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Spectrophotometric and Voltammetric Determination of 2,5-dimethylaniline as Potential Product of Enzymatic Splitting of XY-2,5-dimethylanilide Type Substrates

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A method for determination of 2,5-dimethylaniline is proposed. The procedure enables both spectrophotometrical and voltammetric measurements. The spectrophotometrical determination at λ_{max} =385.8 nm is possible for concentrations up to 1.25×10^{-6} mol l^{-1}. The voltammetric method takes more time, however, due to the adsorptive enrichment of the substrate it is possible to determine concentrations in the range of 10^{-7} mol l^{-1}). In the paper the optimal conditions and parameters for the determination are suggested.

W pracy zaproponowano metodę oznaczania 2,5-dimetyloaniliny. Przedstawiona metoda umożliwia zarówno przeprowadzenie oznaczeń spektrofotometrycznie jak i woltamperometrycznie. Oznaczenia spektrofotometryczne są możliwe przy λ_{max} = 385,8 nm do stężeń ok. 1.25×10^{-6} mol l^{-1}. Z kolei pomiary woltamperometryczne, choć nieco bardziej czasochłonne, pozwalają na oznaczanie niższych stężeń (nawet rzędu 10^{-7} mol l^{-1}) przy wykorzystaniu możliwości adsorpcyjnego zatężania. W pracy zaproponowano optymalne warunki i parametry oznaczeń.

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As the substrates for the activity determinations of dipeptidyl peptidases the dipeptides with aromatical amines (dipeptid-amide) are used. Commercially made substrates for direct spectrophotometrical determinations are mostly the dipetid-amide derivative of 4-nitroaniline [1–6]. The amide compounds with 7-amino-4-methylcoumarin [1,5,18], β-naphthylamine [1,5,7–17] and 4-metoxy-ß-naththyl-amine [5] were used for the direct fluorimetric determinations, and dipetidyl--ß-naphthylamide for the indirect voltammetric determination [12]. The activity of the enzymes can be indirectly determined by spectrophotometric method using ß-naphthylamide – substrates after the released amine is transformed into a coloured azo compound [5,13,14], or after the condensation reaction with 4-dimethylamino-benzaldehyde is completed [15–17]. To reach high sensivity in the activity determinations the substrate should exhibit high affinity to the enzyme (low K_M constant) and the released amine or the products of their transformation should be stable compounds with high molar absorption coefficient. Substrates for fluorimetric determinations seem to fulfil this claim [1,5,7,8,11,18] in contrast with the substrate for spectrophotometrical ones. As commercial chemicals the 4-nitroanilides are poorly savable and often polluted by the decay products, what negatively influences the accuracy of the determination method. For the substrates based on ß-naphtylamine (β-NA) the indirect spectrophotometrical determination of the ß-naphthylamine is laborious. Moreover, the condensation reaction with 4-methylaminobenzaldehyde delivers no stable products. The disadvantages of the substrates available for spectrophotometrical determinations, as well as the demand for simple and quick tests for the clinical diagnostics [10,19] inspires the search for new substrates and new transformation methods of amines (products of the enzymatic hydrolysis) in coloured and (or) electroactive compounds.

The new substrate Gly-Pro-2,5-dimethylanilide planned for the activity determinations of DAP IV sets free due to the enzymatic reaction the 2,5-dimethylaniline, (DMA), similarly as ß-naphthylamine, DMA is an aromatic amine. Therefore a similar behaviour as for ß-NA can be expected [20]. But in the case of DMA we have decided to modify essentially the procedure of sample preparation (see: Experimental). Namely no sample is taken from the reaction mixture , but after breaking of the reaction, the excess of diazo-salt solution is introduced directly to the mixture.This salt will be coupled with the DMA.

The diazo-derivative obtained in this way should exhibit electroactivity what our examinations have proved. However, for using this procedure in analytical practice the optimization of some parameters and conditions is required. This is the purpose of our paper.

EXPERIMENTAL

Apparatus

Zeiss Specord M40 spectrophotometer; Quartz cuvettes S10.00 mm.

Digital pehameter PM–1 (Polmed, Warszawa). PP–04 pulse polarograph (Unitra Telpod Kraków) with digital acquisition system [21]. Hanging mercury drop electrode (HDME) with surface area of 0.12 cm^2. Saturated calomel electrode was used as the reference. Other conditions and details are presented in references [12] and [20].

Chemicals

2,5-Dimethylaniline (DMA); TRIS; sulfanilic acid diazo-salt; sodium hydrocarbonate $NaHCO_3$. All chemicals were of analytical grade (p.a). Doubly distilled water was used throughout experiments. Hydrochloric acid was made by absorption of hydrogen chloride in doubly distilled water and then diluting the solution to the desired concentration with water.

Preparation of the DMA diazo-derivative

A 1.25 ml 0.2 mol l^{-1} Tris-HCl buffer (pH = 7.8) was mixed with 0.25 ml DMA ethanolic solution (of variable concentration, depending on examined parameters) and 0.5 ml diazo salt of 4×10^{-2} mol l^{-1} in hydrochloric acid 1 mol l^{-1} solution.

To this mixture, before the measurement, 2 ml of 1 mol l^{-1} $NaHCO_3$ solution were added to achieve the pH = 7.6 (or when the influence of pH was examined).

Spectrophotometric measurements

The reaction was carried out as described above. The control sample contained 0.25 ml bidistilled water instead of DMA. In the measurements the quartz cuvettes (10.00 mm) were used.

Voltammetric measurements

The normal pulse voltammetry (NPV) with a HMDE was used. For all experiments the start potential (E_o) and pulse base-potential (E_R) were –0.20 V. Temperature was 293 K (20°C) and the pulse duration 40 ms. All potentials are quoted *vs* SCE. The voltammetric curves were evaluated by linear extrapolation of the background current across the bottom of the peak, and peak height was measured from this baseline.

RESULTS AND DISCUSSION

Spectrophotometry

Effect of pH. When the reaction is carried out in acidic solutions, an azo compound with maximum absorption at the wavelength of 491.6 nm is formed. A linear relationship between the absorbance and DMA concentration was found, but the reaction runs slowly and the obtained dyestuff was unstable (large changes of the absorbance), what could affect negatively the determinations. After changing the pH -value by addition of $NaHCO_3$, the maximum moved up to 395.8 nm and remained in time stable. From analytical point of view this is an advantage. For the determination

of the optimal hydrogen ion concentration of the sample (coupling reaction) the $NaHCO_3$ solution with variable concentration (between 0.15 and 1.5 mol l^{-1}) was added causing the pH change from 2 to 8. The observed influence of pH on the absorbance is shown in Figure 1. High and stable absorbance values as well as optimal molar absorption coefficient were achieved at pH = 7.6.

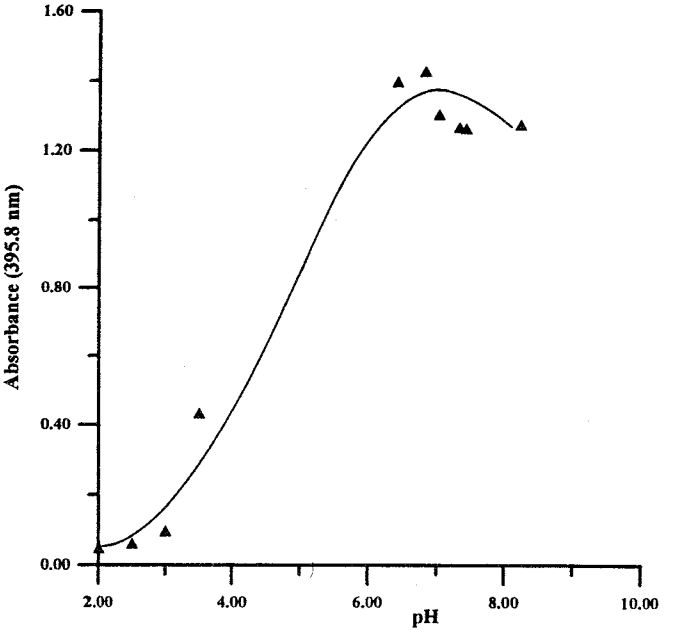

Figure 1. Dependence of dye solution absorbance on pH of reaction mixture. $C_{DMA} = 4 \times 10^{-4}$ mol l^{-1}, 0.2 mol l^{-1} Tris-HCl (pH = 7.6), T = 293 K

Effect of concentration. A linear relationship between DMA concentration and the absorbance was found within a relatively wide concentration range (10^{-4} to 10^{-6} mol l^{-1}) with a very good correlation coefficient, $r = 0.999$. The lowest determined concentration was 1.25×10^{-6} mol l^{-1}. Below this concentration the measuring is no longer possible, because the background absorbance is too large. So high background response may be probably explained by the presence of the self-reacting diazo salt. The determined molar absorption coefficient was 4.26×10^4 l mol^{-1} cm^{-1}.

Voltammetry

Effect of pH. pH is proved as the most important parameter. In acidic as well as neutral solutions the reduction currents of the DMA-diazo-derivative are not convenient for the determination. Also the background current is rather high since probably the reduction of the diazo salt occurs. In that case we have observed a very wide (*ca*

200 mV) current-maximum with the peak potential at –0.40 V. With the increase in pH this maximum disappears slowly and at *ca* –0.58 V a new, sharp maximum becomes to grow, the height of which stabilizes above pH = 7.5. Then there is only one, good shaped maximum, corresponding without doubt to the reduction of the DMA-diazo-derivative. The appearance of the derivative in these conditions was also proved in the spectrophotometrical investigations. The peak potential is located near the of ß-NA-diazo-derivative peak (*ca* –0.60V).

Effect of time. The concentration influence is decisive for analytical purpose, however the measured signal should be possibly stable in time. The influence of time could be observed in two cases, *i.e.* when the coupling reaction is slow, or the product unstable (decay) in the solution. As can be seen in Figure 2, the measurements are time dependent. We have noticed, that the slow rarte of the coupling reaction is responsible for this effect. The current stabilizes after about 20 min. This effect was also confirmed spectrophotometrically. In this way the obtained DMA-diazo-derivative is very stable. The stability was confirmed by one-week long observation of unchanged current. Therefore such stable samples can be examined after any period, provided that the reaction is terminated, *i.e.* the equilibrium is reached after approximately 20 min.

Effect of concentration. From analytical point of view the influence of concentration is decisive. It is related to the determination limit, accuracy and sensivity. In Figures 3, 4 and 5 it is shown how the concentration of DMA affects the reduction cur-

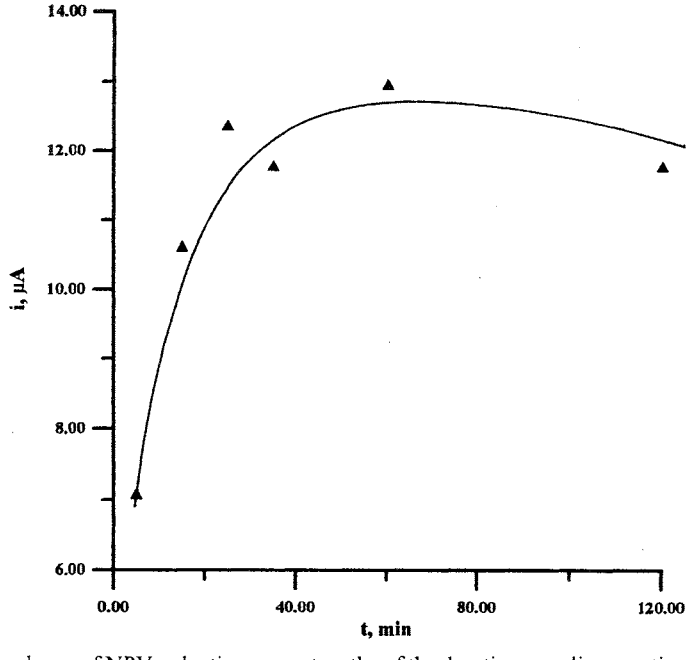

Figure 2. Dependence of NPV reduction current on the of the duration coupling reaction. $U_0 = -0.20$ V, without preconcentration, $C_{DMA} = 4 \times 10^{-4}$ mol l^{-1}, 0.2 mol l^{-1} Tris-HCl (pH = 7.6), T = 293 K

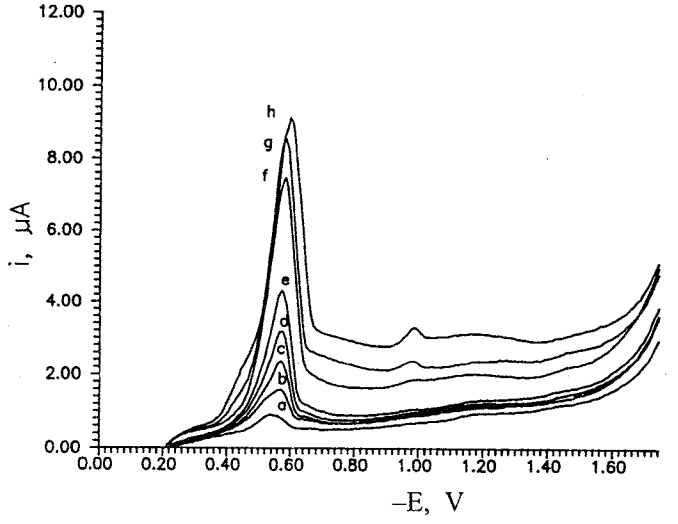

Figure 3. Dependence of NPV reduction current on DMA concentration. E_o = –0.20 V, without preconcentration, 0.2 mol l^{-} Tris-HCl (pH = 7.6), T = 293 K, DMA concentrations (µmol l^{-1}): a) 1.25; b) 2.5; c) 3.75; d) 5.0; e) 6.25; f) 12.5; g) 25.0; h) 37.5

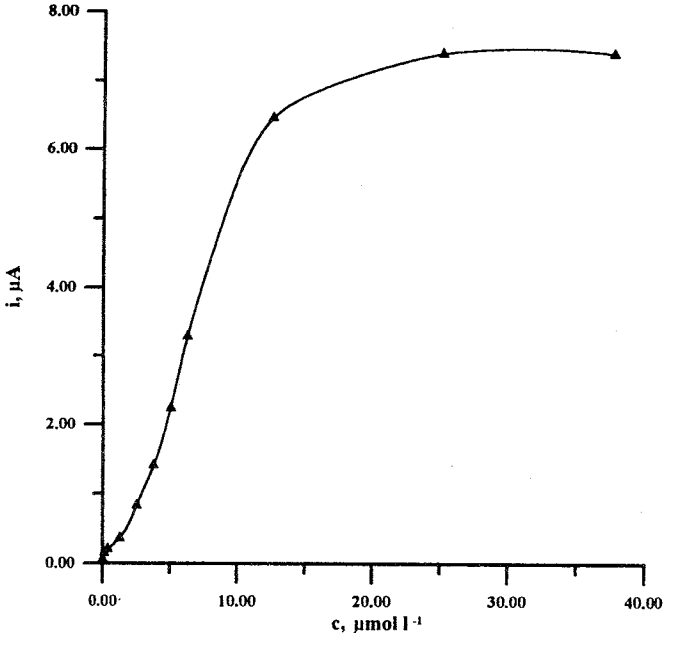

Figure 4. Dependence of reduction current on DMA concentration. Conditions as in Figure 3

rent of DMA-diazo-derivative. Figure 3 shows the voltammograms, registered using the NPV method. The relationship between DMA concentration and the reduction current is clearly shown in Figure 4. Unfortunately, compared to the spectrophotometric methods, there is no linear relationship in so wide concentration range. The shape of curve in Figure 4 is similar to the adsorption isotherm, *i.e.* is typical for the cases when a depolarizer an adsorbs on the electrode surface. For the diazo compounds this can be expected and we have already observed this effect [12,20].

Table 1. Statistical evaluation of the examined methods, ($n = 5$)

C μmol l^{-1}	Method					
	Spectrophotometry r = 0.999 Sen = 0.043 ± 0.0014, mol^{-1} l			NPV r = 0.993 Sen = 0.52 ± 0.055, μA mol^{-1}		
	C_{est} μmol l^{-1}	SD μmol l^{-1}	RSD %	C_{est} μmol l^{-1}	SD μmol l^{-1}	RSD %
0.125	C_{bdl}	–	–	0.596	0.097	16.3
0.375	C_{bdl}	–	–	0.704	0.096	13.6
1.250	1.317	0.306	23.27	1.010	0.093	9.17
4.000	4.587	0.288	6.27	–	–	–
5.000	–	–	–	4.615	0.083	1.79
10.000	9.958	0.267	2.68	–	–	–
12.500	–	–	–	12.75	0.249	1.95
22.000	21.985	0.263	1.20	–	–	–

Where:
C_{est} – Estimated concentration.
C_{bdl} – Concentration below the detection limit.
SD – Standard deviation of the mean.
Sen – Slope of the calibration plot.
r – Correlation coefficient.

At the lowest concentrations (or after dilution of the sample) the relationship $i = f(C)$ is practically linear. Figure 5 shows a part of the curve in Figure 4 for the concentration range below 1.25×10^{-5} mol l^{-1}. The correlation coefficient for this line equals 0.993. The curve can be used as a calibration line. Since the adsorption has also an important influence on the $i = f(C)$ relation, we can expect that the adsorption should be helpful at the lowest concentrations.

Possibility of adsorptive accumulation. If would be helpful to use a preconcentration method for determination of extremely small concentrations. One of the preconcentration methods is the adsorptive enrichment. The effectiveness of the enrichment depends on the concentration of the determined compound in the sample. For the concentrations which correspond to the plateau of the curve in Figure 4 the

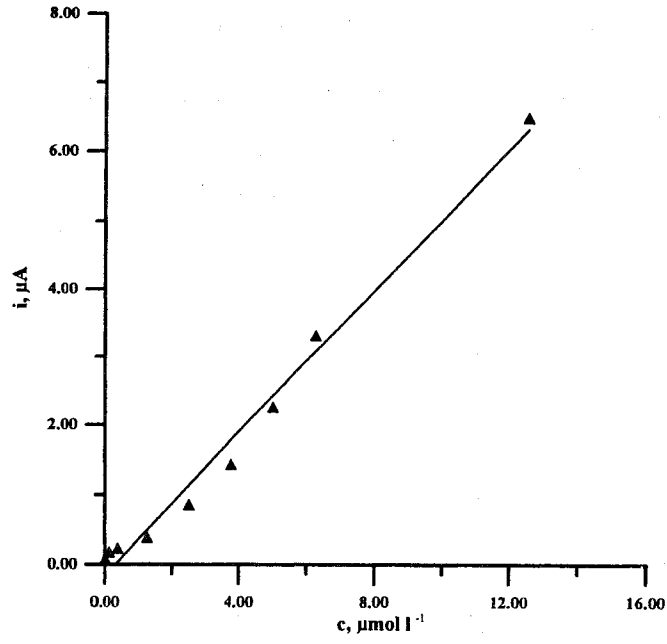

Figure 5. Dependence of reduction current on DMA concentration – calibration curve. Conditions as in Figure 3; statistical evaluation – see Table 1

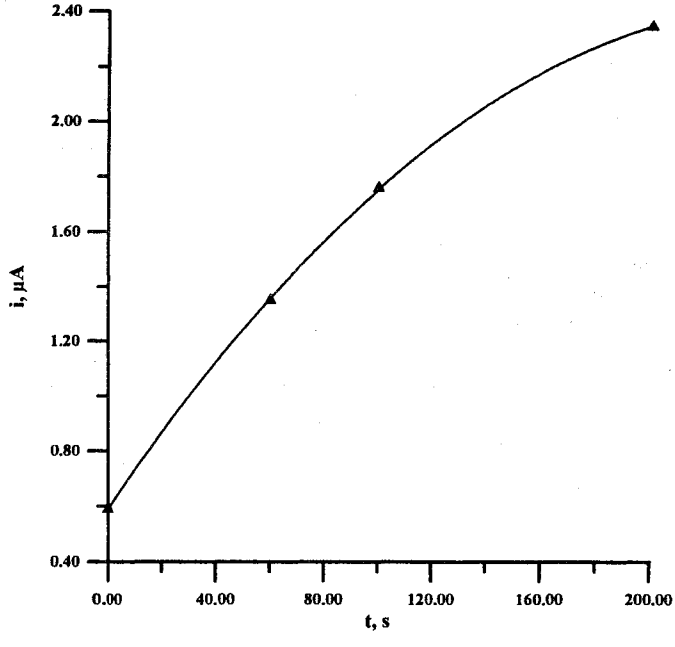

Figure 6. Dependence of NPV reduction current on preconcentration time. $E_o = -0.20$ V, $C_{DMA} = 1 \times 10^{-5}$ mol l^{-1}, 0.2 mol l^{-} Tris-HCl (pH = 7.6), T = 293 K

enrichment can not be effective and is useless. On the other hand, for concentrations below 10^{-5} mol l^{-1} the enrichment is strong, see Figure 6. A short 3–4 min enrichment caused a 5-fold increase of the current (at the maximum) for the sample with 1.25×10^{-5} mol l^{-1} DMA. The longer deposition periods are unnecessary.

CONCLUSIONS

It is possible to determine DMA in aqueous solutions indirectly as a diazo-derivative both spectrophotometrically and voltammetricaly. The spectral method is based on the measurement of absorbance at λ_{max} = 395.8 nm (pH=7.6). The voltammetric method employs the measurement of the reduction currents at E_p = –0.58 V. The voltammetric method has an advantages. It gives a possibility of the adsorptive enrichment of DMA. It is particulary useful for concentrations below 1×10^{-5} mol l^{-1}. Obviously the samples can be measured approximately after 20 min (this time is needed for the coupling reaction at pH > 7.5), but instead the samples are very durable.

For large number of samples the delay of 20 min has no importance. The method in this paper proposed is more favourable than the previous one for ß-NA, because it requires less preparation steps and less reagents. Also, it accelerates the operation and decreases the risk of mistakes. The two methods are effective enough for the intended investigations. Also, they are much cheaper and simplier than the usually used chromatographic methods of amine determination [22–25].

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