molecular weight [20-22]. IgM with molecular weight around 150,000 g/mol is a molecule approximately 258 times heavier than ergotamine therefore the interaction is more easily recorded by the piezoelectric platform. Limit of detection according signal to noise equal to three rule (S/N = 3) was set to be 12.6 µg/ml respectively 9.55 µmol/l in molar scale. Coefficient of determination for the calibration was equal to 0.991.

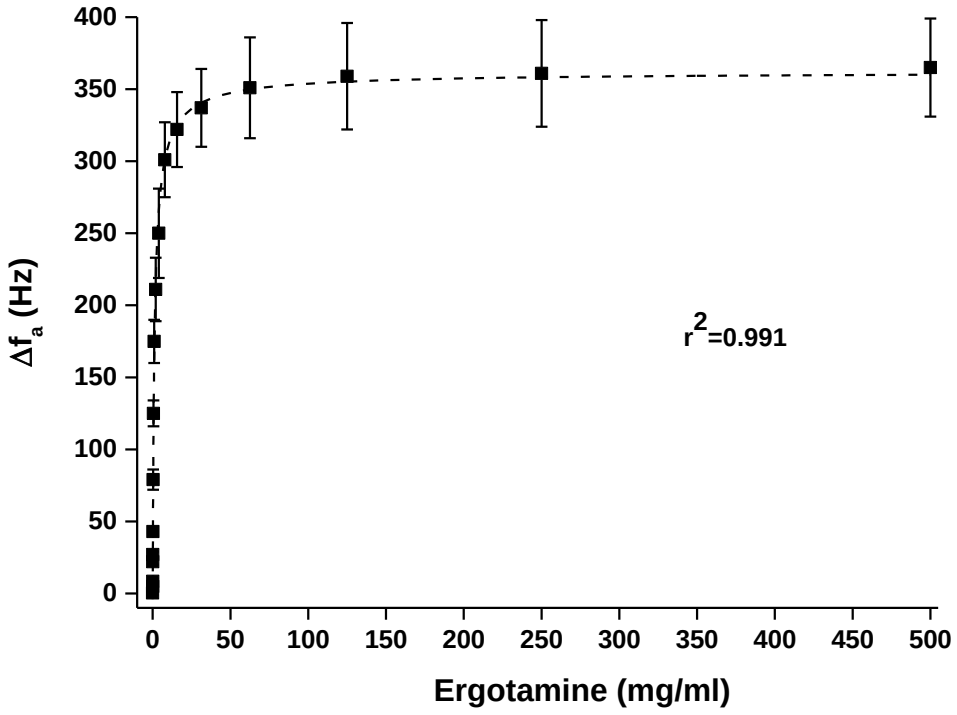

Figure 3. Calibration curve for biosensor and assay where a secondary antibody used for ergotamine measurement. Error bars indicate standard deviation for n = 5.

The same samples used for biosensor calibration were also measured by ELISA and the both assays were compared mutually. Validation of biosensor to ELISA is graphically presented as figure 4. Regression of the two methods was linear with coefficient of determination 0.964. The comparison of the two methods does not show that one of them is more sensitive to ergotamine than the other. On the other hand, biosensor assay was quite simple and only one reagent (antibody) was given, no further chemicals or samples processing is necessary. While limit of detection for the assay by biosensor was equal to 12.6 µg/ml, ELISA exerted limit of detection 29.4 µg/ml. Comparing to biosensor, ELISA is more elaborative as chromogenic reagents including instable hydrogen peroxide and harmful sulfuric acid are common in the assay protocol.

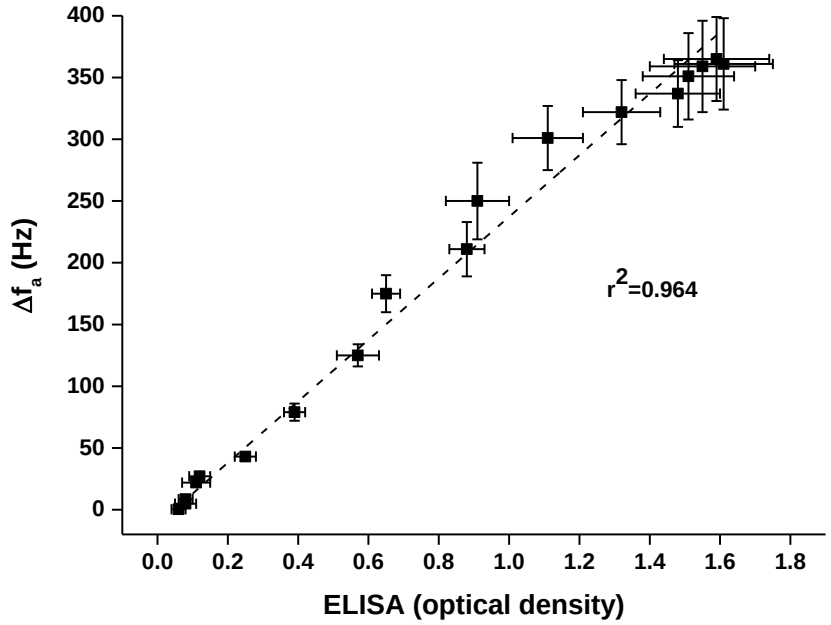

Figure 4. Validation of ergotamine assay by biosensor to ELISA method using following concentration of ergotamine: 0 (pure water), 0.00763, 0.0153, 0.0305, 0.0610, 0,122, 0.244, 0.488, 0.977, 1.95, 3.91, 7.81, 15.6, 31,3, 62,5, 125, 250 and 500 mg/ml. Error bars indicate standard deviation for n = 5.

The assay by biosensor was tested for interference by various compounds that can have relevance in the assay. Samples containing ergotamine tartrate 10 mg/ml was compared with samples of human serum albumin 10 mg/ml, ergometrine maleate 10 mg/ml, tartrate 10 mg/ml and caffeine 10 mg/ml. While signal Δf_a for assay of ergometrine had signal 312±19 Hz, signals for ergometrine, albumin, tartrate and caffeine were insignificant (ANOVA, P 0.05) to blank assay (water). The specificity of biosensor-based assay is a substantial parameter and the fact that the assay does not suffer from false positive results is crucial for practical performance because pertinent malfunction in this regard would be fatal.

Table 1. Specifications of ergotamine assay by biosensor.

Parameter	*Value*
limit of detection for ergotamine – mass concentration	12.6 µg/ml
limit of detection for ergotamine – molar concentration	9.55 µmol/l
time per one assay	approximately 1 hour
sample volume	50 µl
coefficient of determination for the calibration	0.991
coefficient of determination for validation between biosensor and ELISA	0.964

interference by human serum albumin, ergometrine maleate, tartrate, caffeine	not significant in concentration 10 mg/ml

Experiment on matrix effect and potential false negativity was another test that was accomplished. Ergotamine tartrate solution 10 mg/ml was prepared in water, saline solution, phosphate buffered saline solution pH 7.4, milk 1.5 % fat content and suspension of flour in water 50 mg/ml. All samples were assayed in pentaplicates and data mutually compared by ANOVA tests. While assay of ergotamine in water provided signal 312±19 Hz, ergotamine in the saline solution 317±24 Hz, in phosphate buffered saline 305±23 Hz, in milk 293±25 Hz and 299±21 Hz for flour suspension. The data were mutually insignificant on the probability level 0.05. No sensitivity of the assay to water media or other interference of the media can be implicated and the biosensor appears to be suitable tool for the measurement of ergotamine in the extracts on water basis and food samples. Overview of biosensor specifications is written in table 1.

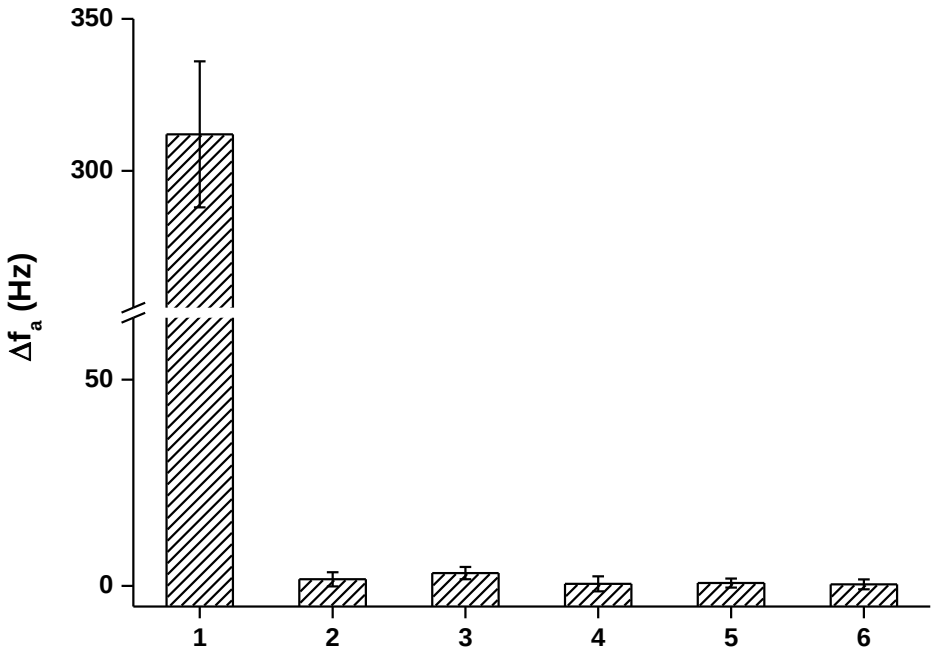

Figure 5. Interference testing: (1) ergotamine tartrate 10 mg/ml, (2) human serum albumin 10 mg/ml, (3) ergometrine maleate 10 mg/ml, (4) tartrate 10 mg/ml, (5) caffeine 10 mg/ml, (6) water. Error bars indicate standard deviation for n = 5.

The biosensor presented in this paper is fully comparable with standard assay and does not have any significant disadvantage when compared to ELISA. Contrary, there are advantages like shorter time per assay, lower number of steps per one assay and lower number of reagents necessary to finish the measurement is necessary. In a total one reagent is applied in the assay. The fact that the biosensor was not able to work as a label free method is not surprising. Piezoelectric assays based on QCM are typically suitable for analytes with molecular weight above 1,000 g/mol [19,20,23-26] while ergotamine has

molecular weight 581.11 g/mol. The biosensor would be used as a laboratory device but is also suitable for a field assay which make the biosensor highly competitive to the standard assays. When the limit of detection presented here for the biosensor compared with methods known from published papers like automated sequential injection fluorometric assay exerting limit of detection limit of detection 0.01 µg/ml [27], cloud point extraction with fluorometric detection with limit of detection 0.11 pg/ml [28], and capillary electrophoresis with limit of detection 0.5 µg/ml [29], the cited papers describe methods with better limit of detection. On the other hand, the biosensor presented here is significantly simpler for a routine performance. Comparison of the results from the assays presented here and the cited papers are given in table 2.

Table 2. Comparison of the biosensors and other assays.

Parameter	*Value*	*References*
limit of detection for ergotamine by biosensor	12.6 µg/ml	biosensor in this work
limit of detection for ergotamine by ELISA	29.4 µg/ml	
automated sequential injection fluorometric assay	0.01 µg/ml	[27]
cloud point extraction with fluorometric detection	0.11 pg/ml	[28]
capillary electrophoresis	0.5 µg/ml	[29]

4. CONCLUSION

In this paper, a simple, reliable, easy to perform and cheap analytical device was invented. Though analytical specifications of the biosensor are not superior to the standard methods, the biosensor provides an advantage to the common standard methods in simple designs allowing mass and cheap production. The assay is also convenient for performance outside laboratories though analytical performance is not worse than the standard immunoassays. The beneficial properties are an outcome from physical principle of the assay where interaction on the biosensor surface are directly recorded. The assay is quite fast and does not need specific reagents for this reason. Practical impact of findings presented here is expected and applicability of the biosensor in food control and clinical toxicology laboratories would be the main users.

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