Scientific paper

Voltammetric Determination of Folic Acid Using Liquid Mercury Free Silver Amalgam Electrode

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Abstract

The electrochemical behavior of folic acid (FA) on a polished silver solid amalgam electrode (p-AgSAE), as a liquid mercury free electrode, has been studied in this paper. Differential pulse voltammetry (DPV) was found as a suitable method for voltammetric determination of folic acid. A limit of detection as 5.88×10^{-10} M (after 60 s of accumulation) was calculated for FA determination in an acetate buffer (pH 5). P-AgSAE in connection with DPV with optimized parameters was successfully applied for the determined folic acid compound in two types of vitamin preparations and two kinds of fruit juices. It was found that p-AgSAE is an appropriate tool for voltammetric determination of this vitamin in these real samples and it can replace mercury electrodes in voltammetric analysis of folic acid.

Keywords: Polished silver solid amalgam electrode, folic acid, differential pulse voltammetry

1. Introduction

Folic acid (FA, CAS: 59-30-3, *N*-(4-{[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino}benzoyl)-L-glutamic acid, pteroyl-L-glutamic acid, Figure 1) is an essential water soluble vitamin which belongs to the B-vitamin group (B_9). The human body can not synthesize 4-aminobenzoic acid or link glutamate with pteridine, which is why enrolling folic acid from food is necessary. Folic acid after ingestion is transformed into its active form (tetrahydrofolate) in the small intestine. The enzyme dihydrofolatreductase (tetrahydrofolate dehydrogenase) catalyzes this reaction.[1,2]

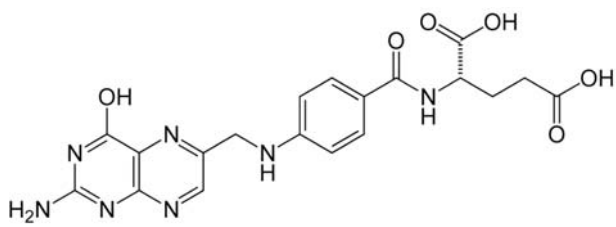

Figure 1: The structure of folic acid

Folic acid as a significant bioactive compound attends many processes in a human body like synthesis of methionine, transferring of one-carbon groups (necessary for DNA and RNA synthesis), formation of red blood cells and differentiation of cells. Sufficient dietary intake of FA during pregnancy prevents neural tube defects of the fetus (e.g. anencephaly or spina bifida).[1–3] The protective effect of FA was also observed in the treatment of some diseases such as stroke,[4] ischaemic heart disease[4] or colorectal cancer.[1,5] Deficiency of folic acid causes some types of anemia and increases the risk of cardiovascular diseases.[1] The recommended daily intake of FA is 0.2 mg for adults and 0.4 mg for pregnant women (The European Food International Council). Foods rich in FA are especially yeast, green vegetables, oranges, nuts, liver and kidney.[1]

Folic acid, as it was described above, plays a very important role in the human body, i.e. many analytical methods have been developed for its determination. One of the very often used analytical methods for FA determination is liquid chromatography (LC) in various performances such as HPLC (high performance LC) with UV[6,7] or diode array detection,[8] LC with tandem mass spectroscopy[9] or microemulsion electrokinetic chromatography.[10] Some other analytical methods like capillary electrophoresis[11] or ELISA (enzyme-linked immunosorbent assays)[12] have also been used for determination of this vitamin.

The electrochemical behavior of folic acid (and its derivates) has been studied for more than 40 years due to its biological relevance and the necessity to find simple, reliable and sensitive methods for its determination. It was found that FA undergoes three two electrons/two protons ($2e^-/2H^+$) reduction steps in an acidic medium and one in an alkaline medium on mercury electrodes. The first step in an acidic medium is a quasi-reversible reduction of FA to 7,8-dihydrofolic acid via an unstable 5,8-dihydrofolic acid derivate, which is subjected to tautomerization. The electrochemical cleavage of the C(9)-N(10) bond of 7,8-dihydrofolic acid to 6-methyl-7,8-dihydropterin and *p*-aminobenzoylglutamic acid represents the second step of electrochemical reduction of FA. The last step is an irreversible reduction of 6-methyl-7,8-dihydropterin to 6-methyl-5,6,7,8-tetrahydropterin. Conversion of FA to 5,8-dihydrofolic acid, which is stable and is not subjected to tautomerization, describes the reduction of FA in an alkaline medium.[13] Mercury electrodes in connection with various polarographic and voltammetric methods have been successfully used for FA determination in various samples. Jacobsen and Bjørnson applied a. c. polarography with DME (dropping mercury electrode) as a working electrode for the determination of FA in pharmaceutics in 1978.[14] Alvarez at al. studied voltammetric behavior of FA on HMDE (hanging mercury drop electrode) using AdSV (adsorptive stripping voltammetry). The limit of detection (LD) was calculated as 1×10^{-11} M after 10 minutes of accumulation.[15] HMDE as a working electrode in connection with AdSV was also applied for determination of FA and riboflavin in vitamin preparations.[16] HMDE was successfully applied for determination of FA in sea water.[17,18,] Besides mercury electrodes, carbon electrodes modified with some complicated organic compounds like phosphomolybdic-polypyrrole film[19] or *p-tert-butyl*-calix[*n*]arenes (where $n = 4$, 6 or 8)[20] were successfully used for the determination of FA in real samples. Electrochemical behavior of FA on single-walled carbon nanotube paste coated glassy carbon electrode was described by F. Xiao et al.[21] Using glassy carbon electrode (as a substrate) with lead film for studying of voltammetric behavior of FA and its determination in two pharmaceutical preparations was described by Korolczuk and Tyszczuk in.[22] They found limit of detection of FA as 7×10^{-10} M after 120 s of accumulation. The direct electrochemistry of FA was studied on 2-mercaptothiazole self-assembled gold electrode by Wan and Yang.[23] They also used mentioned modified gold electrode for FA determination in pharmaceuticals.[23]

Previously mentioned electrodes show high sensitivity for FA determination but their construction and preparation for each measurement is often complicated or they contain liquid mercury, which is prohibited for analytical use in some countries (like in Sweden). Therefore, some new electrode materials are still been looked for. Silver solid amalgam electrodes are relatively new electrodes which were introduced by Novotný and Yosypchuk[24] and independently by Mikkelsen and Schrøder.[25] The one, which was originally prepared by Novotný and Yosypchuk[24] and which has been used in this paper, is made from a narrow glass tube (inner diameter about 0.5 mm) which was filled with silver powder (another metal like copper, indium or gold can be used as well) and platinum wire. Then it was immersed into the mercury for 2 hours (the time required for amalgamation). The upper part of the platinum wire was connected to the supply of electrical contact and the upper part of the electrode was sealed with a plastic cap. The electrode can be used as a polished silver solid amalgam electrode (p-AgSAE) after polishing the working surface, but the polished surface can be modified with a mercury meniscus (m-AgSAE) or mercury film (MF-AgSAE) as well.[24] Advantages of these electrodes are: high hydrogen overvoltage, good mechanic stability, possibility of electrochemical surface regeneration and no presence of the toxic liquid mercury (especially p-AgSAE).[24] AgSAEs have been already used for determination of various inorganic compounds,[24,26,29,30] organic compounds[27,28,31,32] and bioactive compounds like phytochelatins[33–35] or nucleic acids.[36,37]

The voltammetric behavior of FA on m-AgSAE (mercury meniscus modified silver solid amalgam electrode) has been described in our previous paper.[27] This presented paper has been focused on voltammetric behavior of FA on p-AgSAE (polished silver solid amalgam electrode) and the study of electrochemical reduction of FA on p-AgSAE using EVLS. Furthermore, the optimal parameters of differential pulse voltammetry were found for FA determination using model solution and proposed method was successfully applied for FA determination in two types of pharmaceutical preparations and two fruit juices.

2. Experimental

2. 1. Reagents and Standards

All chemicals used for the preparation of supporting electrolytes, standard solution and other stock solutions were p.a. pure. All solutions were prepared in bidistilled water. The Britton–Robinson (B–R) buffer of pH value from 3 to 10 was prepared from an alkaline component consisting of 0.2 M NaOH (Lachema, Brno, Czech Republic) and an acidic component which consists of 0.04 M H_3PO_4, 0.04 M H_3BO_3 and 0.04 M CH_3COOH (all Lachema, Brno, Czech Republic). 0.05 M acetate buffer of pH 5 was prepared by mixing the stock of solutions of acetic acid and sodium acetate (both Lachema, Brno, Czech Republic). Folic acid (Sigma-Aldrich) was dissolved in 0.01 M NaOH (Lachema, Brno, Czech Republic). The solution of 2 M KCl was prepared by dissolution of an appropriate amount of KCl (Lachema, Brno, Czech Republic). Tablets "Folic acid forte" were purchased from Naturvita, Czech Republic and "GS Mamavit" tablets ori-

ginated from Green Swan Pharmaceuticals, Czech Republic. The fruit juice "HELLO VIVA multivitamin" was purchased from LINEA Nivnice, Slovak Republic and the fruit juice "TOMA multivitamin" originated from Pepsi-Americas/General Bottlers CR s. r. o., Czech Republic.

2. 2. Instrumentation

All voltammetric measurements were carried out with the computer controlled Eco-Tribo Polarograph PC-ETP (Eco Trend Plus, Prague, Czech Republic)[38] which was equipped by software POLAR.PRO (version 5.1) for Windows XP. All measurements were provided in a 3-electrodes set up where p-AgSAE (Eco Trend Plus, Prague, Czech Republic) served as a working electrode, silver/silver chloride/saturated KCl (Ag/AgCl/KCl (satur.)) as a reference and platinum wire as an auxiliary electrode (both from Monokrystaly, Turnov, Czech Republic). The pH values were measured by pH-meter Hanna 221 (Hanna Instruments, Inc., USA). Solutions of tablets containing FA were prepared by applying ultrasonic bath Bandelin Sonorex (Schalltec GmbH, Germany). All the measurements were carried out at laboratory temperature.

2. 3. Preparation and Pretreatment of the p-AgSAE

The polished silver solid amalgam electrode (type described in the paper[24]), was ground on soft emery paper and then polished using a polishing kit (Electrochemical Detectors, Turnov, Czech Republic), which consisted of a polishing polyurethane pad, Al_2O_3 suspension (particle size 1.1 μm) and Al_2O_3 powder (particle size 0.3 μm). The polishing was undertaken once per week. Before the first measurement of the day as well as after every pause longer than one hour the electrode surface was electrochemically activated in the solution of 0.2M KCl by applying the potential –2200 mV for 300 s while stirring.[28] Electrochemical regeneration of the amalgam electrode surface before each measurement was inserted into the measuring program. Optimal surface regeneration for p-AgSAE in acetate buffer (pH 5) was found as 30 regeneration cycles between 0 and –2000 mV in jumps (one jump took 0.3 s) and optimal conditions for regeneration in B-R buffer (pH 8) as one regeneration cycle for 20 s at the potential –1500 mV.

2. 4. Voltametric Measurements

CV (cyclic voltametry) was used for study of voltammetric behavior of FA depending on the pH value (B-R buffer of pH value from 3 to 10). DCV (direct current voltammetry) was utilized for studying the influence of the scan rate on voltammetric behavior of FA. DPV (differential pulse voltammetry) was used for all other measurements. Two media were further used as a supporting electrolyte: 0.05M acetate buffer (pH 5) and Britton-Robinson buffer (pH 8). Parameters of the DPV for each medium differ especially in how to regenerate the electrode surface (described in the chapter 2.3.). Other parameters of DPV were almost the same for both used electrolytes: pulse height –50 mV, pulse width 80 ms, initial potential E_{in} = 0 mV (E_{in} = –100 mV for pH 8), final potential E_{fin} = –800 mV (–1000 mV for pH 8), scan rate v = 20 mV s^{-1}, potential of accumulation E_{acc} = 0 mV. The time of accumulation t_{acc} depends on concentration of FA in the medium.

2. 5. Elimination Voltammetry with Linear Scan

This method is often used for explanation of the reaction processes on electrode surfaces and for the increase of current sensitivity. Elimination voltammetry with linear scan (EVLS) is based on elimination of some particular currents from linear voltammetric curves recorded at various scan rates.[39,40] In EVLS, there is assumed that the measured current is the sum of particular current contributions, such as the charging current I_c, the diffusion controlled current I_d, the kinetic current I_k and the so-called irreversible current I_{ir}.

$$I = \sum j I_j = I_k + I_c + I_d + I_{ir} + \ldots \quad (1)$$

Each particular current must be expressible in the form:

$$I_j = W_j(v) \cdot Y_j(E) = v^x Y_j(E) \quad (2)$$

where I_j is a particular current, v is the scan rate, $W_j(v)$ is a function of the scan rate and $Y_j(E)$ is a function of the potential.

The diffusion current is proportional to the square root of v ($I_d = v^{1/2}\, Yd(E)$), the charging current is directly proportional to v ($I_c = v\, Y_c(E)$), the kinetic current is independent of v ($I_k = v^0\, Y_k(E)$) and the irreversible current is inversely proportional to the square root of v ($I_{ir} = v^{-1/2}\, Y_{ir}(E)$). As all n particular currents exhibit their specific dependence on the scan rate, all measurements must be done with n different scan rates. One scan rate is selected as a reference (ref) and the others are compared with it (v/v_{ref}).[39–41]

In this work the following current functions (3)–(9) for calculation of elimination current functions, have been used for scan rates 50, 100, 200 and 400 mV s^{-1}.[40] Only one current was conserved and the others were eliminated.

$$f(I_d) = 17.4857\, I - 11.657\, I_{1/2} - 5.8284\, I_2 \quad (3)$$

$$f(I_k) = 2.4142\, I_2 + 6.8284\, I_{1/2} - 8.2426\, I \quad (4)$$

$$f(I_c) = 3.4142\, I_2 + 4.8284\, I_{1/2} - 8.2426\, I \quad (5)$$

$$f(I_d) = 43.213\ I - 48.041\ I_{1/2} - 11.657\ I_2 + 16.485\ I_{1/4} \quad (6)$$

$$f(I_c) = 16.485\ I_{1/2} - 16.485\ I + 5.282\ I_2 - 5.282\ I_{1/4} \quad (7)$$

$$f(I_k) = 43.213\ I_{1/2} - 33.970\ I + 8.243\ I_2 - 16.485\ I_{1/4} \quad (8)$$

$$f(I_{ir}) = -11.657\ I_{1/2} - 8.243\ I - 1.867\ I_2 + 5.282\ I_{1/4} \quad (9)$$

I_2, $I = I_1$, $I_{1/2}$ a $I_{1/4}$ are currents measured at the particular scan rates. The equations published in paper[39] served for calculation of the coefficient αn (where α denotes the coefficient of the charge transfer and n is the number of exchanged electrons per molecule of reactant involved in the rate-determining step).

2. 6. Preparation of Samples Solutions

One tablet was powdered in a grinding mortar and then the whole powdered amount was transferred to a 100ml standard flask and it was diluted with 0.01M NaOH. The solution was filtered after 30 minutes of sonication. The filtered solution was filled to 100 ml with 0.01M NaOH and it was used for an analysis. 220 µl of solution of "Mammavit" or 110 µl of a prepared solution of "Folic acid Forte" were added to 10 ml of 0.05M acetate buffer to the polarographic cell and 200 resp. 50 µl of standard FA solution ($c_{FA} = 1 \times 10^{-4}$ M) were added as a standard addition. Both fruit juices were not pretreated but it was necessary to stir them right before analysis. 500 µl of stirred juice was added into the polarographic cell with supporting electrolyte and 10 µl of standard solution of 1×10^{-4} M FA was added as a standard addition.

3. Results and Discussion

3. 1. Voltammetric Behavior of Folic Acid on p-AgSAE

The reduction mechanism on mercury electrodes was described in[13] and it was found that FA provides 3 reduction peaks in an acidic medium and 1 peak in an alkaline medium. Voltammetric behavior of FA on p-AgSAE in dependence on pH was initially investigated. Figure 2 shows cyclic voltammogram after addition of FA ($c_{FA} = 5 \times 10^{-6}$ M) into the supporting electrolyte of pH value of 3 (medium thick line), 5 (the thickest line) and 8 (thin line).

Only two cathodic peaks (1 and 2) corresponding to the reductions and one anodic peak (1') corresponding to the oxidation process were observed in an acidic medium. The third supposed reduction peak was not able to be recorded because the potential window available on p-AgSAE is narrower than on HMDE, which is why the most negatively situated peak was probably overlapped by the signal of supporting electrolyte decomposition. The oxidation peak, which was recorded, corresponds to oxidation of the dihydrofolic acid to folic acid. The same results were also observed on m-AgSAE and HMDE.[13,27] The first reduction peak was recorded in the widest range of pH – from 3 to 9. The highest current response of this peak was recorded in the medium of pH equal to 5 (Figure 3). The second reduction peak was recorded only in the acidic medium (pH from 3 to 6, Figure 3). The highest current response was observed in B-R buffer of pH equal to 4 (Figure 3) and the current responses in media of pH 5 and 6 were very low. The position of both mentioned peaks changed with value of pH. All peaks linearly shifted to more negative potentials with increasing values of pH (Figure 4). The obtained linear dependences in Figure 4 can be described with equation (10) for the first reduction peak and equation (11) for the second reduction peak. The correlation coefficient is 0.9985 for equation (10) and 0.9995 for (11).

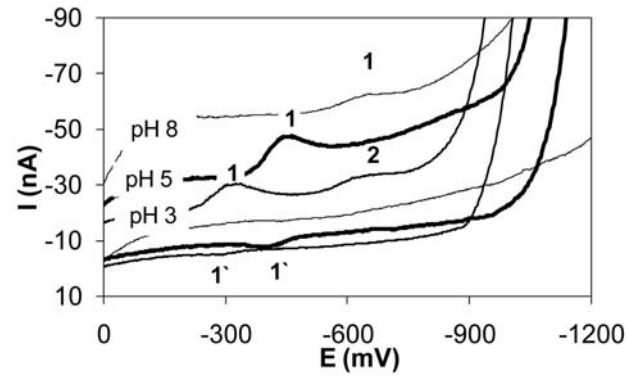

Figure 2: Cyclic voltammograms of FA recorded in acidic and alkaline supporting electrolyte on p-AgSAE. Method: CV, B-R buffer with FA (pH 3 – medium thick line, 5 – the thickest line and 8 – thin line), $c_{FA} = 5 \times 10^{-6}$ M, $E_{in} = 0$ mV, $E_{fin} = -1400$ mV, $v = 100$ mV s^{-1}; 1 – first reduction peak, 2 – second reduction peak, 1' – oxidation peak

$$E_p\ (\text{mV}) = -62.92 \pm 3.8\ \text{pH} - 140 \pm 24 \quad (10)$$

$$E_p\ (\text{mV}) = -116.5 \pm 11\ \text{pH} - 282 \pm 52 \quad (11)$$

Peak 1 was chosen for all other experiments because this peak was observed in the widest range of pH, it was well developed and easily evaluable. Its highest current response was recorded in the medium of pH 5, which is why we chose this value of pH for all subsequent analysis. 0.05 M acetate buffer and B-R buffer of pH 5 were tested as supporting electrolytes. FA provided higher current response in the acetate buffer, p-AgSAE also showed higher sensitivity and the regeneration of electrode's surface was more efficient in this medium. Therefore, 0.05 M acetate buffer (pH 5) was selected as the supporting electrolyte. Some measurements were also carried out in an alkaline medium (B-R buffer of pH 8) for comparison of p-AgSAE's sensitivity in media of various values of pH.

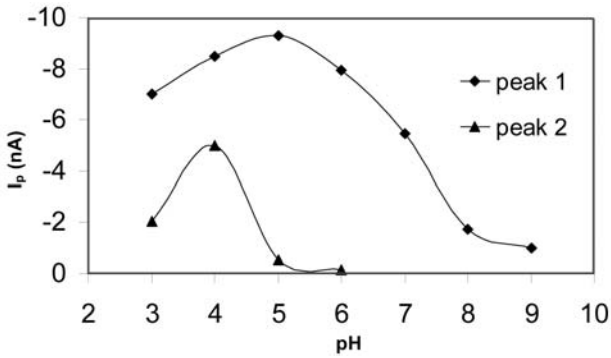

Figure 3: The dependence of current response (I_p) on pH. Method: CV, sup. electrolyte: B-R buffer (pH 3 – 9), c_{FA} = 5 × 10^{-6} M, E_{in} = 0 mV, E_{fin} = –1400 mV, v = 100 mV s^{-1}

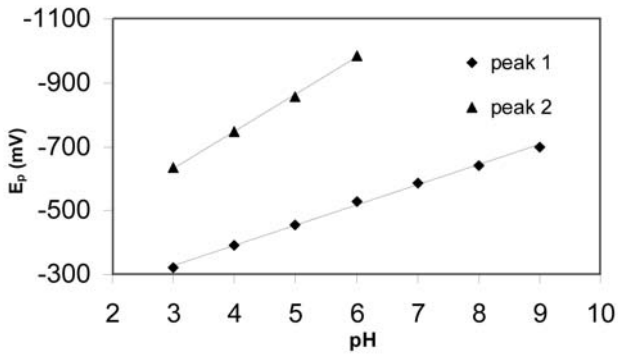

Figure 4: The dependence of peaks' positions (E_p) on pH. Method: CV, sup. electrolyte: B-R buffer (pH 3 – 9), c_{FA} = 5 × 10^{-6} M, E_{in} = 0 mV, E_{fin} = –1400 mV, v = 100 mV s^{-1}

The dependence of height of the first reduction peak on scan rate (v) in acetate buffer is shown in Figure 5. The current response increased linearly in the range of v from 20 to 100 mV s^{-1} and the linear dependence can be described by following equation with correlation coefficient R = 0.998:

$$I_p\ (\text{nA}) = -0.035 \pm 0.002\ v\ (\text{mV s}^{-1}) - 0.99 \pm 0.12 \quad (12)$$

This dependence corresponds with an adsorption-controlled process. The second reduction step and the oxidation step can also be described as an adsorption-controlled process, which is in positive agreement with our previous paper[27] where voltammetric behavior of FA on m-AgSAE and HMDE has been investigated.

As it was mentioned in chapter Experimental, EVLS is based on an elimination of some particular currents from linear voltammetric signals recorded at different scan rates. For this paper, signals were recorded by applying the following four scan rates: 50, 100, 200 and 400 mV s^{-1}; scan rate 200 mV s^{-1} was chosen as a reference rate and the scan rates were in the ratio of 1/4:1/2:1:2. For calculation of particular currents (and the simultaneous elimination of the other currents) equations (3)-(9) were applied. The first observed reduction signal in pH 5 (about the potential (E_p) –450 mV) corresponds to the reduction in an adsorbed state, which is obvious from the course of the calculated elimination curves – I_d with the shape "peak/counter-peak" and I_c as well as I_k with the inverse shape "counter-peak/peak". As can be derived from the ratio between the height of the peak and of the counter-peak of the diffusion current curve (function), the adsorption is relatively weak (in comparison with the mercury electrode[27]) and the diffusion is the controlling process of this FA reduction. The second reduction step (recorded at about –850 mV) represents the reduction in an adsorbed state too, but the adsorption is much weaker than at the first reaction. As it was mentioned above, EVLS enables the elimination of particular components of the recorded signals, e.g., elimination of the kinetic component under simultaneous conservation of the diffusion controlled signal. Such a kinetic process can be the decomposition of the supporting electrolyte, i.e., hydrogen evolution. Nevertheless, the application of this technique was not sufficient in the case of the third reduction step, which is totally overlapped by the signal of the hydrogen evolution. The obtained anodic peak corresponds also with the diffusion controlled process with very weak adsorption.

From the differences of the peak potentials recorded in different elimination voltammograms and in original voltammogram, the product αn could be evaluated for the most positively situated peak (where α denotes the charge transfer coefficients and n number of exchanged electrons per molecule of reactant involved in the rate-determining step). This product amounted to 0.85 on average (from 0.65 to 0.97 for four different elimination equations) for the most positive situated peak. Therefore, it could be concluded that the number of exchanged electrons is equal to 2 and the charge transfer coefficient to 0.42 and the supposed reaction mechanism described in the literature[13] could be confirmed.

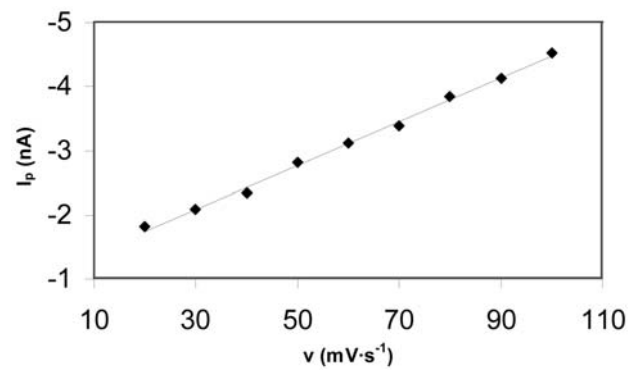

Figure 5: The dependence of current response (I_p) on scan rate. Method: DCV, supporting electrolyte: 0.05 M acetate buffer (pH 5), c_{FA} = 5 × 10^{-6} M, E_{in} = 0 mV, E_{fin} = –1000 mV, v = 20–100 mV s^{-1}

3. 2. Determination of Folic Acid in Model Solution

Differential pulse voltammetry (DPV) was used for examination of the following parameters: initial potential (E_{in}), potential of accumulation (E_{acc}) and time of accumulation (t_{acc}) for both mentioned supporting electrolytes. It was found that the initial potential in the range from 0 to –300 mV (0 to –500 mV for pH 8) had no significant effect on the height of the first reduction peak of FA. Current responses differed not more than 10%, i.e. E_{in} = 0 mV was chosen for the acetate buffer and E_{in} = –100 mV for the B-R buffer as an adequate for the measurements. As well as the current responses did not change with potential of accumulation from 0 to –300 mV for acetate buffer of pH 5 and from 0 to –500 mV for B-R buffer of pH 8 but with more negative values of E_{acc} the current responses started to decrease, i.e. E_{acc} = 0 mV was selected for both used supporting electrolytes. The time of accumulation depended on the concentration of FA. The appropriate time of accumulation was found for all concentration ranges in which FA was determined. For example the height of the first reduction peak of 2×10^{-8} M folic acid increased linearly in the range of t_{acc} from 0 to 40 s and the obtained linear dependence can be described by the equation (13) with correlation coefficient equal to 0.992:

$$I_p\,(\text{nA}) = -0.019 \pm 0.005\; t_{acc}\,(\text{s}) - 0.28 \pm 0.11 \quad (13)$$

Conditions of solid the electrode's surface regeneration are very important because the surface is not renewed and it gets older after every measurement, unlike the mercury drop electrodes' surface. The regeneration step was inserted in the measurement software right before every analysis. Some options of optimal regeneration like an insertion of some tens of regeneration cycles between positive and negative potential or an insertion of one very negative potential, were tested. Insertion of one very negative potential (–1500 mV and more negative) as a regeneration step before each measurement regenerated the surface very well but caused decreasing of reproducibility (especially in medium of pH 5). No decreasing of reproducibility or other disturbing effects were observed using 30 regeneration cycles between 0 and –2000 mV and these parameters were also sufficient for regeneration of the surface, i.e. these parameters were used for all other measurements in the acetate buffer. Conversely, insertion of only one negative potential (–1500 mV) for 20 s was sufficient for regeneration of the surface in the alkaline medium and no disturbing effects were observed. Applying the proposed optimal regeneration parameters, the relative standard deviations of 11 times repeated measurements ($RSD_M(11)$) were amounted to 0.76% ($c_{FA} = 5 \times 10^{-7}$ M) in the acetate buffer and 2.05% ($c_{FA} = 5 \times 10^{-6}$ M) in the B–R buffer of pH 8. Both achieved values of $RSD_M(11)$ are lower than 3%, which confirm good reproducibility of repeated measurements on p-AgSAE in both mentioned pH values and show well-made surface regeneration.

Another statistical parameter as a reproducibility of repeated determinations (RSD_S) was investigated. Every determination was 5 times repeated. RSD_S were calculated for various concentration levels, which were determined in both supporting electrolytes of used pH. The standard addition method was applied for determination of FA in these model solutions. Obtained results are summarized in Table 1 and it was found that all RSD_S are lower than 5%, which demonstrates good reproducibility of repeated determinations. The example of the concentration dependence of FA in model solution in the acetate buffer (A) and in B-R (B) is shown in Figure 6. The current response increased linearly in the range from 2×10^{-8} to 12×10^{-8} M of FA in the acetic medium (Figure 6 A) and from 2×10^{-7} to 10×10^{-7} M of FA in the alkaline medium (Figure 6 B). The obtained linear dependences can be described by the equation (14) with R = 0.999 for the acetate buffer and by the equation (15) with R = 0.9989 for the B-R buffer:

$$I_p\,(\text{nA}) = -0.0146 \pm 0.001\; c\,(\text{nM}) - 0.045 \pm 0.061 \quad (14)$$

$$I_p\,(\text{nA}) = -1.38 \pm 0.012\; c\,(\mu\text{M}) - 0.011 \pm 0.078 \quad (15)$$

The linear dynamic range of the first FA reduction peak was recorded from 1×10^{-9} to 2×10^{-7} M in the medium of pH 5 and from 5×10^{-8} to 4×10^{-6} M in the medium of pH 8.

Table 1: Relative standard deviations of 5 times repeated measurements for various concentrations levels of FA achieved on p-AgSAE in media of pH 5 and pH 8

	pH 5			pH 8	
added (nM)	found* (nM)	$RSD_S(5)$ (%)	added (nM)	found* (nM)	$RSD_S(5)$ (%)
20	20.28 ± 0.33	2.48	200	202.4 ± 2.95	2.20
10	10.20 ± 0.20	2.91	100	102.4 ± 2.99	4.40
5	5.03 ± 0.06	1.64	50	50.1 ± 0.17	0.50
2.5	2.54 ± 0.05	2.75	–	–	–
1	1.02 ± 0.03	4.00	–	–	–

*average of 5 determinations

A limit of detection (L_D) was calculated using ADSTAT software.[42] L_D for FA in acetate buffer is equal to 5.88×10^{-10} M (t_{acc} = 60 s) which is more than 20 times lower than L_D for FA determined in an alkaline medium ($L_D = 1.65 \times 10^{-8}$ M, t_{acc} = 40 s), i.e. only acetate buffer, due to higher sensitivity of p-AgSAE in this medium, was chosen as a supporting electrolyte for determination of FA in real samples. Voltammetric behavior of FA on m-AgSAE and on HMDE was investigated in our former paper.[27] Obtained limits of detection and electrodes' working surfaces (WS) are summarized in Table 2. L_D(p-AgSAE) is approximately 1.15 times higher than L_D(m-AgSAE) but the working surface of p-AgSAE is about 1.39 times smaller than the working surface of m-AgSAE, which confirms that both mentioned amalgam electrodes show almost the same sensitivity for determination of FA.

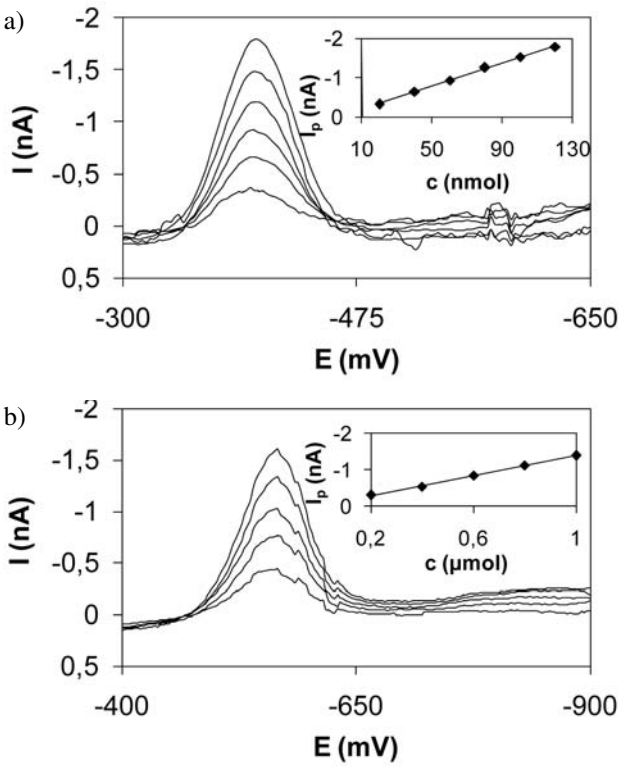

Figure 6: Concentration dependences of FA recorded on p-AgSAE in acetate buffer of pH 5 (**A**) and in Britton-Robinson buffer of pH 8 (**B**) – baseline corrections. **A**: method: DPV, sup. electrolyte: 0.05M acetate buffer (pH 5), $c_{FA} = 2 \times 10^{-8} - 12 \times 10^{-8}$ M, E_{in} = 0 mV, E_{fin} = –800 mV, E_{acc} = 0 mV, t_{acc} = 5 s, v = 20 mV s^{-1}; **B**: method: DPV, sup. electrolyte: B-R buffer (pH 8), $c_{FA} = 2 \times 10^{-7} - 10 \times 10^{-7}$ M, E_{in} = –100 mV, E_{fin} = –1000 mV, E_{acc} = 0 mV, t_{acc} = 5 s, v = 20 mV s^{-1}.

Table 2: Working surfaces (WS) and limits of detection (L_D) of p-AgSAE, m-AgSAE[27] and HMDE[27]

	p-AgSAE	m-AgSAE	HMDE
L_D (nM)	0.588	0.51	0.036
WS (mm^2)	0.28	0.39	0.73

3. 3. Determination of FA in Real Samples

Conditions of DPV, which were optimized on model solutions of FA and are described above, were used for voltammetric determination of FA in vitamin preparations (Folic acid Forte and GS Mamavit) and in fruit juices (Hello VIVA multivitamin and TOMA multivitamin). 0.05M acetate buffer (pH 5) was chosen as a supporting electrolyte for all analysis of real samples due to high sensitivity of p-AgSAE in this medium. The solution of each kind of tablet was prepared as was described above in the chapter 2.6. Preparation of samples solutions. 220 μl of solution of "Mammavit" or 110 μl of a prepared solution of "Folic acid Forte" were added to 10 ml of supporting electrolyte in the polarographic cell and it was analyzed. Then 200 μl (in case of "Mammavit") or 50 μl (in case of "Folic acid Forte") of standard solution of FA (c_{FA} = 1×10^{-4} M) were added as a standard addition. At least two standard additions were added to every analyzed solution. Every determination was repeated 5 times. The standard addition method was successfully applied for determination of FA in the tablets and obtained results are summarized in Table 3. Measured average concentrations of FA in vitamin preparations differ by less than 2% from values of producers, which demonstrates the accuracy of our determinations. Analogically, the FA content in two fruit juices (Hello VIVA multivitamin and TOMA multivitamin) was determined. Samples of fruit juices were not pretreated; they only had to be stirred right before analysis. Initially, the applicability of the found voltammetric method was tested. The known amount of the standard solution (20 μl) of 1×10^{-4} M FA was added into the support electrolyte with 0.5 ml of one of the juices and then the content of FA in the added standard solution was determined (the electrolyte with the juice was taken as a blank) using the standard addition method. Determined amount of FA differs about 9% compared to the added amount of FA (Tab. 3), i.e. the described voltammetric method is applicable for determination of FA in fruit juices. 0.5 ml of stirred juice was added into the polarographic cell with 10 ml of supporting electrolyte and this solution was analyzed. 10 μl of standard solution of 1×10^{-4} M FA was added as a standard addition. At least two standard additions were added to every analyzed solution of fruit juice. Every determination was repeated 5 times. The declared FA content from the producer is more than 400 μg L^{-1} (which corresponds to 0.91×10^{-6} M) in both juices. Determined FA content (Table 3) was more than 2 times higher than the declared content but we obtained basically the same results for the mentioned juices on m-AgSAE and on HMDE and they are published in our former paper.[27] Results obtained on m-AgSAE and HMDE for VIVA multivitamin differ by 4% and results for the second juice differ by 10%. This demonstrates that the content of FA is much higher than the producers declare. Determination of FA in "Hello VIVA multivitamin" is showed in Figure 7.

Table 3: Results of FA acid determination in vitamin preparations and fruit juices on p-AgSAE

	Declared (µg/tablet)	Found* p-AgSAE (µg/tablet)	RSD (%)	Found*[27] m-AgSAE (µg/tablet)	RSD (%)	Found*[27] HMDE (µg/tablet)	RSD (%)
GS Mamavit Folic Acid Forte	400	395.5 ± 6.1	1.2	399 ± 12	2.3	399 ± 19	3.9
	200	202.6 ± 4.8	1.9	199.7 ± 4.8	1.9	200.1 ± 5.8	2.3
	(mg L^{-1})	(mg L^{-1})	(%)	(mg L^{-1})	(%)	(mg L^{-1})	(%)
Hello Viva Juice(blank)	4.41 (added)	4.58 ± 0.18 (Recovery 99.8–107.9)	3.2	4.62 ± 0.21 (Recovery 99.9–109.3%)	3.6	4.60 ± 0.23 (Recovery 99.7–109.9%)	4.0
TOMA Multivitamin Juice (blank)	4.41 (added)	4.55 ± 0.16 (Recovery 99.5–106.8)	1.8	4.75 ± 0.36 (Recovery 99.5–115.7%)	6.1	4.48 ± 0.96 (Recovery 99.4–103.7%)	1.7
Hello Viva Juice	>400	0.940 ± 0.05	4.7	0.909 ± 0.047	4.1	0.945 ± 0.049	4.2
TOMA Multivitamin Juice	>400	1.130 ± 0.028	1.9	1.006 ± 0.037	3.0	1.036 ± 0.034	2.6

*average of 5 determinations

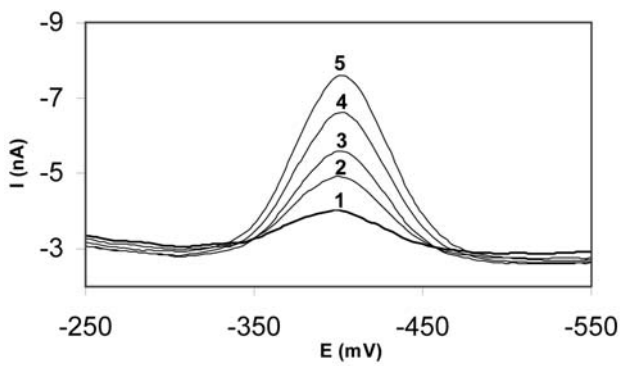

Figure 7: FA determination on p-AgSAE in fruit juice »Hello VIVA multivitamin« using standard addition method. Method: DPV, electrolyte: acetate buffer (pH 5), E_{in} = 0 mV, E_{fin} = –800 mV, v = 20 mV s^{-1}, no. of regeneration cycles = 30, E_{reg1} = 0 mV, E_{reg2} = –2000 mV, $t_{reg1} = t_{reg2}$ = 0.3 s, E_{acc} = 0 mV, t_{acc} = 0 s. Curve 1 – supporting electrolyte with 0.5 ml of juice, curves from 2 to 5 – standard additions of 1×10^{-4} M FA.

4. Conclusion

The aim of our paper was to examine voltammetric behavior of folic acid on a liquid mercury free polished silver solid amalgam electrode (p-AgSAE) and to find appropriate conditions for the voltammetric determination of FA in some real samples. One oxidation and two reduction peaks were observed in an acidic medium using p-AgSAE. This results are consistent with results obtained on m-AgSAE and HMDE[13,27] (the third reduction peak observed on m-AgSAE and HMDE was not able to recorded because of narrower potential window of p-AgSAE). The mechanism of both reduction steps of FA as $2e^-/2H^+$ reduction was confirmed using EVLS and it corresponds with literature,[13] The first reduction peak was chosen for all subsequent analysis because this peak was well developed and easily evaluable and it was recorded in the widest range of pH. The highest current response of this peak was recorded in the acetate buffer of pH 5, which was identified as a suitable medium for FA determination. The optimal parameters of DPV determination were found using model solution of FA and this proposed method was successfully applied for FA determination. Table 1 shows that a nanomolar concentration of FA was correctly determined using p-AgSAE. Polished silver solid amalgam electrode was also successfully applied for determination of FA in two types of vitamin preparations and two kinds of fruit juices. The L_D(p-AgSAE) for FA determination was calculated as 5×10^{-10} M. If we compared the L_D of p-AgSAE and L_D of m-AgSAE[27] and these electrodes' working surfaces, we find that both mentioned amalgam electrodes have almost the same sensitivity. The limit of detection of HMDE published in our former paper[27] is 10 times lower than L_D(p-AgSAE) but the sensitivity of p-AgSAE is sufficient for determination of FA in common real samples, as it was shown above. It can be concluded that p-AgSAE, as a liquid mercury free electrode, can replace mercury electrodes in voltammetric analysis of FA.

5. Acknowledgements

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Povzetek

V članku so opisane elektrokemijske lastnosti folne kisline (FA) na polirani srebrovi amalgamski elektrodi (p-AgSAE) pri uporabi diferencialne pulzne voltametrije (DPV). Pri 60 sekundni akumulaciji je bila v acetatnem pufru (pH 5) meja zaznave za folno kislino $5{,}9 \times 10^{-10}$ M. Metoda je bila uspešno uporabljena pri določevanju folne kisline v vitaminskih preparatih in sadnih sokovih. Srebrova amalgamska elektroda lahko uspešno nadomesti živosrebrovo elektrodo pri voltametričnem določevanju folne kisline.