A new approach for studying the stability and degradation products of ascorbic acid in solutions

Bogusław Pilarski (bpilarski@cerko.pl)
University of Gdańsk
Dariusz Wyrzykowski
University of Gdańsk
Janusz Młodzianowski
University of Gdańsk

Research Article

Keywords: Potentiometric titrations, Conductometric measurements, Electrospray ionization mass spectrometry, Ascorbic acid, Vitamin C, 2,3-Diketogulonic acid

Posted Date: July 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1884225/v1

A new approach for studying the stability and degradation products of ascorbic acid in solutions

Bogusław Pilarski[a], Dariusz Wyrzykowski[b], Janusz Młodzianowski[a]

[a]*Cerko Sp. z o.o. Sp.K, Al. Zwycięstwa 96/98, 81-451 Gdynia, Poland;*
[b]*Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

Abstract

The potentiometric titration (PT) method has been applied for the first time to investigate the stability of L-ascorbic acid (H_2Asc) and to determine its degradation products in aqueous solutions. The presented electrochemical procedures can be considered to be a fast, simple and inexpensive way to control the stability of H_2Asc in a regular analytical practice as well as in the chemical and pharmaceutical industries. Experimental data as well as modeling suggest that the enolic form of 2,3-diketogulonic acid predominates in a solution as the main product of dehydroascorbate (DHA) degradation. Furthermore, the PT results supported by conductometric measurements (CM) and electrospray ionization mass spectrometry (ESI/MS) data enable us to propose the putative mechanism of the H_2Asc decomposition. Moreover, it has been proven that among different types of investigated electrolytes (KNO_3, $KClO_4$ and KSCN), the thiocyanate ions (SCN^-) reveal the stabilizing effect against the degradation of H_2Asc. Thus, the presence of SCN^- in the H_2Asc solution is proposed as an alternative way for some organic solvents earlier used. Finally, a new paraffin-protection-layer procedure has been recommended for studying as well as storage of the solutions comprising components sensitive to external factors (e.g. O_2, CO_2) and to evaporation.

Keywords:

Potentiometric titrations; Conductometric measurements; Electrospray ionization mass spectrometry; Ascorbic acid; Vitamin C; 2,3-Diketogulonic acid

1. Introduction

L-ascorbic acid (known as vitamin C) is a water-soluble molecule commonly present in plants (fruits and vegetables) [1]. However, humans are not able to synthesize this acid endogenously [2, 3]. On account of the well-documented health benefits, vitamin C is widely used in food, cosmetics and pharmaceutical industries. The acid and ascorbates are involved in many biochemical processes that have a proven protective potential against diseases of various etiology including cancer, cardiovascular or diabetes [4]. Furthermore, due to its high reactivity towards reactive oxygen and nitrogen species (RONS) [5], ascorbate is considered to be an outstanding antioxidant, co-antioxidant (enhancing the antioxidant action of vitamin E in lipid peroxidation). On the other hand, ascorbate can also act as a pro-oxidant, capable of the catalytic reduction of some biologically important metal ions such as Fe^{3+} to Fe^{2+}. These redox processes are found to contribute indirectly to the oxidation of the single state organic molecules [6, 7].

From the acid-base equilibria point of view, in aqueous solutions ascorbic acid behaves as diprotic acid (denoted as H_2Asc), namely a keto lactone form with two ionizable OH groups: $pKa_1 = 4.2$ and $pKa_2 = 11.6$ [1, 8]. In aqueous solutions with $pH < pK_{a1}$ ascorbic acid exists mainly in a non-ionized form (H_2Asc). At a physiological pH (7.4) ascorbate species, $HAsc^-$, dominate (99.95%), whereas the content of Asc^{2-} is negligibly small. This suggests that analyzing the stability of vitamin C in water both forms i.e. H_2Asc and $HAsc^-$ should be considered. A pH of a solution affects the types of the ascorbic acid species as well as their concentration (Figure 1. The increase in pH of a solution results in a decrease in the stability of $HAsc^-$ ions. Under physiological conditions, the $HAsc^-$ ions are recognized as reducing agents and readily undergo two-electron oxidation and deprotonation process leading through ascorbyl free radical $HAsc^{\bullet}$, ascorbate free radical ($Asc^{\bullet -}$) to dehydroascorbic acid (DHA) (Figure 1). Moreover, the formation of DHA is also possible in a highly basic environment through two-electron oxidation process of Asc^{2-} anions [9]. Redox processes of ascorbic acid species are light and temperature-sensitive and also depend on other factors, among which the presence of oxygen and metal ions plays a crucial role.

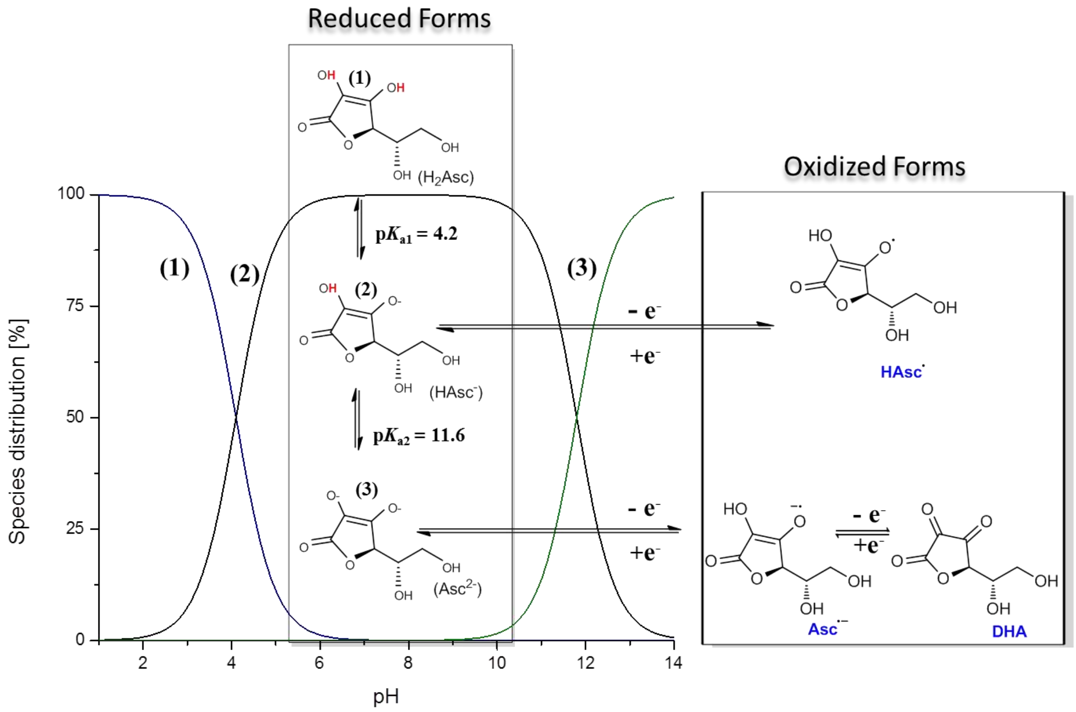
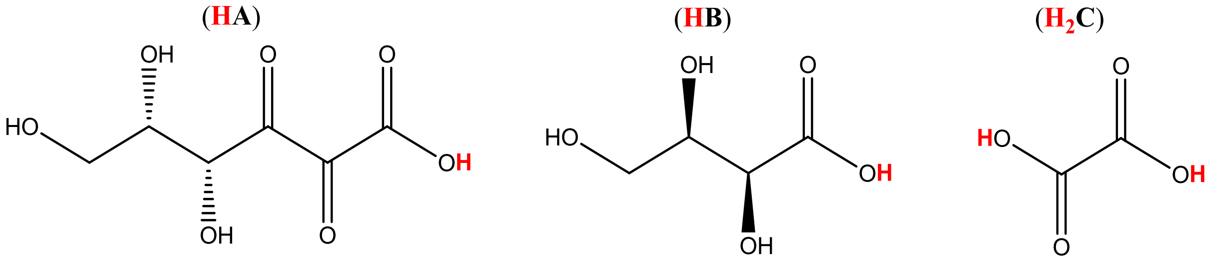


Fig. 1. Acid–base and electrochemical equilibria of ascorbic acid in an aqueous solution.

DHA is a neutral compound with a lactone structure and a carbonyl moiety (3 -C=O) that makes this structure very sensitive to different types of reagents: nucleophiles, oxidants and others. DHA can be reduced to H_2Asc or irreversible hydrolysed to 2,3-DKGA (2,3-diketogulonic acid) which loses biological activity. Interconversion between H_2Asc and DHA via radical ($HAsc^{\bullet -}$) has been the subject of interest of many research groups [10, 11]. However, there is still little evidence that this phenomenon could take place in vivo [12].

In general, the stability of ascorbic acid solutions depends on the composition of a system, namely the presence of catalytic metals ions, the type of inorganic anions, a pH of a solution as well as storage method. These factors affect the quality of a stock solution of ascorbic acid and thus the reproducibility of the results. It has been reported that most of the degradation products of H_2Asc are organic acids (carboxylic and OH-acids) with the general formula: H_nL (where n denotes the number of protons undergoing dissociation: n = 1, 2, …). They were identified by ESI/MS method as 2,3-diketogulonic acid (2,3-DKGA), threnoic acid (ThA) and oxalic acid (OxA) Fig. 2.



2,3-diketogulanic acid (**2,3-DKGA**) threonic acid (**ThA**) oxalic acid (**OxA**)

Fig. 2. Chemical structures of the degradation products of L-ascorbic acid.

Other analytical techniques intensively employed for studying the stability of ascorbic acid are polarography [13, 14], voltametric methods [15] spectrometric UV-VIS methods [16], capillary electrophoresis (CE) [17], thin layer chromatography (TLC) [18] gas chromatography (GC) and HPLC methods [19].

In contrast to these previous studies, in this paper the potentiometric titration method supported by conductometric measurements and electrospray ionization mass spectrometry (ESI/MS) has been employed for the first time to describe H_2Asc degradation products in aqueous solutions. In addition, we have focused our attention on the stabilizing effect of thiocyanate on ascorbic acid in aqueous solutions. Based on experimental data a putative mechanism of thiocyanate action has been proposed.

2. The application of potentiometric titration technique for studying degradation products of L-ascorbic acid – A Theory

Potentiometric titration technique supplemented by theoretical calculations (simulations of titration curves) has been proposed as a useful tool for studying the stability of L-ascorbic acid in an aqueous solution as well as for a quantified assessment of its degradation products.

In the system under study, L-ascorbic acid (H_2Asc) and acidic species formed as a result of the H_2Asc degradation, namely 2,3-diketogulonic acid (HA), threnoic acid (HB) and oxalic acid (H_2C) are presented. All these species have been taken into consideration during the analysis of the composition of a mixture of L-ascorbic acid (H_2Asc) (Fig. 2). The acid dissociation constants of components are available in the literature and were used in calculations. The individual equilibria for all species in the mixture are presented in (Table 1).

Table 1

The equilibria in water solution and pK_a value of present species.

No.	Equilibrium model	Equilibrium expression	pK_a value
1	$H_2Asc = H^{+1} + HAsc^{-1}$	$K_{a1} = [H^{+1}][HAsc^{-1}]/H_2Asc]$	4.2$^{(a)}$
2	$HAsc^{-1} = H^{+1} + Asc^{-2}$	$K_{a2} = [H^{+1}][Asc^{-2}]/[HAsc^{-1}]$	11.6$^{(a)}$
3	$HA = H^{+} + A^{-}$	$K_{a3} = [H^{+1}][A^{-}]/[HA]$	2.3$^{(b)}$
4	$HB = H^{+1} + B^{-1}$	$K_{a4} = [H^{+1}][B^{-1}]/[HB]$	3.4$^{(c)}$
5	$H_2C = H^{+} + HC^{-}$	$K_{a5} = [H^{+1}][HC^{-}]/[H_2C]$	1.4$^{(d)}$
6	$HC^{-} = H^{+1} + C^{-2}$	$K_{a6} = [H^{+1}][C^{-2}]/[HC^{-1}]$	4.1$^{(d)}$
7	$H_2O = H^{+1} + OH^{-1}$	$K_{H2O} = [H^{+1}][OH^{-1}]$	13.99$^{(e)}$

(a) [1], (b) [20], (c) ChemAxon, (d) [21], (e) [8].

Based on the above equilibria model, theoretical potentiometric titrations curves have been simulated and compared with obtained from experimental data. Calculations were carried out with a new CerkoLab System EQSOL software.

2.1. CerkoLab System EQSOL software description

CerkoLab EQSOL (EQSOL) program runs on a Windows operating system and has a user friendly graphics interface similar to other CerkoLab System products [22]. The implemented numerical procedure: EQSOL is based on a revised by Liwo cvequid algorithm earlier presented by Kostrowicki and Liwo [23, 24].

The equilibrium state can be described in terms of an ideal solution model, Debye-Hüeckel or Davies models. Based on a model, EQSOL builds a stoichiometric matrix which is subsequently used in numerical procedures.

Sample parameters are fitted to the model by minimalization of a sum of squares of a target function [24]. EQSOL algorithm allows for all, or specific model parameters to be evaluated. The result of the minimalization procedure is in a form of a listing of evaluated parameters and a plot of fitted titration curves. In addition to fitting titration parameters, EQSOL can present plots of equilibrium concentrations, activity coefficients and ionic strength. Additionally, the EQSOL simulation module can be used to predict the titration curve for a specific concentration of reagents and their pK_a values.

2.2. Titration curves simulation

The titration curves for H_2Asc titration with 0.1 M NaOH (C_T) simulated in the EQSOL are shown in Fig. 3. Calculations were performed for two models:

Model (1) – for the first dissociation step

$H_2Asc = H^{+1} + HAsc^{-1}$ $pK_{a1} = 4.2$

$H_2O = H^{+} + OH^{-}$ $pK_{H2O}=13.99$

Model (2) – for the first and the second dissociation steps

$H_2Asc = H^{+1} + HAsc^{-1}$ $pK_{a1} = 4.2$

$HAsc^{-1} = H^{+1} + Asc^{-2}$ $pK_{a2} = 11.6$

$H_2O = H^{+} + OH^{-}$ $pK_{H2O}=13.99$

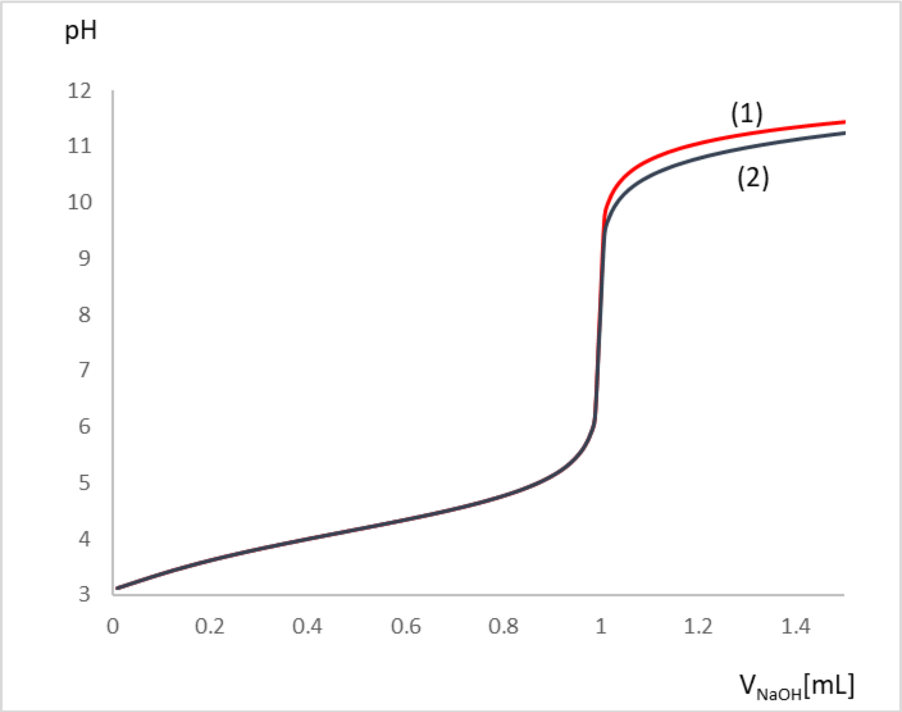


Fig. 3. Theoretical titration curves of H_2Asc in an aqueous solution: $V_0 = 0.01$ L, $C_0 = 0.01$ M ($HAsc^-$ or H_2Asc) and $C_T = 0.1$ M NaOH: (1) H_2Asc as a monoprotic species ($HAsc^-$), (2) H_2Asc as a diprotic acid (H_2Asc).

At the pH ranging from 1 to 9, the shape of the PT simulated curves for both employed models are the same (Figure 3). However, above a pH of 9, the shape of the PT curves differ~~s~~ depending on the model used. The inspection of the PT curves suggests that an equilibria model with H_2Asc as a diprotic acid (H_2L) should be taken into consideration.

Additionally, the titration curves for the above two systems with different types of possible degradation products have been simulated with the EQSOL software and the equilibrium models with the components listed below. DHA is coming from approx. 2% degradation of H_2Asc. pK_a values for HA = 2,3-diketogulonic acid, HB = threnoic acid, H_2C = oxalic acid are taken from Table 1.

Model (3) – the model comprises H_2Asc and its degradation components (Fig. 2.)

$H_2Asc + HA + HB + H_2C$

Model (4) – the model additionally contains DHA as an oxidation product of H_2Asc

$H_2Asc + HA + HB + H_2C + DHA$

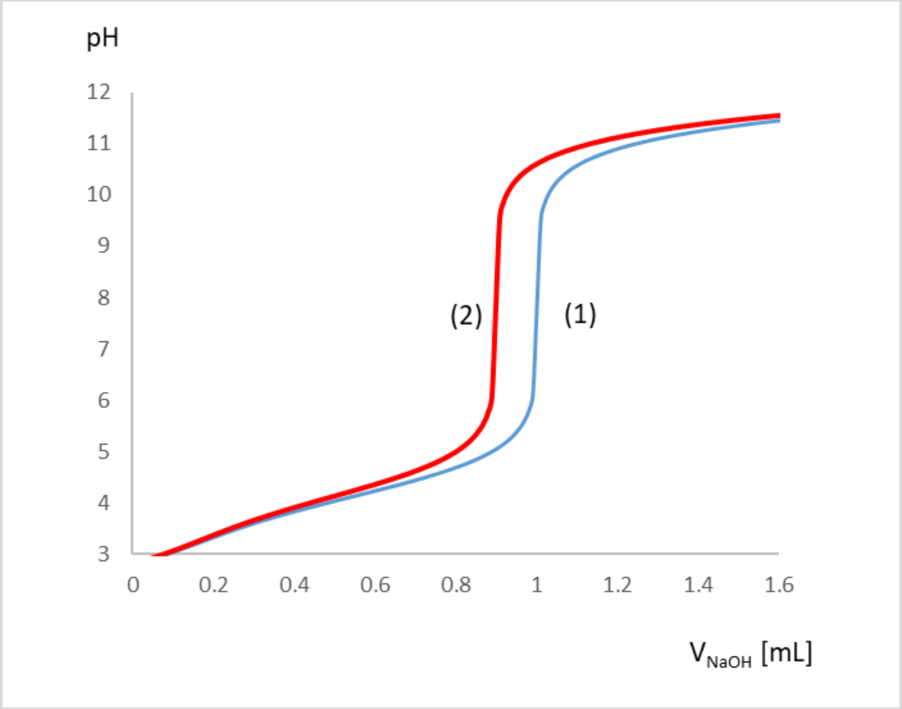


Fig. 4. Theoretical PT curves: (1) the mixture of H_2Asc (0.007 M); HA (0.001 M); HB (0.001 M); H_2C (0.0005 M), (2) the mixture of H_2Asc (0.006 M); HA (0.001 M); HB (0.001 M); H_2C (0.0005 M) and DHA (0.001 M). Experimental conditions: V_0=0.010 L, C_T = 0.1 M NaOH.

Calculated PT curves, Fig. 3 have subsequently been applied for the investigation of the stability of H_2Asc in an aqueous solution, both in the absence and in the presence of different types of electrolytes (KNO_3, $KClO_4$ and KSCN). Additionally, two PT curves: (1) for H_2Asc and (2) for the mixture of H_2Asc and its degradation products (HA, HB and H_2C) were simulated and discussed with respect to the influence of the types of components present (i.e. the differences in the PT curve shape) in a low pH region (pH 2.8- 5.5) Fig. 4.

3. **Materials and methods**

3.1. Materials

L-Ascorbic acid, H_2Asc, (analytical grade) was purchased from Chempur. Potassium salts of chloride, nitrate, perchlorate and thiocyanate (>99.5%) were supplied by Sigma – Aldrich, Poland. Sodium hydroxide solution as titrant was prepared from fixanal delivered by Avantor Performance Materials Poland S.A. All reagents were used as obtained without further purification. Deionized water with conductivity not exceeding 0.18 $\mu S\ cm^{-1}$ (a Hydrolab water purification system) was used for preparation of aqueous solutions.

3.2. Preparation of solution and incubation conditions

The ascorbic acid solution C_o=0.01 M was prepared by dissolving an appropriate amount of analytical grade H_2Asc in distilled water or in a 0.005M solution of electrolyte (KNO_3, $KClO_4$ and KSCN). Prepared solutions were stored in 0,025 L volumetric flasks at laboratory temperature for 0-3-6-24-48-72 hrs.

3.3. Titration experiments

Titration experiments were performed in a 30-mL thermostated (298.15±0.1 K) cell using the Cerko Lab System microtitration unit fitted with the 5-mL Hamilton's syringe. The temperature of measured solutions was controlled by circulating water through a jacketed beaker (Julabo-25 circulation thermostat).

3.3.1. Potentiometric titration (PT)

A pH electrode (Hydromet – ERH-13-6 type) was calibrated with the use of buffer solutions: potassium hydrogen phthalate (pH 4.00), citric acid/Na_2HPO_4 (pH 7.00) and boric acid/KCl/NaOH (pH 10.00). The titrant (T) (0.1 mol L^{-1} NaOH) was standardized according to general analytical procedure and protected from carbon dioxide. The titrand (D) solutions (V_0 = 0.005 or 0.010 L): ascorbic acid (approx. 0.01 mol L^{-1}) in the absence or the presence of an appropriate potassium salt MeX (MeX = KNO_3, $KClO_4$ and KSCN with concentration of approx. 0.005 M) were potentiometrically titrated in the pH range from 2.5 to 11.

Potentiometric titration procedure: The titrant (T) was added to the titrand (D) in increments of 0.01 or 0.005 $L\cdot10^{-3}$, with a pause of 20 s. Each titration was repeated a least twice in order to check the reproducibility of the data.

3.4. Conductometric measurements (CM)

Conductometric measurements were accomplished immediately after a solution preparation on the CerkoLab System – kinetic unit in a measuring cell equipped with a magnetic stirrer. The conductometric sensor (CD 201 from Hydromet) with constant k = 0.096 cm^{-1} was standardized with conductivity standards (aqueous KCl solutions: 1410 μS cm^{-1}) purchased from Hamilton Company.

3.4.1. Standard procedure

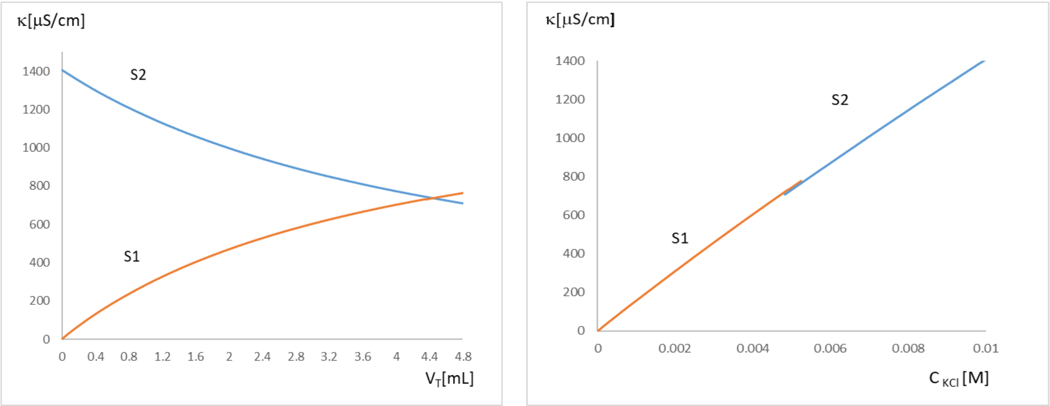
Measured samples of H_2Asc/H2O and $H_2Asc/MeX/H_2O$ were prepared using V_0 = 0.005 L of an appropriate solution with a concentration of 0.005 and 0.01 M of H_2Asc and an electrolyte concentration of approx. 0.005 M. The conductivity measurements were registered every one minute from the start point up to 24 hrs. and every 15 seconds when the measurement was run for 2-3 hrs.

3.4.2. Paraffin-protection-layer procedure

To protect the titrand solution from the components present in the laboratory air (especially CO_2 and O_2) and to eliminate evaporation of a solvent (here H_2O) a special procedure was developed. On the top of the titrand (D) a 0.5 cm thick layer of paraffin was deposited from a syringe fitted with a Teflon tube. The conductivity measurement was registered by CerkoLab System as described in the standard procedure.

3.5. Validation of conductometric procedure data

The linearity test of a conductometric cell was performed by using a standard solution of KCl (1410 µS cm^{-1}) acting as titrant (T) or titrand (D) with V_0=0.0045L, according to the general scheme: $KCl/H_2O \leftarrow \rightarrow H_2O$. Both types of titration procedures have been carried out under the paraffin layer. The results are presented in Fig. 5. The intersection point of the titration curves, namely $KCl/H_2O \rightarrow H_2O$ and $H_2O \rightarrow KCl/H_2O$ at V_T=V_D = 0,0045 (±0.02) L confirm~~s~~ the linearity of the measurement system Fig.5A.



(A) (B)

Fig. 5. Titration curves of KCl standard solution 0.01M (1410 µS cm^{-1}), V_0=0,0045 L according to the scheme: $KCl/H_2O \leftarrow \rightarrow H_2O$ presented as: (A) $\kappa = f(V_T)$ where S1 represents $KCl/H_2O \rightarrow H_2O$ and S2; $H_2O \rightarrow KCl/H_2O$; (B) $\kappa = f(C_{KCl})$ respectively for S1 and S2 data points.

Additionally, we have compared the conductometric data obtained according to the standard procedure and the proposed paraffin-protection-layer procedure (Fig. 6.). It has been proven that the paraffin layer effectively protects the titrand (D) from external environmental conditions and thus guarantees the reproducibility of the data irrespective of the measurement time.

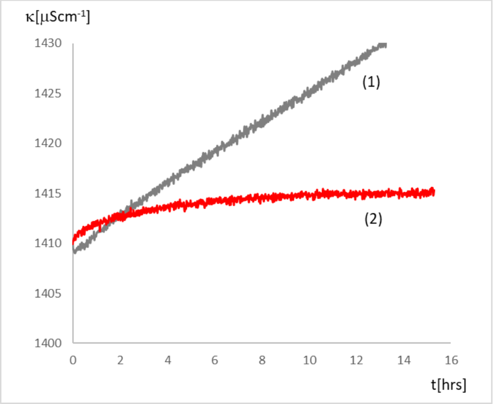


Fig. 6. The conductivity of the standard solution of KCl (1410 μS cm^{-1}) was registered for 16 hrs according to standard procedure (1) and under paraffin layer (2).

3.6. Electrospray ionization mass spectrometry (ESI/MS) measurements

Agilent liquid chromatograph series 1290 (Agilent Technology, Santa Clara, CA, USA) with quaternary pump G4204A, autosampler G4226A, thermostated column compartment G1316C, and triple quadrupole mass spectrometer G6460C with AJS electrospray ionization source was used for flow injection analysis. The chromatographic system was controlled with Agilent MassHunter software B 06.01. Samples (5 μL) were directly injected. The mobile phase flow rate was 0.2 mL/min, and elution was carried out in water with 10 mM ammonium formate. All mass-spectrometric scan data were collected in negative ionization scan mode at 45 psi nebulizer pressure, 5 L/min nitrogen flow, 573,15 Knitrogen temperature, 11 L/min sheath gas (nitrogen) flow, and 623,15 K sheath gas temperature. The fragmentation voltage was 100 V and the capillary voltage was 3.5 kV. The collision cell radio frequency voltage was deactivated, the first quadrupole was in total transmission ion mode, and the second quadrupole was scanning, resulting in a scan mode. The fragmentor voltage controlled the rate at which ions pass through a medium pressure zone and are fragmented by collisions with nitrogen molecules in this mode.

3.7. Analysis of pH-metric titration data - numerical and modeling procedure

The numerical procedure described before [22] for pK_a calculation, the composition of titrand D and the modeling of titration curves is based on the EQSOL.

4. Results and discussion

4.1. Potentiometric titration analysis (PT)

The potentiometric titration method was applied for qualitative investigations of degradation products of ascorbic acid in aqueous solutions in the presence and the absence of some potassium salts, namely KNO_3, $KClO_4$'and KSCN (Hofmeister series). The representative titration curves of ascorbic acid solutions prepared immediately before the titrations as well as after the incubation time at room temperature protected against CO_2 and O_2 present in a laboratory atmosphere for 24-48 hrs are collected in Fig. 7. Shape~~s~~ of pH titration curves differ with the incubation time. This phenomenon indicates the different compositions of investigated solutions due to the oxidation of ascorbic acid.

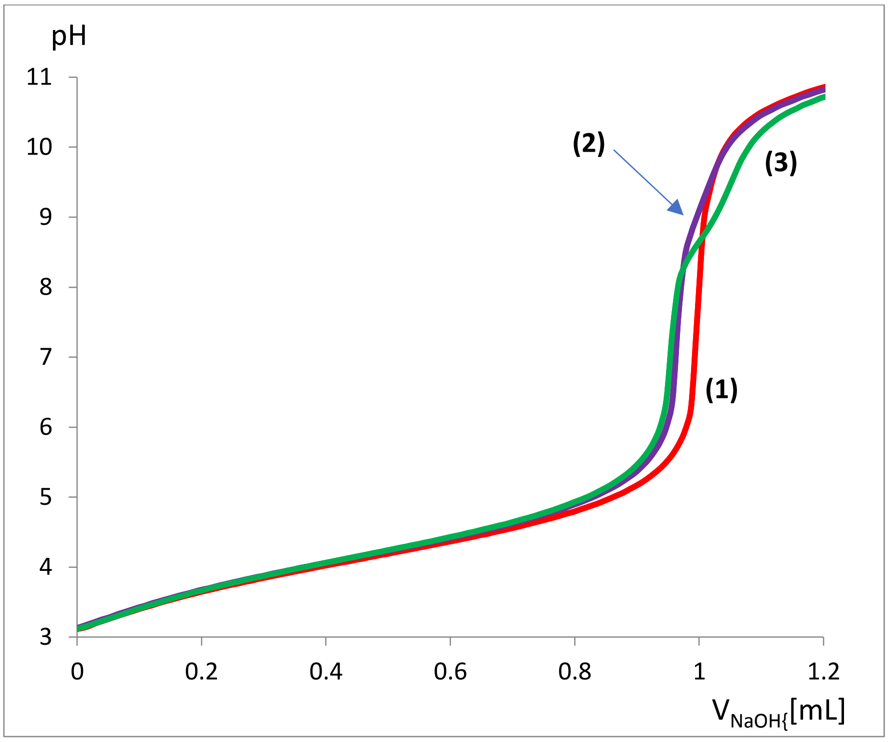


Fig. 7. Titration curves of H_2Asc; $V_0 = 0.010$ L, $C_0 = 0.01$ M with titrant T = 0.1 M NaOH in water : (1) a fresh solution, (2) after 24 hrs and (3) after 48 hrs at 298.15 K.

The composition of examined ascorbic acid solutions was assessed based on the differences in the acid-base properties (their pK_a values) of the resulting degradation species. The general procedure elaborated previously for the pK_a's determination in a mixture of compounds has been applied [22]. The calculated pK_a's values were subsequently assigned to the functional groups of particular species (Table 2).

Table 2

The pK_a values of H_2Asc and its degradation products in aqueous solutions and the aqueous solution of electrolytes (KNO_3, $KClO_4$ and KSCN) with a concentration of approx. 0.005 M at 298.15 K (± standard deviation in the parenthesis).

<table>
<tr><td colspan="4">H_2Asc in water solution
(prepared immediately before the titrations)</td></tr>
<tr><td>pK_{a1}</td><td>pK_{a2}</td><td>pK_{a3}</td><td>pK_{a4}</td></tr>
<tr><td>4.13 (±0.01)</td><td>11.48 (±0.04)</td><td>-</td><td>-</td></tr>
<tr><td colspan="4">H_2Asc in 0.005 M $KClO_4$
(after 24 hrs of the incubation)</td></tr>
<tr><td>4.10 (±0.01)</td><td>11.74 (±0.03)</td><td>2.94 (±0.06)</td><td>8.94 (±0.03)</td></tr>
<tr><td colspan="4">H_2Asc in 0.005 M KNO_3 solution
(after 72 hrs of the incubation)</td></tr>
<tr><td>4.13 (±0.01)</td><td>11.75 (±0.03)</td><td>2.83 (±0.03)</td><td>8.99 (±0.03)</td></tr>
<tr><td colspan="4">H_2Asc in 0.005 M KSCN solution
(prepared immediately before the titrations)</td></tr>
<tr><td>4.09 (±0.01)</td><td>11.41 (±0.02)</td><td>-</td><td>-</td></tr>
<tr><td colspan="4">H_2Asc in 0.005 M KSCN solution
(after 24 hrs of the incubation)</td></tr>
<tr><td>4.07 (±0.01)</td><td>11.27 (±0.02)</td><td>-</td><td>-</td></tr>
<tr><td colspan="4">H_2Asc in 0.005 M KSCN solution; average pK_a values calculated from 3 curves registered at the time: 0, 24 and 48hrs</td></tr>
<tr><td>4.08 (±0.08)</td><td>11.32 (±0.02)</td><td>-</td><td>-</td></tr>
</table>

*pK_{a3} -COOH, pK_{a4} -OH (enol form), Fig. 13.

Calculated dissociation constants of H_2Asc (pK_{a1} and pK_{a2}) stay in line with literature values (Table 1). The oxidation of H_2Asc leads to the formation of acidic products. This manifests itself in the change of the shape of the pH curves. Fitting of calculated curves to experimental data reveals some additional equilibrium reactions denoted as pK_{a3} and pK_{a4} which can be assigned to either diprotic species of the type H_2A or to two monoprotonated species HA+HB (Table 1). The EQSOL algorithm adopted in the CerkoLab software has been employed to calculate theoretical potentiometric titration curves for two systems: H_2Asc+ H_2A and H_2Asc + HA+HB+H_2C, Fig. 8. Obtained theoretical results have subsequently been compared with the experimental data. It has been found that the oxidation of H_2Asc leads to the formation of H_2A species which fits well to 2,3- diketogulonic acid (2,3-DKGA) present in a solution as an enol form (2,3-DKGA) with pKa$_3$ and pK_{a4} values close to 3 (-COOH) and 9 (-OH enol form)~~,~~ respectively.

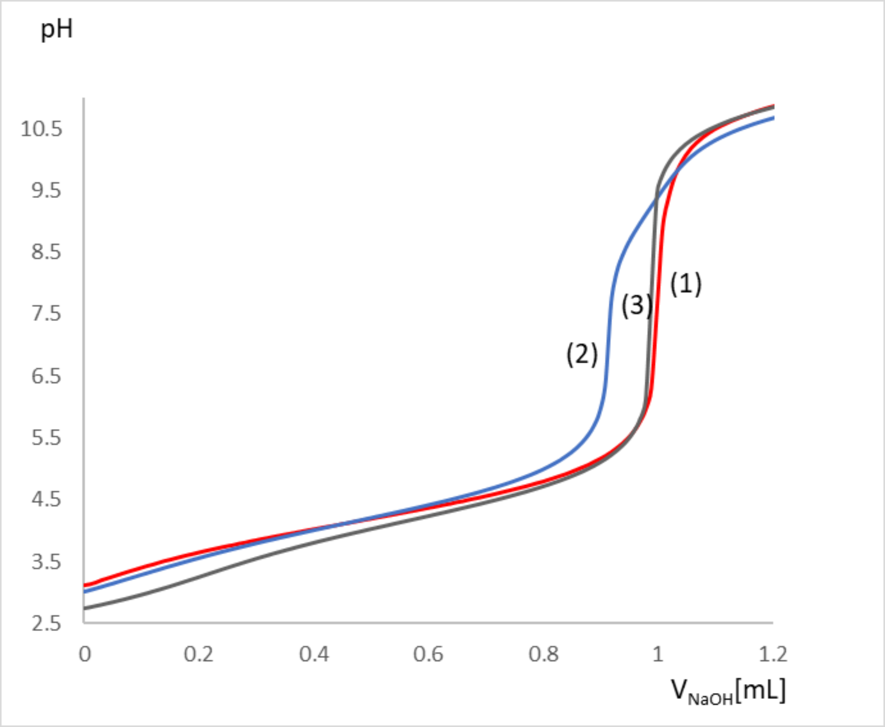


Fig. 8. The experimental titration curves of H_2Asc; V_0 = 0.010 L, C_0 = 0.01 M with titrant T = 0.0995 M NaOH in water (1) and theoretical titration curves for two models: H_2Asc, (0.0082 M)+H_2A (0.00105 M); (2) H_2Asc (0.006M) + HA (0.001M) + HB (0.001M) + H_2C (0.001M); (3) The concentration of titrant T and V_0 value are as for (1).

Theoretical titration curves were generated from CerkoLab EQSOL using pK_a data for H_2Asc, HB and H_2C as in (Table 1) and pK_{a3}=3.0 and pK_{a4} =9 for H_2A = 2,3-DKGA. Experimentally determined pK_a values for 2,3- DKGA as a degradation product of H_2Asc in water solution and in the presence of electrolyte are presented in (Table 2). Potentiometric titration data revealed that the thiocyanate ions effectively protect the ascorbate species

($HAsc^-$) against oxidation. On the other hand, the protection action has not been observed for KNO_3 and $KClO_4$ (Fig. 9). The shape of titration curves of H_2Asc in the presence of electrolytes: $KClO_4$ and KNO_3 is similar to curves registered for water solution of H_2Asc without electrolytes, Fig. 7 and Fig.9. In addition, calculated pK_a values of mixture components are practically the same (Table 2). This proves the presence of the same oxidative degradation products. The only differences are found in the concentration of particular components of titrand D. Furthermore, the concentration of ascorbic acid gradually decreases in time with the increase of the 2,3,- DKGA (enol form) concentration (Table 3).

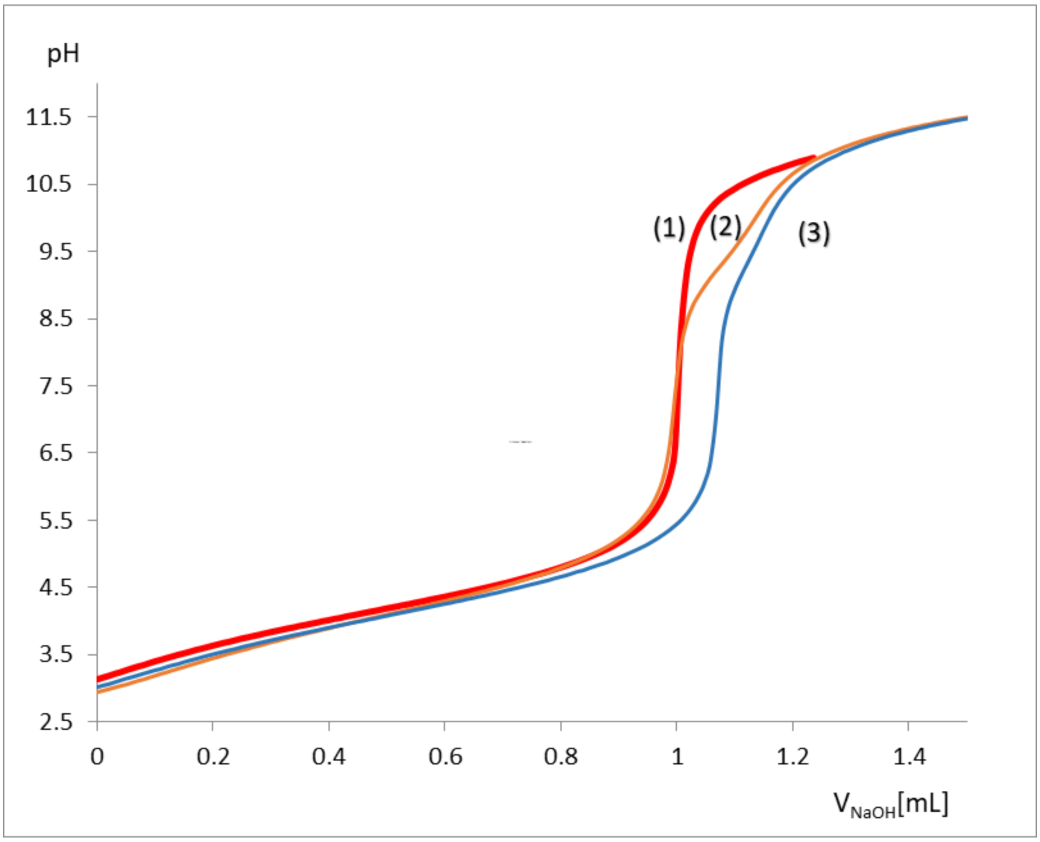


Fig. 9.The experimental titration curves of H_2Asc (0.01 M) with NaOH (0.0965 M) in the presence of the investigated electrolytes (V_0 = 0.010 L, T = 298.15 K): (1) 0.005 M KSCN (incubation time: 48 hrs), (2) 0.005 M KNO_3 (incubation time: 72 hrs) and (3) 0.005 M $KClO_4$ (incubation time: 28 hrs).

Table 3

The concentration of H_2Asc* and 2,3-DKGA** in titrand D (M) in water and water/electrolyte solution determined by using CerkoLab EQSOL software.

Time[hrs]	H_2Asc/H_2O	$H_2Asc/KClO_4/H_2O$	$H_2Asc/KNO_3//H_2O$	$H_2Asc/KSCN/H_2O$
0	$1.07\cdot10^{-2}$	$1.02\cdot10^{-2}$	$1.03\cdot10^{-2}$	$0.99\cdot10^{-2}$
28	-	$0.92\cdot10^{-2}$	-	-
48	$0.85\cdot10^{-2}$		$0.82\cdot10^{-2}$	$1.01\cdot10^{-2}$
Time[hrs]	2,3-DKGA/H_2O	2,3-DKGA/H_2O/ $KClO_4$	2,3-DKGA/H_2O/ KNO_3	2,3-DKGA/H_2O KSCN
0	0	0	0	0
28	-	$0.10\cdot10^{-2}$	-	-
48	$0.11\cdot10^{-2}$	-	$0.15\cdot10^{-2}$	0

*the standard deviation for $H_2Asc \pm 1.8\text{-}4.5\cdot10^{-4}$

** the standard deviation for 2,3-DKGA $\pm 2.8\text{-}4.0\cdot10^{-5}$

The $HAsc^-$ ion is a highly effective reducing agent which easily undergoes one-electron oxidation to ascorbate radical ($HAsc^{\bullet}$) and then to dehydroascorbic acid (DHA) according to the following electron transfer process:

(A) $HAsc^- = HAsc^{\bullet} + e$

(B) $HAsc^{\bullet} = Asc^{\bullet-} + H^+$

(C) $Asc^{2-} = Asc^{\bullet-} + e$

(D) $Asc^{\bullet-} = DHA + e$

A surprising protective action of thiocyanates remains to be elucidated. Nevertheless, it can be supposed that the putative mechanism of action of SCN^- ions in the protection of the ascorbic acid against oxidation involves the formation of the $SCN^{\bullet}$ radicals which participate in the scavenging of ascorbate free radical ($Asc^{\bullet}$), the precursor of DHA:

$$SCN^- + HAsc^{\bullet} = HAsc^- + {}^{\cdot}SCN^{\bullet} \quad \text{step (1)}$$

$$SCN^{\bullet} + Asc^{\bullet-} = {}^{\cdot}SCN^- + Asc^{2-} \quad \text{step (2)}$$

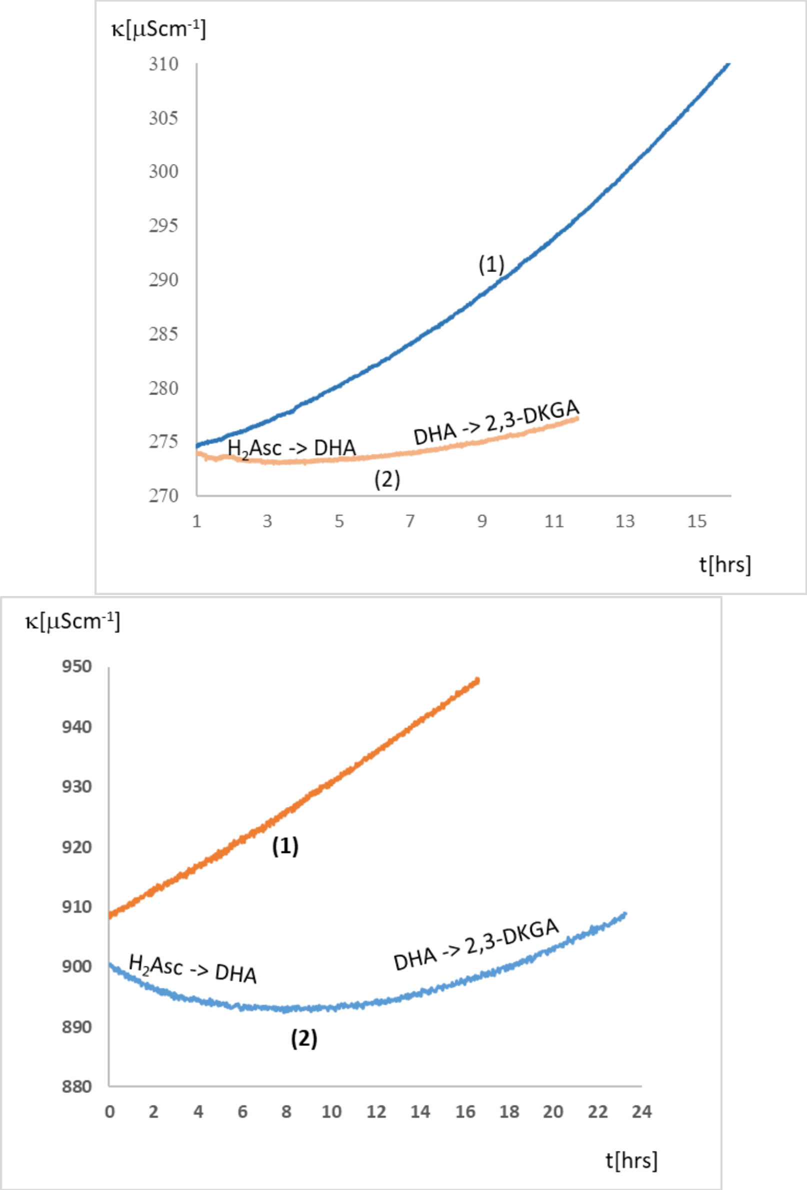
Overall the reaction

$$HAsc^{\bullet} + Asc^{\bullet-} = {}^{\cdot}\, HAsc^- + Asc^{2-}$$

The reaction of thiocyanate ion (SCN^-) with $HAsc^•$ is proposed by analogy to the reaction of SCN^- with other radicals for example with ($OH^•$), [25]. Moreover, $SCN^•$ can react with the excess of $SCN^−$ resulting in the formation of the $(SCN)_2^{•−}$ radical [26, 27].

4.2. Protective action of KSCN and a paraffin layer against the L-ascorbic acid degradation in an aqueous solution - conductometric measurements (CM)

The oxidation of H_2Asc to dehydroascorbic acid (DHA, approx. 1.2%) initially manifests itself in the decrease in the conductivity of a solution. After a time, the conductivity increases slightly, Fig. 10 which is due to the DHA ring opening reaction leading to the formation of 2,3-DKGA, Fig. 13. This phenomenon stays in line with the results of potentiometric titration of H_2Asc samples prepared immediately before the experiments and after storage of solutions for 6 hours in the volumetric flask at laboratory temperature, Fig. 11. The same phenomena were observed in conductometric curves obtained for mixtures of H_2Asc and 0.005 M KNO_3 or $KClO_4$ stored for 24 hrs under the same conditions. On the other hand, a protective effect against the degradation has been found for the H_2Asc solution stored under the paraffin layer in the presence of KSCN (Fig. 12). Taking in mind the influence of gaseous species (O_2 and CO_2) and also evaporation of the solvent (H_2O) we have elaborated a special procedure for conductometric measurements based on a 100% protection of a solution against above mentioned physical and chemical factors. When the experiments were carried out under the paraffin layer (see exp. section) we observed a stable value of conductivity for KSCN solution ($\kappa = 917 \mu S \cdot cm^{-1}$) and an increased value of conductivity for aqueous solution, Fig. 10A and 10B, Fig. 12. It is a new procedure recommended for different types of potentiometric and conductometric measurements (titrations as well as kinetic studies conducted for a long period of time) and especially for O_2, CO_2 sensitive systems.



(A) (B)

Fig. 10. The conductivity changes k=*f*(t) registered at 298.15 K for 16 hrs: (A) H_2Asc solution C=0.01 M, V_0=0.05 L under standard conditions (1) and (2) the same solution under paraffin layer; (B) H_2Asc (0.01 M)/ KNO_3 (0.005 M) solution, V_0=0.05 L registered for 24 hrs, the meaning of (1) and (2) are the same as for (A).

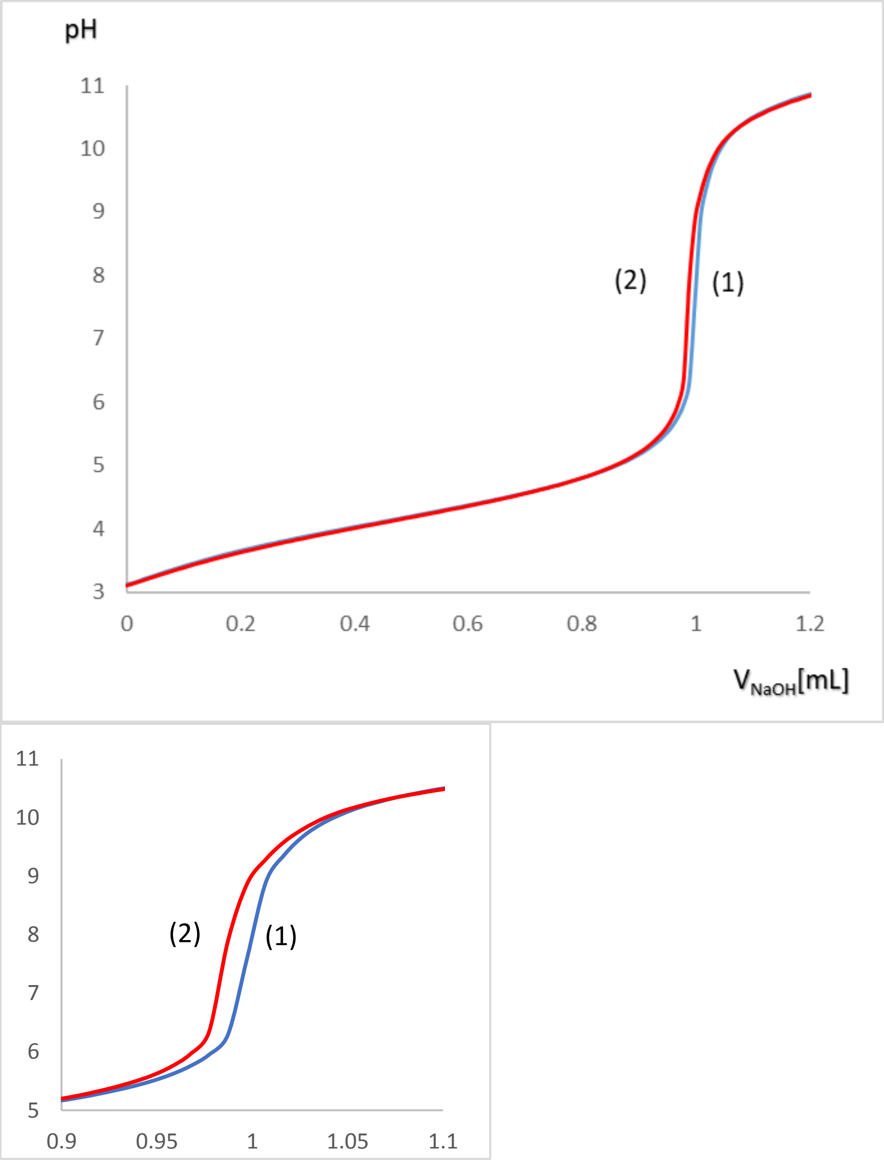


Fig. 11. The potentiometric titration curves of the 0.01M H_2Asc solution, at 298.15 K: (1) fresh solution, (2) the solution after 6 hrs. of the incubation.

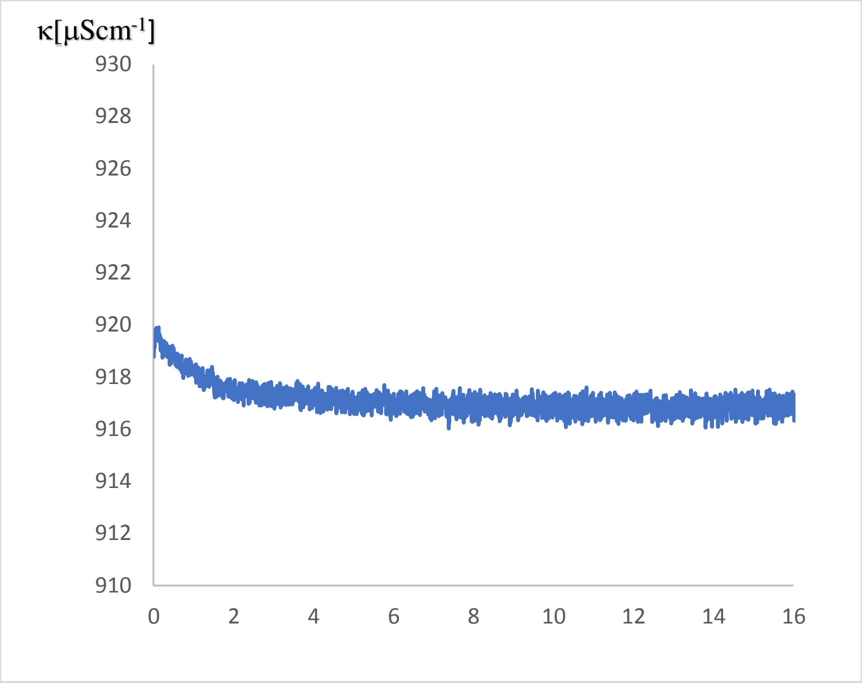


Fig. 12. The conductivity changes as a function of time κ=*f*(t) under the paraffin layer of the 0.005L mixture of H_2Asc (0.01 M) and KSCN (0.005 M), over 16 hrs, at 298.15 K.

4.3. The ESI(-)/MS analysis

The putative mechanism of the H_2Asc decomposition presented in this study is in good agreement with the chemistry of ketolactone and α- keto carboxylic acids. The first step of the degradation of H_2Asc is assigned to the oxidation of H_2Asc to DHA. The next steps of chemical transformations suggested by different authors are shown in Fig. 13. However, the decomposition pathways of H_2Asc depend on different factors and are still the subject of discussion by many research teams. A few important discrepancies in the nomenclature as well as in the structure of the degradation products proposed mainly based on ESI/MS analysis are found in literature. Among these, a lot of discussion and controversy can be found in [28] (Table 4).

Table 4

The main degradation products of H_2Asc with its M.W. and *m/z* values according to different authors in comparison with our data.

Product (Molecular weight)	[27] *m/z*	[28] *m/z*	[29] *m/z*	[30] *m/z**	This work
Ascorbic acid H_2Asc (176.12)	175 115 87	175 115 87	175 115 87		175 115 87
Dehydroascorbic acid DHA (174.11)	173	-	-	-	-
2,3-diketogulonic acid, 2,3-DKGA (191)	210	-	191		191
Monohydrate-2,3-diketogulonic acid MH-2,3-DKGA (210.14)	191	-	209	209	209
Dihydrate 2,3-diketogulonic acid DH-2,3-DKGA (228.15)	209	-		227	
Monohydrate-dehydroascorbic acid MH-DHA (192.12)		-	-	191	-
Dihydrate-dehydroascorbic acid DHDHA (228.15)		-	-	227	-

*DHA hydrolysis was investigated

Based on mass spectra registered after 24-96 hrs we have confirmed only peaks m/z=175, 115 and 87 as described for H_2Asc [28-30] and m/z=191 – for 2,3 DKGA plus m/z=209 for 2,3-DKG acid monohydrate (MH-2,3-DKGA) when the ESI/MS were registered after direct injection of the sample prepared and stored as described in the experimental part. The analysis of H_2Asc under the same conditions after 24 hrs of incubation in KNO_3 and $KClO_4$ solutions resulted in a new observation. We have observed 3 peaks of $HAsc^-$ anions as described above plus 3 peaks: m/z=62, 237,8 and 275,9 for potassium nitrate solution and m/z=99, m/z=237, m/z=275 plus m/z=313 for $KClO_4$ solution. The peak m/z=62 correspond~~s~~ to NO_3^- ion [31,32], m/z=237.8 can be assigned to $[HAsc^- NO_3^-]H^+$ whereas m/z=275.9 to the cluster which includes $[HAsc^- NO_3^-]K^+$. The MS spectra of H_2Asc in $KClO_4$ solution show peak m/z=99 for ClO_4^- plus M+2 peak with relative intensity to isotope Cl^{37} distribution [31, 32]. The isotopic peaks M+2 are also present for clusters: m/z=237, 275 and 313. The registered peaks m/z 237, 275 and 313 correspond to [$ClO_4^-K^+ClO_4^-$], [HAsc$^-$ ClO_4^-]H$^+$ and [HAsc$^-$ ClO_4^-]K$^+$.

It is worth noting that for the mixture of H_2Asc and KSCN (0.005 M) there was a lack of signals characteristic for 2,3-DKGA (m/z=191), monohydrated 2,3-DKG (m/z=209) and dihydrated 2,3-DKG (m/z=227) as well as oxalate (m/z=89) and threonate (m/z=135). Observed stabilization effect of the thiocyanate ion on H_2Asc is in good agreement with potentiometric and conductometric results. Additionally, we have observed the peak with m/z=272 that confirms the cluster formation of the ascorbyl ion with structure: [HAsc$^-$ SCN$^-$]K$^+$ (Table 5). According to our knowledge, the formation of clusters of the type [HAsc$^-$ A$^-$]K$^+$ (A$^-$ denotes SCN$^-$, NO_3^-, ClO_4^-, SCN$^-$) has been proven for the first time in this paper. The analytical importance of this cluster formation will be the subject of our further study**.**

Table 5
The [HAsc$^-$ A$^-$]K$^+$ clusters data (A- denotes SCN$^-$, NO_3^- ,ClO_4^- , SCN$^-$).

KNO_3		**$KClO_4$**		**KSCN**	
	m/z		***m/z****		***m/z***
[NO_3^-]	62	[ClO_4^-]	99	SCN$^-$	59
[HAsc-NO_3^-]	238	[ClO_4-K+ClO_4^-]	237	[Asc-SCN$^-$]K$^+$	272
[HAsc-NO_3^-]K$^+$	276	[HAsc-ClO_4^-]H+	275		
		[HAsc-ClO_4^-]K+	313		

* The isotopic peaks M+2 were also registered

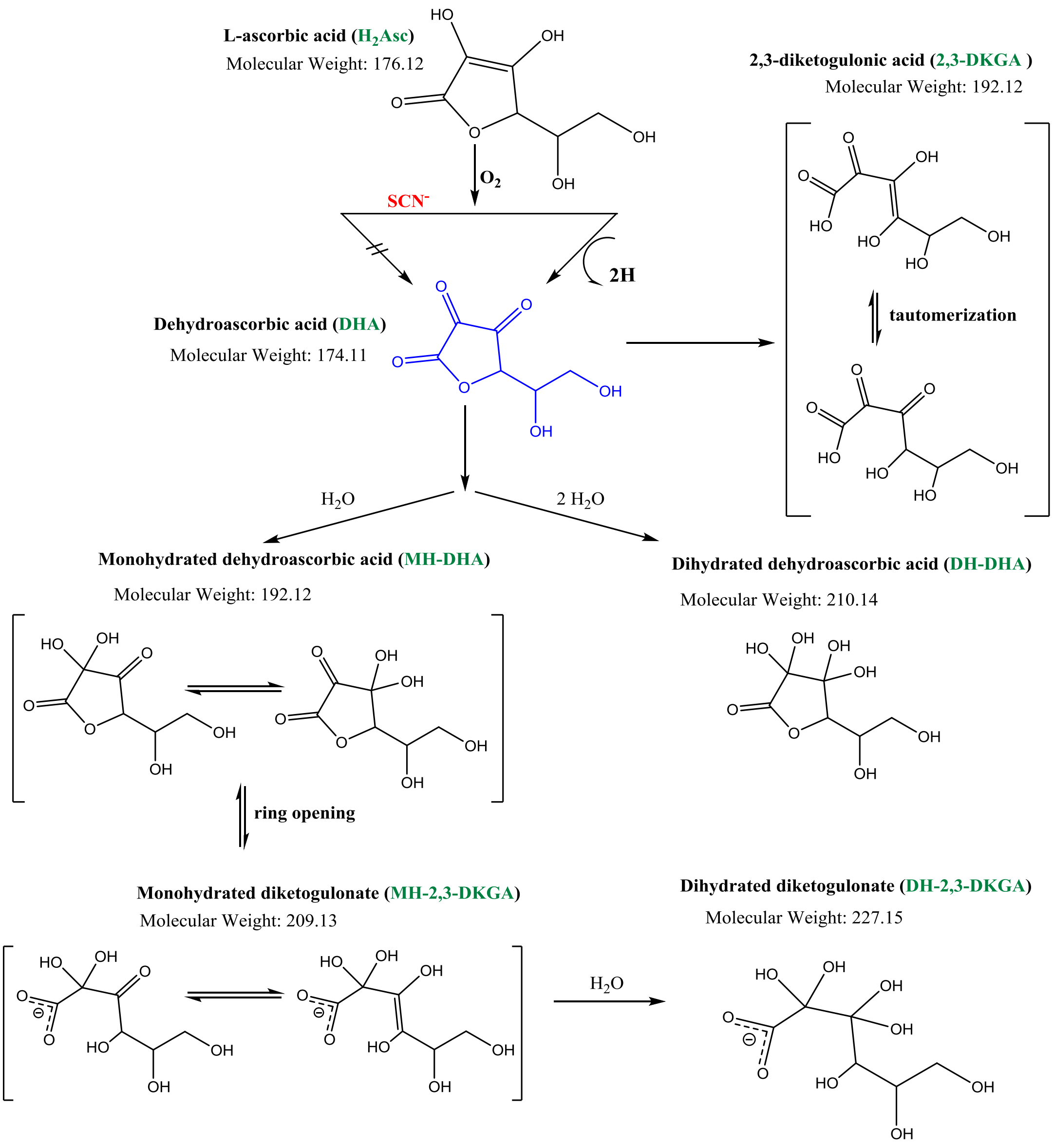


Fig. 13. The putative mechanism of the H_2Asc decomposition/stabilization in water solution and in the presence of electrolyte under study.

5. Conclusion

In this paper new electrochemical procedures have been elaborated for the first time to investigate the stability of ascorbic acid (H_2Asc). It has been proven that the potentiometric titration (PT) experiments supported by the theoretical (modelling) calculations can be successfully applied in a regular analytical practice as well as in the chemical and pharmaceutical industries to control the quality of the aqueous solutions of H_2Asc. In addition, the elaborated paraffin-protection-layer procedure has been proposed as a good laboratory practice for protecting the solutions against external factors exemplified by: O_2,

CO_2 and temperature (evaporation). Furthermore, the potentiometric titration (PT) method enables us to get some insight into the degradation products of the investigated acid. It has been found that 2,3-diketogulonic acid (2,3-DKGA) is the main product of dehydroascorbic acid (DHA) hydrolysis. Experimental data and the numerical EQSOL procedure confirmed for the first time that 2,3-DKGA exists in an aqueous solution mainly in an enol form with the values of dissociation constants equal $pK_{a3} \sim 3.0$ (-COOH) and $pK_{a4} \sim 9.0$ (the -OH enol). Finally, it has been well documented that in aqueous solutions of different electrolytes (KNO_3, $KClO_4$ and KSCN) SCN^- ions are most effective to protect H_2Asc against degradation (oxidation). The stabilizing effect of SCN^- has subsequently been confirmed by conductometric measurements (CM) and ESI/MS data.

Acknowledgments

The authors would like to thank Dr Marek Ślebioda, Perlan Technologies Polska Sp. z o. o. for registration of ESI/MS spectra. Special acknowledgment to Prof Adam Liwo for consulting Cerko and elaborating on the EQSOL algorithm.

References

1. Davey, W., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I., Strain J., Favell, D., Fletcher, J.: Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J. Sci. Food Agric. **80**, 825-860 (2000)

2. Loewus, F.A.: Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. Phytochemistry **52**, 193-210 (1999)

3. Hacisevki, A.: An overview of ascorbic acid biochemistry. J. Fac. Pharm. Ankara **38**, 233-255 (2009)

4. Du, J., Cullen, J.J., Buettner, G.R.: Ascorbic acid: Chemistry, biology and the treatment of cancer. Biochim. Biophys. Acta **1826**, 443–457 (2012)

5. Shen, J., Grifths P.T., Campbell, S.J., Utinger, B., Kalberer, M., Paulson, S.E.: Ascorbate oxidation by iron, copper and reactive oxygen species: review, model development, and derivation of key rate constants. Sci. Rep. **11**, 7417 (2021)

6. Buettner, G.R., Jurkiewicz, B.A.: Catalytic metals, ascorbate, and free radicals: combinations to avoid. Radiat. Res. **145**, 532-541 (1996)

7. Vethanayagam, J.G., Green, E.H., Rose, R.C., Bode, A.M.: Glutathione-dependent ascorbate recycling activity of rat serum albumin. Free Radic. Biol. Med. **26**, 1591-1598 (1999)

8. Brittain, H.G.: Critical compilation of pKa values for pharmaceutical substances 33,18 and 66. Profiles Drug Subst. Excip. Relat. Methodol. Academic Press (2007)

9. Tu, Y.J., Njus, D., Schlegel, H.B.: A theoretical study of ascorbic acid oxidation and HOO•/O2•− radical scavenging. Org. Biomol. Chem. **15**, 4417 (2017)

10. Van der Zee, J., Van der Broke, P.J.A.: Detrermination of the ascorbate radical concentraion in mixture of ascorbate and dehydroascorbate. Free Radic. Biol. Med. **25**, 282-286 (1998)

11. Schweinzer, E., Goldenberg, H.: Monodehydroascorbate reductase activity in the surface membrane of leukemic cells. Eur. J. Biochem. **218**, 1057-1062 (1993)

12. Nishikawa, Y., Kurata, T.: Interconversion between Dehydro-L-Ascorbic acid and L-Ascorbic Acid. Biosci. Biotechnol. Biochem. **64**, 476-483 (2000)

13. Amin, D.: Application of differential pulse polarography to the assay of ascorbic acid. Microchem. J. **28,** 174-179 (1983)

14. Bujak, K.S., Eder-Trifunović, J., Kniewald, G.: Determination of l ascorbic acid in fresh and processed fruit and vegetables by differential pulse polarography. Fresenius Z. Anal. Chem. **329**, 760-763 (1988)

15. Suliborska, K., Baranowska, M., Bartoszek, A., Chrzanowski, W., Namieśnik, J.: Determination of Antioxidant Activity of Vitamin C by Voltammetric Methods. Proceedings **11**, 23 (2019)

16. Abera, H., Abdisa, M., Washe, A.P.: Spectrophotometric method to the determination of ascorbic acid in M. stenopetala leaves through catalytic titration with hexavalent chromium and its validation. Int. J. Food Prop. **23**, 999-1015 (2020)

17. Cancalon, P.F.: Routine Analysis of Ascorbic Acid in Citrus Juice Using Capillary Electrophoresis. J. Aoac Int. **84**, 987-992 (2001)

18. Pyka-Pajak, A., Dołowy, M., Parys, W., Bober, K., Janikowska, G.A: Simple and Cost-Effective TLC-Densitometric Method for the Quantitative Determination of Acetylsalicylic Acid and Ascorbic Acid in Combined Effervescent Tablets. Molecules **23**, 3115 (2018)

19. Novakova, L., Solich, P., Solichova, D.: HPLC methods for simultaneous determination of ascorbic acid and dehydroascorbic acids. Trends Anal. Chem. **27**, 942-958 (2008)

20. Dewhirst, R.A., Fry, S.C.: The oxidation of dehydroascorbic acid and 2,3-diketogulonate by distinct reactive oxygen species. Biochem. J. **475**, 3451–3470 (2018)

21. Bjerrum, J.: Stability constants. In Chem. Soc. pp. No-7 (1958)

22. Pilarski, B., Kaliszan, R., Wyrzykowski, D., Młodzianowski, J., Balińska, A.: General analytical procedure for determination of acidity parameters of weak acids and bases. J. Anal. Methods Chem. 1-8 (2015)

23. Kostrowicki, J., Liwo, A.: A general method for the determination of the stoichiometry of unknown species in multicomponent systems from physiochemical measurements. Comput. Chem. **11**, 195-210 (1987)

24. Kostrowicki, J., Liwo, A.: Determination of equilibrium parameters by minimization of an extend sum of squares. Talanta **37**, 645-650 (1990)

25. Tyler, G.S.D., Vecchio, D., Zadlo, A., Rineh, A., Sadasivam, M., Avcib, P., Huang, L., Kozinska, A., Chandranb, R., Sarna, T., Hamblin, M.R.: Thiocyanate potentiates antimicrobial photodynamic therapy: *In situ* generation of the sulfur trioxide radical anion by singlet oxygen. Free Radic. Biol. Med. **65**, 800–810 (2013)

26. Milligan, J.R., Aguilera, J.A., Paglinawan, R.A., Ward, J.F.: Mechanism of DNA damage by thiocyanate radicals. Int. J. Radiat. Biol. **76**, 1305-1314 (2000)

27. Herrmann, H., Hoffmann, D., Schaefer, T., Brauer, P., Tilgner, A.: Tropospheric aqueous-phase freeradical chemistry: radical sources, spectra, reaction kinetics and prediction tools. Chemphyschem **11**, 3796–3822 (2010)

28. Szultka, M., Buszewska-Forajta, M., Kaliszan, R., Buszewski, B.: Determination of ascorbic acid and its degradation products by high-performance liquid chromatography-triple quadrupole mass spectrometry. Electrophoresis **35**, 585-592 (2014)

29. Bottegal, M., Lang, L., McCord, M., McCord, B.: Analysis of ascorbic acid based black powder substitute by high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. **24**, 1377–1386 (2010)

30. Lavoie, J.C., Chessex, P., Rouleau, T., Migneault, D., Comte, B.: Light-Induced Byproducts of Vitamin C in Multivitamin Solutions. Clin. Chem. **50**, 135-140 (2004)

31. Peng, L., Hua, L., Wang, W., Zhou1, Q., Li, H.: On-site Rapid Detection of Trace Non-volatile Inorganic Explosives by Stand-alone Ion Mobility Spectrometry via Acid-enhanced Evaporization. Sci. Rep. **4**, 6631 (2014)

32. Pavlov, J., Attygalle, A.B.: Direct Detection of Inorganic Nitrate Salts by Ambient Pressure Helium-Plasma Ionization Mass Spectrometry. Anal. Chem. **85**, 278–282 (2013)