Ascorbic Acid Determination by an Amperometric Ascorbate Oxidase-based Biosensor

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The aim of this work is to develop a method for ascorbic acid determination in some fruit juices, by using an ascorbate oxidase-based amperometric enzyme electrode. The enzyme was immobilized on a nylon (Biodyne A) membrane by glutaraldehyde linking. The enzyme-membrane was consecutively attached to the polyethylene membrane of a Clark oxygen electrode, which functioned as transducer. The analytical characteristics of the biosensor (linear range, sensitivity, selectivity, response time, stability) were investigated, as well as the influence of the enzyme loading. The value of the current intensity was monitored, as a function of time, for different ascorbic acid concentrations. The obtained calibration graph was linear within the range 0.10-0.55 mM. The optimum biosensor response was obtained for an ascorbate oxidase amount immobilized on the membrane of 100 U. The values of vitamin C content of the analysed fruit juices ranged between 0.48 and 1.98 mM.

Keywords: amperometric biosensor, ascorbic acid, ascorbate oxidase, oxygen electrode

Ascorbic acid is an antioxidant with therapeutic properties, which plays an important role in activating the immune response, in wound healing, in osteogenesis, in detoxifying the organism, in iron absorbtion, in collagen biosynthesis, in preventing the clotting of blood vessels, and in many other metabolic processes [1-3].

Vitamin C can be easily oxidized, its degradation being accelerated by by heat, light and the presence of heavy metal cations [4, 5]. Thus, due to its content variation, vitamin C represents an important quality indicator of foodstuffs [6] and contributes to the antioxidant properties of food [7, 8].

Traditional methods for ascorbic acid assessment involve titration with an oxidant solution, such as dichlorophenol indophenol (DCPIP) [9], potassium iodate [10] or bromate [11]. Volumetric techniques can suffer from lack of specificity [12].

Chromatografic methods provide a more accurate and sensitive tool for vitamin C determination: HPLC with electrochemical detection has turned out to be a selective and sensitive method for ascorbic acid assessment in foodstuffs and biological fluids [13, 14].

Fluorimetric methods are based on dehydroascorbic acid reaction with o-phenylene diamine [15, 16]. UV-VIS absorbance-based assays were used for quantification of ascorbic acid in horticultural products [17]. Other optical methods for vitamin C estimation are based on flow injection analysis [18-20], spectrophotometrical determination of iodine reacted with ascorbic acid [21] and chemiluminescence [22].

Electrochemical methods have the advantage of being sensitive, require only small volumes of samples, the dynamic range being quite extended [23].

The vitamin C content of apple juices has been monitored by cyclic voltammetry using a Pt working electrode [6, 24]. Recently, the use of various voltammetric techniques has been combined with modified ascorbic acid sensors: square-wave voltammetry was used to determine ascorbic acid, based on its oxidation at a zeolite modified carbon paste electrode [25]. Cyclic and differential pulse voltammetry were used for electrocatalytical ascorbic acid determination, at a carbon paste electrode, modified with 2, 7-bis (ferrocenyl ethynyl) fluoren-9-one [26]. Cyclic voltammetry studies performed on Pt electrodes proved that the growth of Pt surface oxides and the anodic response of a variety of interferrents (glucose, cystine, oxalate) was greatly surpressed by the use of fluorosurfactant-modified Pt electrodes [27]. Ascorbic acid was determined in the presence of SO_2 and acetaldehyde by pulsed voltammetry at interdigitated Pt microelectrodes [28].

A potentiometric biosensor [29] for ascorbic acid was constructed by ascorbate oxidase immobilization in a polymeric matrix, fixed on a graphite-epoxi composite electrode. The potentiometric determination of ascorbic acid was also performed by a sensor array fabricated by screen printing, with a RuO_2 film deposited on the working area of each sensor. The potential response of the enzyme-based biosensor depends linearly on L-ascorbic acid concentration between 0.02 mM and 1 mM [30].

Amperometric biosensors were obtained by ascorbate oxidase immobilization on a nylon net [31] or on a collagen membrane, using a Clark oxygen electrode as transducer [32]. Vitamin C analysis was also performed using a glassy carbon working electrode as transducer, incorporated in a flow system [33]. Ascorbic and uric acid were determined by coupling an amperometric technique with flow analysis [34].

An amperometric sensor for ascorbic acid determination, from foodstuffs and pharmaceutical preparations was developed which was constructed by aniline electropolymerisation on a glassy carbon or screen-printed working electrode [12].

Simultaneous determination of vitamin C and glucose has also been performed using a voltammetric biosensor integrated in an automated SIA system [35].

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Table 1
THE ENZYME ELECTRODES DEVELOPED AND STUDIED FOR ASCORBIC ACID AMPEROMETRIC DETERMINATION AND THE VALUES OF THE ANALYTICAL SIGNAL OBTAINED AT 0.25 MM VITAMIN C CONCENTRATION

Enzyme electrode	Ascorbate oxidase amount-units* (volume of enzyme solution deposited on the membrane)	The value of the analytical signal- the measured current intensity (nA) for 0,25 mM ascorbic acid
1	25 (0.05 mL)	-2.01
2	50 (0.1 mL)	-1.90
3	100 (0.2 mL)	-1.42
4	150 (0.3 mL)	-1.68
5	200 (0.4 mL)	-1.89

*Immobilized on a Biodyne A (nylon) membrane, 0.45 μm porosity

Biamperometric methods [36, 37] could be utilized with good results for the determination of vitamin C in natural juices.

An amperometric sensor for simultaneous determination of uric acid and ascorbic acid using 2-[bis(2-aminoethyl)amino]ethanol, 4,4'-bipyridine bridged dicopper(II) complex was developed [38].

The determination of nanomolar uric and ascorbic acids was performed by using a gold nanoparticles modified electrode [39].

The aim of this study is the development and the study of the analytical characteristics of an ascorbate oxidase-based amperometric biosensor for ascorbic acid. The enzyme was immobilized on a nylon semipermeable membrane, fixed on an oxygen electrode.

The developed biosensor was applied to ascorbic acid content assessment in natural fruit juices (commercial and obtained by fruit pressing) and soft drinks.

Experimental part

Reagents and instrumentation

Ascorbate oxidase (Sigma Aldrich, 1000U/g), ascorbic acid (Merck), monopotassium phosphate (Riedel de Haën), disodium phosphate (Riedel de Haën), glucose (Chiompar), citric acid (Chimopar), sodium benzoate (Sigma Aldrich), Clark oxygen electrode (Loligo Systems Denmark, 7 cm length, 3 mm diameter of the active part), nylon membrane 7/7cm (Biodyne A), 0.45 μm porosity, 0.15 mm thickness, potentiostat galvanostat KSP (laboratory made by professor Slawomir Kalinowski, University Warmia and Mazury, Olsztyn), as well as the respective soft (Chronoamperometry).

The stock ascorbic acid solution 1 mM was prepared in phosphate buffer solution 0.1M, *p*H=6.0. The working solutions were obtained by diluting the stock solution with the buffer solution 0.1M, *p*H=6.0. The ascorbic acid concentration in the working solutions varied between 0.10 and 0.60 mM, as follows: 0.1, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55 and 0.60 mM.

The ascorbate oxidase solution (500 U/mL activity) was prepared by dissolving the respective amount of enzyme in the phosphate buffer solution 0.1M, *p*H=6.0.

The dichlorophenol indophenol (DCPIP) solution, 0.001 n, was prepared by dissolving 145 mg DCPIP, sodium salt (Merck), in 100 mL hot distilled water and a subsequent addition of 300 mL phosphate buffer, 0.066 moles L^{-1}, *p*H= 6.98, previously prepared by mixing the respective volumes of potassium dihydrogen phosphate and sodium monohydrogen phosphate solutions (2/3 ratio). Distilled water was added to the final volume of 1000 mL. After homogenisation, the solution was kept in a dark place (protected from light) and filtered [9].

The phosphate buffer solution 0.1M, *p*H=6.0, used for ascorbate oxidase, as well as for acid ascorbic solubilisation, was prepared by mixing the potassium dihydrogen phosphate 0.1 M and sodium monohydrogen phosphate 0.1 M solutions in a 7.13/1 ratio

The glutaric dialdehyde solution (25%) was purchased from Sigma Aldrich.

Biosensor construction

A volume comprised between 0.05 and 0.4 mL (table 1) of the enzyme solution (500 U ascorbate oxidase/mL), prepared in phosphate buffer 0.1 M, *p*H=6.0, was poured in the center of a nylon membrane (7/7cm).

After complete drying of the enzyme layer (4 h at 4°C) the reticulation of ascorbate oxidase was performed by depositing 0.4 mL of glutaric dialdehyde 0.05 % in the center of the membrane [32].

After complete drying, 4 h at 4°C, the enzyme membrane was immobilized on the outer polyethylene membrane of the oxygen electrode and fixed with an O-ring. Five such enzyme membranes were obtained (table 1).

Working procedure for vitamin C assessment by using the ascorbate oxidase-based biosensor

The ascorbic acid concentration in the working solutions varied as follows: 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55 and 0.60 mM.

The current intensity variation as a function of time, due to the the reduction of the dissolved molecular oxygen non-consumed in the enzymic reaction, was followed using the programme Chronoamprometry.

The variation of the current intensity was registered as a function of time, for 120 s, for the standard solutions as well as for the analysed samples. The measurements were performed at 22°C, in stirred solutions. The sensor response corresponding to a time of 30 s after immersion in the solution (or sample), was taken into account. The volume of the analysed samples was 50 mL. A phosphate buffer solution, 0.1 M, *p*H=6.0 was used for analyte and enzyme dissolution.

Results and discussions

Investigation of the analytical characteristics of the obtained biosensor

The current intensity variation in time was followed for each of the five enzyme electrodes (table 1), at different ascorbic acid concentrations. The working procedure described at Experimental part was applied. Figure 1 illustrates a typical chronoamperogram recorded with the KSP potentiostat, at ascorbic acid determination in a grapefruit juice (Santal), using the enzyme electrode 3 (table 1).

The chronoamperograms obtained at different ascorbic acid concentrations are presented in figure 2 (enzyme electrode 3, table 1).

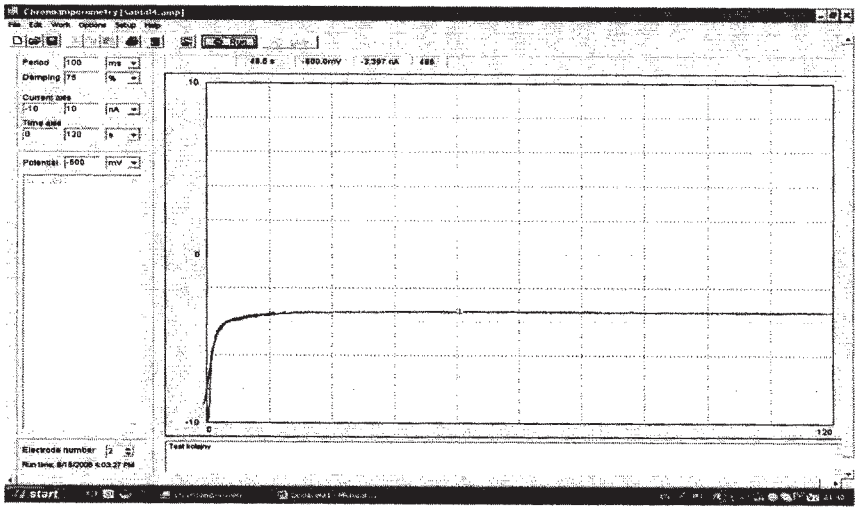

Fig. 1. A typical chronoamperogram recorded with the KSP potentiostat, at ascorbic acid determination in a grapefruit juice sample (Santal), using the enzyme electrode 3. The working procedure described at Experimental part was observed

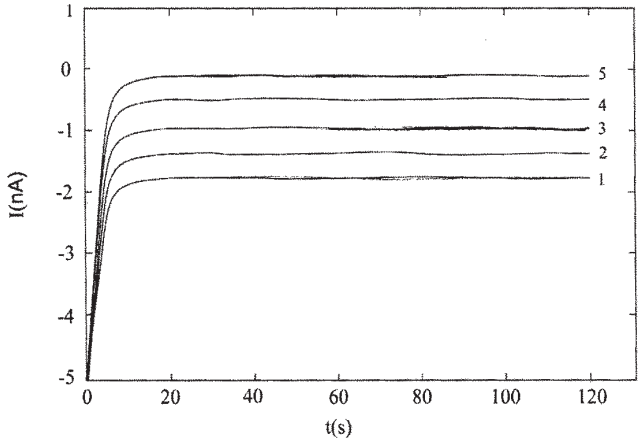

Fig. 2. The amperometric biosensor response (enzyme electrode 3) obtained for different ascorbic acid concentrations: (1) 0.15 mM, (2) 0.25 mM, (3) 0.35 mM, (4) 0.45 mM, (5) 0.55 mM; the working procedure described at Experimental part was used

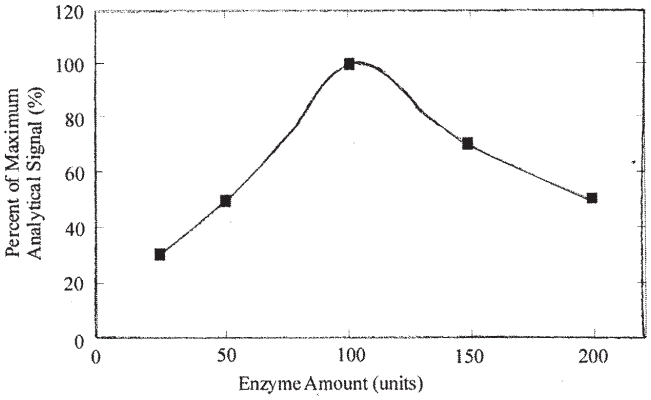

Fig. 3. Influence of the ascorbate oxidase amount, immobilized on the nylon membrane fixed on the oxygen electrode, on the response of the amperometric biosensor. The working procedure described at Experimental part was used.

For the study of the influence of the enzyme loading on the analytical signal (fig. 3), the ascorbate oxidase amount immobilized on the nylon membrane varied between 25 and 200 U. By analysing figure 3, it can be noticed that the highest value of the analytical signal was obtained for an ascorbate oxidase amount of 100 U.

The calibration graphs

The calibration graphs obtained for vitamin C determination by using the enzyme electrodes 3 and 5 (table 1) are presented in figure 4. A linear dependence between the measured current intensity and the ascorbic acid concentration can be noticed within the range 0.10-0.55 mM ascorbic acid. The equations of the calibration graphs are:

$$y = 4.151\,x - 2.465;\ r^2 = 0.9988,\ \text{for enzyme electrode 3} \quad (1)$$

and

$$y = 2.020\,x - 2.401;\ r^2 = 0.9963\ \text{for enzyme electrode 5,} \quad (2)$$

where x represents the ascorbic acid concentration, expressed as mM and y represents the measured current intensity, expressed as nA.

The slopes of the calibration graphs obtained for enzyme electrodes 3 and 5 were 4.15 + 0.114 nA/mM and 2.02 + 0.0545 nA/mM respectively. The maximum value of the sensitivity was obtained for enzyme electrode 3, which was subsequently used for real sample analysis.

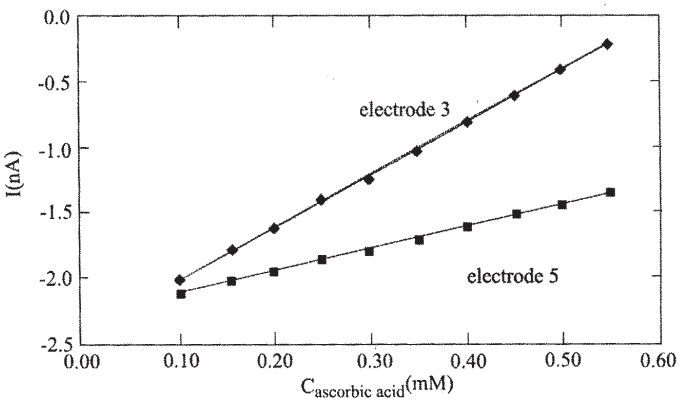

Fig. 4. The calibration graph obtained for the ascorbate oxidase-based biosensor: enzyme electrode 3 (♦) and enzyme electrode 5 (■). The working procedure described at Experimental part was used

The precision of the determination obtained for the enzyme electrode 3 (table 1) was proved by a RSD value of 2.67% (c=0.15 mM, n=10). The analytical signal obtained at 30 s after immersion of the enzyme electrode in the analysed sample was taken into consideration.

The amperometric biosensor stability was verified by recording the analytical signal every three days, for a 0.15 mM ascorbic acid solution, using the enzyme electrode 3 (table 1).When not used for amperometric determinations, the enzyme electrode was kept in a 0.1 M phosphate buffer, *p*H=6.0 at 4^0C.

The amperometric biosensor response was constant for 10 days. For this period, the decrease of the analytical

Table 2
RESULTS OF THE INTERFERENCE STUDY PERFORMED ON SOME ORGANIC COMPOUNDS COMMONLY FOUND IN FRUIT JUICES

Interferent	Interferent/analyte (molar ratio)	Influence on the analytical signal
Glucose	100	< 3.0%
	150	1.6% increase
	200	4.3% increase
Citric acid	150	< 3.0%
	200	3.0% decrease
Benzoate anion	200	< 3.0%

Table 3
DILUTION DEGREES AT FRUIT JUICES ANALYSIS BY THE AMPEROMETRIC BIOSENSOR

Product	Dilution degree
Prigat peach	1/5
Prigat orange	1/5
Fanta lemon	1/4
Santal grapefruit	1/13
Tymbark orange	1/8
Frutia orange	1/8
Cappy grapefruit	1/3
Frutti fresh Tutti frutti	1/5
Lemon juice (fruit pressing)	1/13
Orange juice (fruit pressing)	1/11

signal was smaller than 3%. The stability studies were not performed for periods longer than ten days. Nevertheless, we expect the analytical signal to diminish: the data published in literature [32] indicate a decrease of the analytical signal of 14%, after five weeks.

The detection limit calculated for enzyme electrode 3 was of 0.023 mM and the limit of quantification was 0.076 mM.

The detection limit was calculated as LOD= 3 s/m, where s represents the square mean error, calculated for 10 determinations of the blank and m represents the slope of the calibration graph. The limit of quantification was calculated as LOD= 10 s/m, where s and m were given above.

Interference studies

The interference studies proved that glucose and citric acid have no influence on the analytical signal (error<3%), in concentrations up to 100 times and, respectively, 150 times higher than that of the analyte. A citric acid concentration 200 times higher than that of ascorbic acid determines a 3% decrease of the analytical signal, while a glucose concentration 150 times higher than that of vitamin C produces a 1.6% increase of the analytical signal. A glucose concentration 200 times greater than that of ascorbic acid determines a 4.3% increase of the analytical signal. Benzoate anion has no influence on the analytical signal in concentrations up to 200 times higher than those of vitamin C. The results of the interference study are presented in table 2 and represent the average of three determinations. The ascorbic acid concentration for the interference studies was 0.20 mM.

Glucose, citric acid and benzoate anion do not give significant interferences at ascorbic acid determination with the developed amperometric biosensor, up to an interferent/analyte molar ratio of 200/1. Our results are similar to those reported in literature for this type of electrode [32].

Analysis of real samples

The developed amperometric biosensor (enzyme electrode 3, table 1) was used for ascorbic acid content assessment in several real samples: soft drinks, natural commercial fruit juices, as well as natural juices freshly prepared by fruit squeezing.

Sample preparation before analysis consisted in centrifugation of natural juices which contain fruit pulp: commercial fruit juices (ex. Santal, Tymbark), as well as natural juices obtained by fruit squeezing. Soft drinks, such as Fanta, Cappy, Frutti Fresh, did not necessitate centrifugation, only dilution of the clear sample with the phosphate buffer solution being necessary.

All clear samples were diluted with the phosphate buffer solution, 0.1 M, *p*H=6.0. The dilution degrees applied to the analysis of different samples are presented in table 3.

The experimental conditions and working procedure used for standard solutions were also applied to real sample analysis.

The results, presented in table 4, were compared to those obtained by the volumetric method with dichlorophenol indophenol.

Working procedure for vitamin C titrimetric determination with dichlorophenol indophenol (DCPIP):

A sample of 2.5 mL clear juice was diluted with distilled water to a final volume of 10 mL, in order to diminish the colour intensity of the sample. Then it was titrated with the dichlorophenol indophenol (DCPIP) 0.001 n solution, until a pink tint appears that persists for about 30 s.

The results obtained at ascorbic acid determination by the volumetric method with DCPIP and with the developed amperometric biosensor, as well as the degrees of recovery of ascorbic acid amounts added to the analysed sample are presented in table 4. The results obtained by the amperometric biosensor represent the average of five determinations and the results obtained by the titrimetric method represent the average of three determinations.

For verifying the degrees of recovery of added ascorbic acid amounts, different volumes (comprised between 12.5 and 65 μL, which corespond to amounts ranging from 2.2 to 11.4 mg vitamin C) from the 1M stock solution, were added to 50 mL juice. Given the small volumes added from the concentrated ascorbic acid solution, no corrections of

Table 4

RESULTS OBTAINED AT ASCORBIC ACID DETERMINATION IN FRUIT JUICES, BY THE TITRIMETRIC METHOD WITH DCPIP, WITH THE AMPEROMETRIC BIOSENSOR AND THE DEGREES OF RECOVERY OF KNOWN AMOUNTS OF VITAMIN C ADDED TO THE ANALYSED SAMPLE

Analysed sample	Ascorbic acid concentration (mM) determined by the volumetric method with DCPIP	Ascorbic acid concentration (mM) determined with the developed amperometric biosensor	Ascorbic acid amount (mM) added to the sample (Ist addition)	Ascorbic acid concentration (mM) determined with the amperometric biosensor Ist addition	Degree of recovery (Ist addition) %	Ascorbic acid amount (mM) added to the sample (IInd addition)	Ascorbic acid concentration (mM) determined with the amperometric biosensor IInd	Degree of recovery (IInd addition) %
Prigat orange	$0.85 \pm 0.03^{*}$	$0.79 \pm 0.02^{**}$	0.25	$1.02 \pm 0.02^{**}$	$92.0 \pm 2.1^{**}$	0.50	$1.24 \pm 0.03^{**}$	$90.0 \pm 2.3^{**}$
Prigat peach	$0.70 \pm 0.02^{*}$	$0.76 \pm 0.02^{**}$	0.25	$1.0 \pm 0.02^{**}$	$96.0 \pm 2.1^{**}$	0.50	$1.25 \pm 0.03^{**}$	$98.0 \pm 2.0^{**}$
Fanta lemon	$0.61 \pm 0.02^{*}$	$0.62 \pm 0.01^{**}$	0.20	$0.81 \pm 0.02^{**}$	$95.0 \pm 2.5^{**}$	0.40	$0.99 \pm 0.02^{**}$	$92.5 \pm 1.8^{**}$
Cappy grapefruit	$0.47 \pm 0.02^{*}$	$0.48 \pm 0.01^{**}$	0.15	$0.64 \pm 0.02^{**}$	$106.7 \pm 2.7^{**}$	0.30	$0.765 \pm 0.02^{**}$	$95.0 \pm 2.5^{**}$
Frutti fresh tutti frutti	$0.82 \pm 0.03^{*}$	$0.79 \pm 0.02^{**}$	0.25	1.025 ± 0.02	$94.0 \pm 1.8^{**}$	0.50	$1.275 \pm 0.02^{**}$	$97.0 \pm 1.8^{**}$
Fruttia orange	$1.20 \pm 0.03^{*}$	$1.26 \pm 0.02^{**}$	0.40	$1.65 \pm 0.05^{**}$	$97.5 \pm 2.7^{**}$	0.80	$1.99 \pm 0.05^{**}$	$91.2 \pm 2.1^{**}$
Tymbark orange	$1.26 \pm 0.04^{*}$	$1.28 \pm 0.03^{**}$	0.40	$1.70 \pm 0.04^{**}$	$105.0 \pm 2.02^{**}$	0.80	$2.04 \pm 0.04^{**}$	$95.0 \pm 2.5^{**}$
Santal grapefruit	$1.80 \pm 0.05^{*}$	$1.89 \pm 0.04^{**}$	0.65	$2.50 \pm 0.05^{**}$	$93.8 \pm 1.7^{**}$	1.30	$3.12 \pm 0.08^{**}$	$94.6 \pm 1.8^{**}$
Orange juice (fruit pressing)	$1.73 \pm 0.06^{*}$	$1.64 \pm 0.04^{**}$	0.55	$2.14 \pm 0.04^{**}$	$90.9 \pm 1.7^{**}$	1.10	$2.77 \pm 0.05^{**}$	$102.7 \pm 2.0^{**}$
Lemon juice (fruit pressing)	$2.0 \pm 0.06^{*}$	$1.98 \pm 0.05^{**}$	0.65	$2.57 \pm 0.07^{**}$	$90.8 \pm 2.5^{**}$	1.30	$3.22 \pm 0.07^{**}$	$95.4 \pm 2.0^{**}$

*The standard deviation calculated for three determinations

**The standard deviation calculated for five determinations

volume were applied to the analytical signal obtained after addition and for calculating the degrees of recovery.

By analysing the results presented in table 4, it can be noticed that the values of ascorbic acid concentration (comprised between 0.47 and 2.0 mM) obtained with the amperometric biosensor are in good agreement with those obtained by the titrimetric method with DCPIP. This correspondence, along with the results of the interference study (table 2) and with the values of the degree of recovery (comprised between 90.0 and 106.7%) confirm the accuracy of the method and the lack of matrix effects.

Conclusions

Five amperometric enzyme electrodes were developed for ascorbic acid determination.

An optimum enzyme loading of 100 U ascorbate oxidase immobilized on the enzyme membrane was obtained.

The analytical response obtained for the most sensitive developed enzyme electrode was linear between 0.10-0.55 mM ascorbic acid: $y = 4.151 x - 2.465$; $r^2 = 0.9988$, where x represents the ascorbic acid concentration, expressed as mM and y represents the measured current intensity, expressed as nA. The linear range obtained for the most sensitive developed enzyme electrode is similar to the one reported in literature [32]. The sensitivity (given by the slope of the calibration graph) was 4.15 + 0.114 nA/mM for the best enzyme electrode.

The RSD value was 2.67% (c=0.15 mM, n=10), for the best enzyme electrode.

The developed enzyme electrode was applied to vitamin C determination in fruit juices.

The good agreement with the results obtained by the titrimetric method with DCPIP, as well as the values of the degrees of recovery of ascorbic acid amounts added to the analysed samples, ranging between 90.0 and 106.7% indicate the absence of matrix effects at vitamin C determination by the developed amperometric biosensor.

Glucose and organic acids, at concentrations commonly found in fruit juices, do not give significant interferences at ascorbic acid determination with the developed amperometric biosensor.

The values of vitamin C content of the analysed fruit juices ranged between 0.48 and 1.98 mM. The highest values of ascorbic acid were obtained for natural juices obtained by squeezing of fruits.

The results obtained at the analysis of juice samples, e.g. 1.64 mM (28.86 mg/100 mL) vitamin C, for orange juice freshly obtained by fruit pressing and 1.98 mM (34.85 mg/100 mL), for lemon juice obtained by fruit squeezing are close to the values reported in literature, namely 47.7 mg /100 mL lemon juice [32] or 48 mg/100 g fruit [40], and for orange juice 40.5 mg/100 mL juice [32].

The values obtained for grapefruit juice (Florida), obtained by fruit pressing, ranged between 38 and 56 mg/ 100 mL juice [41].

The researchers [25] obtained for orange juice (fruit pressing) an ascorbic acid content of 34.67 mg /100 mL juice and for sweet lemon juice (fruit squeezing) a vitamin C content of 58.78 mg/100 mL juice.

The investigated method allows a specific, rapid and sensitive determination of ascorbic acid in fruit juices.

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