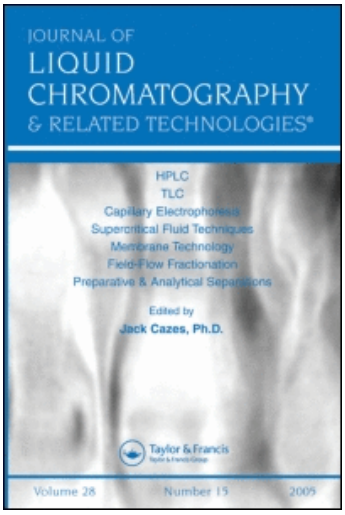

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Determination of Dissociation Constants of Resverastrol and Polydatin by Capillary Zone Electrophoresis

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Abstract: A method to determine the dissociation constants of resverastrol and polydatin by capillary zone electrophoresis was developed. First, resverastrol and polydatin have to be baseline separated to measure their effective electrophoretic mobilities accurately. Second, the first order dissociation constants of resverastrol and polydatin, which are 9.49 and 9.40, can be obtained from the non-linear regression between their effective electrophoretic mobilities and the H^+ activities of the running buffer. The correlation coefficients of the non-linear regression are 0.9956 and 0.9982, respectively. The reliability of the method is validated by the result of the hydroquinone of which the first order dissociation constant is known.

Keywords: Resverastrol, Polydatin, Dissociation constant, Non-linear regression, Capillary zone electrophoresis

INTRODUCTION

Resverastrol (3,5,4′-trihydroxy-stilbene, res) is a type of phytoalexin, which is relatively rich in *vitis vinifera L.* and *rhizoma polygoni cuspidate*. Res can also consist in the plants in the form of glucoside (3,5,4′-trihydroxy-stilbene-3-β-mono-D-glucoside, polydotin, pd). Pd has the same biological activity as res, and it can be converted into res by the glycosidase in the intestinal tract. Res and pd exist in plants in trans configuration and show many biological

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functions, such as scavenging oxygen free radical,[1] cancer prevention,[2,3] inhibiting lipid peroxidation,[4] phytoestrogen action,[5] controlling microbial flora, [6] and protecting the liver,[7] etc. It is very important in the development of techniques in extracting and purifying res and pd from plants to understand their dissociation constants (pK_a).

The pK_a values of res and pd can be obtained from the non-linear regression between their effective electrophoretic mobilities and the H^+ activities of a running buffer. Their effective electrophoretic mobilities can be calculated based on the migration times of res and pd in capillary zone electrophoresis (CZE). In this paper, the separation conditions of res and pd are optimized first; their pK_a values are determined second. So far, it has been not reported that the pK_a values of res and pd are determined by CZE.

THEORY

There are 3 phenolic hydroxyl groups in the structures of res and pd, so they should be treated as ternary acid. Their effective electrophoretic mobility can be written as follows:[8]

$$\mu_{eff} = \alpha_0\mu_{H_3A} + \alpha_1\mu_{H_2A^-} + \alpha_2\mu_{HA^{2-}} + \alpha_3\mu_{A^{3-}} \quad (1)$$

where μ_{H_3A}, $\mu_{H_2A^-}$, $\mu_{HA^{2-}}$, and $\mu_{A^{3-}}$ are the absolute electrophoretic mobility of H_3A, H_2A^-, HA^{2-}, and A^{3-}, α_0, α_1, α_2, and α_3 are the distribution coefficient of H_3A, H_2A^-, HA^{2-}, and A^{3-}. The expressions of α_1, α_2, and α_3 are as follows:

$$\alpha_1 = \frac{[H_2A^-]}{[H_3A] + [H_2A^-] + [HA^{2-}] + [A^{3-}]},$$

$$\alpha_2 = \frac{[HA^{2-}]}{[H_3A] + [H_2A^-] + [HA^{2-}] + [A^{3-}]}$$

$$\alpha_3 = \frac{[A^{3-}]}{[H_3A] + [H_2A^-] + [HA^{2-}] + [A^{3-}]}$$

Under the determination conditions for pK_{a_1}, the second and third order dissociation is so weak that $[HA^{2-}]$ and $[A^{3-}]$ can be ignored. Because $\mu_{H_3A} = 0$, and

$$K_{a_1} = \frac{\gamma_{H_2A^-}[H_2A^-]a_{H^+}}{[H_3A]} \quad (2)$$

μ_{eff} can be simplified as

$$\mu_{eff} = \frac{K_{a_1} \cdot \mu_{H_2A^-}}{a'_{H^+} + K_{a_1}} \quad (3)$$

where a_{H^+} is the activity of H^+ in buffer, $a'_{H^+} = \gamma_{H_2A^-}\, a_{H^+}$, $\gamma_{H_2A^-}$ can be calculated by

$$-\lg\gamma = \frac{0.5085Z^2\sqrt{I}}{1 + 3.281d\sqrt{I}}$$

where I is the ionic strength of the background electrolyte, Z is the number of charge for solute ion, and d is its hydrated ion diameter assumed as 0.5 nm.

When variable μ_{eff} and a'_{H^+} are known by measurement, based on Eq. (3), fit constant K_{a_1} and $K_{a_1} \cdot \mu_{H_2A^-}$ will be obtained by their non-linear regression analysis. The negative logarithm of a'_{H^+} can be calculated as follows:

$$-\lg a'_{H^+} = pH' = pH + \frac{0.5085Z^2\sqrt{I}}{1 + 3.281d\sqrt{I}} \tag{4}$$

and μ_{eff} can be obtained with the following equation:

$$\mu_{eff} = \frac{L_t L_d}{V}\left(\frac{1}{t_{EOF}} - \frac{1}{t_m}\right) \tag{5}$$

where L_t is the total length of the capillary, L_d the length from the capillary injection end to the detection window, V the operating voltage, t_m the migration time of the solute and t_{EOF} the migration time of a neutral solute, respectively. μ_{eff} and a'_{H^+} can be acquired by Eqs. (4) and (5).

EXPERIMENTAL

Instruments and Chemicals

The separation was carried out on a BioFocus 3000 capillary electrophoresis system (Bio-Rad Inc. U.S.A.) equipped with a UV detector and an uncoated fused silica capillary (50 μm i.d. × 50 cm total length, 45.4 cm effective length, Yongnian Optical Fiber Factory, Hebei province, China). PHS-4 acidimeter (Jiangsu Electronic Analysis Instrument Factory, Jiangsu province, China) was used for pH determination.

Res was purchased from Sigma Company (U.S.A., 99%) and pd from Tianjin Jianfeng Natural Production Company (Tianjin, China, 99%). Sodium hydroxide and boric acid (guaranteed grade), hydroquinone, anhydrous alcohol, hydrochloric acid (analytical grade) were purchased from Beijing Reagent Company (Beijing, China). Water used for buffer solution was deionized to the resistivity of 150 kΩ · m.

Standard Preparation

The standard solutions of 2.50 g/L res and 2.75 g/L pd was prepared by dissolving them in anhydrous alcohol. The interim solution was prepared by diluting the standard solution 50 fold with anhydrous ethanol. The sample solution was prepared by mixing 25 μL 1 M NaOH into 175 μL interim solution.

Electrophoresis Conditions

The running buffer was 40 mM borate solution adjusted to pH ranging from 8.23 to 10.46. Electrokinetic injection mode was applied. Injection voltage was 10 kV, and injection time was 10 s. Separation voltage was 15 kV. Detection wavelength was set at 308 nm.

The temperature of cartridge and carousel was both 25°C. The buffers and sample were filtered through a 0.45 μm filter before use. Between the runs, the capillary was purged with deionized water (60 s), 1 M HCl (120 s), deionized water (60 s), 1 M NaOH (120 s) and deionized water (120 s), respectively.

RESULTS AND DISCUSSION

Optimization of Separation Conditions

The accurate migration time of analyte is necessary in the measurement of its first order dissociation constant. Then the resolution of analytes has to meet the requirement of determination. Besides the pH of the running buffer, the concentration of electrolytes in the running buffer, injection voltage, and time can influence the resolution of analytes in CZE. These parameters should be optimized in order to obtain the better resolution between res and pd.

Electrophoresis is carried out with 40 mM borate buffer adjusted to pH ranging from 8.23 to 10.46 under the condition of injection voltage 10 kV and injection time 3 s. The results show that the resolution between res and pd decreases with the reduction of pH values under the same injection condition. The resolution is 1.0 and meets the requirement at the lowest pH 8.23. In order to consider the effect of buffer concentration on the resolution, electrophoresis is also carried out with the borate buffer ranging from 40 mM to 100 mM and adjusted to pH 9.02. The results show that the resolution is approximately increased with the increase of the concentration. The higher the concentration is, the larger the separation current is, so 40 mM is chosen.

Under the condition of 40 mM buffer adjusted to pH 9.02, the injection time of 3 s and the injection voltages ranged from 10 to 15 kV are applied respectively. The results show that the higher the voltage is, the worse the

Table 1. μ_{eff} and a'_{H^+} of three solutes

		μ_{eff} ($m^2\ s^{-1}V^{-1}$)		
pH	a'_{H^+} (mol/L)	Hydroquinone	Res	Pd
8.23	3.41×10^{-9}	5.82×10^{-8}	1.00×10^{-6}	1.89×10^{-6}
8.47	1.91×10^{-9}		1.59×10^{-6}	3.00×10^{-6}
8.63	1.30×10^{-9}	9.80×10^{-8}	2.11×10^{-6}	3.95×10^{-6}
8.85	7.70×10^{-10}	1.20×10^{-7}	3.14×10^{-6}	5.32×10^{-6}
9.02	5.14×10^{-10}	1.77×10^{-7}	3.98×10^{-6}	6.66×10^{-6}
9.46	1.83×10^{-10}	3.82×10^{-7}	5.97×10^{-6}	1.27×10^{-5}
9.79	8.52×10^{-11}	5.76×10^{-7}	7.22×10^{-6}	1.23×10^{-5}
10.07	4.45×10^{-11}	6.74×10^{-7}	8.60×10^{-6}	1.42×10^{-5}
10.46	1.81×10^{-11}		9.68×10^{-6}	1.50×10^{-5}

resolution is, and the low voltage can result in the long analytical time. The voltage of 10 kV is selected. The injection time ranged from 1 to 20 s is applied at 10 kV. The results show that the longer the injection time is, the higher the peak height of analytes is, and the worse the resolution is. Considering both the peak height and resolution, 10 s is chosen. The higher injection voltage and the longer injection time can result in the larger sample size, and this may be the origin of which the resolution decreases with the increase of injection voltage and time.

Determination of pK_{a_1}

The migration time of res, pd, and hydroquinone is determined by the buffers adjusted to pH ranging from 8.23 to 10.46 under the operating conditions. The

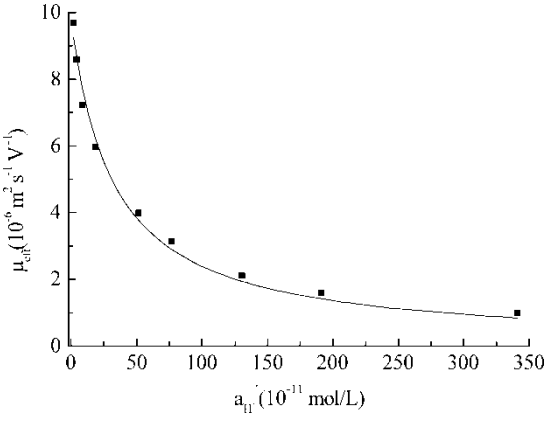

Figure 1. Non-linear regression curve of resverastrol.

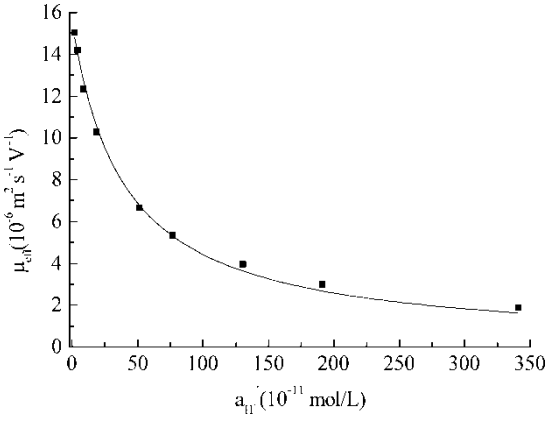

Figure 2. Non-linear regression curve of polydotin.

migration time of the analytes and neutral solute is measured for 4 times at every pH values. The μ_{eff} averages of the analytes can be calculated based on Eq (5). Table 1 shows the a'_{H^+} of the buffer and the μ_{eff} of the analytes. Based on Table 1 data, the fit curve (see Figure 1–Figure 3) and pK_{a_1} of three solutes are obtained.

The pK_{a_1} of hydroqninone (25°C) is 9.91,[9] the pK_{a_1} of non-linear regression analysis is 9.87, and correlation coefficient is 0.9979. The result approaches to the value of literature, and demonstrates the accuracy of the method. The pK_{a_1} of res and pd is 9.49 and 9.40, and correlation coefficient is 0.9956 and 0.9982.

For the CZE method, it is not necessary to know the concentration of the analyte, the sample volume is hardly needed, and the high sensitivity can be

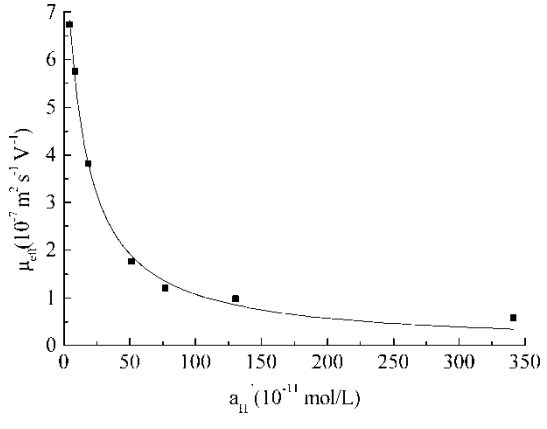

Figure 3. Non-linear regression curve of hydroquinone.

obtained for hydrophobic analytes. In order to obtain the exact dissociation constant, the effect of activity coefficient must be considered and non-linear regression analysis must be used. The error of measurement is mostly from the pH of buffer.

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