POLAROGRAPHIC DETERMINATION OF VITAMIN C AFTER DERIVATIZATION WITH *o*-PHENYLENEDIAMINE

José António Rodrigues[1], Inês Maria Valente[2], Luís Moreira Gonçalves[3], João Grosso Pacheco[4] and Aquiles Araújo Barros[5,*]

Requimte – Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre No. 687, 4169-007 Porto, Portugal; e-mail: [1] jarodrig@fc.up.pt, [2] inesmariavalente@sapo.pt, [3] luis.goncalves@fc.up.pt, [4] joao.pacheco@fc.up.pt, [5] ajbarros@fc.up.pt

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A differential pulse polarographic (DPP) method has been developed for the determination of ascorbic acid (AA) and dehydroascorbic acid (DHA), the two main forms of Vitamin C. The method consists of the DPP analysis of a quinoxaline obtained by the derivatization of DHA with *o*-phenylenediamine. Results using the proposed method correlated well with those obtained by two reference methodologies: the common iodometric method and a published chromatographic methodology. It was also used in the study of Vitamin C degradation in fruit juices, showing that it involves an initial oxidation of AA to DHA, followed by hydrolytic degradation of the latter.

Keywords: Ascorbic acid; Electrochemical techniques; Fruit juices; Polarography; Voltammetry; Vitamin C.

The term Vitamin C is usually used to identify ascorbic acid (AA). However, dehydroascorbic acid (DHA), resulting from the oxidation of AA, has the same biological activity as it can be converted into AA in the human body. So, a simple definition of Vitamin C should be AA + DHA [1]. Although it occurs naturally in various concentration levels in a broad range of fruits and vegetables, AA is intentionally added to foods, including fruit juices, to improve the nutritional quality and for its potent antioxidant capacity to act as a singlet oxygen quencher[2]. The joint commission of Food and Agricultural Organization (FAO) and the World Health Organization (WHO) recommend a daily intake of 45 mg of Vitamin C for adults[3]. In fresh foods, Vitamin C is usually found as AA. However, during industrial processing and conservation of food products, oxidation of AA to DHA can occur. AA can be easily degraded by enzymes and atmospheric oxygen. Oxidation reactions can be accelerated by high temperatures, high pH, light and pres-

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ence of metals. Thus, a method that selectively determines the levels of AA and DHA in food samples would be useful in monitoring the oxidative degradation of the food.

Numerous analytical techniques have been proposed for Vitamin C determination in different matrices and at different levels, including titrimetry, voltammetry, potentiometry, fluorometry, flow injection analysis (FIA), spectrophotometry and chromatography[4,5]. Particularly, chromatographic methods are very efficient in Vitamin C assay of complex materials such as fruit juices, vegetables and beverages[6]. High-performance liquid chromatography with UV detection (HPLC-UV) is currently the most commonly used technique for the analysis of AA and DHA in food. Some HPLC methods with fluorimetric and electrochemical detection have been reported as well[1]. Electrochemical detection is an attractive alternative method for detection of electroactive species, because of its inherent advantages of simplicity, ease of miniaturization, high sensitivity and relatively low cost[7,8].

In this paper, an indirect determination of AA is proposed, involving a previous oxidation to DHA with *N*-bromosuccinimide (NBS), followed by a derivatization with *o*-phenylenediamine (OPDA)[9–12] and differential pulse polarographic (DPP) determination of the resulting quinoxaline[13]. The method allows the determination of both AA and DHA and it is applied to their determination directly in some fruit juices and tablets with no need for any previous separation procedures. The direct electrochemical determination of AA by oxidation on a conventional electrode is too difficult because of its large overpotential and fouling by the oxidation products[14].

EXPERIMENTAL

Equipment

Cyclic voltammetry (CV), square-wave voltammetry (SWV) and DPP were performed using a Metrohm 663 VA voltammetric stand (Herisau, Switzerland) with a platinum wire as an auxiliary electrode and a reference AgCl|Ag (3 M KCl) electrode. The system was connected to an Autolab PGSTAT 10 voltammetric system (Eco Chemie, Utrecht, The Netherlands). All measurements were made at room temperature. CV and SWV were performed with a hanging mercury drop electrode (HMDE) and DPP with a dropping mercury electrode (DME), drop size of 0.024 mm^3, drop time of 1 s. CV used a sweep rate of 100 mV s^{-1}. SWV and DPP measurements were performed with a potential step of 6 mV and amplitude of 25 mV and 50 mV, respectively.

The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low pressure quaternary gradient unit (model PU-2089 Plus), a UV-Vis detector (model UV-2070 Plus) and a manual injector Rheodyne (model 7725i). Separations were achieved on a Hypersil GOLD HPLC column (Thermo Scientific, Waltham, USA) RP C_{18} (100 mm × 4.6 mm, 3 μm) in isocratic conditions, 0.01% solution of sulfuric acid adjusted to pH 2.6 for 25 min (ref.[15]).

The flow rate was 1 ml min^{-1} and the wavelength used for detection was 245 nm. 20 µl of the sample were injected into the chromatographic column kept at room temperature.

Chemicals and Samples

All chemicals were of analytical grade and were used without further purification. AA, NBS, *o*-phosphoric acid, potassium iodate, starch and sodium thiosulfate were purchased from Merck (Darmstadt, Germany), dehydroascorbic acid (DHA), OPDA, DL-dithiothreitol (DTT), potassium iodide and the activated charcoal (Norit) were purchased from Sigma–Aldrich (St. Louis, USA). Ultra-pure water from a Millipore Simplicity 185 water purification system (Millipore, Billerica, USA) was used. 0.1 M Phosphate buffer solutions of pH 2, 3, 6, 7 and 8 were prepared by dissolving disodium phosphate (Merck), adjusting the pH with HCl (Merck). Acetate buffer solutions of pH 4 and 5 were prepared in the same way, however, sodium acetate (Merck) was used instead. Solution of pH 1 consisted of a 0.1 M HCl solution. The OPDA and NBS solutions were daily prepared by dissolving an appropriate amount of those compounds in the buffer solution used for derivatization and kept in the dark. Suitable precautions were taken when using OPDA as it is toxic and may also cause allergic reactions. The juice and enriched milk samples were purchased in a local supermarket and the tablets in a local pharmacy.

Recommended Procedures

DHA analysis. In a voltammetric cell, 50 µl of the sample were added to 25 ml of 0.1 M acetate buffer solution, pH 5. After removing oxygen with nitrogen for 10 min, 1 ml of OPDA (1.25%) was added and the derivatizing reaction took place during 5-min period, wherein nitrogen was continuously bubbled.

AA + DHA analysis. Equal to the DHA analysis with the sole difference that 200 µl of NBS (250 mg l^{-1}) were previously added and let react for 5 min in order to oxidize all AA into DHA, before the addition of OPDA. AA was determined by calculating the difference between the results of these two analyses.

The method for Vitamin C chromatographic analysis was performed following the procedure reported earlier[15]. Diluted juice samples were centrifuged at 4 °C for 15 min. The supernatant was filtered through a 0.45 µm Nylon filter and 20 µl of the filtrate were directly injected in the chromatographic system to determine AA. The tablets were dissolved in ultra-pure water and filtered prior to HPLC analysis. To determine total Vitamin C, 200 µl of DTT solution (20 mg ml^{-1}) were added to 1 ml of the filtered sample. DHA was calculated as the difference between the Vitamin C and AA contents.

The determination of AA using the iodometric method was performed as follows: 2 g of KI were weighed and placed in an Erlenmeyer flask along with 50 ml of 0.5 M H_2SO_4, 50 ml of 0.3 mM KIO_3 and about 10 g of juice (rigorously weighed), then 2 g of starch were added, and the solution immediately became blue; subsequently, the mixture was titrated with 4 mM $Na_2S_2O_3$, until the blue colour disappeared.

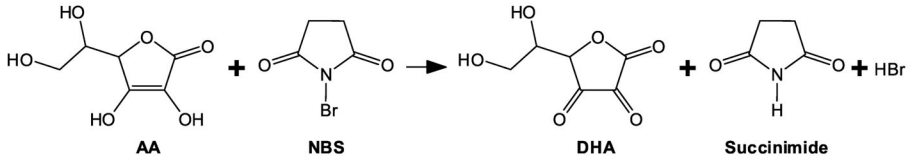
RESULTS AND DISCUSSION

Oxidation of AA and Derivatization of DHA

Activated charcoal was the first oxidant tested to convert AA into DHA. In spite of its use in the spectrofluorimetric method of determination of AA adopted by the AOAC [16], its application in the DPP method was not successful because of the variation of the polarographic signal with the amount of activated charcoal added (data not shown). However, the oxidation of AA to DHA was successfully achieved using NBS [17], as shown in Scheme 1. Although there is no direct polarographic signal of NBS alone, a polarographic interference was found in the presence of OPDA, due to a product formed by reaction between the excess of NBS and OPDA. The amount of OPDA did not have a critical effect on the height of the interfering peak and the concentration of 0.05% OPDA was adopted, large enough to obtain a complete derivatization of solutions up to 20 mg l^{-1} of DHA in less than 5 min; in the case of NBS, it has to be in excess to completely oxidize AA but not too much since, as mentioned, the reaction product with OPDA starts to interfere in the polarographic measurement[18]. Accordingly, different concentrations of NBS had to be stipulated for different calibration ranges of DHA: 1, 5 and 20 mg l^{-1} of NBS for DHA concentrations up to 0.8, 4 and 20 mg l^{-1}, respectively. Good linear calibrations were obtained in the three regions, as can be observed in Table I, these calibration curves also set the method limit of detection (LOD) and quantification (LOQ) at 3 and 10 μg l^{-1}, respectively. The obtained relative standard deviations (RSD) were around 3% in all three calibration curves.

Voltammetric Determination of DHA

According to Ohmori et al.[19], the reaction between DHA and OPDA gives rise to several products as shown in Scheme 2. In water, DHA (**A**) is predominantly found as an hydrated bicyclic hemiacetal (**B**), 3,3,3a,6-tetra-



SCHEME 1
AA oxidation with NBS

hydroxytetrahydrofuro[3,2-*b*]furan-2-one[20,21], which with time is further hydrated to a lactone (**C**), 5-(1,2-dihydroxyethyl)-3,3,4,4-tetrahydroxy-dihydrofuran-2-one[22]. Compound **B** reacts with OPDA giving rise to 3-(2,3,4-trihydroxytetrahydrofuran-2-yl)quinoxalin-2-one (**D**). On the other hand, **C** reacts with OPDA to give the fluorescent 3-(1,2-dihydroxyethyl)-furo[3,4]quinoxalin-1-one (**E**). Both **D** and **E** can further react with OPDA, when such is present at a quantity large enough[19], producing 2-(2-amino-phenyl)-1-[3-(1,2,3-trihydroxypropyl)quinoxalin-2-yl]ethanone (**F**). These products originate different voltammetric peaks: P1, P2 and P3 in Fig. 1 should correspond to species **E**, **F** and **D** in Scheme 2, respectively. All three peaks shift to the more negative potentials with pH increase and P2 tends to disappear, which can be explained because a higher pH kinetically unfavours the reaction with OPDA [18]. Compound **E** (P1) is a fluorescent species used in the spectrofluorimetric method[16]. In this work, peak choice for quantitative purposes was P3, i.e. compound **D**.

Peak P3 increases when lowering the pH of the supporting electrolyte, with a clear linear behaviour[23–26]. However, at a pH lower than 4, the voltammetric peak shifts to a more positive potential and the resulting product from the reaction between NBS and OPDA starts to interfere with the voltammetric measurement. Moreover, the derivatization reaction is faster at a pH around 5 and the derivatized solution is stable for a longer

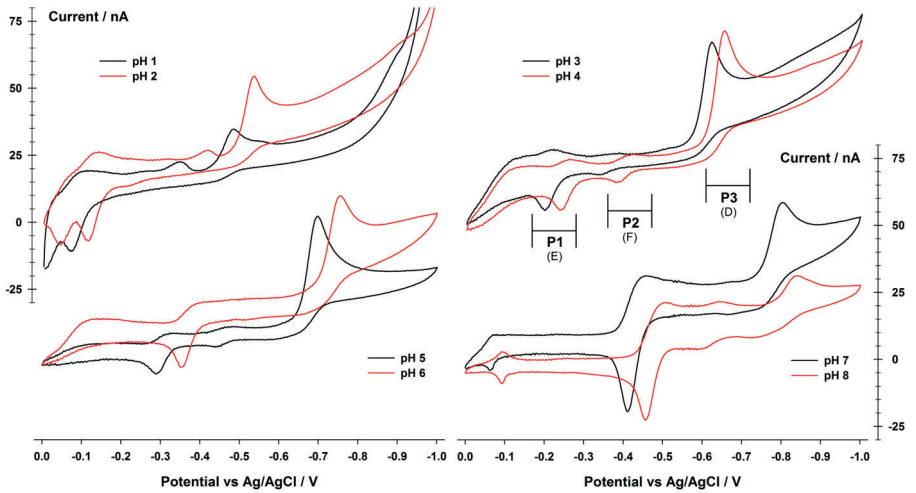
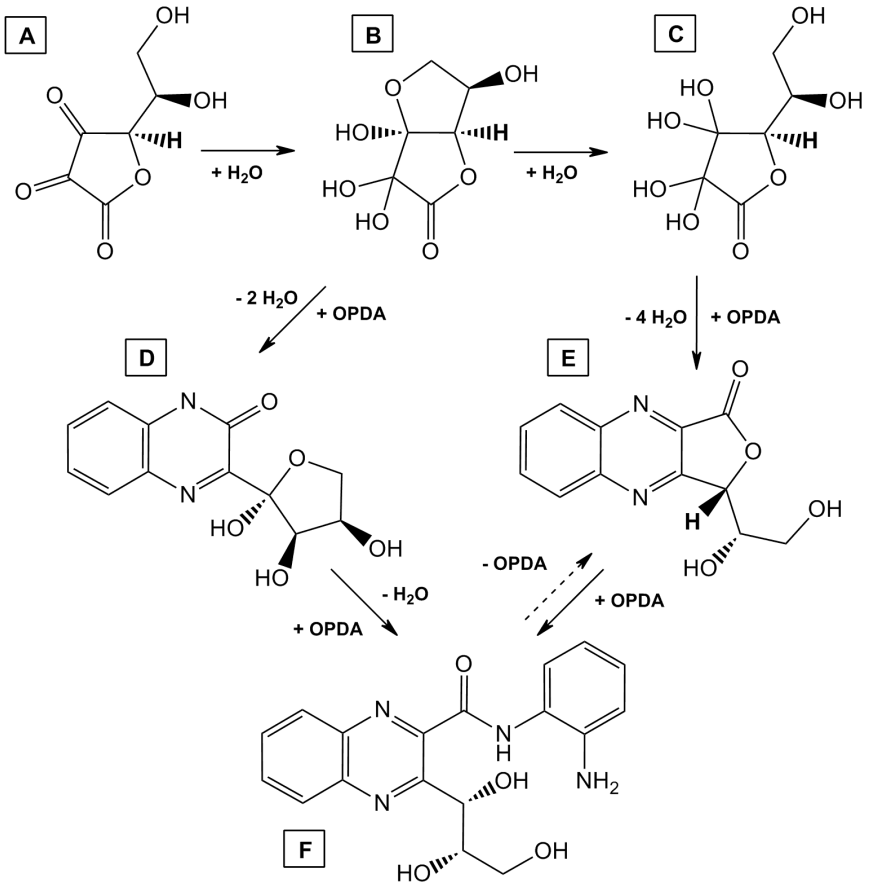


FIG. 1
Cyclic voltammograms at different pH, scan rate of 100 mV s^{-1}, [DHA] = 1 mg l^{-1}

TABLE I
Linear calibration curves, $i_p = f([DHA]/10^{-3}\ mg\ l^{-1}) = m[DHA] + b$, and regression coefficients, r^2, for the DPP analysis of DHA with different NBS concentrations used

[NBS], mg l^{-1}	[DHA], mg l^{-1}	r^2 (n)	i_p, nA
1	0.01–0.8	0.9999(7)	0.171[DHA] + 0.5
5	0.05–4.00	0.9998(9)	0.161[DHA] – 2
20	0.2–15.0	0.9999(7)	0.135[DHA] + 22



SCHEME 2
Reaction between DHA and OPDA and resulting quinoxalines with suggested stereochemistry

period of time[18,26]. Therefore, pH 5 was used in the subsequent determinations.

SWV gives greater intensity peaks, particularly at higher frequencies, than DPP (Table II). Yet, the difference was not as substantially as it might have been if P3 was not, unmistakably, an irreversible peak. However, DPP was used preferably because it is more immune to the interference from the product of the reaction between NBS and OPDA. Therefore, if an analyst only wishes to analyse DHA (it does not require NBS), and not also AA, SWV may be the preferable technique.

It is also known that at several pHs, including 5, part of the total DHA is rapidly hydrolyzed to 2,3-diketogulonate[21,27], and this chemical species also reacts with OPDA since it is a vicinal diketone[13]. However, it has no influence in the developed methodology given that their wave potentials, at pH 3.44, are –0.40 and –0.27 V vs normal calomel electrode (NCE)[28], i.e. they overlap P1 and P2 but do not influence P3.

Determination of Vitamin C in Several Matrices

In the determination of AA and DHA in commercial fruit juices, two different sample pre-treatments were tested. The pre-treatments involved filtration and centrifugation. The results obtained were the same with no pre-treatment (data not shown), showing that the analysis can be performed with the addition of a small quantity of fruit juice directly to the polarographic cell containing 25 ml of the buffer solution with NBS concentration of 5 mg l^{-1}.

In order to evaluate the method accuracy, different samples were analysed in triplicate by the developed methodology, using the method of standard additions (Fig. 2). The validation was carried out using a volumetric (iodometric) method commonly used[29–31] and a chromatographic method[15]. As can be seen from Fig. 3, the results obtained are statistically

TABLE II
Results comparison between DPP and SWV

[DHA], mg l^{-1}	DPP, nA	SWV (100 Hz), nA	SWV (200 Hz), nA	SWV (400 Hz), nA
0.200	5.8	10.0	23.8	49.1
0.400	16.6	24.9	47.8	96.4

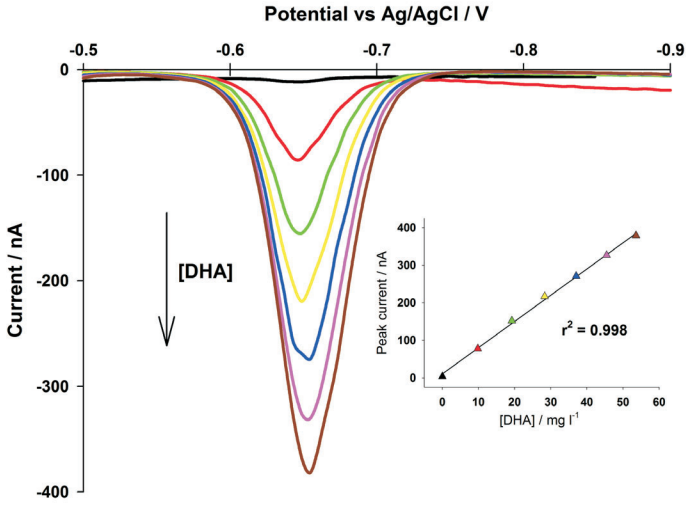


FIG. 2

Differential pulse voltammograms of several standard additions of DHA, with concentration from 0 to 53.6 mg l^{-1}. Inset: Plot of the calibration curve obtained

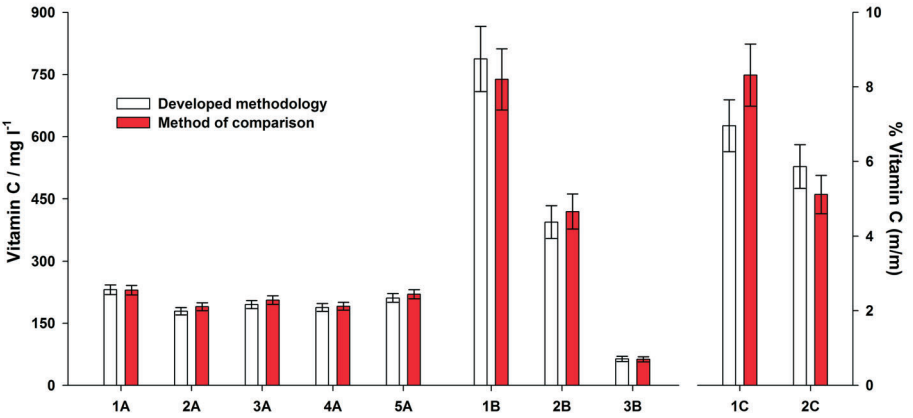


FIG. 3

Determination of Vitamin C by the developed methodology and a reference methodology; samples **A** were compared with an iodometric procedure and samples **B** and **C** with a recognized HPLC procedure; samples **1A–5A** as well as **1B** and **2B** are several kinds of fruit juices, sample **3B** is an enriched milk, samples **1C** and **2C** are tablets. In both methodologies: prior to analysis, 5% of *o*-phosphoric acid was added to sample **3B**, to promote protein coagulation; prior to analysis, samples **1C** and **2C** were diluted in water

consistent, confirming the accuracy of the developed voltammetric method.

AA and DHA contents were measured in three different juice samples during 4 days following the opening of their packages (Fig. 4). This showed the usefulness of the methodology in evaluating AA resistance to deterioration by oxidation and subsequent DHA appearance. By analysis of Fig. 4, it is possible to conclude that the disappearance of AA is quite fast and that the

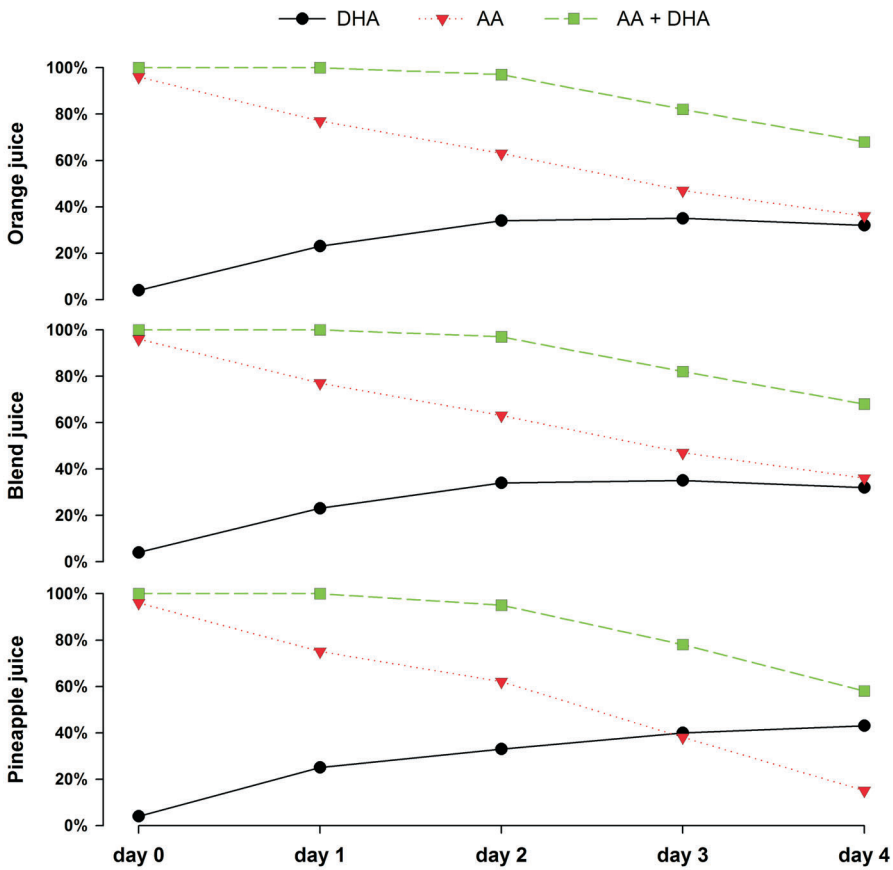


FIG. 4
Evolution of AA and DHA of three different fruit juices kept at room temperature after the package opening

first product of its degradation is DHA, as it would be expected. It can also be noticed that the sum of AA and DHA is practically unchanged during the first two days and then starts to decrease. These observations are in agreement with the results reported earlier[32–35]. Another remark is that the juice stability is higher with lower pH: orange juice – pH 2.8; blend juice ("tuttifruti") – pH 3.0 and pineapple juice – pH 3.8, is related, among other factors, with the different stability of ascorbic acid and ascorbate (pK_{a1} = 4.2). Again, these results were coherent with bibliographic data[36,37].

CONCLUSIONS

A DPP method was developed for the determination of Vitamin C (AA + DHA). The proposed method consists of the voltammetric analysis of one of the quinoxalines formed by the DHA derivatization with OPDA. Total Vitamin C was determined by previous AA oxidation to DHA with NBS; AA content was calculated by the difference between total Vitamin C and DHA. This method was successfully applied to the determination of Vitamin C in several matrices with no sample pre-treatment and the results agreed with the chromatographic method widely used. Degradation studies of Vitamin C in fruit juice samples were also performed, showing that AA is readily converted to DHA over time along with the expected overall decrease of Vitamin C. The developed methodology can be a good alternative to other analytical methods for Vitamin C determination due to its specificity and simplicity, low reagent consumption and no required sample pre-treatment.

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