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Voltammetric Biosensor Based on a Modified Chitosan Membrane Enzyme Peroxidase

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The determination of hydrogen peroxide (H_2O_2) has a significant meaning in environmental, pharmaceutical, healthcare and food analyses. The current work is focused on construction of a fast but reliable and accurate voltammetric biosensor enabling detection of H_2O_2 giving opportunity to its application into practice. This biosensor was based on screen printed electrode covered with chitosan membrane entrapping horseradish peroxidase (HRP), magnetic particles (MPs) and Prussian blue (PB). MPs an inorganic substrate having pseudo-peroxidase activity together with HRP catalyzed reduction of H_2O_2 and PB as mediator of electron transfer enabled highly sensitive detection at low applied potential. Square wave voltammetry (SWV) was chosen as a detection device. Average area peaks of each H_2O_2 concentration created calibration curve replying Michaelis-Menten equation with correlation coefficient 0.999. Limit of detection was equal to 8.2 µmol/l of H_2O_2. Uric acid, ascorbic acid, water soluble derivate of vitamin E (trolox), acetaminophen and reduced glutathione were measured as possibly interfering substances replacing H_2O_2 in the reaction and no significant influence on the assay was observed when presented in equivalent concentrations as the H_2O_2. Different personal care matrix substances (tooth paste, hand cream and skin tonic) were spiked with H_2O_2 and no effect of matrix on the determination was detected. On the basis of the presented results, , the proposed assay was considered to be highly sensitive, accurate and fast assay for detection of H_2O_2 so this platform can be applied in wide spectrum of research and clinical fields.

Keywords: Voltammetry; hydrogen peroxide; horseradish peroxidase; Prussian blue; magnetic particles

1. INTRODUCTION

Hydrogen peroxide (H_2O_2) can be found as a residue from production or a component of non-food product (textiles, toys, personal care products etc.), it is used as an antibacterial agent added to milk products or as a disinfection of equipment related to food and beverage. It is also a natural part of

rainwater and the other environmental samples [1-7]. However, toxic impact of H_2O_2 in concentrations exceeding 6 % (1.96 mol/l) on human organism is well known so the accurate determination of H_2O_2 in products contacting it which are entering human body is crucial and the level is strictly controlled in the most countries [7]. Moreover, H_2O_2 is very important analyte increasing during many reactions used for determination of biochemical markers (glucose, cholesterol, NAD(P)H, uric acid etc.) enabling diagnosis of pathological condition including poisoning and it is a part in immunoassays where peroxidase is chosen for labeling of molecules [8-12]. All of the above mentioned facts make the determination of H_2O_2 a required analysis in the scientific, industrial and clinical field.

Horseradish peroxidase (HRP) has been found as one of the most appropriate mean how to determine H_2O_2 a decades ago [13,14]. On the other hand, some drawbacks connected with use of HRP have revealed: difficulties with HRP immobilization onto electrode surface, low stability, activity loss, poor pH and thermal stability or relatively high price [15,16]. So the materials with pseudo-peroxidase activity and their optimal combination should be found. Purpose of this study was to prepare fast, sensitive and accurate peroxidase biosensor, where disadvantages of usual peroxidase sensors will be improve by electrode modification by chitosan membrane entrapping combination of enzyme HRP and pseudo-peroxidase catalysts which could make the peroxidase sensor maximally effective.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were obtained from commercial sources. They were used without further purification because they were achieved in analytical grade. Iron oxide magnetic micro particles (MPs) carboxy-functionalized (20 mg/ml), peroxidase from horseradish type VI (4.0 mg/ml water solution), chitosan, Prussian blue (PB), o-phenylenediamine dihydrochloride (3.5 mg/ml) and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Acetic acid (0.2 mol/l), sodium hydroxide (1 mol/l), ethanol (60 % water solution) and hydrochloric acid (0.2 mmol/l) were bought from Penta (Prague, Czech Republic). Skin toner with lotus extract was bought from Balea (Karlsruhe, Germany), moisturizing hand cream with almond oil and shea butter was purchased from Cien (Neckarsulm, Germany) and whitening toothpaste was gained from Clinomyn (Camberley, Great Britain). Phosphate buffer was prepared by dissolution of one tablet in 200 ml of demineralized water. PB was prepared by dissolution of 3 mg of powder in 950 µl of 60 % ethanol and 50 µl of 0.2 mol/l hydrochloric acid. Reverse osmosis process using device Aqua Osmotic 2 (Aqua Osmotic, Tisnov, Czech Republic) was used for preparation of demineralized water.

2.2. Apparatus

Square wave voltammetry (SWV) was performed by Electrochemical Sensor Interface PalmSens (PalmSens, Utrecht, The Netherlands). Assay utilizes three-electrode screen printed sensors based on carbon working electrode (circle shaped with 1 mm diameter), platinum counter electrode

(circle around working and reference electrodes) and silver reference electrode (circle over the working electrode) covered with silver chloride. Whole screen printed electrode sensor was sized 25.4 × 7.26 × 0.63 mm. The electrodes were obtained from BVT Technologies (Brno, Czech Republic). SWV was performed in potential range from -1.5 to 0 V with potential step equal to 0.007 V, amplitude of potential 0.01 V and frequency 1 Hz. The colorimetric assay was performed in 96-multiwell polysorb plates from Nunc (Roskilde, Denmark) by ELISA spectrophotometer Sunrise (Tecan Salzburg, Austria).

2.3. Data processing

All measurements were done in pentaplicate under standard ambient and pressure conditions. PS Trace 4.8 software was used for handling with electrochemical device. The achieved results were processed in Origin 9.1 (OriginLab Corporation, Northampton, MA, USA) and non-linear curve fitting using Michaelis-Menten equation was chosen for concentration curves construction. Peak areas were determined using Peak Analyzer (Peak Fitting module) from Origin 9.1 (OriginLab Corporation, Northampton, MA, USA) as voltage in V×µA. Signal to noise ratio equal to three (*S/N=3*) was considered as limit of detection.

2.4. Membrane modifications

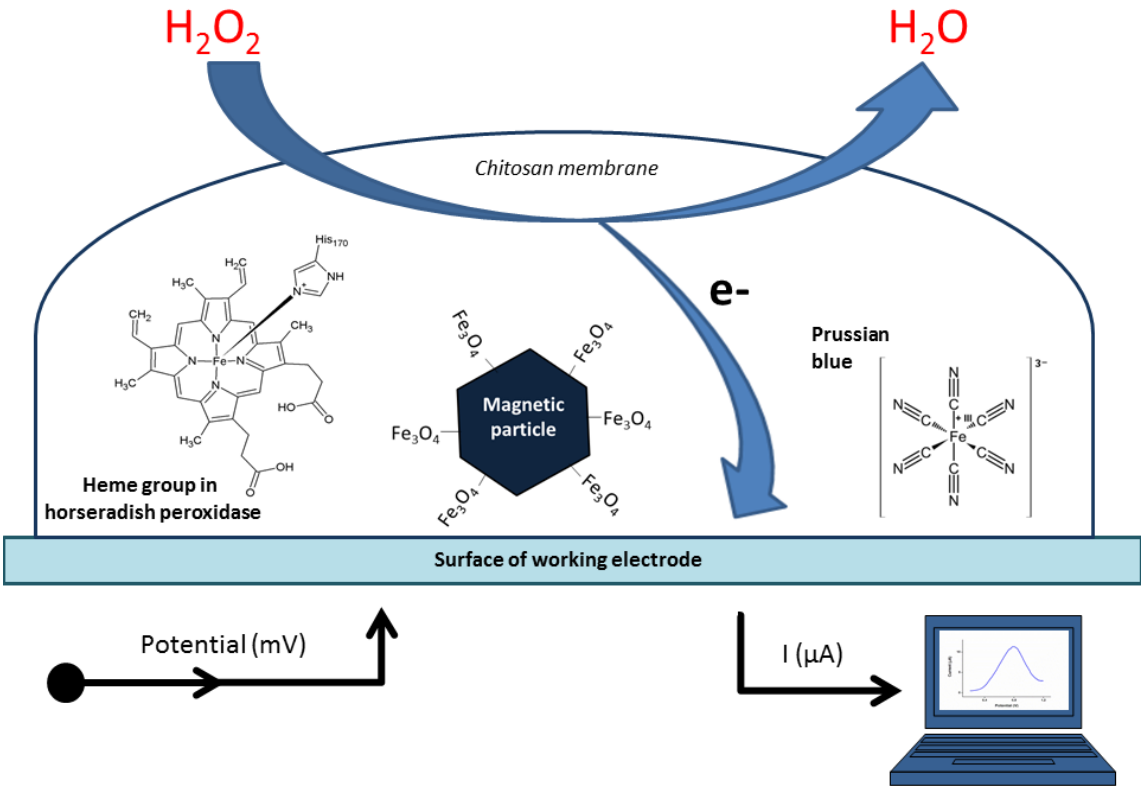

Figure 1. Principle of H_2O_2 measurement performed on screen-printed electrodes modified by chitosan membrane using SWV like detection method.

Chitosan solution was prepared by mixing 18 mg of chitosan powder together with 1 ml of acetic acid for approximately one hour until homogenous solution was achieved. Different types of chitosan membrane modifications were prepared and 1.5 µl of the solutions was dropped onto surface of working electrode. Modifications of chitosan membrane were produced by mixing of 14 µl of chitosan solution together with followed substances: A – 6 µl of water, B – 6 µl of HRP, C – 6 µl of PB, D – 6 µl of MPs, E – 3 µl of HRP and 3 µl od PB, F – 3 µl of HRP and 3 µl od MPs, G – 3 µl of PB and 3 µl of MPs, H – 2 µl of HRP, 2 µl od PB and 2 µl of MPs. Freshly made membrane was left for 30 minutes and after that, 5 µl of 1 mmol/l sodium hydroxide solution was dropped onto them. Redundant sodium hydroxide solution was washed by demineralized water after 15 minutes. Dried electrodes with bounded membranes were used for measurement of 0.8 % solution of H_2O_2 using SWV (Figure 1). Assay was performed by dropping 21 µl of PBS solution together with 7 µl of H_2O_2 onto electrode surface and by recording of electrical current signal (SWV curves).

2.5. Concentration curve

H_2O_2 in concentration range from 0 to 3.2 % was measured by SWV using electrodes with the one selected chitosan membrane (with the best measured properties) described in chapter "Membrane modifications". PBS in amount 21 µl was dropper onto electrode surface together with 7 µl of H_2O representing 0 % concentration of H_2O_2 or together with 7 µl of H_2O_2 in increasing concentrations (0.05, 0.10, 0.20, 0.40, 0.80, 1.6 and 3.2 %). SWV curves were recorded.

2.6. Interferences

Substances considered as possible interferents (reduced glutathione, ascorbic acid, trolox, uric acid and acetaminophen) in concentration 0.8 % were used in the same way like analyte and they contained no H_2O_2 in the measurement for gaining the information about assay specificity. Water and 0.8 % H_2O_2 was used as an analyte as well representing negative and positive control respectively. Electrode with chitosan membrane and the best properties prepared according procedure described in chapter Membrane modifications were used for SWV analysis. Solution made of 21 µl of PBS and 7 µl of interfering substances or one of the controls was dropped onto electrode surface and electrical current signal was recorded.

2.7. Matrix effect

Influence of different matrices, where H_2O_2 is usually determined, was studied. Different matrices including skin toner, hand cream and toothpaste was spiked by 1.6 % H_2O_2 in ratio 1:1. Hand cream and toothpaste were diluted twice by PBS before spiking. Spiked samples as well as 0.8 % H_2O_2 (positive control) and water (negative control) were measured by SWV on electrodes with chosen type of modified membrane prepared the same way as it was described in chapter "Membrane modifications".

2.8. Selectivity of membrane

Selectivity of membrane was analyzed using both electrode with chosen chitosan membrane and bare electrode. 7 µl of 0.8 % ascorbic acid (representing antioxidants possibly affecting reaction) was mixed together with 21 µl of PBS and dropped onto both types of electrodes and measured by SWV. SWV curves were recorded.

2.9. Comparison of novel and reference method

Concentration curve results (average peak areas) were compared with results of standard H_2O_2 colorimetric assay (absorbance) measured in compliance with the protocol from previous work using HRP as catalyzer and o-phenylenediamine dihydrochloride as reaction substrate [16]. Colorimetric assay was performed at 450 nm in concentration range from 0 to 3.2 % H_2O_2 (0, 0.05, 0.10, 0.20, 0.40, 0.80, 1.6 and 3.2 %).

3. RESULTS AND DISCUSSION

3.1. Membrane modifications

SWV curves measured on differently modified electrodes created peaks of diverse areas (Figure 2). Average areas measured on each type of membrane are showed in Figure 3. On the base of the gained results, chitosan membrane H (Table 1) with all three modifiers (PB, MPs and HRP) was selected as the one with the best properties. It showed the largest peak area significantly different from the areas of other membranes and one of the smallest standard deviations. Visual differences between unmodified and modified electrode are showed in Figure 4. HRP is an enzyme catalyzing H_2O_2 reduction. Although enzymes are highly specific, effective materials with better thermic and chemical stability are sought for analytical purposes [16-18]. MPs as the one of the most well-known and attractive nanomaterials are usually applied in drug delivery, biological separation and catalysis for their low toxicity and exquisite chemical properties [19]. Especially, their pseudo-peroxidase activity proved in many former studies provides another interesting application of MPs as peroxidase replacing catalysts [16,17,20]. PB consisting of Fe(III) and Fe(II) three dimensional structure has gained attention for its low cost, easy preparation and great electrochemical, photo-physical, and magnetic properties as well as for its possibility of wide application in electrochemical devices, fuel cells, magnetic materials and in the bioelectrocatalytic fields. Their application as “artificial peroxidase” and “mediator of electron transfer” has been known as well [21,22]. While MPs and PB are known as more stable catalysts HRP has proved higher catalytic activity [22,23]. So the combination of HRP, MPs and PB as catalysts is more convenient than using only one or two of these modifiers.

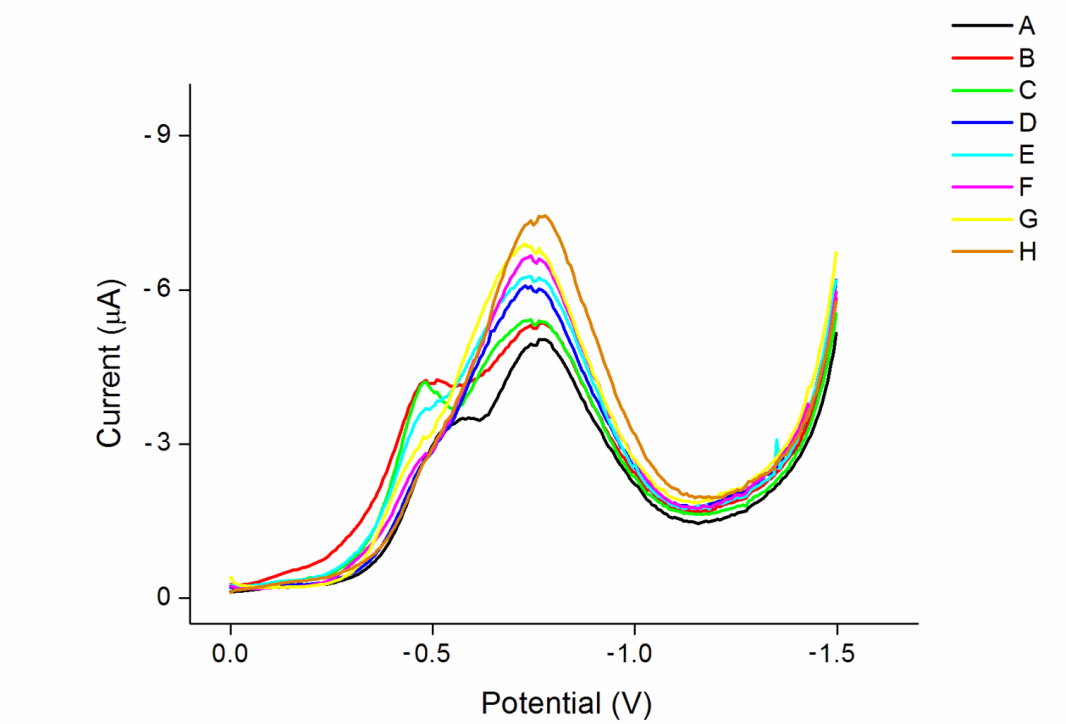

Figure 2. SWV curves recorded on differently modified electrode surface using 0.8 % H_2O_2 as measured analyte. A – chitosan; B – chitosan + HRP; C – chitosan + PB; D – chitosan + MPs; E – chitosan + HRP + PB; F – chitosan + HRP + MPs; G – chitosan + PB + MPs; H – chitosan + HRP + PB + MPs.

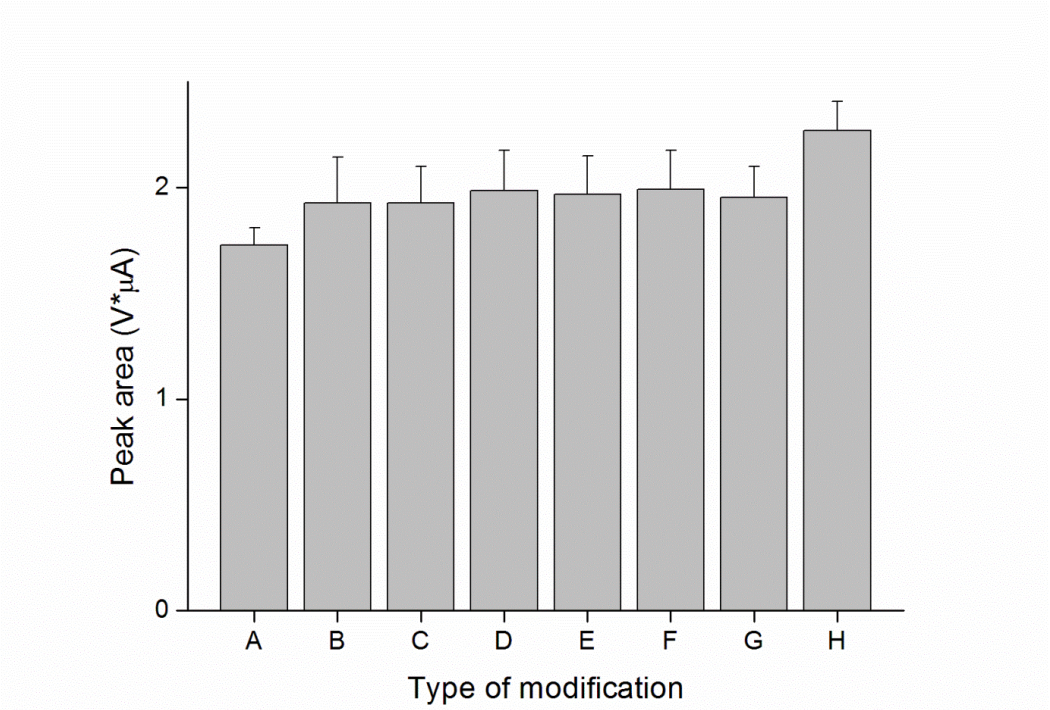

Figure 3. Comparison of different modification of chitosan membranes immobilized onto surface of screen printed electrode for 0.8 % H_2O_2 determination by SWV. A – chitosan; B – chitosan + HRP; C – chitosan + PB; D – chitosan + MPs; E – chitosan + HRP + PB; F – chitosan + HRP + MPs; G – chitosan + PB + MPs; H – chitosan + HRP + PB + MPs. Error bars indicates standard deviation for n = 5.

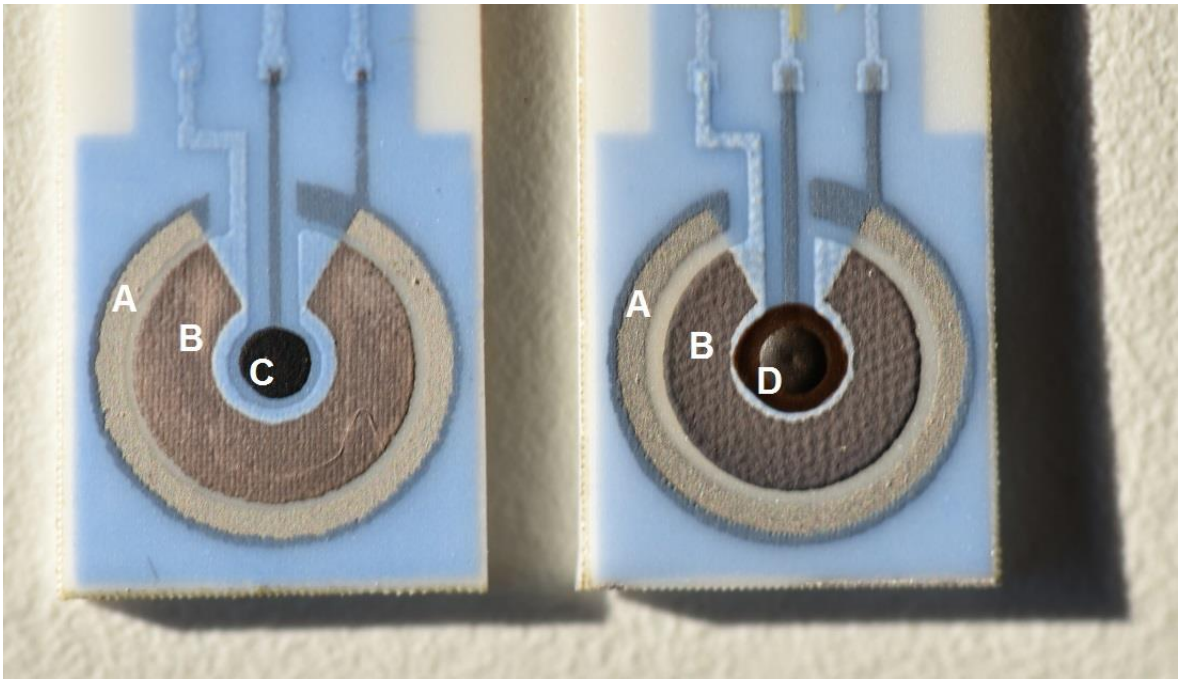

Figure 4. Visual differences between bare screen-printed electrode and screen-printed electrode modified by membrane type H. A – counter electrode; B – reference electrode; C – bare working electrode; D – working electrode covered by chitosan membrane type H entrapping HRP; MPs and PB.

3.2. Concentration curve

Concentration curve was constructed on the base of average peak area results measured on electrodes with chitosan membrane type H and it is showed in Figure 5. Curve replying Michealis-Menten equation has Michaelis constant equal to 0.25 % (81 mmol/l) of H_2O_2. Correlation coefficient was calculated to be 0.999 and limit of detection was set to be 2.5×10^{-5} % (8.2 µmol/l) of H_2O_2. Curve showed linear dependence in concentration range from 0 to 1.6 % (0.52 mmol/l) of H_2O_2 with correlation coefficient equal to 0.998. Summary of assay statistical parameters have been summarized in Table 1 and compared with another selected electrochemical methods for H_2O_2 determination in Table 2. SWV record of changing electrical current depending on increasing potential is showed in Figure 6 where H_2O_2 created peak at potential level -670 mV. According European regulations concentrations of H_2O_2 higher than 0.1 % (33 mmol/l) are not allowed in non-food products [7]. In the USA, Food and Drug Administration allows contain of H_2O_2 in milk lower than 0.05 % (16 mmol/l) and residual contain of H_2O_2 in finished food packages even lower than 0.5 mg/l (15 µmol/l) [6], so limit of detection of our assay was low enough for detection of observance of H_2O_2 contain limitations in non-food and food products. The limit of detection of presented assay is also appropriately low for detection of H_2O_2 levels in biological samples such as aqueous humor, where H_2O_2 is presented in concentrations in a range from 25 to 60 µmol/l [1]. In comparison with current methods, our limit of detection also keeps up with the other assays.

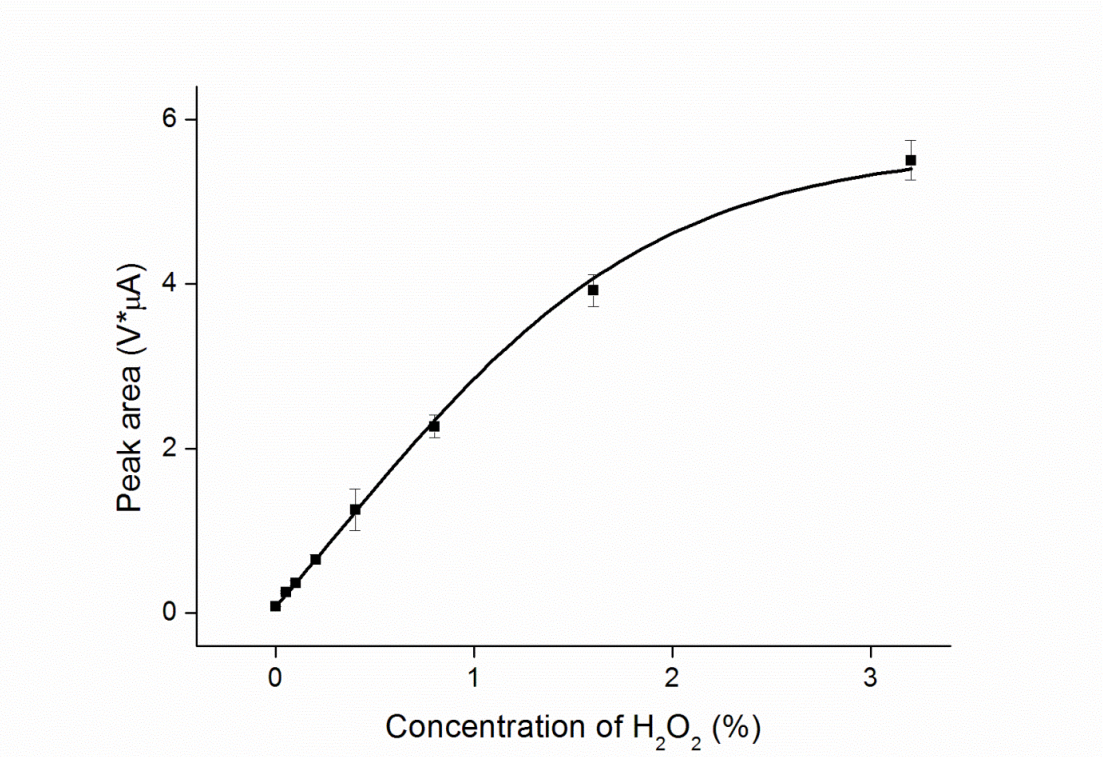

Figure 5. Concentration curve of H_2O_2 measured by SWV using screen printed electrodes modified by chitosan-HRP-MPs-PB membrane. Error bars indicates standard deviation for n = 5.

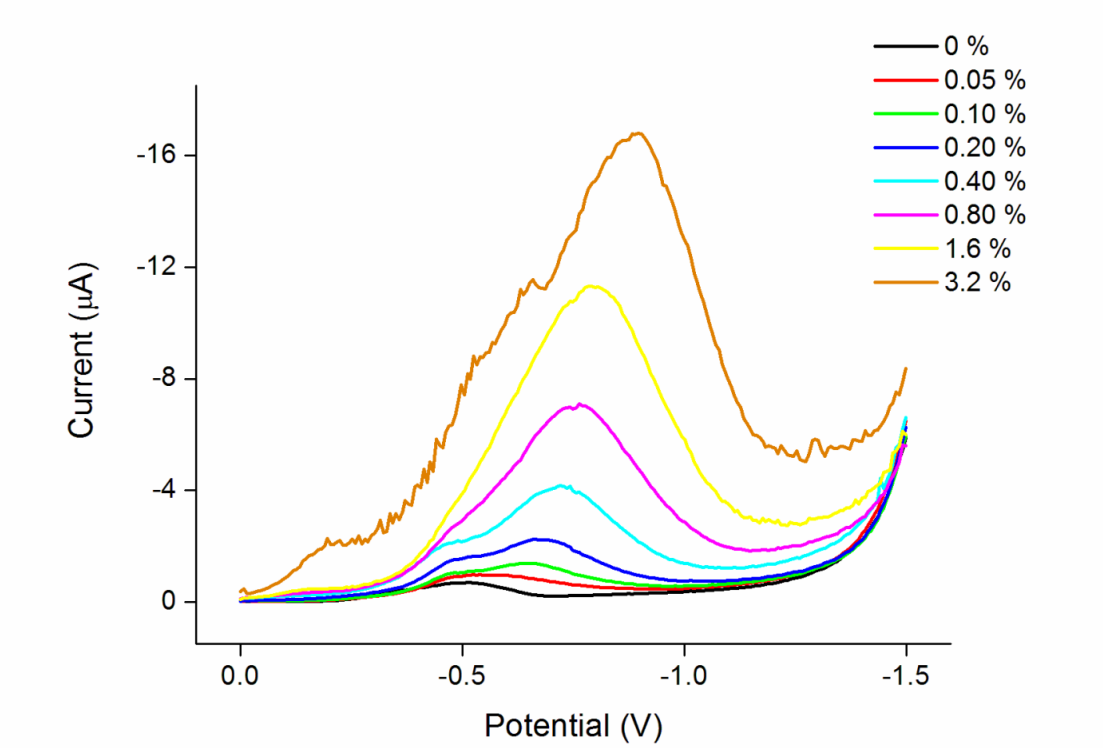

Figure 6. SWV curves of H_2O_2 in the used concentrations from 0 % (water) to 3.2 % where dependency of electrical current on increasing potential is showed. Peaks were observed around -0.67 V with increasing area depending on H_2O_2 concentration.

Nogueira and coauthors gained the limit of detection of their spectrophotometric method equal to 143 µmol/l and Chekin and coauthors (2015) reached limit of detection of their voltammetric method in range from 0.4 to 0.98 µmol/l according type of used carbon nanotubes [24,25].

3.3. Interferences

Possibly interfering substances replacing peroxidase in the reaction were and recorded SWV curves are showed in Figure 7. Average peak areas of the interfering substances were compared with average peak area of positive and negative control (Figure 8). Positive control was represented by 0.8 % H_2O_2 which showed the largest average peak area. The finding was in compliance with our expectation. The average peak areas of the interfering substances were significantly lower than average peak area of positive control and they were not significantly different from average peak area of negative control. That means that the possibly interfering analytes could not be used as substrates for the reaction (Table 1). Reduced glutathione and uric acid was tested in an interference study by Chekin and coauthors (2015), ascorbic acid, acetaminophen and uric acid was tested as possibly interfering substances in the study of Yao and coauthors (2012) and all these compounds in both studies did not interfere to detection of H_2O_2 [22,25]. On the other hand, in the study of Abbas and coauthors (2010) ascorbic acid did interfere unlike our results [6].

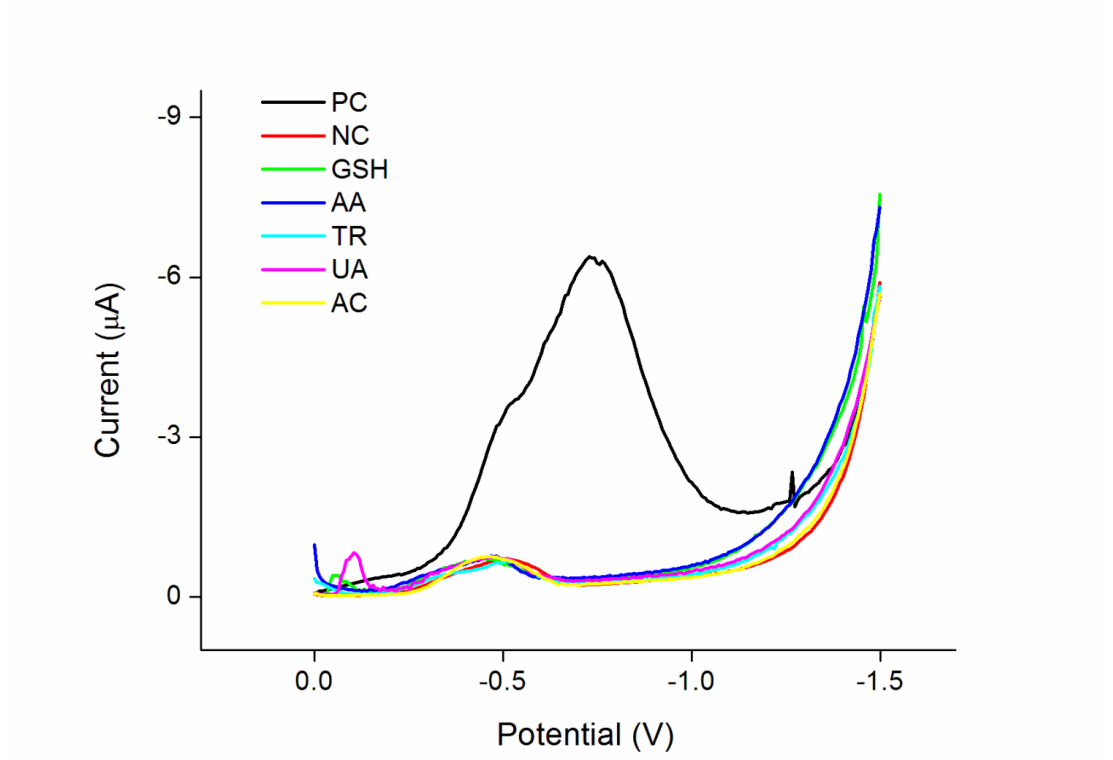

Figure 7. SWV curves of possibly interfering substances measured on screen-printed electrodes covered with chitosan membrane type H. PC–positive control; NC–negative control; GSH–reduced glutathione; AA–ascorbic acid; TR–trolox; UA–uric acid, AC–acetaminophen.

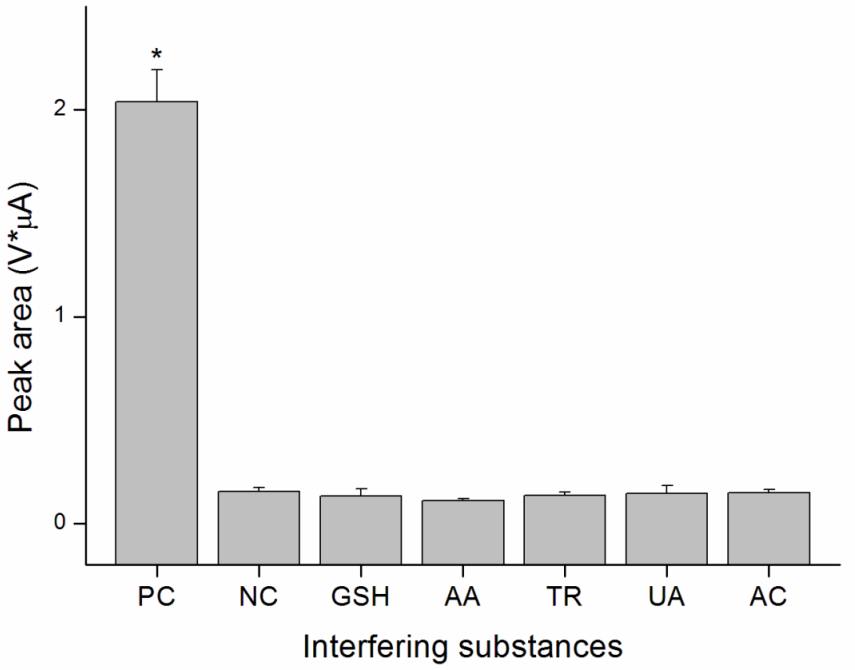

Figure 8. Comparison of average peak areas of possibly interfering substances with positive (H_2O_2) and negative controls (PBS) on screen-printed electrodes covered with chitosan membrane type H. PC–positive control; NC–negative control; GSH–reduced glutathione; AA–ascorbic acid; TR–trolox; UA–uric acid, AC–acetaminophen. Results significantly different from negative control are marked by symbol * above the column. Error bars indicates standard deviation for n = 5.

3.4. Matrix effect

Effect of different matrices (toothpaste, hand cream and skin tonic) on the assay was determined in this part of experiments. Selection of matrices was based on the works of Gimeno and coauthors and Campanella and coauthors [2,7]. In the first study, H_2O_2 was determined in personal care products such as toothpaste, mouthwash, whitening cream or hair days using high performance liquid chromatography [7]. In the second study, the commercial samples for cosmetic or pharmaceutical purposes such as creams, emulsions or aqueous solutions of disinfectants were analyzed [2]. On the basis of these works we selected commercially available personal care products as matrices for our measurements. Recorded SWV curves and spiked matrices and positive and negative control are showed in Figure 9. Average peak areas of spiked matrices were compared with the peak area of positive control (H_2O_2) and negative control (PBS) and the comparison is showed in Figure 10. According measured results, average peak areas of matrices were not significantly different from average peak area of positive control and they were significantly different from negative control so our results indicated that H_2O_2 can be analyzed by our method in all of these products without any affection (Table 1) and the concentration of H_2O_2 was not falsely higher or lower.

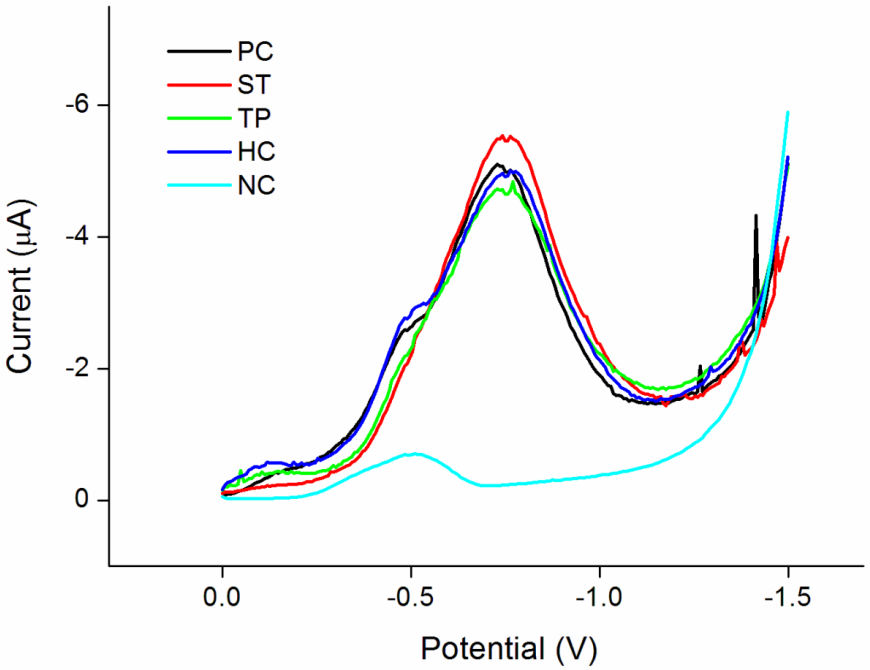

Figure 9. SWV curves of different cosmetic matrices spiked by H_2O_2, positive and negative control. PC – positive control (0.8 % H_2O_2 without matrix), NC – negative control (PBS without matrix), ST – spiked skin tonic; TP – spiked toothpaste; HC – spiked hand cream.

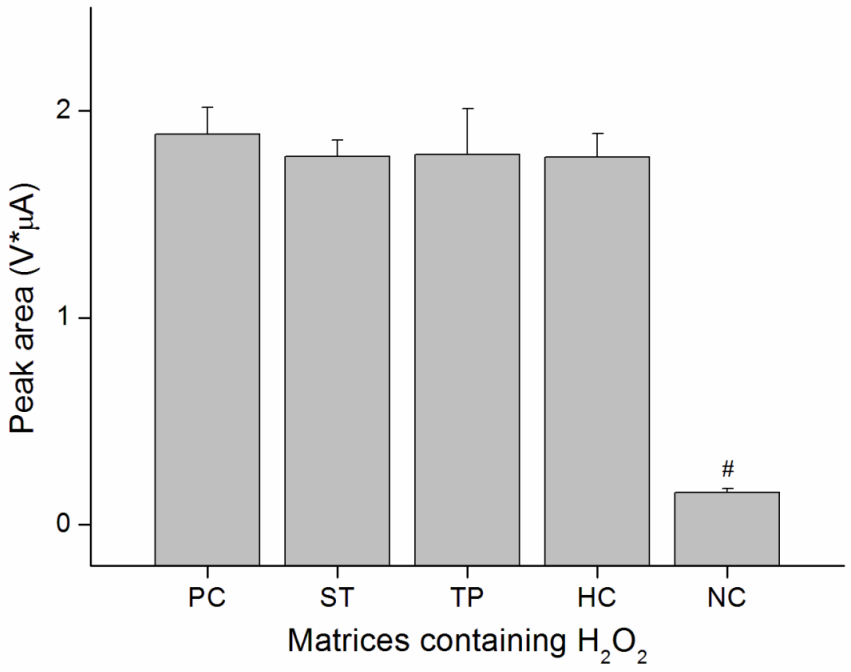

Figure 10. Comparison of average peak area of different matrices (ST – skin tonic; TP – toothpaste; HC – hand cream) diluted by 1.6 % H_2O_2 twice with positive (PC – 0.8 % H_2O_2 without matrix) and with negative control (NC – PBS). Results significantly different from positive control are marked by symbol # above the column. Error bars indicates standard deviation for n = 5.

3.5. Selectivity of membrane

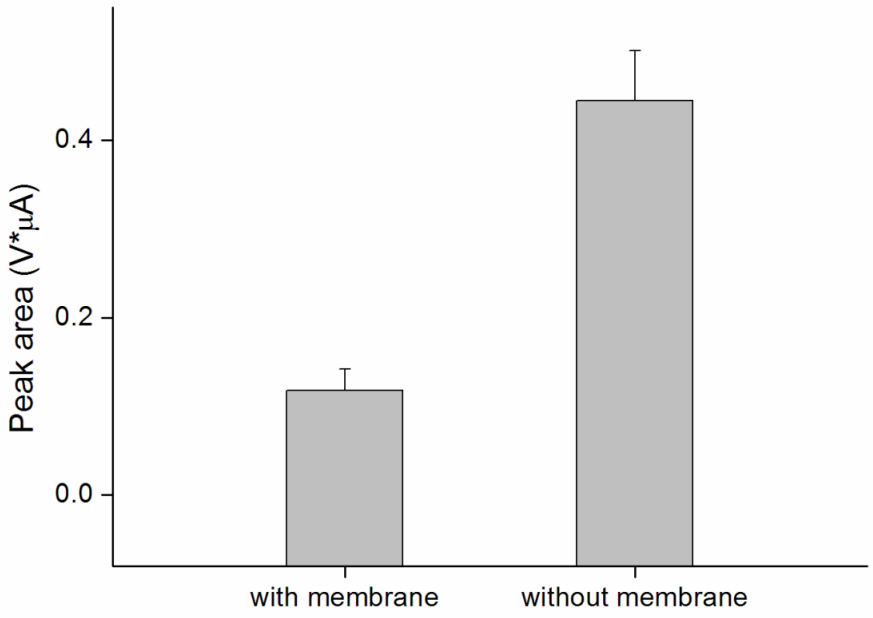

Figure 11. Comparison of average area of ascorbic acid peaks measured on the bare electrode and on the electrode modified by chitosan-MPs-HRP-PB membrane. Error bars indicates standard deviation for n = 5.

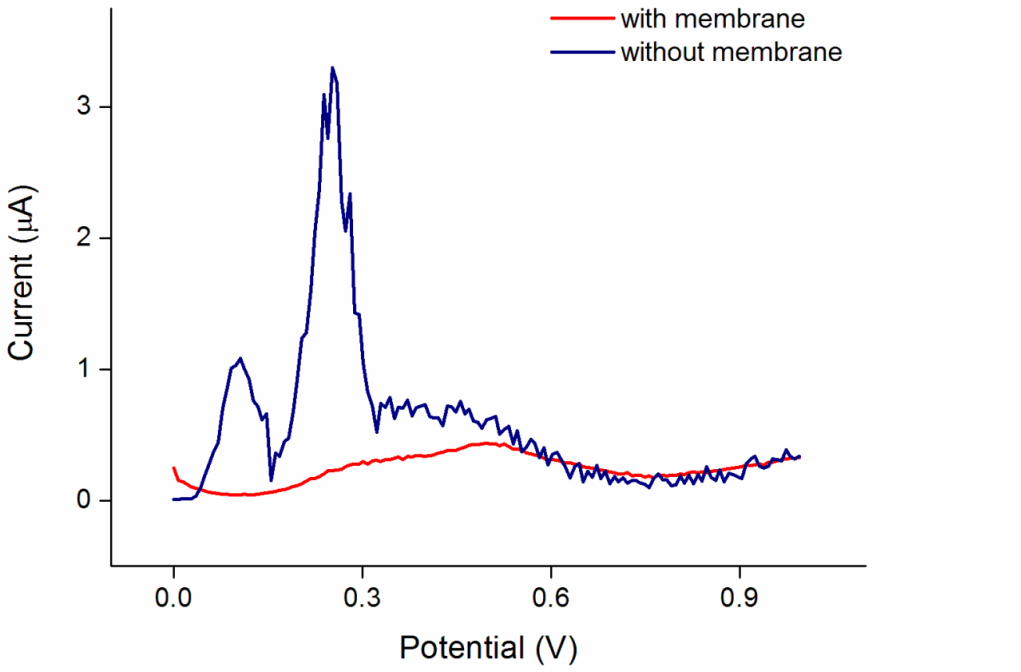

Figure 12. SWV curves of ascorbic acid (representing antioxidants) measured on both bare screen-printed electrode and screen-printed electrode modified by chitosan membrane type H.

Selectivity of membrane for H_2O_2 determination was tested using ascorbic acid on modified and bare electrode and the results are compared in Figure 11. Average area of peaks measured on electrodes modified by chitosan membrane with entrapped MPs, HRP and PB is significantly lower than average area of peaks measured on the bare electrodes.

The area of peaks measured on the modified electrodes is very similar to average area of peaks measured as 0 % concentration of concentration curve (average peak area of H2O was equal to 0.134 V*µA and average area of ascorbic acid peak measured on modified electrode was set to be 0.120). That means the modification of electrode by chitosan membrane lower sensitivity of electrode toward other antioxidants and these antioxidants could not affect H_2O_2 measurement (Table 1). The noise of signal caused by ascorbic acid during measurement was eliminated by electrode modification as it was showed on SWV curves in Figure 12.

3.6. Comparison of novel and reference method

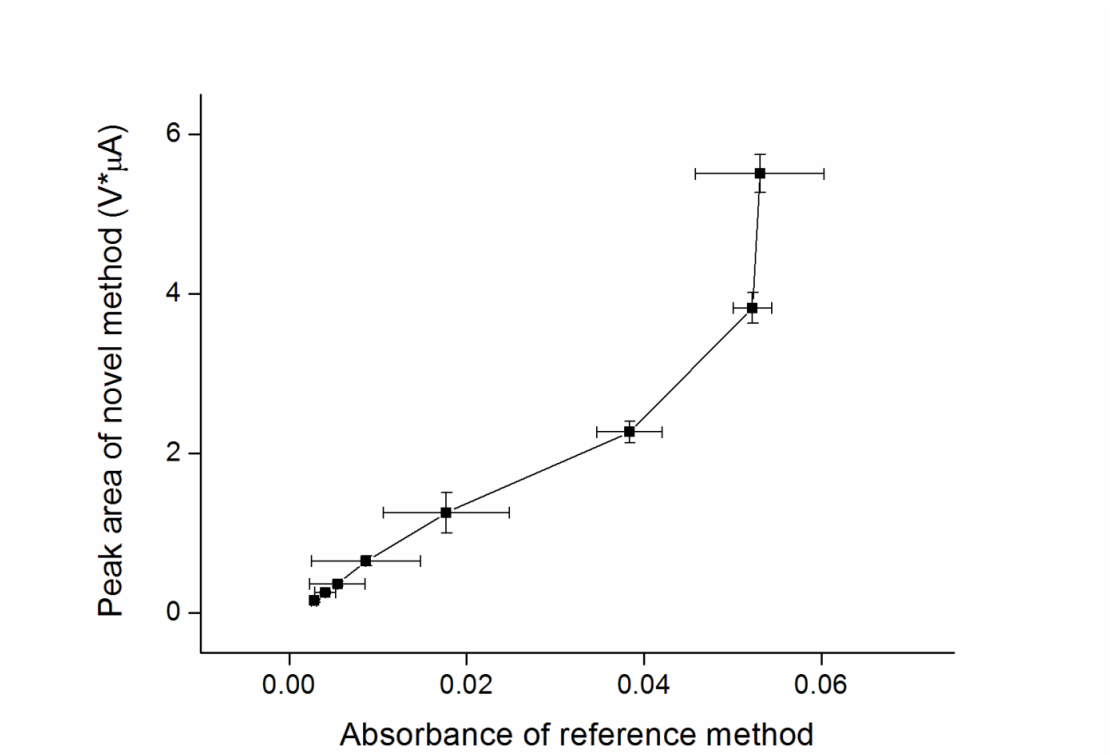

Figure 13. Comparison of voltammetric method for H_2O_2 determination based of HRP-MPs-PB-chitosan membrane modifying electrode with standard spectrophotometric method for H_2O_2 determination. Error bars indicates standard deviation for n = 5.

Results measured as concentration curve by SWV was compared with results measured by standard spectroscopy [16] and the final graph is showed as Figure 13. Linear dependency of novel method on reference one is showed in concentration range from 0 to 0.8 % of H_2O_2. In the whole concentration range, novel method had better sensitivity and lower standard deviation (Table 1). In

compliance with the gained results, we can evaluate voltammetric method based on electrodes modified by chitosan membrane entrapping MPs, HRP and PB as better one than standard colorimetric method measured by spectrophotometry. On the other hand, in comparison with other electrochemical methods for H_2O_2 determination out method showed differing results (Table 2). Concerning limit of detection another mentioned methods gained results in range from 0.4 to 8.0 μmol/l and Michaelis constant was determined between 7.6 μmol/l and 1.38 mmol/l. However it must be said that our methods was optimized in the widest concentration range (from 0 to 1.04 mol/l) so our linear range of concentration curve is also the widest. It follows that our method has the widest spectrum of application which makes it universal for use in broad spectrum of environmental, pharmaceutical, healthcare and food analyses.

Table 1. Summary of all results and findings.

Method	**Findings**	**Figure**
Membrane modification	Chosen membrane type H (HRP, MPs, PB)	2-4
Concentration curve	Michaelis constant = 0.25 % (81 mmol/l) Correlation coefficient = 0.999 Limit of detection = 2.5×10^{-5} % (8.2 μmol/l) Linearity in range 0 - 1.6 % (520 mmol/l)	5, 6
Interferences	Reduced glutathione, ascorbic acid, trolox, uric acid and acetaminophen did not interfere	7, 8
Matrix effect	Matrices toothpaste, hand cream and skin tonic did not affect the measurement	9, 10
Selectivity of membrane	Chitosan membrane containing HRP, MPs and PB was highly selective for H_2O_2 determination	11, 12
Comparison with standard method	Linearity in range 0 – 0.8 %, better sensitivity in whole concentration range.	13

Table 2. Summary of statistical parameters of electrochemical methods for H_2O_2 determination.

Measurement system	**Limit of detection**	**Linear range**	**Michaelis constant**	**Reference**
Chitosan membrane containing HRP, PB and MPs on screen-printed electrode	8.2 μmol/l	0-520 mmol/l	81 mmol/l	This paper
Screen-printed carbon electrodes modified with carboxyl-functionalized single-wall carbon nanotubes and HRP	0.4 μmol/l	0.5–500 μmol/l	7.6 μmol/l	[25]
Screen-printed carbon electrodes modified with carboxyl-functionalized multi-wall carbon nanotubes and HRP	0.48 μmol/l	0.5–250 μmol/l	8.6 μmol/l	[25]
HRP immobilized on screen-printed carbon electrodes	0.98 μmol/l	1–250 μmol/l	16 μmol/l	[25]
Ferrocene-chitosan: HRP: chitosan-glyoxal immobilized onto glassy carbon electrode	8.0 μmol/l	0.035-1.1 mmol/l	0.2 mmol/l	[26]
Glassy carbon electrode modified by	0.078	0.2-680	1.38 mmol/l	[27]

magnetic dextran microsphere entrapping HRP	μmol/l	μmol/l		
Hemoglobin immobilized on a glassy carbon electrode modified with Fe_3O_4/chitosan core-shell microspheres	4.0 μmol/l	0.05-1.8 and 1.8-6.8 mmol/l	0.29 mmol/l	[28]
Screen-printed carbon paste electrodes modified by electropolymerization of pyrrole with entrapped HRP	-	0.1-2.0 mmol/l	-	[29]
Iron oxide nanorods	1.3 μmol/l	0-2.5 mmol/l	-	[30]

4. CONCLUSION

In a summary, the benefits of electrodes covered with chitosan membrane entrapping catalysts and pseudo-catalysts (HRP, PB and MPs) in H_2O_2 determination were successfully proved. The method was showed as highly sensitive and selective unlike the uncovered electrodes, limit of detection, no interferences and no effect of used matrices was also showed. Moreover, in comparison with commonly used colorimetric method our method demonstrated better results. In the base of gained results we can evaluate our method as highly applicable in wide spectrum of industrial, environmental, pharmaceutical and medical branches.

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