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Analytical Methods

Amperometric detection of ascorbic acid in honey using ascorbate oxidase immobilised on amberlite IRA-743

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ABSTRACT

A differential amperometric method for the specific determination of ascorbic acid in honey was developed by association of a flow injection analysis (FIA) system and a tubular reactor containing the ascorbate oxidase enzyme immobilised. A gold electrode modified by electrochemical deposition of palladium was employed as working electrode. Ascorbic acid was quantified in seven samples of commercial honeys using a potential of +0.60 V vs. $Ag/AgCl_{(sat)}$. The linear dynamic range in ascorbic acid extends from 1 to 50 μmol L^{-1}, at pH 7.0. At flow rate of 1.5 mL min^{-1} and injecting 250 μL sample volumes, a sampling frequency of 180 determinations per hour is afforded. The detection and quantification limit of this method are 0.14 and 0.49 μmol L^{-1}, respectively. The samples analyses were compared with the volumetric method, and showed an excellent correlation between the methods.

1. Introduction

Honey is a sweet, viscous fluid, elaborated by bees from the nectar of plants and stored in their combs as food (Matei, Birghila, Dobrinas, & Capota, 2004). Bees and plants are known as the primary sources of components as carbohydrates, water, traces of organic acids, enzymes, amino acids, pigment and other compounds such as pollen and wax (which arise during honey maturation), that ends resulting in the honey complex matrix (Torres et al., 2005).

Because of its high complexity, the chemical analysis of honey implicates a considerable challenge. This analysis is important due to three main purposes: (1) to determine its geographical and botanical origin, (2) to verify adulteration and (3) to identify pharmacological active compounds. The first and second points assist with certification of quality of the product, which is commonly used as a food product; and the third purpose allows the examination of the content for the use of honey in medicinal purposes (Franchini, Matos, Colombara, & Matos, 2008).

One of the most important vitamins present in honey is the vitamin C (ascorbic acid). The ascorbic acid (AA) is known for its reductive properties, for its use as an antioxidant agent in food and drinks, as well as for its importance for therapeutic purposes and biological metabolism. The literature indicates that human beings consume between 15 and 50 mg of ascorbic acid in a period of 24 h (Matos, Augelli, Lago, & Angnes, 2000). Beyond its function in collagen formation, the vitamin C is known to increase absorption of inorganic iron, to help the formation of the connective tissue, bones, teeth, blood vessels walls and to assist the body in assimilating amino acids. Also vitamin C has been used for the treatment of the common cold, mental illnesses, infertility and cancer (Matei et al., 2004).

The determination of ascorbic acid is generally based on its reducing properties or on its capacity to produce coloured substances. In the literature, several methods such as volumetric, chromatographic, enzymatic, eletroanalytical and spectrophotometric (Augustin, Beck, & Marousek, 2006; Ferreira et al., 1997; Matos, Augelli, Pedrotti, Lago, & Angnes, 1998) can be found; the last one is the most used, despite the inconvenience of the simultaneous determination of dehydroascorbic acid, which is one of its oxidation products. Therefore, due to the recent advances in the food and pharmaceutical industries and the need for nutritional assessment, the development of a selective, simple, and accurate method to determine AA has been being researched (Burini, 2007; Kim, Dahlgren, Moroz, & Sweedler, 2002). Due to its selectivity and sensitivity, an electrochemical method to determine ascorbic acid has been a subject of considerable interest. A wide variety of examples of the electrochemical determination of AA have been proposed. These include a glassy carbon electrode (GCE) and a carbon paste electrode with complexes and organic compounds, such as, Naphthol green B doped in polypyrrole film (Mohadesi & Taher, 2007), cobalt phthalocyanine nanoparticles (Wang, Xu, Tang, & Chen, 2005), poly(caffeic acid) (Li, Ren, & Luo, 2007)), octacyanomolybdate-doped-poly(4-vinylpyridine) (Thangamuthu, Senthil Kumar, & Chandrasekara Pillai, 2007), ferrocene and its

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derivatives (Pournaghi-Azar & Ojani, 1999; Raoof, Ojani, Bitollahi, & Hosseinzadeh, 2006; Wang & Du, 2004), vanadium oxide polypropylene carbonate (Tian et al., 2006), ruthenium oxide (Shakkthivel & Chen, 2007) and polyaniline film (Mu & Kan, 2002).

Enzymes have been used to improve the selectivity of many reactions using the amperometric detection. Due to the enzymes high cost, some strategies have been reported to reduce its consumption. Recently, various ion-exchange resins have gained considerable attention not only for separation purposes but also as carriers of catalytic active substances, as enzymes (Franchini et al., 2008). These resins must meet several requirements as having a porous structure that is strong enough to withstand a pressure increase, usually applied in flow bioreactors, and having a chemically and physically resistant membrane material. These requirements can be met by several aromatic and aliphatic polyamides. Therefore, resin prepared from these polymers is a suitable substrate for the immobilisation of enzymes (Watkins et al., 1995). The covalent binding of the enzymes to the polymer matrix is one of the most prospective methods for its immobilisation. It is known that the ascorbate oxidase enzyme catalyses fast and selectively the oxidation reaction of ascorbic acid.

In this work, we describe a differential amperometric determination of ascorbic acid in honey using a gold electrode modified by electrodeposition with palladium, and a tubular reactor containing the ascorbate oxidase enzyme immobilised on amberlite IRA-743. The concentrations of ascorbic acid in each sample were calculated based on the difference between the current measured before and after the enzymatic treatment.

2. Material and methods

2.1. Enzymes immobilisation

The procedure adopted to immobilise the ascorbate oxidase enzyme was quick and simple (Matos, Pedrotti, & Angnes, 2001). Amberlite IRA-173 resin was selected as support, because it has active amine groups in its chemical structure. The enzyme immobilisation process begins with the addition of 100 μl of glutaraldehyde 0.1% to 250 mg of resin, and this mixture was stirred for 5 min. Subsequently, 50 units of enzymes were introduced into the mixture and stirred for an additional time of 10 min. In the next step, the resin was transferred to a tygon tubing (2.5 mm of i.d. and 25 mm long) with its extremities closed with a thin layer of glass wool to assemble the reactor. To adapt the enzymatic reactor to a FIA (flow injection analysis) system, the tubing (0.8 mm of i.d.) was attached in each of its extremities with the aid of a small piece of silicone tubing (1.3 mm i.d. and 5 mm long). Finally, the reactor was washed with 100 mmol L^{-1} phosphate buffer solution (pH 7.0) to remove the excess of ascorbate oxidase.

2.2. Reagents and chemicals

All solutions used were of analytical grade. Ascorbic acid, mono- and di-hydrogen phosphates were obtained from Merck (Darmstadt, Germany). Buffer solution was prepared by dissolving the solids in distilled water that was also treated with a nanopure system. Commercial ascorbate oxidase (EC 1.1.0.3.3–162 U mg^{-1}) was obtained from Sigma (St. Louis, MO, USA). The amberlite IRA-743 ion-exchange resin and glutaraldehyde were obtained from Aldrich (Milwaukee, WI, USA). Diluted solutions of ascorbic acid were prepared daily using phosphate buffer solution (pH 7.0) 100 mmol L^{-1}.

2.3. Sample collection

This work was carried out on seven Brazilian samples. The samples were stored in a dark room at low temperature prior to analysis. For determination of ascorbic acid, about 2 g of honey were dissolved in 25 mL of phosphate buffer solution 100 mmol L^{-1} (pH 7.0), and injected in the flow-injection system. Each sample was injected in triplicate.

2.4. Electrodes and instrumentation

The electrochemical cell consists of a palladium modified gold electrode (3.0 mm diameter). Modification was done by electrochemical deposition of Pd (K_2PdCl_6 2 mmol L^{-1}, pH 4.8, at −1.00 V for 15 min). Microscopic observation of the electrodes after electrodeposition showed uniform palladium deposit, with a very rough surface. The modified electrodes were stable enough to at least a week under intense use. The reference electrode was a miniaturised $Ag/AgCl_{(sat)}$ electrode constructed in our laboratory (Pedrotti, Angnes, & Gutz, 1996) and a stainless steel tube (1.2 mm i.d.) was used as auxiliary electrode.

In this work, a double channel flow system was employed. The flow system used during the development of this work consisted of two lines, in the first one the sample was added in the detection system, and in the second one the sample was inserted in the line that contain the enzymatic reactor before the detection system. A potentiostat (μ-AUTOLAB) operating in the amperometric mode was employed for FIA measurement. The system contained a peristaltic pump, a pinch valve, a sampling loop, a tubular reactor (ϕ = 0.25 and 2.5 cm of length) with ascorbate oxidase chemically immobilised in amberlite IRA-743 resin, an electrochemical cell and the potentiostat.

2.5. Procedure

For amperometric detection of direct ascorbic acid, a +0.60 V (vs. $Ag/AgCl_{sat}$) potential was found as the most favourable to be applied at the gold electrode modified with palladium. The differential determination of the analyte requires two measurements, one containing just the sample and the standards solutions in the channel without the reactor, and a second one involving the sample passage through the enzymatic reactor. In the first case, the signal obtained corresponds to the concentration of ascorbic acid and the interfering components present in honey samples. In the second case, the signal corresponds only to the interfering components. The calculated difference is compared with the calibration plot.

3. Results and discussion

Preliminary tests employing palladium-modified electrodes showed an interesting behaviour in the presence of ascorbic acid. Cyclic voltammograms of the bare gold electrode and of the same electrode after palladium deposition, after increasing concentrations of AA, were obtained. The current enhancement was remarkable when the electrode is modified (Fig. 1). Probably, part of the increase in the current may be attributed to the growth of the effective area of the electrode. Observations with a microscope showed the formation of a very porous surface after the palladium deposition.

The influence of parameters, such as flow rate and sample volume, was studied. Fig. 2a shows the amperometric responses of a gold electrode modified with palladium for injections of 150 μL of AA 50 μmol L^{-1}, as a function of the flow rate (1–4 mL min^{-1}). For high flow rates, the ascorbate oxidase immobilised in the tubular reactor was unable to oxide the AA completely into DAA, and for low flow rates a larger dispersion for the current signal of ascorbic acid is observed. Thus a flow rate of 2.5 mL min^{-1} was chosen as the most favourable, since it combines good reproducibility, high

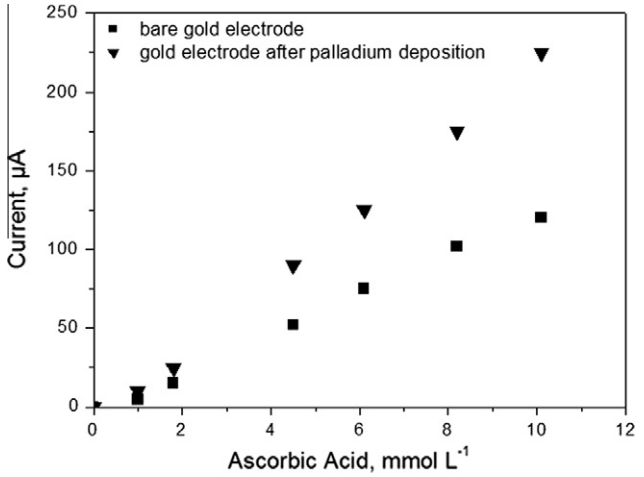

Fig. 1. Current response of a bare gold electrode and the signal obtained for the same electrode after palladium electrodeposited for increasing concentrations of ascorbic acid (1–10 mmol L^{-1}). Applied potencial, +0.60 V vs. $Ag/AgCl_{(sat)}$.

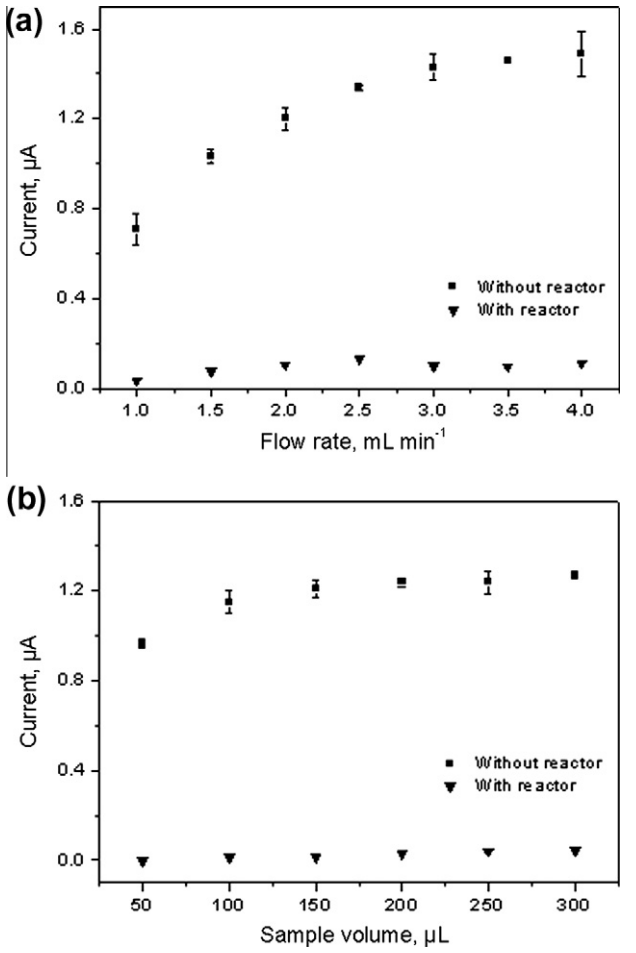

Fig. 2. Repetitive injections of ascorbic acid 50 μmol L^{-1} to find the most suitable working conditions. (a) Flow rate from 1 to 4 mL min^{-1} and (b) samples volume injected from 50 to 300 μL. Measurements made with a gold electrode modified by electrodeposition of palladium. Applied potencial, +0.60 V vs. $Ag/AgCl_{(sat)}$.

efficiency (180-samples h^{-1}), and low consumption of carrier solution, also providing the complete oxidation of AA into DAA.

The influence of the sample volume on the analytical signal was also evaluated. Fig. 2b shows the amperometric responses of a gold

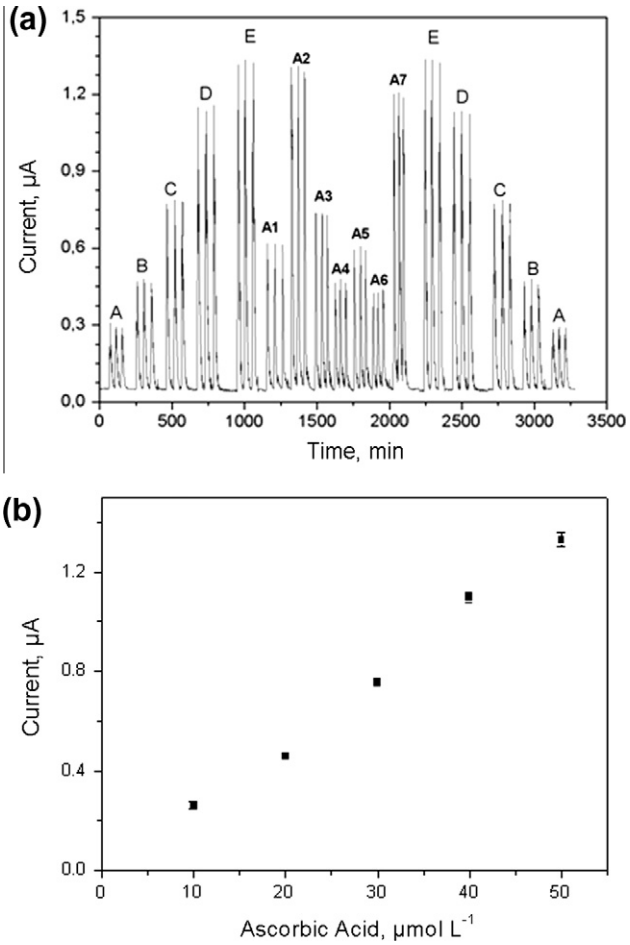

Fig. 3. (a) FIA-amperometric measurements involving injections of 200 μL solutions containing (A) 10 μmol L^{-1}, (B) 20 μmol L^{-1}, (C) 30 μmol L^{-1}, (D) 40 μmol L^{-1}, and (E) 50 μmol L^{-1} of ascorbic acid and seven honey samples (A1–A7) using the tubular reactor containing ascorbate oxidase immobilised. Conditions: sample volume, 150 μL; flow rate, 2.5 mL min^{-1}; applied potential, +0.60 V vs. $Ag/AgCl_{(sat)}$.

electrode modified with palladium for injections of AA 50 μmol L^{-1} and a flow rate of 2.5 mL min^{-1}, as a function of the loop (50–300 μL). When the volume of the sample is increased, the amperometric signal increases such as well as the time required for each analysis. A volume of 150 μL was chosen as the working volume for the subsequent experiments. For all the studied volumes, the ascorbate oxidase immobilised in the tubular reactor was sufficient to oxidise AA completely in DAA.

To examine the efficiency of the rector containing immobilised ascorbate oxidase on amberlite IRA-743, amperometric responses of a gold electrode modified by electrodeposition of palladium involving 50 injections of 150 μL of ascorbic acid 50 μmol L^{-1} for a channel with and without immobilised ascorbate oxidase were performed. The precision for injections of ascorbic acid without immobilised ascorbate oxidase on tubular reactor was 3%. An important characteristic observed in the immobilised enzymes was its storage stability of at last 1 week under intense use with ascorbic acid standard. After this period, a decrease on the order of 50–60% of the enzymes activity was observed. When applied in the determination of ascorbic acid in honey, the enzymatic reactors showed a loss in the enzyme activity after 50 injections, requiring construction of new reactors. When not in use, the reactors were stored in a freezer at −20 °C (Wilson, 1991).

Fig. 3a shows the response of a gold electrode modified by electrodeposition of palladium for successive injections of 150 μL ascorbic acid from (A) 10 μmol L^{-1} to (E) 50 μmol L^{-1} and seven

Table 1
Results obtained in analysis of ascorbic acid (mg (100 g)$^{-1}$) in honey samples.

Sample	Flower	Amperometry (mg/100 g)	Iodometry (mg/100 g)
A1	Citrus *aurantium* var. sinensis	3.10 (±0.01)	3.00 (±0.45)
A2	*Eucalyptus tereticornis*	6.20 (±0.06)	5.50 (±0.40)
A3	*Vernonia polyanthes*	1.50 (±0.02)	1.90 (±0.15)
A4	*Cissus rhombifolia*	1.95 (±0.00)	2.00 (±0.10)
A5	*Croton campestris* A. St.-Hill	3.20 (±0.00)	3.40 (±0.43)
A6	*Julocroton triqueter* wild	1.50 (±0.01)	1.70 (± 0.10)
A7	*Euphobia milii* var. splendens	5.00 (±0.01)	4.30 (±0.11)
Mean	–	3.21	3.11
V_{min}	–	1.50	1.70
V_{max}	–	6.20	5.50

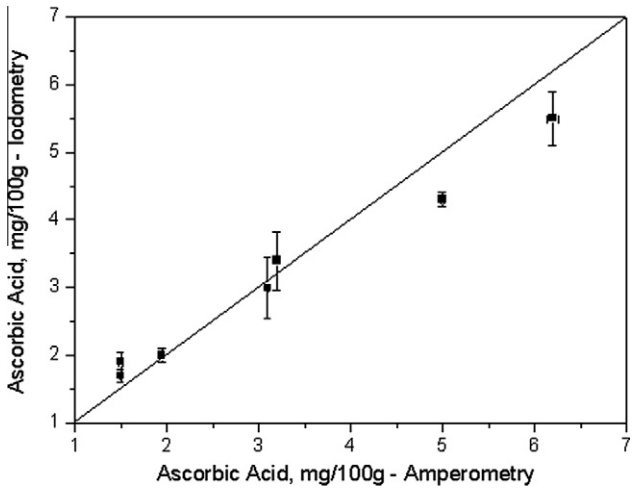

Fig. 4. Comparison of the results obtained by the differential amperometric and the iodometric titration methods for the analysis of ascorbic acid in seven different sample of honey.

samples of honey (A1–A7). Plot calibration (Fig. 3b) implies that the proportionality between the amperometric current and the concentrations of the analyte is Current (μA) = −0.068 + 0.028 [AA] (μmol L^{-1}); correlation coefficient, 0.9938. The proposed system presents a good reproducibility and has a very favourable signal-to noise ratio, demonstrated by the very stable baseline obtained for these low concentrations. The detection limit was calculated on the basis of 3σ (σ being the residual standard deviation of the intercept), yielding a value of 0.14 μmol L^{-1} and the quantification limit was calculated on the basis of 10σ, yielding a value of 0.49 μmol L^{-1}.

Under the optimum conditions, the FIA-amperometric system applied for the determination of ascorbic acid in seven honey samples was based on two steps involving the injection of: (1) the sample and the standards solutions in the channel without the reactor and (2) the sample in the channel containing the enzymatic reactor with ascorbate oxidase immobilised.

Table 1 and Fig. 4 compare the results of the analyses performed by amperometric method, developed in this work, and iodometric titration method (Suntornsuk, Gritsanapun, Nilkamhank, & Paochom, 2002) for seven different samples (in triplicates). The comparison of the amperometry with gold/palladium electrode and the iodometry gives a slope and intercept close to unity and zero, respectively. A good correlation (r^2 = 0.9998) between the amperometric and titration methods was found. The confidence interval for the slope and intercept are (0.77 ± 0.04) and (0.63 ± 0.15) mg (100 g^{-1}), respectively, for a 95% confidence level. A paired Student's t-test showed that the mean values ($t_{exp} < t_{crit}$; 0.5 < 2.5, n = 7, P = 0.95) not significantly differ. Taking into account of these results, do no significant differences between the three methods were observed, which strongly indicates the absence of systematic errors. Recovery experiments on honey solutions spiked with different amounts of ascorbic acid were also carried out. The method recoveries obtained for the ascorbic acid ranged from 92% to 107%; such values confirm the accuracy of the proposed method. The main disadvantage of the present method is the fact that the honey inactivates the ascorbate oxidase after 50 injections, requiring the construction of a new reactor.

4. Conclusions

This work demonstrated the potentiality of the amperometric method using gold electrodes modified with palladium coupled with flow injection analysis techniques for the determination of ascorbic acid in honey using the enzyme ascorbate oxidase immobilised in a tubular reactor. The method developed proved to be more advantageous in comparison with other methods reported in the literature since it seems to be more sensitive, economical, practical and less time consuming. Ascorbic acid determination at low concentrations and in coloured sample is possible by high performance liquid chromatography, for example, although very expensive equipment and chemicals are necessary (Xi & Masanori, 1995). Ion chromatographic and gas chromatographic methods are lengthier and also very expensive for the determination of ascorbic acid (Mura, Maruyama, & Koh, 1995; Silva, 2005). The enzymatic method is known to be a very sensitive, specific, simple and useful method, in which the immobilised form of the enzyme is generally used (Akyilmaz & Dinçkaya, 1999).

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