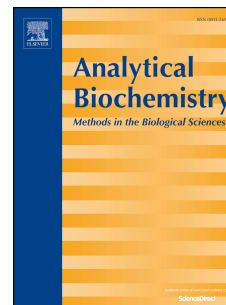


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Nano solid phase micro membrane tip and electrochemical methods for vanillin analysis in chocolate samples

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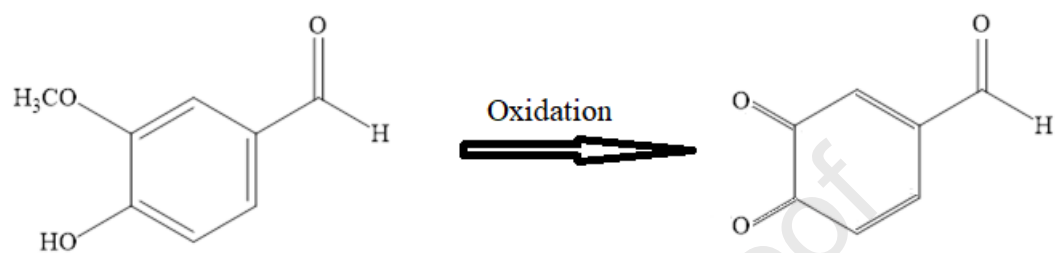
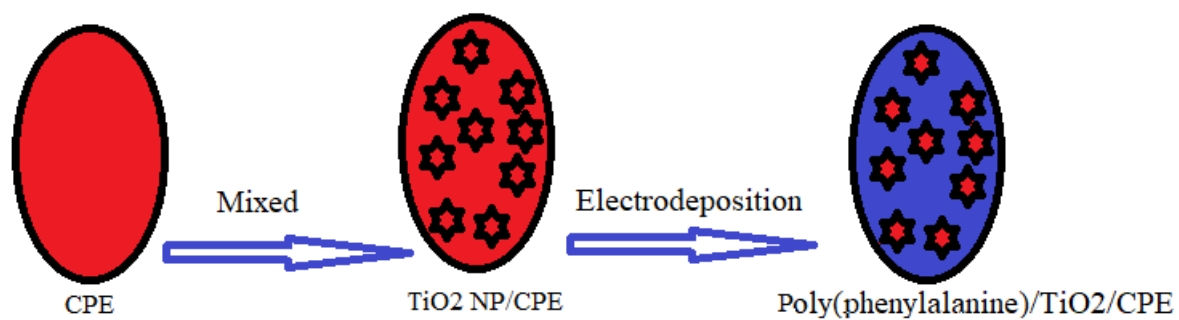
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Authors statement

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Graphical abstract

Nano solid phase micro membrane tip and electrochemical methods for vanillin analysis in chocolate samples

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ABSTRACT

A polymer-based nanosensor and electrochemical methods were developed for the quantitative analysis of vanillin. The sample preparation was done using nano solid phase micro membrane tip extraction (NSPMMTE). A novel poly(phenylalanine)/TiO₂/CPE sensor was built as the working electrode for the first time for the analysis of the vanillin substance. The electrochemical behavior and analytical performance of vanillin were examined in detail by cyclic voltammetry (CV) and differential pulse stripping voltammetry (DPSV) techniques via the oxidation process. The optimized modules of the DPSV technique that affected the vanillin peak current and peak potential were pH, pulse amplitude, step potential, and deposition time. The electroactive surface areas of bare CPE, TiO₂/CPE, and poly(phenylalanine)/TiO₂/CPE electrodes were found to be 0.135 cm², 0.155 cm², and 0.221 cm², respectively. The limit of detection (LOD) was 32.6 µg/L in the 0.25-15.0 mg/L working range at pH 7.0. The selectivity of the proposed DPSV method for the determination of vanillin on the modified electrode was investigated in the presence of various organic and inorganic substances, and the determination of vanillin with high recovery was achieved with less than 5% relative error. The analytical application was applied in chocolate samples and the DPSV method was found highly efficient, reproducible, and selective.

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Keywords: Vanillin determination, Electrochemical determination, Polymer-based nanosensor, Comparison of the results, Real sample analysis.

1. Introduction

Vanillin is one of the most important molecules being used widely used in most food products, such as biscuits, cakes, pudding, chocolate, ice cream, and, wine, because of its unique taste, and good antioxidant and antimicrobial properties [1-3]. It is a unique natural flavoring substance from the phenolic aldehyde compound class and obtained from the vanilla bean or pod [1,2,4,5]. Vanillin is widely used in most food products, such as biscuits, cakes, pudding, chocolate, ice cream, and, wine, because of its unique taste, and good antioxidant and antimicrobial properties [1-3]. Moreover, it is preferred as a fragrance additive in perfumes, cosmetics, and even drugs [2,4]. However, vanillin, which is an attractive substance to consumers, also has toxic effects at high concentrations (over 75 mg/body weight) [6]. In addition, excessive daily consumption can cause important health problems such as headaches, nausea, and vomiting by affecting the main metabolism of the liver and kidney [2,5,7,8]. Moreover, vanillin is not used in baby foods because of its side effects [9]. The acceptable daily intake (ADI) of sweeteners like vanillin, imposed in 1967 by the FAO/WHO Joint Expert Committee on Food Additives (JECFA), is currently maintained at 0-10 mg/kg body weight [5,10]. Therefore, determining vanillin, which is an important food additive for human health, in food, pharmaceutical, and cosmetic products is extremely necessary and routinely required.

The IUPAC name of this molecule is 4-hydroxy-3-methoxy benzaldehyde ($C_8H_8O_3$). Many analytical methods have been developed so far for the qualitative or quantitative determination of vanillin. These methods are capillary electrophoresis [11,12], chemiluminometry [13], optical fluorescence [14,15], and chromatography [16-18]. Although most of these methods are highly sensitive, selective, and have high accuracy and precision yet also have some weak points such as long analysis time, pre-processing, consuming too much solvent, need for expensive equipment, and the device requiring expertise. Therefore, alternative analytical methods are needed for the determination of vanillin in natural samples.

Electrochemical methods are good candidates as an alternative to quantitative determination techniques for numerous compounds due to their excellent electrode performances, superior selectivity, high accuracy, and short analysis time. Therefore, the development of electrochemical sensors is of great importance in rapidly detecting significant foodstuffs with vanillin. In this respect, some indicator electrodes such as glassy carbon (GCE), carbon paste (CPE), gold (AuE), boron-doped diamond electrode (BDDE) [19], platinum electrode [5], and pencil graphite electrode (PGE) [20] were used in the vanillin detection. In addition to these electrodes, various nanomaterials-modified composite sensors such as Au electrodeposited on fluorine-doped reduced graphene oxide modified glassy carbon electrode (Au/F-rGO/GCE) [1], carbon paste electrode - fullerene functionalized multi-walled carbon nanotubes (*f*-MWCNTs-FNTs/CPE) [6], multi-walled carbon nanotubes modified glassy carbon electrode (MWNCT/GCE) [21], NiFe₂O₄/rGO/ionic liquid (IL)/CPE [22], graphene - Au nanoparticles - glassy carbon electrode (AuNPs/GR/GCE) [23], polyaniline, molybdenum disulfide, and graphitic carbon nitride modified glassy carbon electrode (MoS₂/PANI@g-C₃N₄/GCE) [24], active poly (titan yellow) and octoxynol-9 modified carbon nanotube paste electrode (PTOMCNPE) [25], poly(glutamine) film-modified carbon nanotube paste electrode (PGAMCNTPE) [26], and poly(1H-1,2,4-triazole-3-thiol) (poly(T3T))-the gold electrode (Au) [27] have been developed for the investigation of vanillin via its oxidation. However, there is still a need for next-generation sensors that can be compared to traditional analytical methods because of the importance of nanny pharmaceuticals to be used in varied applications [28-32]. In particular, developing more selective and inexpensive sensors with lower detection limits that can respond even in a complex matrix environment is necessary. In addition, the preparation of the sensor to be developed for analyte determinations should be easy and simple.

The widespread use of electrochemical methods and new horizons in sensor development allows for qualitative and quantitative analysis of many substances [33-35]. Numerous nanomaterials have been used in the development of sensors in recent years in the electrochemistry application [36,37]. Furthermore, studies with electrochemical sensors in the determination of drugs, food additives, trace elements, and countless substances have been increasing day by day. Numerous new-generation sensor probes have been developed and various analytes detected in real samples such as blood, serum, environmental, and food samples [38-40]. Among these new-generation sensors, nanoparticles, and polymers are among the first to come to mind. The main reasons for this are nanoparticles, their unique conductivity properties, low toxicity, excellent stability, outstanding catalytic behavior, and low cost [22, 41,42]. Polymers are one of the important materials in sensor construction due to their superior properties [43]. Moreover, these are indispensable materials in the production of portable sensing devices in very small sizes. In addition, they are used in the determination of numerous analytes due to their properties such as very large surface area, conductivity, flexibility, and excellent selectivity [43]. Consequently, electrochemical techniques have proven to be the best candidates for the determination of analytes in foods in recent years by using ultrasensitive sensors [44-46].

This article aims to develop a novel generation nanosensor with a uniquely superior polymer and nanoparticle composite with excellent catalytic effect and to investigate its effective role in the determination of vanillin in real samples. A new sensor included a carbon paste electrode (CPE) treated with TiO₂ NPs and poly(phenylalanine) constructed in two independent steps. Experimental optimization and analytical parameters of the proposed electrochemical method as differential pulse stripping voltammetry (DPSV) for vanillin oxidation were discussed in detail. Demonstration of the validity of the method, determination of its selectivity, and analysis of an unknown vanillin concentration in commercial food

products were performed with the newly constructed poly(phenylalanine)/TiO₂/CPE. Thus, the novelty of this study is a new sensitive, selective, and fast sensor for the determination of vanillin, which is an important food for human health. In addition, we would like to emphasize that the constructed poly(phenylalanine)/TiO₂/CPE composite has advantages over existing studies, thanks to the large surface area of the sensor and low detection limits.

2. Materials and methods

2.1. Apparatus and reagents

Electrochemical measurements were carried out by using an analyzer called the Ivium Vartex model. Its device includes a software-supported potentiostat/galvanostat with a three-electrode system with an electrochemical cell connection. This cell is constructed from a working electrode (BASi MF-2010), a reference electrode Ag/AgCl (BASi, MF-2052), and a counter electrode Pt wire (BASi, MW-1032). The pH value of all the solutions used in the experimental studies was adjusted with a portable ISOLAB-branded pH meter. An ultrasonic bath was used to homogenize the prepared solutions.

The vanillin was purchased from Sigma-Aldrich, while methanol, HCl, boric acid, acetic acid, and phosphoric acid were purchased from Merck, Germany, at chromatographic purity. Vanillin was prepared daily as 500 mg/L by weighing 0.0050 g with analytical precision and dissolving in 10 mL of methanol. While 0.1 M HCl was preferred for pH 1.0 as the supporting electrolyte, the 0.04 M Britton Robinson (BR) buffer solution was used for pH 2.0 to pH 12.0. The BR buffer solution contains boric acid (99.8%), acetic acid (Glacial, ReagentPlus[®], ≥ 99%), and phosphoric acid (85%). Furthermore, the supporting electrolyte was prepared precisely at the desired pH values by using 2.0 M NaOH or 2.0 M HCl solutions. Distilled water was used in the preparation and cleaning of all solutions. Stock solutions were stored in a dark refrigerator when not in use.

2.2. Sample preparation

To avoid the interference of uric acid and phenol in the electrochemical detection of vanillin, the sample preparation was carried out. The sample preparation was done by using nano solid phase micro membrane tip extraction (NSPMMTE) as described somewhere else [47, Figure S1]. The nanomaterial used was N-methyl butyl imidazolium bromide ionic liquid copper oxide nanocomposite as described somewhere else [48]. The solutions of vanillin, uric acid, and phenol of 25.0 mg /L were prepared in Britton Robinson buffer of pH 7.5. To prepare the extraction procedure, 5 mg of nano-composite adsorbent was carefully added to the cone-shaped membrane. The open edge of the membrane was then heat-sealed to prevent any loss of the adsorbent material. Next, each tip was thoroughly cleaned in acetone for 10 minutes. Finally, the cone-shaped membrane was inserted into the end of the tip to complete the setup.

The solid-phase membrane tip was placed in a 15 mL solution of vanillin, uric acid, and phenol in a Britton Robinson buffer of pH 7.5. The solution was stirred on a magnetic stirrer for 30 minutes. A dynamic extraction procedure was carried out for 30 minutes from the same solution. A 200 μ L of the sample was withdrawn into the micro tip using a micropipette at an interval of 15 seconds. The withdrawn sample was released from the micro tip back into the sample. This procedure was repeated for 30 minutes and the micropipette was detached from the micro tip. After the extraction, the cone-shaped membrane was removed. The cone was dried in air and the adsorbed vanillin, uric acid, and phenol were extracted with 20 mL methanol containing 0.1% acetic acid. 20.0 mL methanol was concentrated to 1.0 mL under vacuum. The presence of vanillin, uric acid, and phenol was ascertained by HPLC.

2.3. General lines for the production of working electrodes

For the manufacture of bare carbon paste electrode (CPE), 30% by mass of mineral oil was accurately weighed and 70% by mass of graphite powder from Sigma-Aldrich was added. This mixture was press-treated for approximately 2 hours to be completely homogeneous. The

prepared CP paste was firmly placed on the BASi MF-2010 hollow Teflon electrode, which was thoroughly cleaned, and dried with water, methanol, and acetone with the help of a plastic syringe needle. Finally, CPE was polished with zero belt sandpaper to obtain a smooth electrode surface.

For the preparation of TiO₂ nanoparticle modified electrode (TiO₂/CPE), a mixture containing 30% mineral oil, 65% graphite powder and 5% TiO₂ nanoparticles was prepared. As with the bare electrode, the prepared mixture of TiO₂/CPE was placed in the cavity of the BASi MF-2010, and then polishing was applied to the electrode surface like the bare electrode. For poly(phenylalanine)/TiO₂/CPE, in the second step, 0.04 M 10 mL of the prepared phenylalanine solution was transferred into the electrochemical cell and three electrodes were immersed. The poly(phenylalanine) was coated on TiO₂/CPE by electrodepositing 50 cycles with cyclic voltammetry (CV). As a final process, the produced poly(phenylalanine)/TiO₂/CPE was washed with distilled water and the composite electrode surface was activated with CV before measurement.

2.4. Preparation of food samples

For the analytical application of DPSV on poly(phenylalanine)/TiO₂/CPE for the vanillin analysis in natural samples such as chocolate, and vanillin powder were purchased from the market. The supplied chocolate samples were weighed precisely at 5 g. Then, after transferring and dissolving in a 100 mL flask, it was filled with methanol so that the final volume was 100 mL. For the vanillin powder, a 1 g sample was weighed and dissolved in 10 mL methanol. Prepared real samples were mixed in an ultrasonic water bath for 10 minutes at room temperature. Thus, vanillin in natural samples was extracted as described in the sample preparation method and ready for direct measurement.

3. Results

3.1 Sample preparation

The analysis of the extracted samples of vanillin, uric acid, and phenol was carried out by using HPLC under the condition of a C28 reversed-phase column with water and methanol (35:65) as mobile phase at 220 nm detection. Only one peak at 2.0 retention time was observed. This peak was confirmed by running the standards of vanillin, uric acid, and phenol and it was found that the observed peak was of vanillin. It means that the reported extraction method was capable to extract vanillin only leaving behind phenol and uric acid. The observed ionization of N-methyl butyl imidazolium bromide can be attributed to its pK_a value, which falls within the range of 7.0-7.4. This indicates that at a pH of 7.0-7.4, N-methyl butyl imidazolium bromide undergoes ionization, resulting in the formation of a cationic N-methyl butyl imidazolium ionic liquid. On the other hand, the pK_a value of vanillin is 7.4 and available in the form of anion and, consequently, vanillin interacted with N-methyl butyl imidazolium bromide cation via electrostatic force of extraction. On the shaking of the used adsorbent, the sorbed vanillin was extracted in the methanol. On the other hand uric and (pKa = 5.6) and phenol (pKa = 10.0) did not exist as anions at pH 7.5 of Robinson buffer and, consequently, did not interact with the cation of N-methyl butyl imidazolium ionic liquid; leading no extraction. In this way, the reported extraction method is selective for vanillin in a mixture of vanillin, uric acid, and phenol.

3.2. Comparison of working electrodes

The bare carbon paste (CPE), modified TiO₂/CPE, and composite poly(phenylalanine)/TiO₂/CPE as working electrodes were compared and the results of differential pulse voltammograms are shown in Figure 1. Firstly, the measurement was taken with a bare electrode in pH 5.0 Britton Robinson buffer solutions and the anodic peak signal

was measured as 1.96 μA for the 5 mg/L vanillin on the CPE. Then, using the CPE electrochemical sensor modified with TiO_2 , which showed unique catalytic properties, the oxidation current was 2.68 μA at the same concentration as the vanillin. Also, using the composited poly(phenylalanine)/ TiO_2 /CPE indicator electrode; which was developed thanks to the polymers' catalytic and conductivity; the anodic signal of 5 mg/L vanillin was measured at an intensity of 3.20 μA . Compared to bare CPE, the polymer-based nanosensor increased the vanillin peak intensity by 63.46% and increased it by 19.32% against modified TiO_2 /CPE. It caused an increase in vanillin peak signal; especially by acting synergistically with poly(phenylalanine) modified TiO_2 /CPE. In conclusion, a highly sensitive poly(phenylalanine)/ TiO_2 /CPE working electrode was developed for the first time for the electrochemical determination of vanillin.

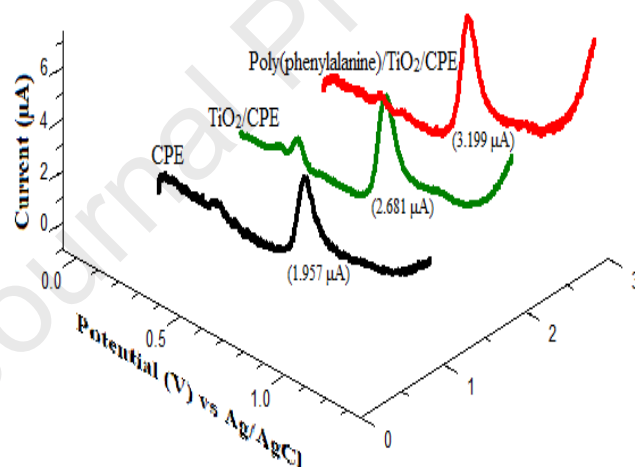


Fig. 1. Differential pulse voltammograms obtained from different electrodes (Bare CPE, Modified TiO_2 /CPE and Composited poly(phenylalanine)/ TiO_2 /CPE) for the determination of 5 mg/L vanillin.

3.3. pH effects

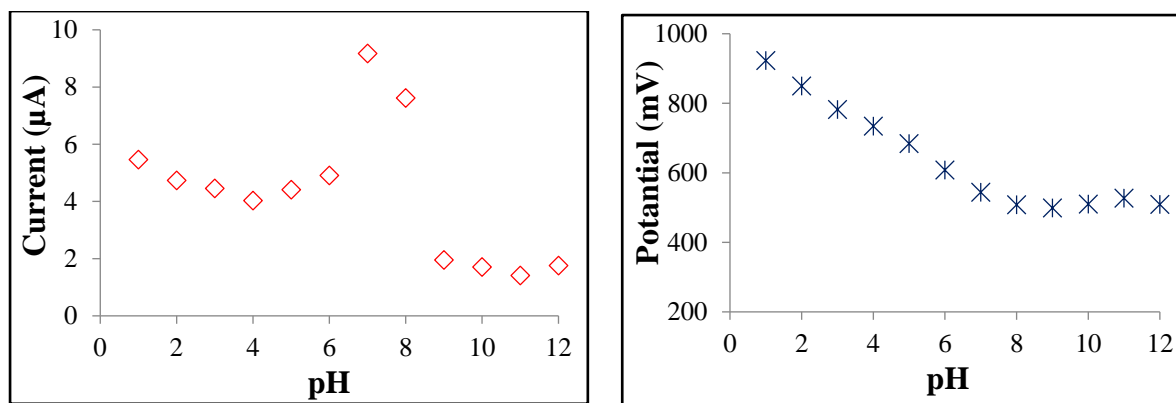
The supporting electrolyte is one of the most important parameters that have an extremely important effect on the analytical peak current and the peak potential. For this reason, DP voltammograms were obtained in a wide pH range between pH 1.0 and pH 12.0 to

investigate the effect of the supporting electrolyte on the composited poly(phenylalanine)/TiO₂/CPE. For this, 0.1 M HCl solution was used as a pH 1.0 support electrolyte. Britton Robinson (BR) buffer containing mix buffer solutions (0.04 M boric acid, phosphoric acid, and acetic acid) was preferred between pH 2.0 and pH 12.0. While the peak current value of vanillin was high in a strong acid medium, it was low in a strongly alkaline medium. The highest peak current was obtained in the neutral regions. In particular, pH 7.0 was chosen as the optimum supporting electrolyte, since the highest peak current and the well-defined anodic peak were obtained by DPV. All subsequent studies were carried out at this pH value.

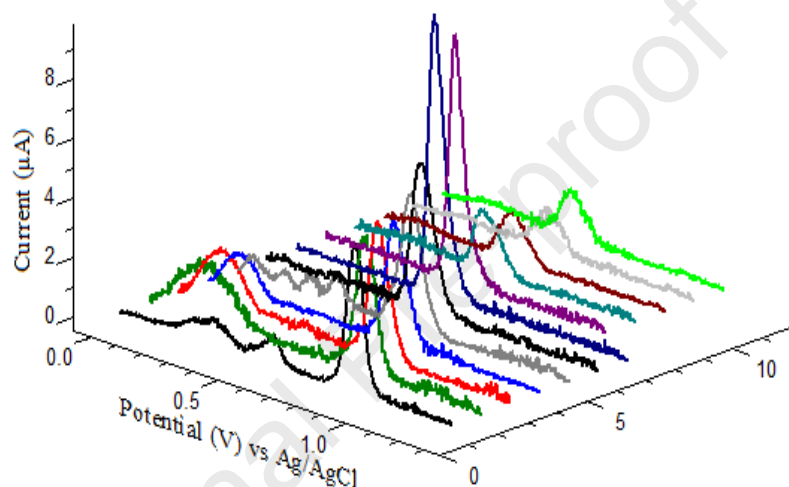
In addition, the vanillin anodic peak shifted less positive regions due to increasing pH. This proves that the hydronium ion (H⁺) as a proton is involved in the vanillin oxidation reaction on the composited poly(phenylalanine)/TiO₂/CPE. While net shifts were observed in the vanillin peak current up to pH 8.0, little changes occurred between pH 8.0 and pH 12.0. In addition, two different linear lines, pH 1.0 – pH 7.0 and pH 8.0 – pH 12.0, were obtained depending on the pH by using composited poly(phenylalanine)/TiO₂/CPE. The point where these two lines meet was approximately 7.5 and this value is very close to the true pK_a (7.4) of vanillin. The results are given in Figures 2a and b.

$$E_p \text{ (mV)} = -60.06 \text{ pH} + 910.5; r = 0.9975 \text{ (First slope equation (pH 1.0 – pH 7.0))}$$

$$E_p \text{ (mV)} = +25.16 \text{ pH} + 506.5; r = 0.6847 \text{ (Second slope equation (pH 8.0 – pH 12.0))}$$



(a)



(b)

Fig. 2. (a): Effects of pH (1.0-12.0) of vanillin determination by DPV on composited poly(phenylalanine)/TiO₂/CPE and (b): differential pulse voltammograms of vanillin determination at different pHs (1.0-12.0).

3.4. Electrochemical behavior of Vanillin

Cyclic voltammetry is one of the advanced analysis techniques that provide important information about both qualitative and quantitative aspects of an electroactive analyte. In particular, it provides detailed data on the electrochemical behavior of matter to researchers. To investigate the electrochemical behavior of vanillin, cyclic voltammograms at different scanning rates between 5.0 mV/s and 1000 mV/s were recorded on the poly(phenylalanine)/TiO₂/CPE (Figure 3). A cyclic was carried out in the anodic scanning direction from 0 to +1000 mV and in the cathodic direction from +1000 to 0 mV. Depending

on the scan rate, the oxidation peak was obtained at nearly +570 mV in the cyclic voltammogram in the anodic direction, while no signal was observed in the cathodic direction. This indicates that the vanillin oxidation electrode reaction is irreversible. In addition, vanillin anodic peak potentials shifted linearly to positive regions with increasing scanning speed. This showed that the electro-oxidation reaction vanillin took electrons (e^-).

In addition, the cyclic voltammetric method provides kinetic models as well as lethal information about substance transport. The slope value of $\log v$ (V/s) and $\log I_p$ (μA) obtained graph based on cyclic voltammograms at different scan rates between 50 mV/s and 1000 mV/s was found to be 0.773 on the poly(phenylalanine)/TiO₂/CPE (Figure 3). In substance transport processes, there is a general perception that this slope value was considered diffusion-controlled when it was 0.5 and adsorption-controlled when it was 1.0 [49]. Since the 0.773 calculated value for vanillin was between these two values, the substance transport process was controlled by both adsorption and diffusion that was mixed as adsorption/diffusion on the poly(phenylalanine)/TiO₂/CPE.

$$E_p \text{ (V)} = -0.051 \log v \text{ (Vs}^{-1}\text{)} + 0.625; r = 0.9836$$

$$\log I_p \text{ (}\mu\text{A)} = 0.773 \log v \text{ (V s}^{-1}\text{)} + 1.510; r = 0.9940$$

$$I_p \text{ (}\mu\text{A)} = 30.28 v^{1/2} \text{ (V s}^{-1}\text{)} - 3.231; r = 0.9935$$

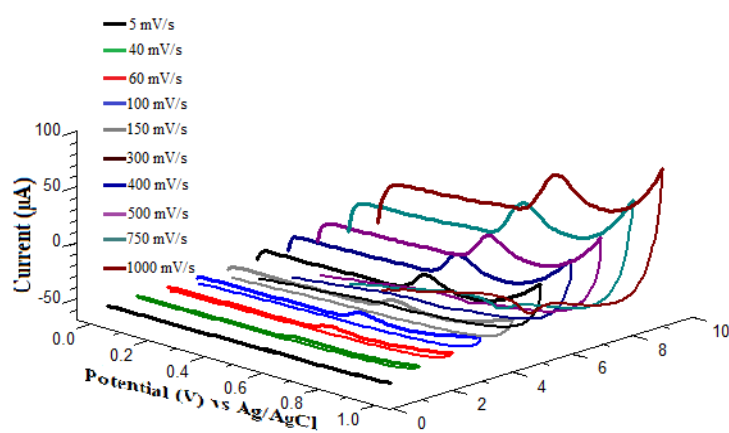


Fig. 3. Cyclic voltammograms for the 5 mg/L vanillin at different scan rates from 5 mV/s to 1000 mV/s on the poly(phenylalanine)/TiO₂/CPE.

Randles-Sevcik equation ($I_p = 2.69 \times 10^5 n^{3/2} A C D^{1/2} \nu^{1/2}$) [50] was preferred to calculate the active surface areas of the developed electrodes (bare CPE, modified TiO₂/CPE, and composited poly(phenylalanine)/TiO₂/CPE) for vanillin determination. In this equation, n , I_p , A , C , D , and ν icons the number of electric transfer, the reduction peak current (A), the electroactive surface area (cm²), the concentration of ferrocyanide solutions as 1.0 mM, the diffusion coefficient of K₃[Fe(CN)₆] in solution (7.6×10^{-6} cm²/s), and the scan rate (100 mV/s), respectively. The electroactive surface areas of bare CPE, TiO₂/CPE, and poly(phenylalanine)/TiO₂/CPE electrodes were found to be 0.135 cm², 0.155 cm², and 0.221 cm², respectively. This shows that the modified poly(phenylalanine)/TiO₂/CPE has a larger surface area than other electrodes.

4. Discussion

4.1. Optimum module of DPSV

Validation studies have gained great importance in an analytical method developed for the first time. Certainly, analytical studies such as precision, accuracy, and selectivity are required to show the validity of the method. Parameters such as pulse amplitude, scanning rate, and pulse time in the proposed differential pulse stripping voltammetry (DPSV) electroanalytical method for the determination of 5 mg/L vanillin were optimized one by one on the composited poly(phenylalanine)/TiO₂/CPE at pH 7.0 BR buffer solutions.

To examine the optimum pulse time, DP voltammograms were obtained at different pulse times between 1 and 10 ms on the poly(phenylalanine)/TiO₂/CPE in the pH 7.0 BR buffer solutions (Figure 4a). While significant increases were observed in the vanillin anodic peak up to 5 ms. This increase continued unevenly between 5 ms and 9 ms pulse times. The highest current intensity of the vanillin oxidation peak was obtained at 9 ms (Figure 4a). However, a serious decrease in the vanillin anodic signal was observed at the 10 ms pulse. From this, the 9

ms was chosen as the optimum pulse time, which obtained the highest signal and well-defined peak of vanillin.

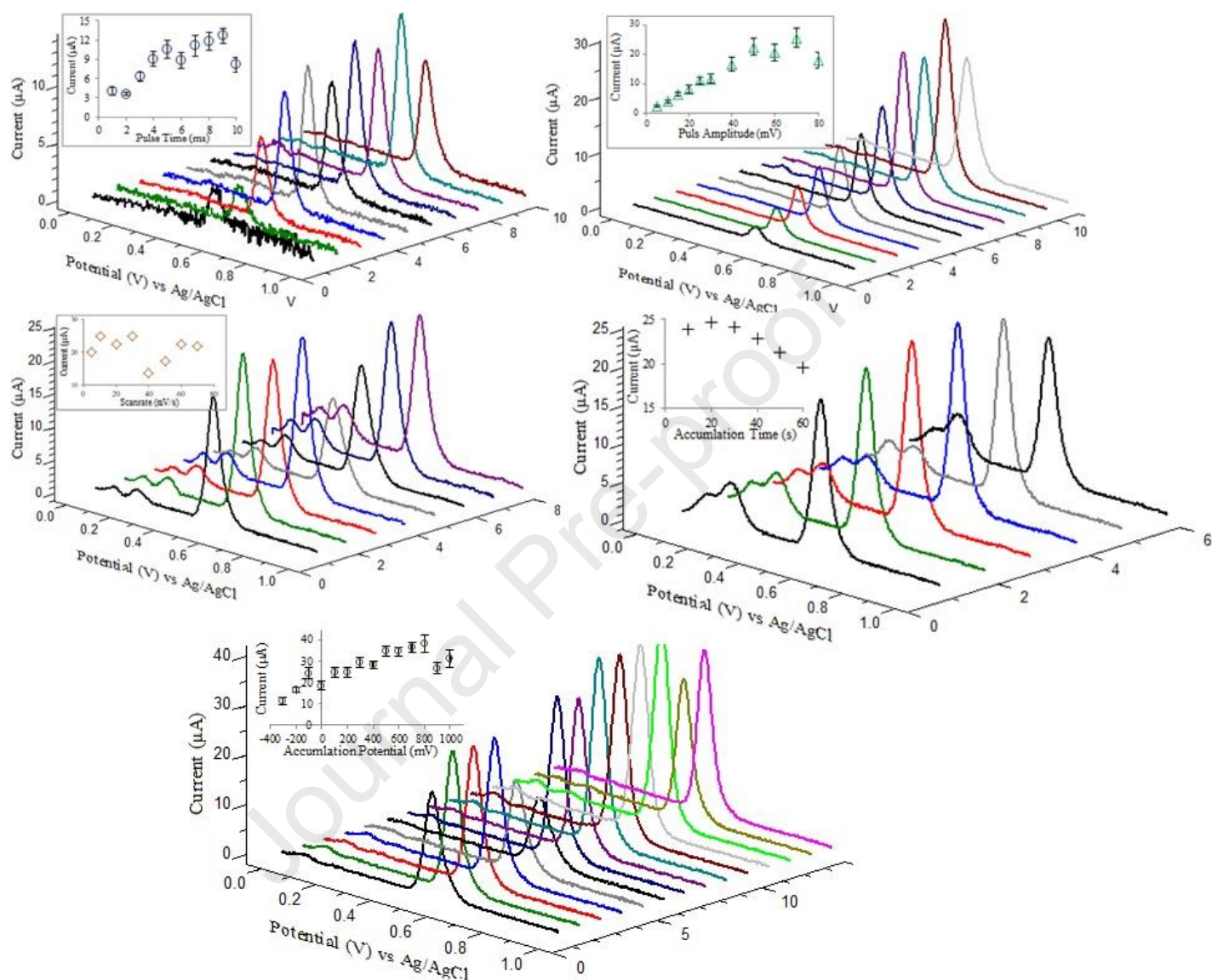


Fig. 4. Differential pulse voltammograms at (a): different pulse times, (b): different pulse amplitude, (c): different scan rates, (d) different accumulation times ranges and (e): different accumulation potential on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions.

Another important parameter affecting the analytical peak current is the pulse amplitude. Different pulse amplitudes between 5.0 mV and 80 mV were applied to determine the effect of pulse amplitude on the anodic signal of vanillin by differential pulse stripping voltammetry (DPSV). Linear increases in the oxidation peak of the analyte were observed up to the pulse amplitude of 50 mV on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer

solutions, as seen in Figure 4b. In pulse amplitudes after 50 mV, these increases continued unevenly. At pulse amplitudes after 70, severe reductions in the peak signal of the analyte were seen. As a result, 70 mV pulse amplitude; where the highest peak current of the analyte was obtained; was chosen as the optimum value at pulse amplitudes between 5 and 80 mV on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions by DPSV.

Scan rate is one of the parameters affecting the analytical peak of the vanillin substance. To examine this effect, DP voltammograms were measured at scan rates ranging from 5 to 70 mV/s on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions. When the obtained voltammograms were examined, irregular currents were observed in the anodic signal of vanillin. Depending on the scanning speed, 30mV/s was chosen as the optimum scanning speed, with the highest peak intensity and the smoothest peak shape. This value was used as the optimum scan rate in DPSV detection. The results are shown in Figure 4c.

In stripping voltammetric techniques, accumulation potential is one of the most influential factors on the peak current of the analyte. To investigate the effect of accumulation potential on the oxidation peak of vanillin, accumulation at different potentials between -300 mV and +1000 mV was applied. Depending on the applied accumulation potential, the anodic signals of vanillin were recorded. As seen in Figure 4d, the peak current of vanillin at negative deposition potentials was approximately 20 μ A, while this value was around 35 μ A in positive regions. In addition, regular increases were observed between 0 mV and +800 mV accumulation potentials, while a decrease was observed in the vanillin peak signal at later potentials. In the electrochemical determination of vanillin with DPSV; the +800 mV, where the highest current was obtained; was chosen as the optimum accumulation potential on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions.

In the electrochemical determination of vanillin, the change in the anodic signal between 0 sec and 10 s was examined for comparison with and without accumulation. It was

observed that the peak increased by 20% with the accumulation time. After that, the effect of accumulation time on finding the highest current for the oxidation peak of vanillin was investigated in detail. For the accumulation time; which is an important parameter in stripping techniques; DPSV voltammograms were obtained by an accumulation between 10 s and 60 s. When these voltammograms were examined, an increase was observed in the anodic peak of vanillin for about 20 s. After 30 seconds, decreases were observed in the vanillin anodic signal. This is an indication that the electrode surface is completely covered with the analyte between approximately 20–30 seconds. As a result, 20 s, in which the highest current intensity of vanillin electrochemical determination was obtained, was accepted as the optimum accumulation time value. The results are shown in Figure 4e.

4.2. Analytical validation

Validation includes large-scale studies such as working range, detection limits, and selective and reproducibility to determine the advantages, provided that they bring innovation to the newly developed or existing methods. In this study, vanillin determination was carried out using a polymer-based nanosensor (poly(phenylalanine)/TiO₂/CPE) in pH 7.0 BR buffer solutions by DPSV, and validation studies were carried out in a detailed manner.

4.2.1. Calibration analysis

The poly(phenylalanine)/TiO₂/CPE was calibrated with standard vanillin with DPSV in pH 7.0 BR buffer solution. The potential Ag/AgCl *versus* 0.0 and 1.0 V was applied under optimum conditions and a concentration current plot was plotted to construct a calibration curve by the standard addition method concerning the anodic signal at about 0.6 V (Figure 5). As can be seen from the obtained calibration graph (Figure 5), vanillin determination was carried out in a wide linear working range between 0.25 mg/L and 15.0 mg/L on the poly(phenylalanine)/TiO₂/CPE.

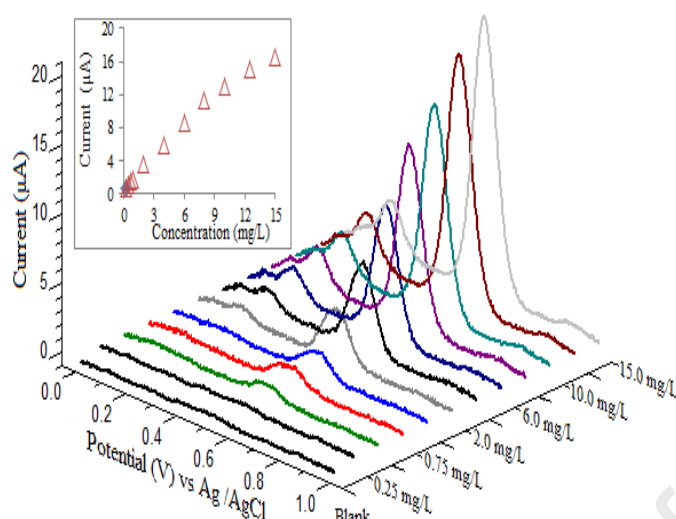


Fig. 5. The DPS voltammograms and calibration plot in the determination of vanillin on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions.

For the determination of the limit of detection (LOD) and limit of quantification (LOQ), which are other important validation parameters, “3s/m” and “10s/m” formulae were applied, respectively [51,52]. While “s” in these formulas is the standard deviation of the response obtained by DPSV at the lowest detectable vanillin concentration, “m” represents the slope of the calibration graph. By substituting all the obtained data in these formulas, LOD and LOQ values were calculated as 32.57 and 108.57 µg/L, respectively (Table S1). Moreover, an intraday reproducibility study was performed to investigate the precision of the constructed modified composite electrode for vanillin determination. For this purpose, the relative standard deviation (% RSD) value was calculated according to the peak signal and potential of vanillin by collecting seven measurements repeated during the day in the presence of 0.5 mg/L with the poly(phenylalanine)/TiO₂/CPE. The % RSD value was calculated as 2.47 and 0.66, respectively by DPSV in the pH 7.0 BR buffer solutions, and these values are at an acceptable level. In conclusion, the %RSD value obtained with the proposed method and composite electrode proved that the precision validation parameter was highly predictive for the analysis of vanillin by DPSV. The variation in vanillin peak current between the newly produced electrode and the previous electrode was 1.47%. In addition, after one month of use with an

electrode, the recording of the vanillin peak signal is within 5% tolerance limits. This proves that the developed poly(phenylalanine)/TiO₂/CPE reproduction can perform a low relative error vanillin determination. Also, all validation data of the DPSV method on poly(phenylalanine)/TiO₂/CPE were given in Table S1 in detail.

4.3. Effects of interference in vanillin sensing

The most important part of the analytical performance is the selectivity of the method. Precise, reliable, and accurate analysis of the target analyte, even in complex matrix environments, demonstrates the superiority of the proposed method. Therefore, interference effects in the determination of vanillin of substances such as ascorbic acid, folic acid, gallic acid, glucose, and Cu (II), Fe (II), Zn (II), Pb (II), and Cr (III) were investigated on the poly(phenylalanine)/TiO₂/CPE by DPSV in pH 7.0 BR buffer solution. Firstly, differential pulse stripping voltammograms were obtained for the presence of 1.0 mg/L vanillin at optimum conditions. Then, the concentrations of interference species were 1:1; 1:5, and 1:10 by mass. It was added and also three repeated electrochemical measurements were taken on the poly(phenylalanine)/TiO₂/CPE by DPSV. The percent change in the vanillin anodic peak signal was calculated as percent recovery without the interfering species and in the presence of the interfering species (Table S2).

The ratio of organic molecules such as ascorbic acid, folic acid, gallic acid, uric acid, and glucose in different concentrations did not seem to have a serious interference effect in the determination of 1 mg/L vanillin. It is understood that vanillin is determined with high recovery in the presence of these co-existence agents. Moreover, the interference effects of some cations were also investigated in detail on the poly(phenylalanine)/TiO₂/CPE with DPSV in pH 7.0 BR buffer solution. It is seen that ionic species in different concentrations acted within the tolerance limit of %5 on the anodic peak signal of 1 mg/L vanillin (Table S2). Among the interference species, only uric acid was found to interfere in the determination of vanillin at a concentration

of 1:10 by mass. There are two main reasons for this: 1) Since uric acid is electroactive and peaks at about 310 mV at pH 7.0, vanillin is affected by the anodic signal at 500 mV 2) Because the electrode surface is limited and when uric acid is oxidized at less potential, the second surface of the electrode may cause an obstacle to the transport of the substance. It was concluded that the accuracy, precision, and selectivity of the proposed method were outstanding by using the new generation composite sensor developed for vanillin detection.

4.4. Analytical application

The analytical application, which is the most important component of the validation indicator, provides highly meaningful information about the accuracy and precision of a developed method. In particular, the analytical performance of the recommended method for the determination of the analyte in natural samples needs to be evaluated. Therefore, the determination of vanillin (indispensable in food samples) was carried out from commercial chocolate samples and vanillin powder. 5 g of the chocolate sample was weighed precisely and methanol was added so that the final volume was 100 mL. The samples were then mixed in an ultrasonic water bath for 10 minutes at room temperature. The same process was applied to vanillin powder. Electrochemical measurements were performed on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions by DPSV in 4 repetitions and the mean and standard deviations were calculated. For this purpose, 0.2 mL of firstly prepared real samples were added in pH 7.0 BR buffer solutions and DPS voltammograms were obtained under the optimum conditions. Then, 1.0 mg/L of standard vanillin solution was added to this solution and DPS voltammograms were recorded. Thus, the amount of vanillin in real samples was calculated quantitatively with the difference in vanillin anodic peak signals. For chocolate sample and vanillin powder samples, 4.97 ± 0.07 , and 4.90 ± 0.06 vanillin determinations were made with DPSV on the poly(phenylalanine)/TiO₂/CPE at a 95% confidence interval, respectively. The determination of vanillin was successfully performed with high recovery and

low relative standard deviations in natural food samples. The results are given in Table 1. The DPS voltammograms for the determination of vanillin in the food sample on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions are shown in Figure 6.

Table 1

Determination of vanillin in real samples.*

Vanillin	Chocolate sample I	Vanillin powder
Labbed (mg/L)	5.0	5.0
Found (mg/L \pm mg/L)	4.97 \pm 0.07	4.90 \pm 0.06
% Recovery	98.99	97.91
% BSS	2.44	3.07
% Relative Error	-1.01	-2.09

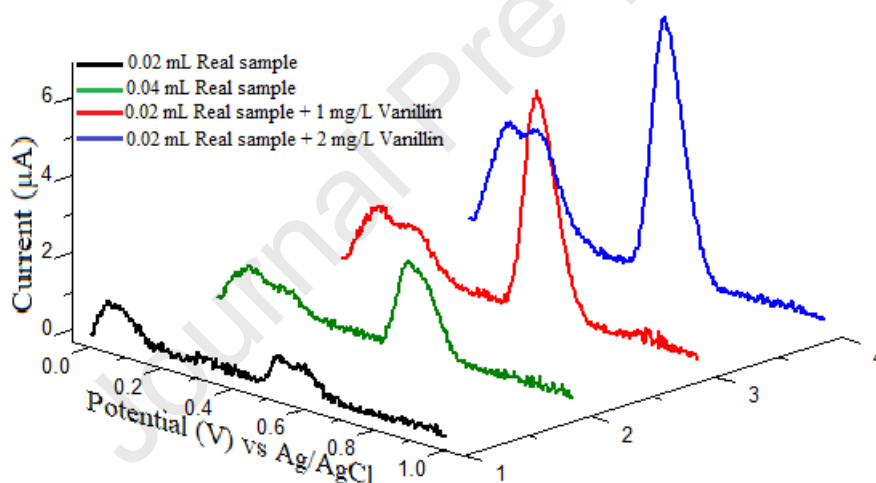
* $n = 4$ ve $t = \% 95$ 

Fig. 6. The DPS voltammograms for the determination of vanillin in the food sample on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions.

A comparison of sensitivities of various electrodes for the determination of vanillin was carried out and summarized in Table 2. It is understood that the developed poly(phenylalanine)/TiO₂/CPE with DPSV has a wide working range compared to the previous electrochemical studies in the literature (Table 2). Moreover, the limit of detection (LOD) value is highly sensitive with DPSV using a new generation sensor for vanillin detection. Being an easy, portable, reproducible, and sensitive indicator electrode in terms of preparation, it is

extremely successful in the determination of vanillin on the poly(phenylalanine)/TiO₂/CPE. Moreover, the low detection limit value obtained on a modified electrode by DPSV is at a level that can compete with both spectroscopic methods and chromatographic techniques. In contrast to spectroscopic (UV-spectroscopy [53], and chromatographic methods (RP-HPLC, titania-based HPLC) [54,55] for the vanillin determination in real samples such as chocolate and vanillin powder was carried out with high accuracy and precision with proposed electrochemical method using a constructed composite electrode, in a fast and inexpensive way, without the need for any preprocessing. In addition, another outstanding aspect of the recommended method is that vanillin determination can be made with DSPV on the poly(phenylalanine)/TiO₂/CPE even at very low sample volumes.

Table 2

Comparison of sensitivities of various electrodes for the determination of vanillin.

Electrodes	Methods	Working range (μM)	Limit of detection (LOD) (μM)	Analytical application	Ref
Au/F-rGO/GCE	DPV	1.0 – 150.0	0.15	Milk samples	[1]
BDDE	SWAdSV	3.3 – 98.6	0.16	Pudding powder samples	[2]
BDDE	SWAdSV	3.3 – 330.0	0.38	Commercial food and beverage samples	[19]
MWCNTs/GCE	DPV	4.2 – 294.1	3.44	Ice cream and cake sample	[21]
GR/AuNPs/GCE	DPV	5.0 – 120.0	1.70	Cookie samples	[23]
Gold (Au) electrode modified with 3-amino-1,2,4-triazole-5-thiol (ATT)	DPV	1.1 – 76.4	0.19	Commercial coffee sample	[56]
GR/GCE	SWAdSV	3.3 – 210.0	0.50	Commercial food and beverage samples	[57]
Anodically pretreated BDDE	SWAdSV	6.6 – 660.0	1.54	Commercial food and beverage samples	[58]
CPB/CNF/GCE	DPV	0.50 – 750.0	0.14	Vanilla sugar, vanilla pods, and cream milk powder samples	[59]
CTABM/GPE	DPV	4.0 – 70.0	1.29	Commercial food samples	[60]
Poly(phenylalanine)/TiO ₂ /CPE	DPSV	1.6 – 98.6	0.21	Chocolate and vanillin powder samples	This work

Cetylpyridium bromide (CPB), Carbon nanofibers (CNF), Cetyltrimethylammonium bromide (CTAB), Graphene paste electrode (GPE)

4.5. Oxidation mechanism of vanillin

Scanning rate study with CV, the kinetic behavior of 5 mg/L vanillin substance was investigated on the poly(phenylalanine)/TiO₂/CPE in pH 7.0 BR buffer solutions. The analyte's peak current and peak potential values at different scanning rates between 5 mV/s and 1000 mV/s with CV were measured for this. The electrochemical response of 5 mg/L vanillin was investigated with CV on poly(phenylalanine)/TiO₂/CPE and a linear graph with a slope of 0.051 was obtained between the logarithm of the different scan rates from 5 mV/s to 1000 mV/s and the anodic potential of vanillin. An application was made to find the number of electrons (n) transferred to the equation given below, which is considered the most ideal for irreversible reactions (Figure 7). When all the obtained data were placed in the Laviron equation, the "αn" value in the vanillin oxidation reaction was calculated as 0.864. In addition, when the value of 0.5 ("α"), which is accepted for irreversible electrochemical reactions, is substituted, the number "n" is found to be "1.728". The "n" was considered 2 when this value was closer to the integer 2. Thus, as in other studies, the number of electrons transferred in the vanillin electrode reaction on poly(phenylalanine)/TiO₂/CPE was found to be "2".

$$E_p = E^0 + \left(\frac{2.303RT}{nF}\right) \log\left(\frac{RTk^0}{nF}\right) + \left(\frac{2.303RT}{\alpha nF}\right) \log v$$

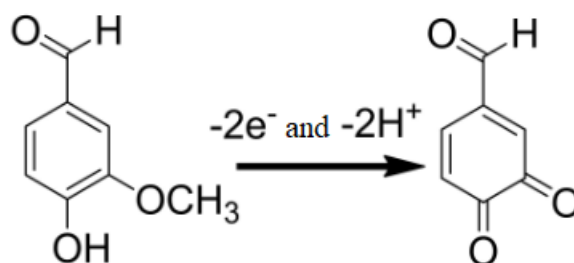


Fig. 7. Possible anodic mechanism of vanillin [61].

5. Conclusion

In this study, a polymer-based sensor (poly(phenylalanine)/TiO₂/CPE) was developed for the determination of vanillin, an indispensable component of foods. The sample preparation was done by using nano solid phase micro membrane tip extraction (NSPMMTE). The modified sensor made the anodic peak signal of vanillin approximately 63.46% more sensitive, compared to the bare CP and TiO₂/CP electrodes. Parameters such as pulse amplitude, step potential, accumulation time, and accumulation potential in the differential pulse stripping voltammetric technique were optimized one by one in detail. The electrochemical behaviors of vanillin were investigated by cyclic voltammetry (CV) on the composited electrode. The vanillin has an irreversible electrode reaction and electron transport process is mixed control, which is called adsorption/diffusion. The selectivity of the developed DPSV method and the poly(phenylalanine)/TiO₂/CPE in the determination of vanillin was achieved with high recovery in the presence of various substances. Finally, the analytical application of chocolate and vanillin powder samples was successfully applied for the accuracy and precision of the method without any preprocessing. As a result, the new generation nanosensor produced with this study has brought an innovation for the determination of an important substance such as vanillin in natural samples. Therefore, the reported methods may be used to analyze vanillin in any chocolate sample after nano solid phase micro membrane tip extraction.

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Ethical statement

Not applicable.

CRedit authorship contribution statement

Imran Ali: Conceptualization, Writing; Murat Mısıır: Methodology, Data collection; Ersin Demir: Methodology, Data collection; Writing; İrem Dinçer: Methodology, Software;

Marcello Locatelli: Writing, Review and Editing; Zeid A. ALothman: Writing, Review and Editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

All data are given in tis manuscript.

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7. Conflicts of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at xxxxxxxxxxxxxxxxxxxxxxx

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Highlights

- Nano solid phase micro membrane tip extraction of vanillin.
- Novel poly(phenylalanine)/TiO₂/CPE for selective detection of vanillin.
- Ultra-sensing DPSV for the vanillin on the poly(phenylalanine)/TiO₂/CPE.
- Poly(phenylalanine)/TiO₂/CPE sensor; a promising candidate for vanillin detection.
- New, simple, sensitive and selective sensor as a poly(phenylalanine)/TiO₂/CPE.
- Fast and economic vanillin determination methods in chocolate samples.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Title: Nano solid phase micro membrane tip and electrochemical methods for vanillin analysis in coffee samples