Voltammetric Iodometric Titration of Ascorbic Acid with Dead-Stop End-Point Detection in Fresh Vegetables and Fruit Samples

Roxana A. Verdini and Claudia M. Lagier*

Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000, Rosario, Argentina

The present work describes a method for determining ascorbic acid, which combines iodometry with a voltammetric technique to detect the end point of the titration. In addition, the validity of the method applied to natural vegetable or fruit samples was assessed. The results were compared with those obtained by an accurate method such as HPLC using UV detection. Similar values of ascorbic acid for different natural samples were obtained by means of this approach ($p > 0.05$). The limit of quantification was 0.1 mg. This technique presents the advantage of other electroanalytical methods such as avoiding filtration or ultracentrifugation steps, with the additional benefit of using the platinum electrodes, which are routinely used in the laboratory. These facts allow a rapid and efficient quantification of ascorbic acid with very low cost of reagents and equipment.

Keywords: *Ascorbic acid determination; vitamin C; voltammetric titration*

INTRODUCTION

A large number of works on vitamin C (mainly ascorbic acid, AA) has been published in the last 40 years since vitamin C is considered to play crucial roles in human health (Byers and Perry, 1992; Hemilä, 1992; Gershoff, 1993). As fresh vegetables and fruits are the most important suppliers of vitamin C in a normal diet, the content of this vitamin is considered a fundamental marker of the quality and value of vegetables, fruits, and their derivative products (Cassella et al., 1989; Jacques and Chylack, 1991; Gershoff, 1993).

A variety of methods for determining AA in vegetables and fruits are being currently used. Among these are colorimetric or spectroscopic methods, which include fluorometric determinations, the chromatographic methods, and the electrochemical ones (Lento et al., 1963; Lau et al., 1985; Washko et al., 1992; Davis, 1993; Bersier and Bersier, 1994). The colorimetric techniques range from those based on the reducing properties of AA regarding 2,6 dichloroindophenol to enzymatic assays using peroxidase or ascorbate oxidase, as well as fluorescing quinoxaline derivative (Marchesini et al., 1974; Speek et al., 1983; Cassella et al., 1989; AOAC, 1990; Moeslinger et al., 1994; Moeslinger et al., 1995; Verma et al., 1996). In recent years, a number of HPLC techniques have been developed using different detection methods for the analysis of AA (Pachla and Kissinger, 1976; Tsao and Salimi, 1982; Speek et al., 1983; Behrens and Madére, 1987; Washko et al., 1989; Nagy and Degrell, 1989; Lazzarino et al., 1991; Washko et al., 1992). However, both of these spectrophotometric and chromatographic methodologies require steps such as ultracentrifugation or filtration to remove particles in suspension resulting from homogenization and extraction of the vitamin. These steps are drawbacks of these methods, not only because of the time spent in this stage, which increases the chance of AA oxidation, but also because of the cost of equipment required. On the other hand, different electroanalytical methods have been proposed for the determination of AA, several of which avoid some laborious steps when preparing the sample (Mason et al., 1972; Khristova et al., 1976; Matsumoto et al., 1981; Falat and Cheng, 1982; Nasser et al., 1987; Park and Shaw, 1989; Lyons et al., 1991; Campiglio, 1993). However, many of these are based on the use of membrane electrodes sensitive to a particular compound, which is the product of reaction between AA and the reagent used as the titrant. For example, it has been proposed using a Copper II Ion Selective Electrode (ISE) for the titration of vitamin C with Cu(II) solution as a titrant (Campiglio, 1993). Similarly, different methods using an iodide ion-selective electrode to determine vitamin C have been described (Khristova et al., 1976; Wring et al., 1990). Matsumoto et al. (1981) determined AA with an electrode having an ascorbate oxidase film attached to a Clark oxygen electrode. Nasser et al. (1987) proposed a potentiometric titration with hexamine Co(II) tricarbonate cobaltate. Other electrochemical methods, such as the voltammetric ones, were proposed, including amperometric determination of AA with a graphite-epoxy composite electrode chemically modified with cobalt phthalocyanine (Hart, 1984; Wring et al., 1990) and a carbon fiber microelectrode with a thin copper-heptacyanonitrosylferrate film on its surface (Gao et al., 1993). The electrochemical methods require fewer steps to prepare the sample than the colorimetric or the chromatographic ones. However, all of the methods mentioned above need, at least, a specific reagent not always available in every laboratory or a specific device such as an electrode, which has a finite working life.

Taking into account that electroanalytical methods present the advantages previously described, the aims of this work are to validate a voltammetric iodometry to determine ascorbic acid. The technique is performed by means of a bipotentiometric titration, improved by end-point detection, carried out with the highly versatile

* To whom correspondence should be addressed. Fax: 54-341-4372704. E-mail: ifise1@citynet.net.ar.

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platinum electrodes. The approach is advantageous, as it avoids the ultracentrifugation or filtration step, since the titration can be made in the presence of particles in suspension. Moreover, it requires very elementary equipment, easily available reagents of low cost, and one pair of nonexpiring platinum electrodes. In addition, redox iodometry was verified to be accurate for quantification of AA in fresh vegetables and fruits. The method has been proved in two different samples of vegetable and fruit. A good correlation has been obtained when the results were compared with those obtained using HPLC method with UV detection.

MATERIALS AND METHODS

General Outline of the Method. A known excess of iodine is generated through the following reaction:

$$9I^- + Cr_2O_7^{2-} + 14H^+ \rightleftharpoons 3I_3^- + 2Cr^{3+} + 7H_2O \quad (1)$$

Ascorbic acid ($C_6H_8O_6$) is a weak reductant, which reacts stoichiometric and rapidly with the generated iodine to give dehydroascorbic acid (DHAA: $C_6H_6O_6$) according to:

$$C_6H_8O_6 + I_3^- + H_2O \rightleftharpoons C_6H_6O_6 + 2H^+ + 3I^- \quad (2)$$

The remainder iodine is then quantified with sodium thiosulfate previously standardized by means of a redox bipotentiometric titration.

$$I_3^- + 2S_2O_3^{2-} \rightleftharpoons 3I^- + S_4O_6^{2-} \quad (3)$$

The proposed titration is carried out in galvanostatic conditions allowing dead-stop end-point detection. The amount of AA can therefore be calculated as the difference between the iodine equivalents generated and the iodine excess titrated with thiosulfate.

Apparatus. The apparatus and instruments used in the different methods were as follows.

Voltammetric Titration. Determination of AA by voltammetric titration was carried out using a Metrohm Herisau Precision Potentiometer E 353B (Switzerland) with a Metrohm 6.0308.100 (Switzerland) double platinum electrode. Although the progress of all the titrations reported in this work was followed with the above-mentioned apparatus, other model of potentiometer-pH Meter (Metrohm 713 pH Meter) was also used to verify the reproducibility of the results.

HPLC Analysis. Determination of AA by HPLC was performed in a Waters 600 HPLC system (Milford, Massachusetts), with a Phenosphere (5 mm; ODS2; 250 × 4.6 mm) (Phenomenex U.K. Ltd.) column. Detection was done by UV absorption at 245 nm (Waters 486 Tunable Absorbance Detector). At a flow rate of 1 mL/min (Waters 600 Pump working at 2800 PSI), the retention time for AA was 3.15 min. Identification of the AA peak was carried out using a highly purified standard solution prepared as described below.

Reagents. The stock and standard solutions were prepared dissolving the highest analytical grade reagent commercially available (Sigma Chemical Co. and Aldrich Chemical Co. Ltd.) in distilled deionized water, which had been boiled in an attempt to remove oxygen to minimize unwanted decomposition reactions such as oxidation of both AA and iodide. The following stock solutions were used: 0.1500 N $K_2Cr_2O_7$, 5% W/V KI, H_3PO_4 (pH 0.5), and ca. 0.04 N $Na_2S_2O_3$. The latter solution was stored under refrigeration and standardized using iodometric titration with voltammetric final end-point detection every other day. Standard ascorbic acid solutions were prepared dissolving the ACS reagent (Sigma Chemical Co.) in distilled-deoxygenated water, immediately before use. Supplementary solutions used were the following: preserving solution of metaphosphoric-acetic acid (3% W/V metaphosphoric acid, 8% V/V acetic acid) and the HPLC mobile phase (HPLC-grade solvents and chemicals). The HPLC mobile phase was a 60:28:12% V/V mixture of potassium dihydrogen phosphate buffer (55 mmol/L, adjusted to pH 3 before mixing), methanol, and 1-hexanesulfonic acid sodium salt (55 mmol/L), filtered through a 0.2-mm pore-size filter (Millipore, Bedford, MA).

Ascorbate oxidase (Sigma Chemical Co.) was used to prove the absence of collateral reactions by means of specificity assays. The enzyme (250 U) was dissolved in 2.5 mL of glycerol plus 2.5 mL of 100 mmol/L monopotasium phosphate solution adjusted to pH 6, divided into portions and stored at −40 °C until use.

Preparation of the Iodine Solution. Two milliliters of 0.1500 N $K_2Cr_2O_7$, contained in the titration glass flask, was treated with 4.0 mL of KI plus 10.0 mL of H_3PO_4. The reaction was allowed to progress for 2 min before adding the sample. The extraction of vitamin C was carried out during this period.

Samples. Tests were carried out on samples of one vegetable (spinach) and of one fruit (kiwi). Fresh fruits and vegetables were obtained from local markets. The operative processes were the usual ones: green spinach leaves were washed in running water, drained, and finally weighed (about 10 g/sample). Kiwi fruits were peeled and cut into suitable, representative portions of the proper weights (about 10 g/sample).

Extraction Method. Different extraction methods were applied, according to the methodology used and the assay performed.

Voltammetric Titration. Each sample was prepared by weighing a sufficient amount of vegetable or fruit to provide ca. 10 mg of AA/sample. Twenty-five milliliters of distilled-deoxygenated water was added, and the sample was homogenized with a Braun knife homogenizer for two minutes. The homogenate was immediately added onto the iodine solution prepared as described above, covered with aluminum foil, and incubated for one minute at room temperature. This solution was then used for titration.

Specificity Assays. Kiwi fruit portions were homogenated with monopotasium phosphate solution (100 mmol/L) and pH was adjusted to 6 before adding the ascorbate oxidase aliquot. In ancillary experiments, studies of enzymatic kinetics were performed to determine the amount of ascorbate oxidase required to degrade, in a period not longer than 15 min, the total amount of AA contained in the samples. Preceding experiments were performed to follow, by UV spectroscopy, the kinetics of the enzymatic reaction in standard solutions of AA. Five units of the enzyme was enough to completely degrade 20 mg of AA in 15 min. Therefore, aliquots of 100 μL of the enzyme solution were systematically used to achieve utter oxidation of the AA present in the samples. The pH of the sample was then adjusted to 0.5−0.8, and this suspension was added into the iodine solution, prepared as described above.

HPLC Analysis. Kiwi fruits were peeled and cut into portions of about 10 g/sample. A total of 25 mL of metaphosphoric-acetic acid solution was added, and the sample was homogenized with a Braun knife homogenizer for 2 min. Homogenates were submitted to 15 000 rpm in a refrigerated centrifuge for 10 min. A portion of 20 μL of the clear supernatant was diluted to 2.0 mL, and an aliquot of this solution was filtered through a 0.2-mm pore-size filter (Millipore, Bedford, MA), before being injected into the HPLC system (loop 50 mL).

Titration. The platinum electrodes were immersed into the problem solution, which was automatically stirred at constant rate during titration. The typical current intensity used was 0.25 μA, although other intensities were also used, ranging from 0.25 to 2.5 μA. The sodium thiosulfate solution was added using a 10.00 mL calibrated microburet. The potential difference between both electrodes was registered against the volume of the titrating agent. In the vicinities of the equivalence point, there is a cathodic polarization, which causes a dramatic instability in the reading device. From that point on, the titration was continued drop by drop, and the end-point was considered to be reached just before the maximum deflection of the potential occurred. Thus, the end-point was obtained deducting the volume of one drop from the volume

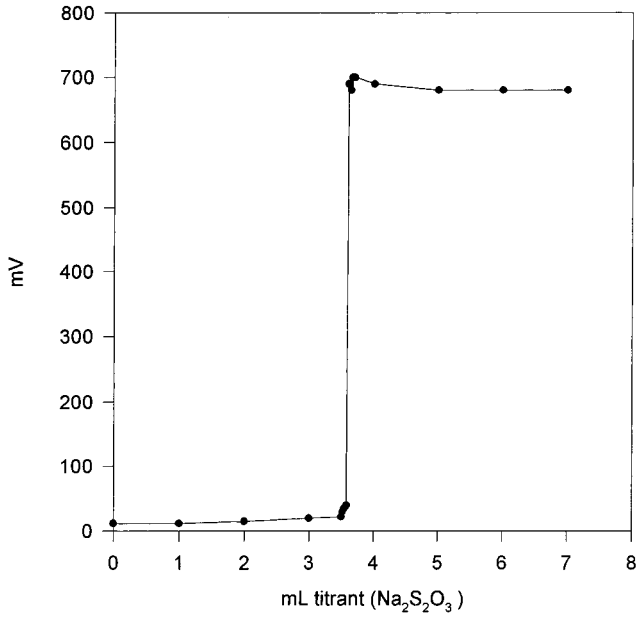

Figure 1. Potential difference as a function of titrant volume added. Typical curve obtained for a sample containing 10 mg of ascorbic acid, working at a current of 2.5 μA.

of titrant used to produce the utmost shift of potential difference. After the end-point, the deflection of potential decreased again (Van Name and Fenwick, 1925; Foulk and Bawden, 1926; Bard and Faulkner, 1980). A typical titration curve of a bipotentiometric dead-stop end-point titration is shown in Figure 1.

RESULTS AND DISCUSSION

Iodometric techniques have been largely used to quantify weak reductants that cannot be determined by primary standards, which are strong oxidizing agents (Harris, 1991; Kolthoff and Elving, 1978). Iodometry has already been used to quantify AA in citrus juices and soluble samples of vitamin C (Stevens, 1938; Bailey, 1974). Many of these methods titrate the ascorbic acid directly with iodine solution. This procedure presents the inconvenience of demanding an oxygen-free atmosphere to carry out the titration, due to instability of ascorbic acid. The approach proposed in this work is an iodometric back-titration with dead-stop end-point voltammetric detection. Although this method does not measure directly AA, the technique has the known benefits of retrotitration, since ascorbic acid reacts very rapidly with iodine, thus avoiding the risks of oxidation caused by the oxygen dissolved. The excess of generated iodine is then quantified, and the end point is easily detected in a few minutes. The technique avoids tedium and is free of potential limitations related with AA degradation, as expected to occur in other indirect titrations.

First, optimum assay conditions were needed to be determined. The preferred chemical for acidification was phosphoric acid. This choice was made on the basis that phosphoric acid not only confers the protons, which take part in reaction 1, but also can complex ferric iron. This ion, commonly present in natural samples, interferes since it reacts with one of the reagents, the iodide, according to:

$$2Fe^{3+} + 3I^- \rightleftharpoons I_3^- + 2Fe^{2+} \qquad (4)$$

Iodide can be oxidized by oxygen in the presence of H^+ according to:

$$^1/_2O_2 + 3I^- + 2H^+ \rightleftharpoons H_2O + I_3^- \qquad (5)$$

To verify whether reaction 5 is negligible or is an actual interference of the method, experiments were carried out working under nitrogen atmosphere or air atmosphere. Two sets of five replicates each were used to titrate sodium thiosulfate, working at pH 0.5. The values obtained under both conditions were not significantly different with each other ($7.77 \times 10^{-2} \pm 3.5 \times 10^{-4}$ N and $7.74 \times 10^{-2} \pm 7.9 \times 10^{-4}$ N under nitrogen and air atmosphere, respectively; $p = 0.288$, as assessed by the unpaired Student *t*-test).

Taking into account that reaction 5 is favored by large amounts of iodide and H^+, titrations were carried out using different working pH, different concentrations of iodide solutions, and different incubation time. These tests led to the establishment of the following conditions: the pH of the H_3PO_4 must range between 0.2 and 0.8, and the acid must be added 2 min before adding the sample. For pH <0.2, reaction 5 becomes not negligible. For pH >0.8, titrations may become tedious, since they can demand a length of time. It is recommended that the redox equivalents of potassium iodide added do not exceed five times the equivalents of potassium dichromate. When greater excesses of potassium iodide were added, spontaneous oxidation of iodide by oxygen became not negligible at the chosen working pH.

The reaction time between AA and iodine was varied from 1 to 10 min, presenting equivalent results for the whole range of time studied, provided the pH of the iodine solution was not extremely low. For H_3PO_4 solutions of pH <0.5, it is recommended to backtitrate soon after the addition of the sample onto the iodine solution, to prevent interferences due to reaction 5. Thus, the kinetics of the reaction proved to be appropriate for practical use. For operational reasons, a typical reaction time of 1 min was considered adequate before starting the titration.

Accuracy, Precision, and Detection Limit. Standard solutions of ascorbic acid were titrated using the described voltammetric end point detection method. Six series of five replicates each, containing the same amount of analytical grade AA, were analyzed using the proposed methodology. The titrations of standard AA solutions were performed for amounts of ascorbic acid ranging from 0.1 to 15 mg. A tight correlation between the measured values of AA and standard solutions was obtained. The results registered for the sets of five replicates at each concentration are presented in Figure 2, the slope being 0.9943 ± 0.0126. However, it should be noticed that the amount of AA to be quantified determined the rate of titration. This is a consequence of the attempt to minimize errors when performing any backtitration. Accordingly, the volume of thiosulfate consumed to evaluate the remnant iodine should be about half of the volume consumed when no AA is present. Hence, the smaller the amount of AA contained in the sample, the longer the time required for the potential to be stabilized after each addition of the titrant, as a consequence of using diluted solutions. For practical reasons, it is advised to use a sample size containing ca. 10 mg of AA to perform the titration in a suitable time. This amount allows a rapid assessing of

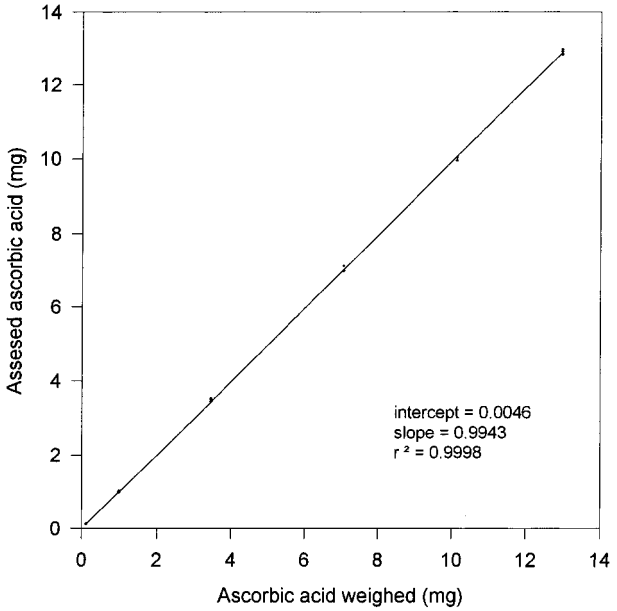

Figure 2. Ascorbic acid (AA) measured as a function of AA weighed. The regression line, as assessed by least-squares method, was $y = (0.9943 \pm 0.0126)\,x + (0.005 \pm 0.017)$.

AA. Lower amounts of AA will make the procedure slower and, therefore, more time consuming. In those samples in which the content of AA is lower than that of spinach or kiwi fruit, for instance red kidney beans, proportionally higher amounts of plant product samples will be required to be blended with 25 mL of distilled water, if a similar time scale for AA determination is to be kept. In case the resulting suspension is too thick to allow, for instance, for an adequate stirring, a lesser amount of plant product sample should be added, but the procedure will become slower. The limit of detection (0.1 mg of AA) is low enough to allow an accurate determination of AA, even in plant product samples with low AA content.

It is noteworthy that standard solutions containing 10.12 mg of AA, i.e., approximately the amount suggested to be titrated by this approach, have a standard deviation of 0.05. These results show not only that a proper stoichiometry for the reaction exists but also the accuracy of the method.

The proposed method uses water as extractant, since the sample processing takes no longer than 3 min. However, as reference techniques use preserving solution, it was necessary to determine that homogenizing the sample with distilled water does not lead to lower results as compared with those obtained when the preparation of the sample was carried out using metaphosphoric-acetic acid as the extracting solution. Two sets of seven replicates of kiwi samples were analyzed for each extraction method. The results are indicated in Table 1. From them, it can be inferred that the absence of preserving solution does not reduce the amount of AA titrated.

Assay Specificity. Vegetables and fruits contain, in addition to AA, various reducing substances such as thiols, sulfydryl compounds, phenols, trioses, reductones or glutathione, which might be titrated together with AA. To assess the validity of the proposed method, it is essential to demonstrate that the presence of those compounds occurring in natural samples produce negligible interference. For this purpose, assays were performed on samples previously treated with ascorbate

Table 1. Comparison of Two Different Extraction Methods[a]

sample	water	preserving solution
1	112.8	109.7
2	112.6	117.1
3	110.8	106.0
4	117.0	115.9
5	108.1	114.2
6	119.5	122.6
7	110.6	107.4
mean	113	113
SD	4	6
p	>0.05	

[a] Ascorbic acid content (mg/100 g) in kiwi fruit, as determined on samples homogenized in methaphosphoric-acetic acid preserving solution and samples homogenized in water.

oxidase. This enzyme uses substrates which possess a cyclic structure with an endiolic system adjacent to a carbonyl group (Dawson, 1966). This property confers the enzyme high specificity toward AA (Casella et al., 1989).

After treating the sample with the enzyme, the titration was carried out, expecting to consume the same amount of titrant as if no AA was present in the sample. The mean value obtained from four kiwi fruit samples treated with the enzyme was only 1.5 mg/100 g, as compared with the average value obtained in untreated samples (113 mg/100 g). As can be seen, the extracts treated with ascorbate oxidase may still cause iodine reduction to a very low extent. This suggests that collateral reactions exist and that other reductants different from AA compete with reaction 2. However, the values determined represent only the 1.3% of the total AA present in the samples. Thus, admitting this as the error of the method, species other than AA can be considered negligible interferences for the titration in the natural samples studied.

This method quantifies AA through its reducing properties, rendering DHAA. The presence of the oxidized form, DHAA, is not expected to alter the results since this compound shows no reducing properties. Even when this possibility is unlikely, it was directly verified by assessing a fixed amount of AA (1 mg) in the presence of variable amounts of DHAA (1, 5, 10, and 20 mg). No change in the values of AA recorded was apparent (data not shown). Hence, changes in the proportion of DHAA in the samples do not influence AA determination using this approach.

To verify the absence of physical interference, AA was determined in the presence of a polysaccharide typically found in vegetable fiber, such as pectin. Different amounts of citrus pectin, ranging from 6 to 8 mg, were added to six different samples containing a known amount of AA (7 mg). This renders a final pectin-to-AA ratio higher than that found in unripe fruits, which are natural products containing high proportion of pectin. The value of AA evaluated by the proposed voltammetric titration was 7.0 ± 0.1 mg. Therefore, the amount of AA determined was virtually the same than the added amount, regardless the presence of pectin in the standard solution. This result indicates that this physical interference is negligible.

Method Comparison. In this study, the results obtained via a voltammetric titration have been compared with those obtained using HPLC analysis, aimed to detect possible differences in the quantification of AA in natural samples.

Table 2. Method Comparison[a]

sample	HPLC	voltammetry
1	138.6	140.0
2	126.6	120.6
3	138.3	140.9
4	126.2	123.7
mean	132	131
SD	7	11
p	>0.05	

[a] Ascorbic acid content (mg/100 g) determined in kiwi fruit samples by means of HPLC−UV detection and voltammetric titration.

Table 3. Recovery Study[a]

	kiwi fruit				spinach			
sample	1	2	3	4	1	2	3	4
amounts								
initial (mg)	9.32	7.29	7.66	7.00	6.45	7.72	5.58	5.21
added (mg)	6.88	7.78	8.56	6.68	4.07	4.32	4.28	4.40
found (mg)	15.66	14.77	15.84	13.79	10.20	11.96	9.54	9.36
recovery (%)	92	96	96	102	92	98	93	94
mean	96%				94%			
SD	4%				3%			

[a] Percent recovery of ascorbic acid in four different samples of kiwi fruit and four different samples of spinach.

Table 2 displays the results for the quantification of ascorbic acid in four different kiwi samples, as determined simultaneously by both HPLC and the voltammetric method. A statistical analysis of the values by a one-way ANOVA (Snedecor, 1969) showed no difference between both methods at $p = 0.05$. These results suggest that, as far as precision and exactitude is concerned, both methods are equivalent.

It should be noticed that this voltammetry allows one to determine AA, but it cannot be used to simultaneously evaluate DHAA, the oxidized form of vitamin C presenting an equivalent antiscorbutic activity. On the other hand, a separative method like HPLC presents the advantage of allowing both AA and DHAA to be determined simultaneously. However, besides its antiscorbutic properties, AA has a number of additional beneficial effects as compared with DHAA. The most noticeable of these benefits is that AA is an antioxidant due to its reductive properties, which are not shared by its oxidized form. Therefore, the relevance of determining AA should not be overviewed. Since AA is the main form of vitamin C in fresh plant products (McCance and Widdowson, 1992) and the proposed method permits to evaluate accurately AA, the amount of this vitamer determined by this voltammetry can be considered a good estimation of the contents of vitamin C in fresh plant products.

Recovery Study. Sets of four different spinach samples and four different kiwi fruit samples were supplemented with AA (ca. 90% of the content determined previously) to evaluate the extent of ascorbic acid recovery using the voltammetric titration technique. Table 3 shows the results obtained for each set of sample. The AA recovered was virtually the same amount of ascorbic acid added, being the average recovery 96 ± 4% in kiwi fruit and 94 ± 3% in spinach. These results further show that the samples present negligible amounts of inhibitors that interfere in the proposed titration method.

Conclusions. A bipotentiometric dead-stop end-point titration is proposed to determine the contents of AA in natural fruits and vegetables. The method comparison, the specificity assay and the recovery studies demonstrated that AA can be accurately determined by this method. The benefits of this technique are (1) simplicity, since it bypasses preparation steps such as ultracentrifugation or filtration, and (2) feasibility, regarding the availability of reagents and apparatus used, which turns out in an efficient and low cost methodology for quantifying ascorbic acid.

ABBREVIATIONS USED

AOAC, Association of Official Analytical Chemists; HPLC, high-performance liquid chromatography; AA, ascorbic acid; DHAA, dehidroascorbic acid; SD, standard deviation; ANOVA, analysis of the variance; IFISE, Instituto de Fisiología Experimental; CONICET, Research Council, Argentina.

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