Electroanalysis may be used in the Vanillin Biotechnological Production

William Giraud · Marie Mirabel · Maurice Comtat

Abstract This study shows that electroanalysis may be used in vanillin biotechnological production. As a matter of fact, vanillin and some molecules implicated in the process like eugenol, ferulic acid, and vanillic acid may be oxidized on electrodes made of different materials (gold, platinum, glassy carbon). By a judicious choice of the electrochemical method and the experimental conditions the current intensity is directly proportional to the molecule concentrations in a range suitable for the biotechnological process. So, it is possible to imagine some analytical strategies to control some steps in the vanillin biotechnological production: by sampling in the batch reactor during the process, it is possible to determine out of line the concentration of vanillin, eugenol, ferulic acid, and vanillic acid with a gold rotating disk electrode, and low concentration of vanillin with addition of hydrazine at an amalgamated electrode. Two other possibilities consist in the introduction of electrodes directly in the batch during the process; the first one with a gold rotating disk electrode using linear sweep voltammetry and the second one requires three gold rotating disk electrodes held at different potentials for chronoamperometry. The last proposal is the use of ultramicroelectrodes in the case when stirring is not possible.

Keywords Vanillin · Eugenol · Gold · Amalgamated electrode · Sensor

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most common molecule used as aroma in agrifood, perfumery, and pharmaceutical industries. The current demand is about 15,000 t a year and only 1 % is extracted from natural vanilla. The price of vanilla varies from 1,200 up to 4,000 dollars per kilo while the synthesized vanillin is only 12 dollars per kilo [1, 2]. So, there are numerous works to propose different processes of synthesis. Vanillin may be synthesized by chemical methods [3] from coniferin, glucoside of coniferyl alcohol oxidized by potassium

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bichromate in acidic media, eugenol by isomerization of isoeugenol and oxidation of eugenol acetate, by oxidation with hydrogen peroxide catalyzed by vanadium pentoxide or by oxidation with nitrobenzene in dimethyl sulfoxide, from lignin present in liquors of the paper industry with catalytic oxidation under pressured air, extraction with butanol, and distillation [3–19].

Vanillin may also be obtained by cell cultures (*Vanilla planifolia* and *Capsicum frutescens*), mushrooms, bacteria, and yeasts to modify precursors such as eugenol, isoeugenol or vanillyl, ferulic acid, coniferyl and veracyl alcohols, and fermentable organisms [20–26].

Alternative sources of vanillin flavor [1] can be based on cultured vanilla plant cells or plant callus tissue, prokaryotic or eukaryotic microorganisms [27–33], or isolated enzymes used as biocatalysts [34–39].

Today, those works benefit from advances in molecular biology and from the better knowledge of metabolism of phenyl propanoids. Bioconversion of ferulic acid to vanillin has not been successful because of the degradation of vanillin into vanillic acid and vanillyl alcohol. New processes have been developed using a variety of bacteria or enzymatically by feruloyl-CoA-synthetase and enoyl-CoA-hydratase/aldolase. Cloning of the genes encoding these two enzymes into *Escherichia coli* yielded 1.1 g L^{-1} of vanillin in 48 h from 0.2 % (*w*/*v*) ferulic acid, with *Streptomyces* and an absorbent resin to capture vanillin it has been possible to convert 45 g L^{-1} of ferulic acid into 19 g L^{-1} of vanillin [40] while recombinant *Escherichia coli* was able to convert ferulic acid to 5 g L^{-1} vanillin in 24 h with a 86 % molar yield [41]. A second way is the conversion of isoeugenol. A strain of *Bacillus pumilus* converted 10 g L^{-1} isoeugenol to 3.75 g L^{-1} vanillin with a molar yield of 40 % in 150 h [42]. With *Bacillus fusiformis* the bioconversion of 50 g L^{-1} isoeugenol lead to 8 g L^{-1} vanillin [40]. *Pseudonomas putida* converted isoeugenol to 16 g L^{-1} vanillin with a molar yield of 75 % in 24 h. A recombinant strain of *E. coli* containing the isoeugenol monooxygenase gene from *P. putida* converted isoeugenol to vanillin 28 g L^{-1} in a 6-h bioconversion with a yield of 81 % [43].

Some reactions of oxidation or reduction could lower the yield of vanillin production. A major risk is the oxidation of vanillin present in the complex media [31, 36, 44, 45]. The presence of bacteria, mushrooms, or yeasts foreign to the biotechnological process can lead to the formation of undesirable products such as guaiacol [33], vanillyl alcohol [28, 30, 31], and other aldehydes [16].

The wide existing variety of biotechnological processes to produce vanillin requires some chemical tools to control the production in real time. As vanillin is systematically present in complex media, it is necessary to detect the chemical intermediates involved in the process of vanillin production to monitor them efficiently. Moreover in front of the growing demand of synthesized vanillin, a controlled quality is required in order to satisfy consumers and avoid the presence of any undesirable aroma or any consequence on health. Different analytical techniques are used to detect vanillin in order to estimate its purity and calculate the yields of production. The methods used, like high-performance liquid chromatography or gas chromatography coupled with mass spectroscopy, are generally time consuming and expensive. Electrochemistry takes some inherent advantages on classical analytical methods in terms of size, cost, and time of analysis. Under equilibrium conditions at electrode–electrolyte interface, no electrochemical reaction occurs and Nernst's law rules thermodynamic potential, an example of application is the pH measurement. When measurement is done out of equilibrium, i.e., in dynamic conditions, the faradaic current is directly related to the kinetics of the electrochemical process. The interfacial electron transfer and the mass transport to the electrode surface control the rate of the global reaction. Conventional electrochemical dynamic measurements are cyclic voltammetry, chronoamperometry, and steady-state measurement at a rotating disk electrode. Voltammetric techniques are associated with the measurement and interpretation of current potential relationships, while potential is changed linearly with time.

Cyclic voltammetry, operated in a non-agitated solution, is a powerful tool to understand electrochemical reactions and follow electron transfer kinetics. Under controlled hydrodynamic conditions, with rotating disk electrode, mass transport is increased in order to have diffusion-limiting transport and current intensity is increased too. When agitation is not possible, signal/noise ratio can also be drastically increased using square wave voltammetry or by the use of ultramicroelectrodes. Few electrochemical studies were done on the molecules involved in the bioproduction of vanillin [46–55].

The present study gives basic knowledge in electrochemistry and some electrochemical ways to control the bioproduction of vanillin. Figure 1 describes some steps involved in a vanillin biotechnological production process. The control of each step of the process requires accurate analytical methods, fast and strong. Some of the molecules mentioned before present electrochemical properties [46–48, 50, 55–61]. The aim of the work is to bring some new results on the electrochemical study of vanillin, its mechanism of oxidation, and the electrochemical behavior of eugenol, ferulic acid, vanillyl alcohol, and vanillic acid in order to propose analytical strategies to control the biotechnological process of vanillin production.

Materials and Methods

Compounds

All compounds and reagents were of analytical grade and solutions were prepared with demineralized water by an Elga cartridge type C114. Ferulic acid, vanillin, eugenol, vanillyl alcohol, and vanillic acid were purchased from Sigma-Aldrich. Absolute ethanol was supplied by VWR and used without further purification. Mono and dibasic potassium phosphate salts were supplied by Acros and potassium hydrogen tartrate KHT by Prolabo.

Preparation of the Electrolytes

Hydroalcoholic solution was a 12 % volume ethanol in water prepared by dissolving 2 g L^{-1} of potassium hydrogen tartrate and adjusting the pH at 3.7; phosphate buffer solution was prepared by dissolving mono and dibasic potassium phosphate to obtain a 0.1 mol L^{-1} concentration. The pH was adjusted with 2 mol L^{-1} potassium hydroxide and 2 mol L^{-1} nitric acid solutions.

Electrochemical Material

The potentiostat used was a μAutolab type III from Metrohm controlled with GPES 4.9 software. The saturated calomel reference electrode (noted sce) was a type 100 Radiometer Analytical and the counter electrode a platinum grid. All potentials are defined versus this reference. The working electrodes were gold, platinum, and carbon disks of respectively 2-, 2-, and 3-mm diameters. The EDI 101 Radiometer Analytical rotating disk electrode was used along with a CTV 101 Radiometer Analytical rate controller. The pH meter was a Hanna HI 98140.

Methods

Cyclic voltammetry was performed using a scan rate of 50 mV s^{-1} while current intensity is recorded between working and counter electrodes. In the case where mass transport is very

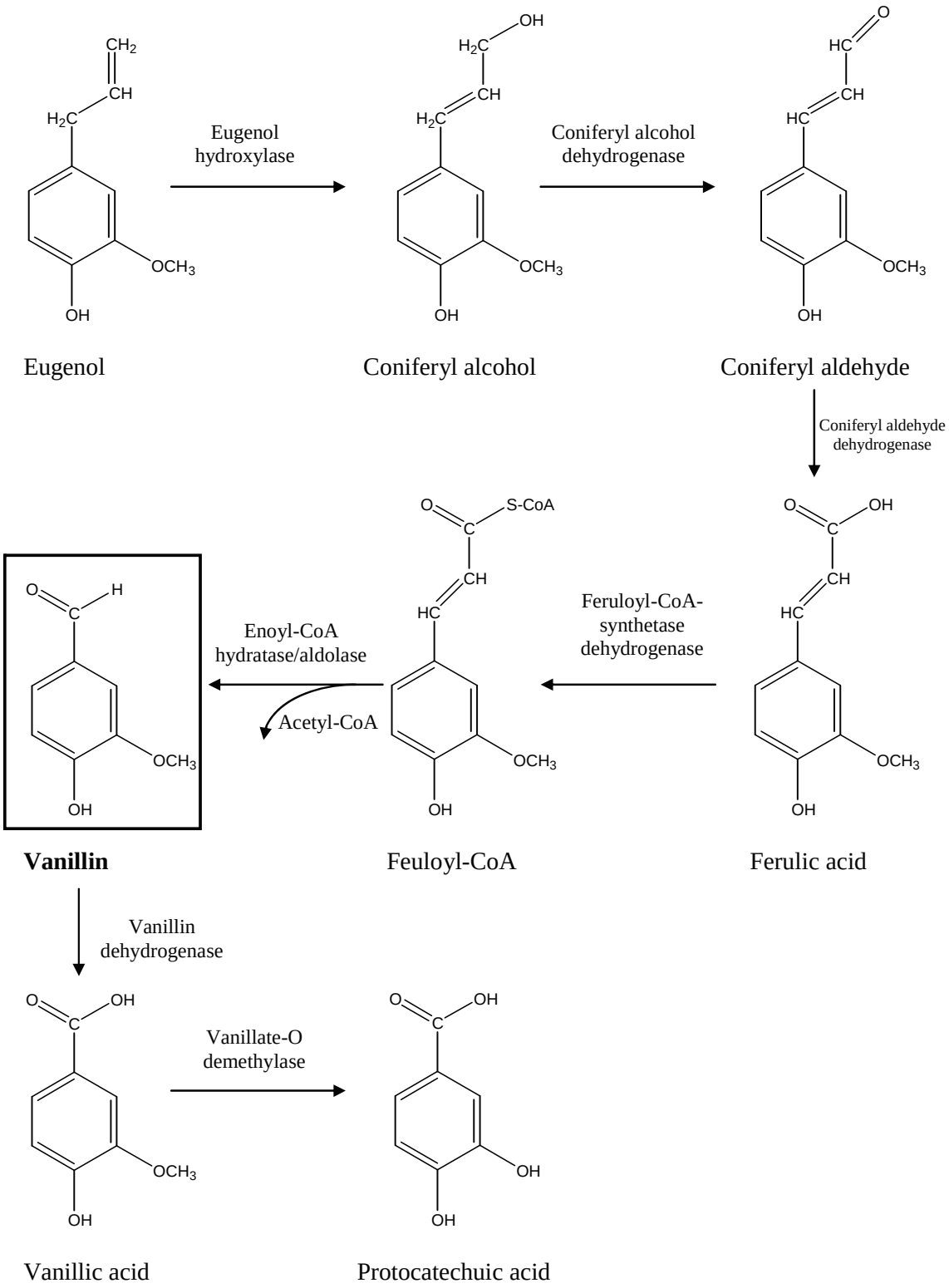

Fig. 1 Some reactions found in the biotechnological production of vanillin [3]

slow in front of the heterogeneous electron transfer rate, the current potential curve is characterized by a peak *Ip* ruled by the Randles–Sevčik's equation [62]:

$$I_p = 2.69 \times 10^5 n^{3/2} A C D^{1/2} v^{1/2}$$

for the potential difference between working and reference electrodes; with n the number of electrons, A (square centimeter) the electrode surface area, C (mole per cubic centimeter) the

concentration of the electroactive species, D (square centimeter per second) the diffusion coefficient of the species and v (volt per second) the scan rate.

The intensity–potential curves with the rotating disk electrode were obtained with a scan rate of 6 mV s^{-1} and a rotation rate for the electrode of 1,500 revolution per minute. The presence of an electroactive species is responsible for a current–potential curve characterized by the limit current intensity I_{lim} theoretically given by Levich's equation [62]:

$$I_{\lim} = 0.62nFAD^{3/2}\omega^{1/2}\nu^{-1/6}C$$

with n, A, D, and C as defined above and F (charge per mole) the Faraday constant, ω (per second) the angular frequency of rotation of the electrode, and ν (cubic centimeter per second) the kinematic viscosity of the solution. Another characteristic is the half-wave potential $E_{1/2}$ at which $I = I_{lim}/2$. The rotating disk electrode method is also useful to calculate the kinetic information βn of the electrochemical reaction from Tafel's equation [62]. The optimized characteristics for square wave voltammetry were: 50 Hz for the frequency, 1 mV for the step potential and 100 mV for the amplitude.

Results and Discussion

Vanillin Electrooxidation at the Steady State

The influence of pH was studied with three different values to study vanillin oxidation on a gold rotating disk electrode; the voltammograms are represented in Fig. 2. The $E_{1/2}$ for the oxidation of vanillin increases of 60 mV/pH means that this mechanism takes place with an exchange of as many protons as electrons. The βn calculated by the Tafel's equation on the first step of oxidation leads to the values 0.9, 1.2, and 2.2, respectively, for pH 5, 6, and 7. The corresponding diffusion coefficient, extracted from Levich's equation [62] is about 6×10^{-6} cm^2 s^{-1} which is coherent with such a molecule in aqueous media.

The oxidation of vanillin is compared on two other materials: glassy carbon and platinum. Figure 3 shows the voltammograms obtained with the rotating disk electrode at pH 5 on gold, platinum, and glassy carbon. The potential of oxidation is similar on the three materials. The limit intensity is slightly higher for gold and lower for platinum, compared to glassy carbon. It

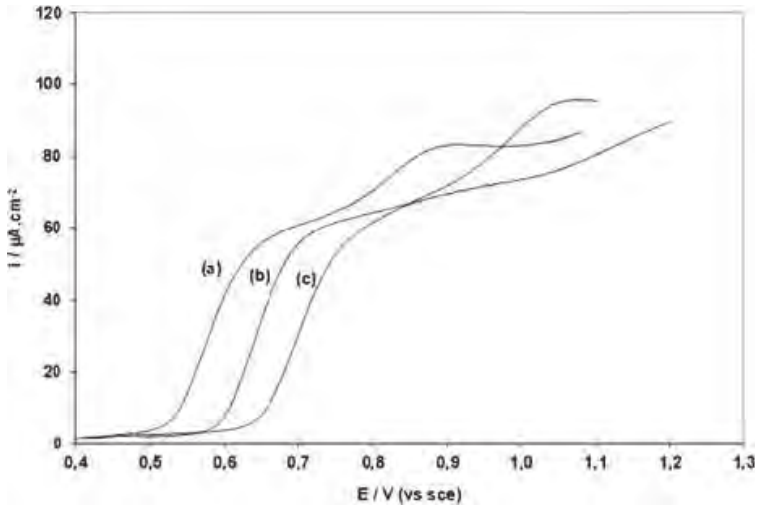

Fig. 2 Rotating disk electrode for vanillin oxidation at 5.10^{-5} mol L^{-1} on a gold electrode in a phosphate buffer solution at *a* pH=7.0; *b* pH=6.0, and *c* at pH=5.0

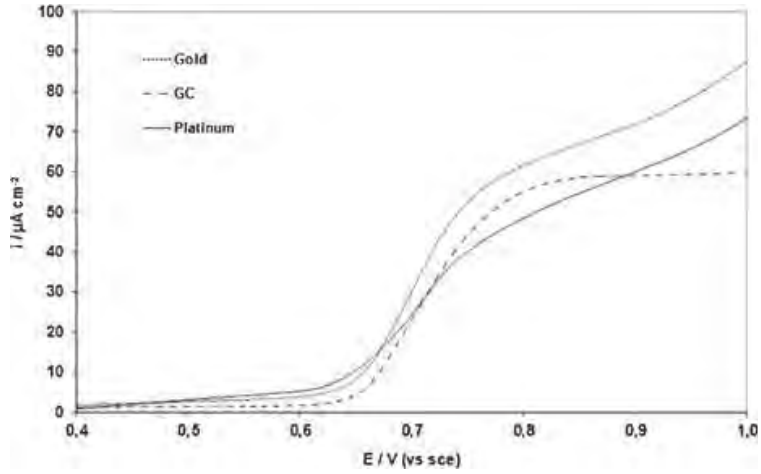

Fig. 3 Rotating disk electrode for vanillin oxidation at 5.10^{-5} mol L^{-1} on gold, GC, and platinum electrodes in a phosphate buffer solution at pH=5.0

can be noticed that an additive resistance is present on gold and platinum voltammograms, estimated at about 10 kΩ cm^{-2}. This resistive layer could be due to a polymerization film on the surface of the electrode.

Cyclic voltammetry, performed on a glassy carbon electrode, gives more information about the mechanism of vanillin oxidation. During cycle 1 of Fig. 4, vanillin is irreversibly oxidized at 0.73 V (vs sce). The presence of a reduction peak at a potential of 0.43 V (vs sce) during the second cycle shows that the byproduct of vanillin oxidation has reacted chemically. This is confirmed during the second cycle because of a significant decrease of the intensity of vanillin oxidation. Influence of scan rate was done (results not shown) and the linear correlation between the peak's intensity and squared root of the scan rate showed that the process is controlled by diffusion.

Vanillin Electroreduction

In a preliminary study, the reduction of vanillin was proposed on a rotating disk-amalgamated electrode. The nature of the material of electrode allows exploration of more negative potentials range, what is useful for vanillin as it presents a carbonyl group, which is quite

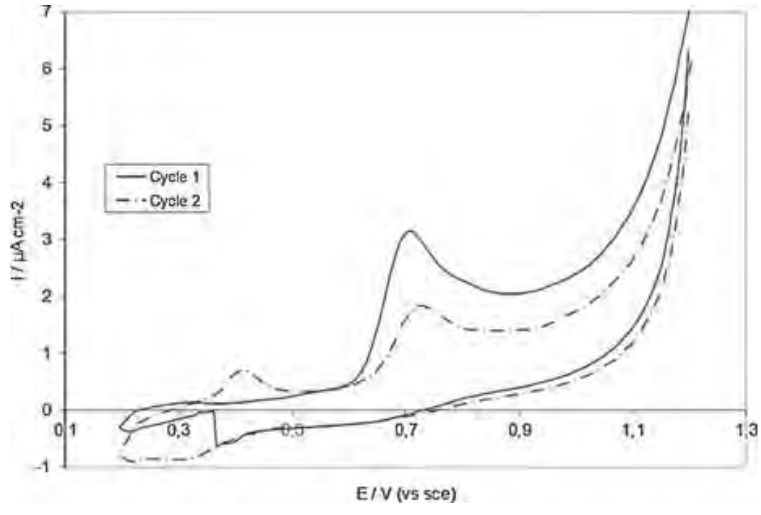

Fig. 4 Cyclic voltammetry for vanillin oxidation at 5.10^{-5} mol L^{-1} on a glassy carbon electrode in a hydroalcoholic model solution, scan rate of 50 mV s^{-1}

difficult to reduce. The voltammograms obtained are presented in full line in Fig. 5. From −1.2 V (vs sce), vanillin is reduced with a limit intensity directly proportional to the concentration of vanillin in solution from 5.0×10^{-4} up to 1.5×10^{-3} mol L^{-1}. Another way to quantify the concentration of vanillin in solution is to form a product that is then reduced on the electrode. So, hydrazine is introduced in excess into the solution and reacts on the carbonyl function to form the corresponding hydrazone:

$$R{-}CHO + H_2N{-}NH_2 = RCH = N{-}NH_2 + H_2O$$

The latter is known to be reducible at a lower potential than the carbonyl group. The voltammograms obtained are shown in Fig. 5 in dashed lines. In the presence of hydrazine, the reduction presents a potential shift of 300 mV towards lower potentials, making the reduction easier, and presents limit intensity linearly proportional to the concentration of vanillin between 5.0×10^{-4} and 3.0×10^{-3} mol L^{-1}.

In order to lower the detection limit for vanillin reduction, square wave voltammetry is used. With similar experimental conditions than for the rotating disk electrode, the voltammograms obtained are presented in Fig. 6. The signals obtained are now some peaks, the potential of which is close to the $E_{1/2}$. The intensity of the peaks is directly proportional to the concentration of vanillin from 2.5×10^{-5} up to 2.5×10^{-3} mol L^{-1}. It has been noticed that in the case where some hydrazine is introduced in excess in the middle, some new peaks appear but those corresponding to the reduction of vanillin are still observed.

Electrochemistry of Some Other Compounds: Eugenol, Ferulic Acid, Vanillyl Alcohol and Vanillic Acid

A rapid overview of the electroactivity of some other compounds involved in the biochemical process of vanillin production is proposed. All the compounds are dissolved at a concentration of 1.0×10^{-5} mol L^{-1} in a hydroalcoholic solution. The presence of ethanol allows dissolving some compounds that are less soluble in aqueous media. The voltammograms obtained for the oxidation of the studied compounds on a gold rotating disk electrode are presented in Fig. 7. All the compounds are electroactive and vanillin, eugenol, ferulic acid, coniferyl aldehyde, and vanillic acid are oxidized with $E_{1/2}$, respectively, equal to 0.26, 0.51, 0.56, 0.62, and 0.64 V (vs

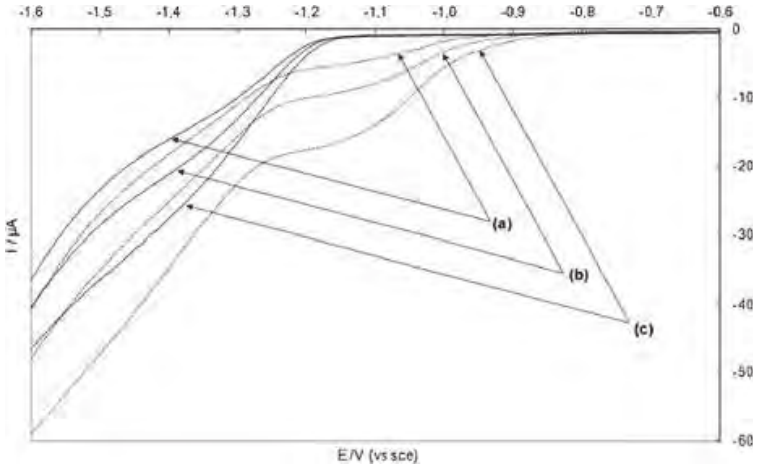

Fig. 5 Rotating disk electrode for vanillin reduction on a silver solid amalgam electrode in a hydroalcoholic model solution. Different concentrations of vanillin: *a* 1.0×10^{-3} mol L^{-1}, *b* 1.5×10^{-3} mol L^{-1} and *c* 2.0×10^{-3} mol L^{-1}, without (*solid lines*) and with (*dashed lines*) an excess of hydrazine in the solution

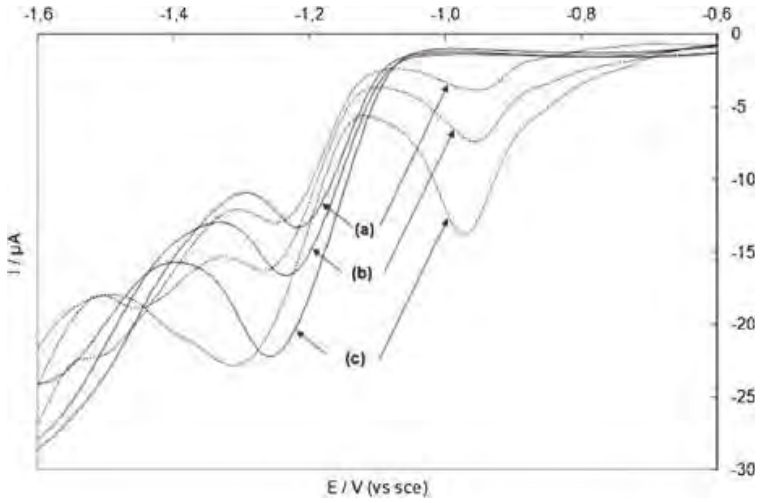

Fig. 6 Square wave voltammetry for vanillin reduction on a silver solid amalgam electrode in a hydroalcoholic model solution. Different concentrations of vanillin: *a* 1.0×10^{-3} mol L^{-1}, *b* 1.5×10^{-3} mol L^{-1} and *c* 2.0×10^{-3} mol L^{-1}, without (*solid lines*) and with (*dashed lines*) an excess of hydrazine in the solution

sce). Those differences are mainly due to the more or less attractive function situated in the ortho position (Fig. 1) and they offer the possibility to obtain a certain selectivity by the potential imposed during chronoamperometry. Moreover, the mechanisms of oxidation looks different like with vanillin or ferulic acid that are oxidized in two steps. The mechanism of oxidation of vanillin seems more complex in hydroalcoholic solution than in phosphate buffer. Ethanol may have a certain electroactivity as its relative high concentration may favor adsorption and electropolymerization in oxidation. However the linearity of limit current intensity versus concentration was confirmed.

Application to the Process Control of the Biotechnological Production of Vanillin

The electrochemical results presented above are interesting to foresee different strategies of sampling and analyses to control and follow the key steps of the process of the biotechnological production of vanillin (Fig. 1). From the differences of oxidation potentials of the essential compounds appearing in Table 1, it is possible to assess a certain selectivity to rate different compounds in the batch reactor during the process. As presented in the introduction, the

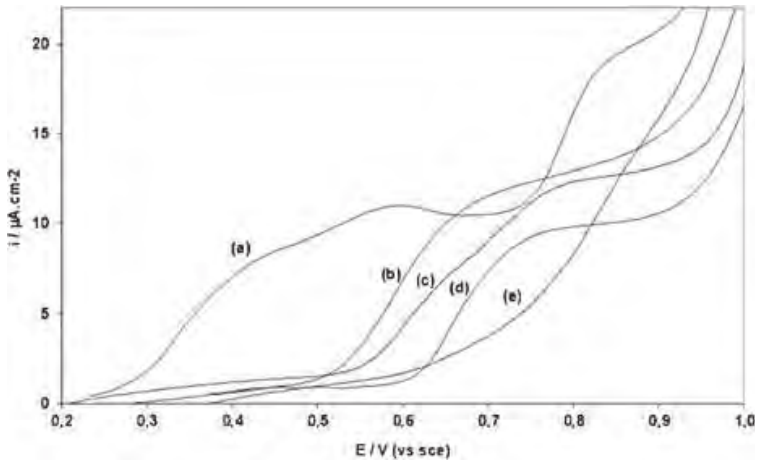

Fig. 7 Rotating disk electrode for vanillin (*a*), eugenol (*b*), ferulic acid (*c*), coniferyl aldehyde (*d*), and vanillic acid (*e*) oxidations, at a concentration of 1.0×10^{-5} mol L^{-1} in a hydroalcoholic solution on a gold electrode

Table 1 Half-wave potential, concentration range, and number of electrons exchanged during oxidation of eugenol, vanillin, and vanillic acid on a gold working electrode

	Eugenol	Vanillin	Vanillic acid	Ferulic acid
$E_{1/2}$ (V)	0.51	0.26	0.64	0.56
Concentration range (μmol L^{-1})	2.5–50	1–200	1–100	1–10
n	2	2	2	2
D (cm^2 s^{-1})	ND	6×10^{-6}	ND	ND

Diffusion coefficient is estimated from the study of vanillin in aqueous media

ND not determined

duration required for biotechnological production of vanillin is at least in the order of magnitude of several hours. As the time required for one electrochemical measurement is, at the very most, a few seconds, it is possible to sample an aliquot in the batch reactor for an external analysis while the process is still running. A current–potential curve can be obtained in the sample with a gold rotating disk electrode to detect vanillin, eugenol, and an eventual presence of vanillic acid. Subsequently, an addition of hydrazine in the sample makes the detection of vanillin possible with an amalgamated electrode. A second approach consists in implementing a rotating disk electrode directly in the bioreactor batch to obtain a current–potential curve with a gold rotating disk electrode; this strategy offers a good reactivity to control the process. As an alternative way, a third approach consists in the implementation of three gold rotating disk electrodes in the batch for chronoamperometric detection. The first electrode, held at a potential of 0.55 V, should maintain a constant current intensity as the decrease of intensity due to eugenol consumption is compensated by an equal increase of intensity due to vanillin production; a second electrode, held at 0.4 V, is indicative of vanillin concentration and should increase all along the process until stabilization; a third electrode, with a potential of 0.7 V, should maintain a constant current intensity (after the step of ferulic acid intermediate production) excepted when vanillic acid is produced with an increase of current intensity. The first electrode is a tool to follow the process while the second and third electrodes act as an alarm to detect any deviation of the correct progress. If no stirring is possible in the batch, for instance due to technical reasons, a fourth strategy requires the use of ultramicroelectrode. Constant potential chronoamperometry performed with disk ultramicroelectrodes gives a theoretical current intensity defined by the following equation [62]:

$$I = nFAC\sqrt{\frac{D}{\pi t}} + 4nFDCr$$

all variables are defined above except for the time of measurement t (second) and the radius of the ultramicroelectrode r (centimeter). Compared to rotating disk electrode, the use of ultramicroelectrodes at the steady state is possible in non-agitated solution and the limit of detection is lower than cyclic voltammetry performed on microelectrodes.

Conclusion

Our work shows that electroanalysis may be used in vanillin biotechnological production. Vanillin is oxidized on different electrodes materials such as gold, vitreous carbon, and platinum, the results obtained with linear sweep voltammetry at a stationary state present few differences

depending on the nature of the material in a 1–200 μmol L^{-1} range of concentration. Vanillin can be reduced with or without addition of hydrazine, on a silver-amalgamated electrode, within a 25–500 μmol L^{-1} concentration range, square wave voltammetry both helps to lower the detection down to 5×10^{-5} mol L^{-1}. The reduction explored on mercury film, covering working electrode, is promising to lower the detection threshold of vanillin, particularly with square wave voltammetry. Other molecules involved in the biotechnological process for vanillin production are electroactive and can be oxidized at a stationary state on a gold electrode: eugenol, ferulic acid, coniferyl aldehyde, and vanillic acid. The results obtained are promising to fulfill an interesting tool to control the biotechnological process of vanillin production.

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