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Ester flavorants detection in foods with a bienzymatic biosensor based on a stable Prussian blue-copper electrodeposited on carbon paper electrode

Maria-Cristina Radulescu, Madalina-Petruta Bucur, Bogdan Bucur*, Gabriel Lucian Radu

National Institute of Research and Development for Biological Sciences, Centre of Bioanalysis, 296, Splaiul Independentei, 060031 Bucharest, Romania

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ABSTRACT

A stable and reproducible layer of Prussian blue (PB) modified with copper was electrodeposited on carbon paper electrodes for the multiple detection of ester flavorants with a bienzymatic biosensor. Carbon fiber composite paper was investigated as high-surface, low-cost substrate for biosensor development. The pre-activation of the electrode surface by cyclic voltammetry was necessary to improve the electrochemical properties before the electrochemical deposition of Prussian blue-copper film (PB-Cu). The stability and the reproducibility of the obtained PB-Cu carbon paper electrode was demonstrated at pH 7.4, optimum for biosensor development. The developed biosensor is based on the immobilization of two enzymes (carboxyl esterase and alcohol oxidase) by cross-linking with glutaraldehyde onto PB-Cu carbon paper electrode. A mixture of key aroma ester compounds (methyl butyrate, ethyl butyrate, methyl cinnamate and ethyl cinnamate) was detected in several food samples with low interferences.

1. Introduction

Processed foods with highly appealing organoleptic properties often rely on added flavorants to modify both the perceived taste and odor. Numerous additive compounds are available for this purpose to the food industry and they have a major contribution to the quality of final product [1]. The widespread use of additives led to the need to control their use in order to ensure consumer safety. While the currently approved flavorants are considered to be safe at normal food intake levels [2], their usage should be in accordance with the *quantum satis* rule, i.e. the smallest amount which is necessary to achieve the desired technological effect and in accordance with good manufacturing practice to obtain the desired effect.

Various esters with a fruity flavor such as: methyl cinnamate, methyl butyrate, ethyl cinnamate and ethyl butyrate (presented in Scheme 1-ESM) are often used as additives in the form of natural extracts or synthetic liquid concentrates to achieve the desired organoleptic properties of food products [3] based on their evaluated relative contribution to the fruity aroma [4]. Due to the high cost or unavailability of natural flavor extracts, most commercial flavorants are "nature-identical", which means that they are the chemical equivalent of natural flavors, but chemically synthesized rather than being extracted from source materials [3].

Highly sensitive analytical methods were developed to accurately quantify the volatile constituents involved in food flavoring using chromatography coupled with mass spectroscopy [4], but these techniques are labor intensive and require sophisticated equipment that is neither adapted for regular monitoring nor available to small food producers. Alternatively, “electronic nose” and “tongue” devices have been proposed for the quality evaluation or for the classification of foods based on sensors arrays with different selectivity for flavorants coupled with chemometric for data interpretation [5]. The employed sensitive elements of such arrays include: insect cells expressing insect odorant receptors [6], polyaniline layers with different dopants [7], molecularly imprinted materials [8], and odorant-binding proteins [9].

We propose in this paper a much simpler approach for the fast sensory evaluation of foods: the quantitative determination of the total content of ester flavorants using a single bienzymatic biosensor with a large detection spectrum. Our proposed biosensor couples the catalytic activities of carboxyl esterase and alcohol oxidase with the sensitivity and simplicity of electrochemical detection. Carboxyl esterase (CaE) catalyses the hydrolysis of ester flavorants into a mixture of alcohols that are further oxidized by alcohol oxidase (AOX) producing thus hydrogen peroxide. In the end, hydrogen peroxide is detected electrochemically using carbon paper sensors modified with a mixed Prussian blue-copper film. Electrochemical analytical systems are used because of their simplicity, miniaturization and low cost. Carbon paper is an inexpensive material, environmental friendly, versatile, with enhanced

* Corresponding author
E-mail address: bucurica@yahoo.com (B. Bucur).

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charge transfer [10], attractive as electrode material for modification with various nanoparticles [11] and high power for fuel cells development [12]. A mixed Prussian blue-copper film (PB-Cu) with enhanced stability was electrodeposited [13] on the carbon paper electrode and was used as an electrochemical mediator due to its high sensitivity towards hydrogen peroxide in the presence of oxygen and low interferences [14]. To the best of our knowledge, this is the first bienzymatic biosensor reported for ester flavorants quantification.

2. Materials and methods

2.1. Reagents

Alcohol oxidase (AOX, from *Hansenula* sp. 7.7 UI/mg solid), carboxyl esterase (CaE, from porcine liver, 17 UI/mg solid), methyl cinnamate (MeCin), ethyl cinnamate (EtCin), methyl butyrate (MeBut), ethyl butyrate (EtBut), potassium phosphate monobasic, bovine serum albumin (BSA), glutaraldehyde (GA, 25% solution in water), potassium chloride, iron(III) chloride, hydrochloric acid 37%, sodium phosphate dibasic, potassium phosphate monobasic, hydrogen peroxide 30%, citric acid, copper(II) sulphate anhydrous (98%), potassium ferricyanide $K_3[Fe(CN)_6]$ and potassium ferrocyanide $K_4[Fe(CN)_6]$ were purchased from Sigma-Aldrich. Methanol was obtained from Merck.

Stock solutions of hydrogen peroxide were prepared daily in supporting electrolyte. Stock solutions of 100 mM ester flavorants were prepared daily in a mixture of 1:1 acetonitrile: 0.1 M phosphate buffer solution pH 7.4 and further dilutions were made in PBS.

The amperometric measurements were made in PBS pH 7.4 supplemented with 0.1 M potassium chloride. The candies, fruit essences and fruity rewards for puppies were purchased from a local supermarket.

2.2. Apparatus

The electrochemical measurements were performed with a galvanostat/potentiostat Autolab PGSTAT302N (Metrohm-Autolab) controlled by a PC with the software Nova 1.11. Toray TGP-H-60 carbon paper which is a composite carbon fiber paper with a thickness of 190 µm from Alfa Aesar was used as working electrode. A square area of 25 mm^2 was delimited by applying a nail polish onto carbon paper. The reference and counter-electrodes were screen-printed from DropSens.

2.2.1. Prussian blue-copper film (PB-Cu) electrodeposition on carbon paper electrode

The carbon paper electrodes (Toray TGP-H-60 from Alpha Aesar) were electrochemically activated by cyclic voltammetry in 0.1 M H_2SO_4, between −0.2 and +1 V, for 50 cycles at 100 mV/s scan rate. After washing with water and drying in air, the activated carbon paper surface was introduced in a 10 mL cell with a freshly prepared solution containing 0.5 mM $FeCl_3$, 0.25 mM $CuSO_4$ and 0.5 mM $K_3[Fe(CN)_6]$ in 10 mM HCl and 100 mM KCl. The PB-Cu film was formed on the electrode surface by cyclic voltammetry (20 scans, from −0.1–0.5 V at 50 mV/s) by adapting a procedure previously optimized for screen-printed electrodes [13]. Then, the electrodes were washed with water and dried at room temperature before use. Carbon fiber composite paper PTFE treated (Toray TGP-H-60-PTFE from Alpha Aesar) were also investigated as electrode material.

2.2.2. Enzymes immobilization onto PB-Cu carbon paper electrodes

The two enzymes were immobilized on the modified PB-Cu carbon paper electrodes by reticulation with GA and using an inert protein for stabilization: 1 mg of CaE (18 IU) solubilized in 30 µL of PBS solution containing 1% GA and 0.5% BSA was mixed with 6 µL containing 1 mg AOX and 5 µL of the resulting enzyme solution (containing 1 IU AOX and 3 IU CaE) were deposited on the delimited surface of the carbon paper electrode [15]. The electrodes were dried for one hour at room temperature and then stored at −20 °C. The same immobilization protocol was also applied for two commercial carbon screen-printed electrodes used for comparison.

2.2.3. Ester flavorants amperometric measurements with bi-enzymatic biosensors

Amperometric measurements were carried out at −50 mV in 10 mL PBS. The solution was magnetically stirred and the filter from ECD module was set to 1 s for noise reduction. After baseline stabilization a small aliquot of ester flavorants standard or sample solution was injected into the cell. The current stabilizes in less than 1 min and the analytical signal is the difference between the reached plateau and baseline values. During the optimization, hydrogen peroxide was injected instead of the ester flavorants and various working electrodes were used (PB-film electrode, PB-Cu film electrode or bi-enzymatic biosensor).

3. Results and discussions

3.1. Optimization of the carbon paper electrodes modified with PB-Cu film

Carbon paper electrodes have attractive properties that recommended them for development of sensors [10] or support of catalyst for fuel cells [11]. Both carbon fiber composite paper (Toray TGP-H-60) and carbon fiber composite paper PTFE treated (Toray TGP-H-60-PTFE) were investigated as low-cost alternative substrates for screen-printed sensor manufacturing. Our experiments showed that higher magnitude of the signals and a better sensibility were obtained with untreated carbon paper electrodes in comparison with those obtained with PTFE treated carbon paper electrodes. This is due to the PTFE layer which may reduce the conductivity and porosity of the electrode so that the electrode performances will be reduced.

The bare carbon paper electrodes displayed poor electrochemical properties when tested by cyclic voltammetry using potassium ferricyanide as redox couple, as shown by the low current intensities and the deformed anodic and cathodic peaks (Fig. 1). Improvement of the electrochemical properties of the carbon paper electrodes was reported by applying harsh electrochemical pre-treatments, but this leads to an important increase of the baseline currents [16]. There are numerous processes that take place within the carbon based electrodes during the activation processes including alterations in the surface area/roughness or modification to functional groups, impurities, defects and edge plane-like sites on the graphite that affect both the electrochemical properties and double-layer capacitance [17]. We have carried out a preliminary activation step by cyclic voltammetry in a solution of 0.1 M H_2SO_4, with 50 cycles from −0.5 to +1 V at 100 mV/s scan rate to obtain high-performing carbon paper electrodes during which it was noticed only a small increase of the capacitive currents (Figure 1-ESM). After the activation step, the two well defined redox peaks corresponding to potassium ferricyanide were observed while the capacitive currents recorded in PBS were still small (Fig. 1). Increasing the number of scans or the scanning potential window during the activation step did not further improve the electrochemical characteristics of the carbon paper electrodes.

After pretreatment in acidic solution, the electrodes were dried at room temperature for one hour and then Prussian blue (PB) mediator was electrodeposited. PB has very good sensitivity for hydrogen peroxide, but suffers from instability issues at neutral pH that could be mitigated by electrodeposition in presence of other metals such as copper [13,18]. Thus, PB-Cu films were electrodeposited from a solution containing $FeCl_3$ together with $CuSO_4$ in $K_3[Fe(CN)_6]$, 10 mM HCl and 100 mM KCl. Different ratios of $FeCl_3$, $CuSO_4$ and $K_3[Fe(CN)_6]$ (1: 1: 1, 0.5: 0.5: 1, 1: 0.5: 1) were tested in order to obtain a stable PB-Cu film with good detection of hydrogen peroxide and to reduce the background noise. Two peaks were observed during PB-Cu electrodeposition on carbon paper electrodes at 0.111 V and 0.036 V due to

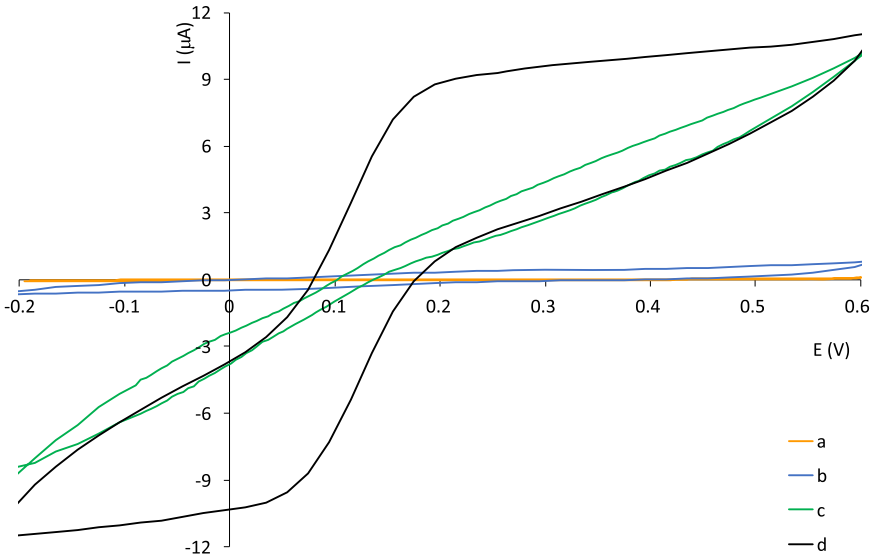

Fig. 1. Cyclic voltammograms recorded at 50 mV/s scan rate in: PBS, pH 7.4 before (a) and after electrode activation (b) and in PBS containing 1 mM ferricyanide, before (c) and after electrode activation (d).

mediator electrodeposition and their current intensity increased with the number of cycles. A stable PB-Cu film was obtained after 20 cycles between −0.1 and 0.5 V at a scan rate of 50 mV/s (Figure 2-ESM). Optimum concentrations used for further work are: 0.5 mM $FeCl_3$, 0.25 mM $CuSO_4$ and 0.5 mM $K_3[Fe(CN)_6]$.

The PB-Cu electrodes were tested by CV in PBS and the following parameters were obtained for a 50 mV/s scan rate for the redox peaks: E_{red} = 0.011 V, E_{ox} = 0.137 V, ΔE_p = 0.126 V, I_{red} = -53.5 μA, I_{ox} = 49.6 μA and I_{ox}/I_{red} = -0.93. For comparison, the peaks for electrodes modified with PB without copper are slightly smaller while the potentials are similar: E_{red} = 0.016 V, E_{ox} = 0.147 V, ΔE_p = 0.131 V, I_{red} = -39.2 μA, I_{ox} = 28.2 μA and I_{ox}/I_{red} = -0.72. The variation of the peak intensity is linear with the square of the scan rate (Figure 3-ESM and 4-ESM), this being characteristic for electrodes modified with PB [17]. The operational stability of the modified electrodes was tested by 50 repeated scans in PBS pH = 7.4 at a scan rate of 50 mV/s between −0.2 and 0.5 V. Four different electrodes were tested: PB-Cu, PB, PB-Cu-enzymes and PB-enzymes in order to investigate the stabilization provided by copper and also by the enzyme layer since the layers provide a supplementary stabilization [19]. From the CV presented in the Figure 5-ESM and the peak currents values presented in Fig. 2 it can be noticed that both copper and the enzyme layer provide an important stabilization effect. Thus, the percentages of the decrease calculated for the cathodic peak currents of the 50th cycle in comparison with the first recorded cycle for various electrodes varies between 14% for PB-Cu-enzyme, 25% for PB-Cu, 44% PB-enzyme and 49% PB. Another set of tests were further carried out by repeated cyclic voltammetry measurements in PBS pH 7.4 for two hours. A set of repeated measurements (n = 10) were registered with a PB-Cu electrode and then at different periods of time of 10 min, 30 min, 1 h and 2 h (Figure 6-ESM). Between each set of measurements the electrode was kept immersed in PBS buffer. A decrease of the analytical signal of about 25% compared to the initial value was observed after a 2 h immersion in buffer. The stability was further improved by the protein layer and was sufficient for the biosensor development. Moreover, the stability of the PB-Cu modified electrodes was significantly improved in comparison with PB films obtained without copper for which the current intensity decreased by 45% from the original value after similar 2 h measurements in PBS and continued to decrease for longer times.

The stability of the PB-Cu film was tested also by repeated amperometric measurement at −0.05 V by injection of 5 μM H_2O_2 in PBS pH 7.4, the cell being washed with purified water between measurements. After a set of 10 repeated measurements the PB-Cu electrode was immersed for 10 min in PBS and another set of 10 amperometric measurements followed. This procedure was repeated over a 2 h period. A mean value (nA) of the amperometric signal was calculated for each set of 10 measurements. Good reproducibility was obtained, the relative standard deviation (RSD) for different sets of measurements ranged between 2.2% and 5.1% and the signal variation over 2 h was less than 5% for PB-Cu modified carbon paper electrode i.e. no significant decrease of the amperometric signals. The average signal for a concentration of 5 μM hydrogen peroxide was 264.1 ± 7.09 nA. The calibration curve for hydrogen peroxide at pH 7.4 using PB-Cu electrodes was linear in the 0.25–30 μM range, also appropriate for the development of biosensors. For comparison, the responses towards hydrogen peroxide were tested in the same concentration domain for all possible configurations and the sensibility of the calibration line increases in the following order: PB-enzyme, PB-Cu-enzyme, PB and PB-Cu (Figure 7-ESM).

From all cyclic voltammetry and amperometric experiments described above it was concluded that addition of the copper allowed the improvement of the PB-Cu film stability and neutral pH, the enzymatic layer further stabilizes PB-Cu and a satisfactory sensitivity is achieved for biosensor development.

3.2. *Ester flavorants detection by using the developed biosensor*

The response of the bienzymatic biosensor depends on the specific kinetic properties of both enzymes for each substrate and a cumulative interaction between overall kinetic processes. Thus, CaE produces different alcohols by the enzymatic hydrolysis of the various ester flavorants, while AOX has a higher affinity and response for methanol in comparison with ethanol, although both alcohols may be detected in good conditions [20]. Furthermore, in case of a mixture of various substrates leading to with the production of a common detected reaction product (e.g. the case of alcohols mixture oxidation by AOX with the production of hydrogen peroxide), the total quantity of the product is higher than for individual substrates and lower than the algebraic sum of individual reactions depending on the AOX affinity for each alcohol [20]. Several ratios between AOX and CaE were tested and 1: 3 (AOX: CaE) was chosen as optimum due to an increase analytical signal. Initial studies aimed to investigate the biosensor response for each analyzed compound when presented alone in solution. The calibration graphs for each analyzed ester flavorant are presented in Fig. 3 and

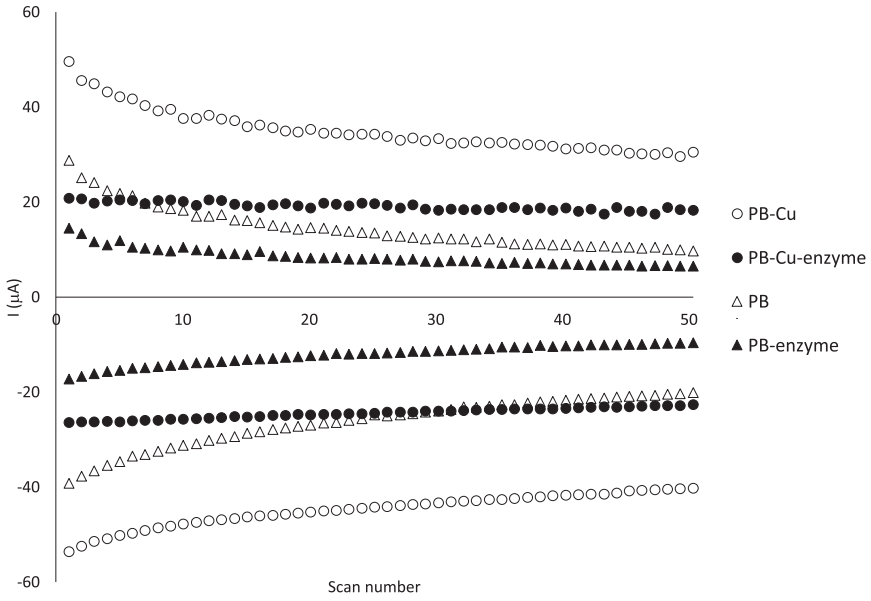

Fig. 2. The peak currents for 50 successive cyclic voltammograms recorded at 50 mV/s in PBS pH 7.4 with electrodes modified with: PB-Cu, PB-enzyme, PB and PB-enzyme.

demonstrate a higher sensitivity for analytes that contain methyl moieties in comparison with the ones with ethyl moieties, in line with AOX higher affinity for the first type of alcohol [20]. Also, a higher sensitivity was obtained for ester flavorants containing butyrate in comparison with the esters of cinnamate with the same alcohol moiety (either methanol or ethanol).

The good stability of the biosensors due to the usage of mixed PB-Cu film coupled with the robust enzyme immobilization allowed numerous repeated measurements. Thus, with the same biosensor it was possible to make a calibration graph (in triplicate) and at least other 40 repeated measurements for various ester flavorants concentrations, separated by washing the electrochemical cell with purified water for a total operation time surpassing 3 h and 30 min. In the end the decrease of the biosensor response was lower than 20% (~18.5%) compared to the initial value. The improved stability of the biosensor in comparison with PB-Cu electrodes is attributed to the protein layer, that membranes abilities to slow the film dissolution being already reported [21]. The reproducibility of the measurements ranged between 3.6% and 6.6% for n = 10 repeated measurements with the same biosensor. In Table 1 are shown the characteristics of the calibration graphs for the

Table 1
Characteristics of the calibration plots for the analyzed ester flavorants by using the developed bienzymatic biosensor.

Analyte	Characteristics			
	Concentration range (μM)	Detection limit (μM)	Equation	R^2
MeBut	2.5–1000	0. 8	y = 1554.2x + 30.6	0.9951
MeCin	5–1000	2	y = 659.4x + 20.2	0.9945
EtBut	10–1000	4	y = 319.98x + 17.1	0.9856
EtCin	25–1000	9	y = 145.3x + 1.4	0.9882

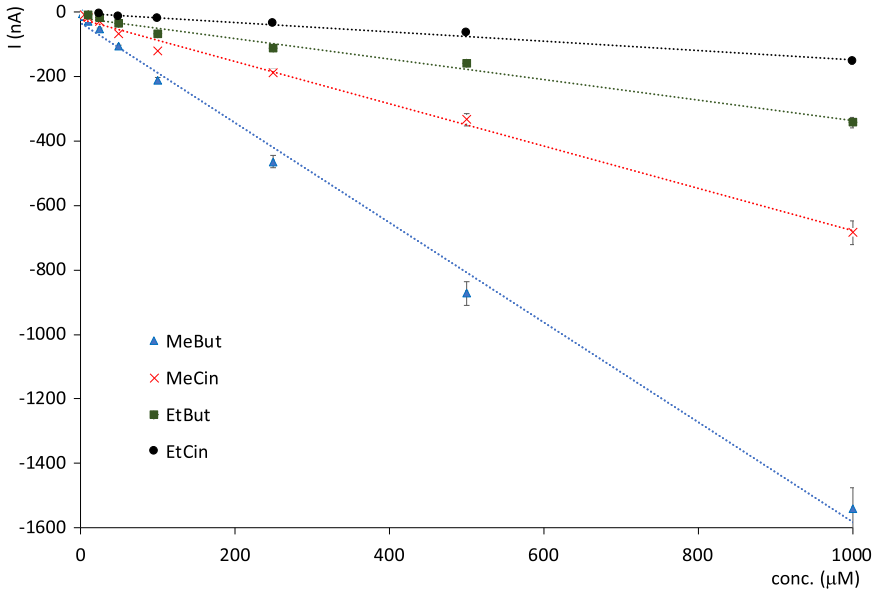

Fig. 3. The calibration curves obtained for the tested analytes using the developed bienzymatic biosensor. The linear part for each analyte starts from different initial concentrations depending on the enzyme affinities for each analyte.

Table 2
Analytical signals recorded for the ester flavorants - individually and in the mixture. Two measurements are presented with the order of ester flavorant injection in solution being reversed.

Analyte (analytical signal for single compound)	100 µM EtBut (68 nA)	25 µM MeBut (52 nA)	50 µM MeCin (66 nA)
50 µM EtBut (35 nA)		83; 94	103; 103
250 µM EtCin (44 nA)	104; 114	92; 101	115; 102
500 µM EtCin (64 nA)	123; 120	109; 112	124; 135
25 µM MeBut (52 nA)	118; 124		
25 µM MeCin (27 nA)		88; 88	
50 µM MeCin (66 nA)	131; 144	122; 123	

individual analyzed ester flavorants: detection limit, linear range, regression equations. The limit of detection is at µM level and the dynamic range is up to mM concentration (highest tested). The highest sensitivity is obtained for MeBut and the lowest one for EtCin, but the response is sufficient for a reliable quantification of all tested ester flavorants. The concentration ranges and limits of detection are suitable for the biosensor purpose, namely a simple, affordable and easy to use evaluation tool suitable for the need of small food producers without access to sophisticated analytical laboratories. A comparison with several other (bio)sensors reported in literature is presented in Table 1-ESM.

Subsequent investigations were carried out with mixtures of ester flavorants. Various concentrations of each ester flavorant were chosen from the linear part of the calibration graph and combinations of two ester flavorants were sequentially injected into the cell. It was observed that the signal obtained for mixtures of two ester flavorants is similar regardless the order of analyte injection in the cell and similar with the sum of the individual analytical signals (Table 2). We attribute this additivity of the individual signals to several factors: (i) we have used an excess of CaE in comparison with AOX, therefore the two ester flavorants are hydrolyzed at a faster rate than the alcohol oxidation reaction rate regardless of the differences between affinities of the CaE for the substrates, (ii) AOX is able to catalyze in parallel both methanol and ethanol with the production of hydrogen peroxide at an overall rate that is dependent on both the relative concentrations and affinities of both alcohols according to equation (1) [20] and (iii) the concentration ranges for the individual calibration lines are shifted to higher concentrations for ethanol and in consequence higher concentrations of ethanol are present at biosensors surface. These results prove that our biosensor is able to quantify the overall concentration of a mixture of ester flavorants. The data interpolation for flavorant mixtures is made using the calibration graph for a single compound.

$$\frac{d[H_2O_2]}{dt} = \frac{V_{max}^{Met}[Met]K_M^{Et} + V_{max}^{Et}[Et]K_M^{Met}}{K_M^{Met}K_M^{Et} + K_M^{Et}[Met] + K_M^{Met}[Et]}, \quad \text{Met = methanol;} \quad \text{Et = ethanol} \tag{1}$$

3.3. Study of interferences

Beside the ester flavorants added to food products to enhance their taste, different compounds are used as stabilizers or emulsifiers. Numerous substances that might interfere during measurements have been investigated: ascorbic acid, citric acid, lactic acid, glutamic acid, cysteine, quercetin, sodium metabisulfite, sodium sulfite, benzoic acid, gallic acid, hesperetin, catechin. No significative changes of the analytical signal were observed for a concentration of 2 mM of interfering compound. Of course, our biosensor is not suitable for analysis of alcohol containing products due to the detection principle (measurement of the produced alcohols). In case of doubt, a mono-enzymatic biosensor based only on AOX should be used to investigate if an unknown sample contains primary aliphatic alcohols that are oxidized by AOX.

3.4. Real samples analysis

The developed biosensor was applied to the detection of total fruity flavors in real samples acquired from local food shops: candies with fruity flavor (blackberries and raspberries flavors), flavoring essences for cooking (banana and pineapple) and fruity reward for dogs (with lemon, marigold or cranberry flavors). The label of solid samples indicated the presence of naturally identical flavors. The solid samples were prepared as follow: 5 g were mixed with 10 mL of water, sonicated for 20 min and centrifugated to collect the clear supernatant. One mL of the extract for candies, respectively 0.1 mL of fruity reward extract were injected in the electrochemical cell and the analytical signal was recorded. The liquid samples for cooking, natural flavoring banana and pineapple essences contain natural aroma, polyetilenglicol, water, tartrazine and sunset yellow as indicated on the label. A volume of 100 µL of essence was injected directly into the electrochemical cell without any sample preparation. In case of the liquid aromas, the absence of alcohols used as solvents was confirmed using a monoenzymatic AOX biosensor.

The analytical signal for the real samples was interpolated using the calibration curve for MeBut. The total ester flavorants content calculated in candies with black blueberries and raspberries flavor and reported as MeBut equivalent was 43.1 mg/kg and 29.9 mg/kg, respectively. A higher quantity of ester flavorants of 212.16 mg/kg reported as MeBut equivalent was founded in the fruity reward for dogs. High concentrations of ester flavorants (especially butyrates) are added to the dogs' diet due to positive effects on the animals health by constituting an essential energy source for epithelial cells and vital intestinal function [22].

In the commercial essences, the total content of esters quantified using the proposed biosensor and expressed as MeBut equivalent was 336 mg/L in flavoring pineapple essence and 199 mg/L in flavoring banana essence. Similar results were obtained by Jordan et al. [23,24] for commercial passion fruit and kiwi essences by using gas chromatography – mass spectrometry (GC-MS) and multidimensional gas chromatography olfactory (GC/GC-O). Also, the authors reported that the aroma profiles of the kiwi and yellow passion fruit essences are rich in esters with low molecular weight than the puree/juice of the fruits and MeBut together with EtBut are found in highest concentrations.

4. Conclusions

A bienzymatic biosensor for ester flavorants analysis was developed by immobilizing CaE and AOX on a carbon paper electrode coated with a PB-Cu mediator film. Carbon paper electrodes were used as an affordable material for biosensor development, good electrochemical properties being obtained after an activation step by cyclic voltammetry in diluted sulfuric acid. The PB-Cu film obtained by electrodeposition in the presence of Cu(II) ions is an efficient mediator for hydrogen peroxide and has an improved stability at neutral pH, appropriate for the development of reusable biosensors. The proposed biosensor is able to detect individual ester flavorants at micromolar level with low interferences. Interestingly, the biosensor is able to perform a quantification of the total ester flavorants present in a food sample without separation of the analytes based on the enzymatic catalysis of multiple substrates in parallel. The results recommend the biosensor as a potential alternative tool to chromatographic methods for determining the total ester flavorants content in alcohol-free food products.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.02.094.

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