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Optimization of Promethazine and Demethylpromethazine Separation Conditions in Capillary Electrophoresis by Response Surface Methodology

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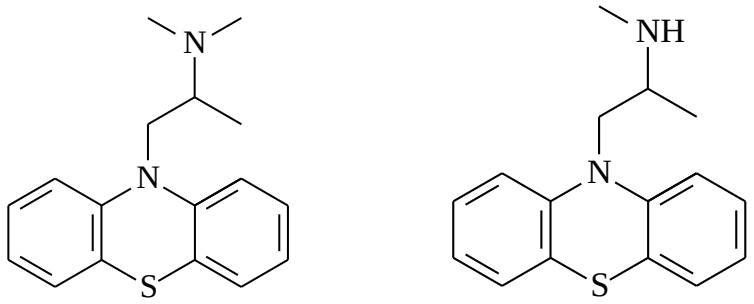
Demethylpromethazine (DMPMZ) is one of the main metabolites of promethazine (PMZ). DMPMZ has only one methyl less than PMZ. In the past, in order to separate them by capillary electrophoresis (CE), it is often necessary to add additives to separation buffer solution. Under the optimized experimental conditions by response surface methodology, however, PMZ and DMPMZ can be separated well without using additives. The optimum CE conditions were 21.846 mmol/L phosphate buffer solution (pH 5.653) and separation voltage of 14.408 kV. The resolution of PMZ and DMPMZ is more than 1.5 without any additives under the optimized experimental conditions. The optimization method is universal for many multiparameter processes.

Keywords: Capillary electrophoresis; Promethazine; Demethylpromethazine; Optimization; Response surface methodology

1. INTRODUCTION

Capillary electrophoresis (CE) is an efficient method for separation of biochemical and medical analytes because of its strong separation ability, short analysis time and less sample consumption. Tris (2,2'-bipyridyl) ruthenium (II) ($Ru(bpy)_3^{2+}$)-based electrochemiluminescence (ECL) for detecting organic amine compounds has attracted much attention due to its inherent high sensitivity and selectivity in the past decades. CE separation with end-column ECL detection (CE-ECL) has been extensively studied and utilized for the analysis of various drugs [1–21], antibiotics [22–24], enzymes [25], alkaloids [26–29], amines [30–34], hormones [35] and pesticide residues [36, 37] in different foods, pharmaceuticals, animals and plants.

Promethazine (PMZ) is widely used as an antihistamine to alleviate allergic symptoms and enhance the analgesic, anesthetic and sedative effects of other drugs [38]. In vivo, PMZ can be biotransformed into a variety of metabolites which most belong to the phenothiazine category which contain tertiary amine groups, such as promethazine sulfoxide (PMZSO), demethylpromethazine (DMPMZ), dioxopromethazine (DOPMZ), dihydroxypromethazine (DHPMZ) and so on. Simultaneous determination of PMZ and its metabolites in blood or urine is extremely important to realize the economic effect and pharmacokinetics of PMZ. DMPMZ has only one methyl less than PMZ (see Figure 1). In the past, in order to separate them by CE, it is often necessary to add additives to separation buffer solution [39, 40], otherwise they could not be completely separated. However, under the optimized experimental conditions by response surface methodology (RSM), DMPMZ and PMZ can be separated well without using additives.



PMZ DMPMZ

Figure 1. Structure of promethazine and demethylpromethazine.

RSM is a statistical method to solve multivariable problems by using appropriate experimental data to find the optimal process parameters [41, 42]. There are many reports in the literature about the optimization of experimental conditions by RSM [43-46]. In this paper, on the basis of single factor experiments, RSM is used to investigate the interactions of different factors. It is expected that PMZ and DMPMZ can be separated directly by optimizing the separation conditions of CE.

2. EXPERIMENTAL

2.1. Materials and Reagents

Tris (2,2'-bipyridyl) ruthenium (II) dichloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$) was purchased from Alfa Aesar (Johnson Matthey, USA). Disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were all of analytical reagent grade and were purchased from Beijing Chemical Factory (Beijing, China). PMZ and DMPMZ standard substances were purchased

from National Institutes for Food and Drug Control (Beijing, China).

2.2. Solutions preparation

$Ru(bpy)_3^{2+}$solutions were prepared with $Ru(bpy)_3Cl_2 \cdot 6H_2O$ and secondary distilled water. Phosphate buffer solutions (PBS) were prepared with disodium hydrogen phosphate, sodium dihydrogen phosphate and secondary distilled water. Standard solutions of PMZ and DMPMZ were prepared with their standard substance and secondary distilled water. All solutions used in the experiments were filtered through a 0.22 μm cellulose acetate membrane.

2.3. Apparatus and ECL detection conditions

CE-ECLwas performed on a MPI - B multi-parameter chemiluminescence analysis test system (Xi'an Remex analytical instruments Co., Ltd., Xi'an, China). Cyclic voltammetry and potentiostatic method were carried out in a three electrodes system with a platinum working electrode of 500 μm in diameter, an Ag/AgCl reference electrode of 300 μm in diameter and a platinum wire auxiliary electrode of 1 mm in diameter. Uncoated capillary (25μm x 40 cm, Yongnian Optical Fiber Factory, Hebei, China) was rinsed respectively with 0.1 mol/L NaOH solution for 20 min, secondary distilled water for 10 min and running buffer for 15 min before use.

ECL conditions in detection cell: Detection potential is 1.2V (vs. Ag/AgCl). Concentration of $Ru(bpy)_3^{2+}$ is 6 mmol/L. Concentration of PBS is 40 mmol/L. The pH of PBS is 6.5.

3. RESULTS AND DISCUSSION

3.1. Selection of capillary electrophoresis parameters by single factor experiments

3.1.1 The pH of separation PBS

The acidity of separation PBS is an important condition affecting the separation effect. In the literature work of CE with PBS medium, the application range of pH value is mostly between 4-8 [4-12,22-24,28-32]. Therefore, we mainly study the influence of pH in this range on the resolution. When the pH of the PBS changes from 4.5 to 6.5, the resolutions of PMZ and DMPMZ under 20 mmol/L PBS and separation voltage 14 kV are shown in Figure 2. With the increase of the pH of separation PBS, the resolution shows a trend of "first increase and then decrease", and reaches the maximum at pH = 5.5. So, we chose 5.5 as the pH value of separation buffer solution.

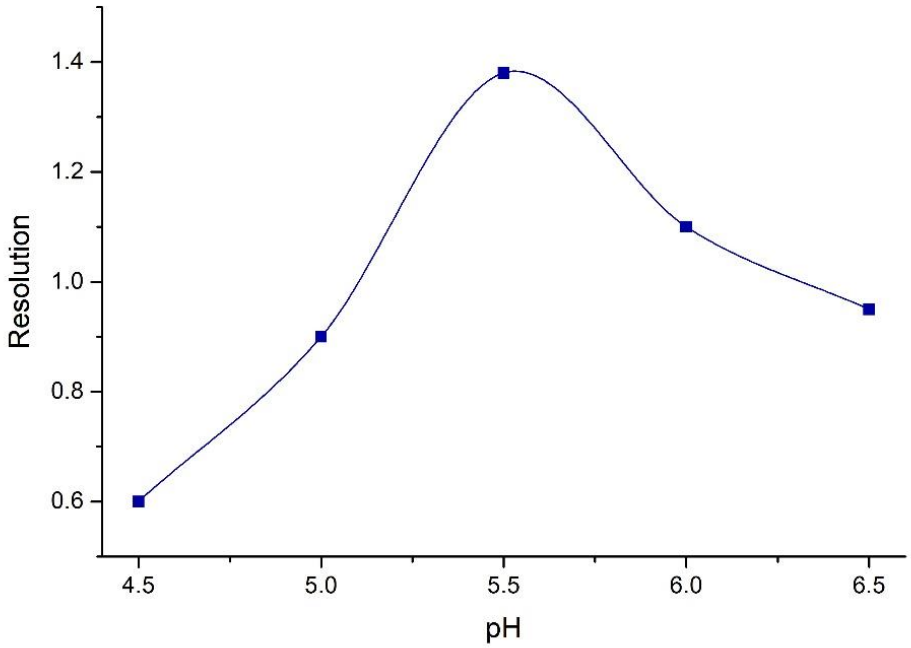


Figure 2. Effects of pH of separation PBS on the resolution of PMZ and DMPMZ.

3.1.2 Concentration of separation PBS

The ionic strength of separation PBS is another important condition affecting the separation effect. PBS with different pH values are prepared by different proportions of Na_2HPO_4 and NaH_2PO_4. When the concentration of the two increased in proportion, the pH value remained unchanged, but the buffer capacity increased, and the total ionic strength of the solution also increased. In this experiment, the effect of ionic strength on resolution was investigated by changing concentration of PBS under pH of PBS 5.5 and separation voltage 14 kV. The results are shown in Figure 3. With the increase concentration of PBS, the ionic strength increased. When the concentration of PBS is 20 mmol/L, the resolution of PMZ and DMPMZ is relatively large. The ionic strength can also be increased by simply adding strong electrolyte into PBS. We used to increase ionic strength by adding NaCl to PBS [40]. Other research groups also use Na_2SO_4, KCl or NH_4Cl to change the ionic strength [8-19, 23, 27, 32, 35]. However, the addition of strong electrolytes can sometimes lead to other unexpected problems.

3.1.3 Separation voltage

The separation voltage affects the migration time of components, and then changes the resolution of components. In the literature work, the application range of separation voltage is very wide. However, it rarely exceeds 20 kV [27], and most of them are between 10-20 kV [5-25,28-34]. In this experiment, the separation voltages from 12 kV to 16 kV are investigated under 20 mmol/L PBS

(pH 5.5). The results are shown in Figure 4. As you can see, 14 kV is the best separation voltage.

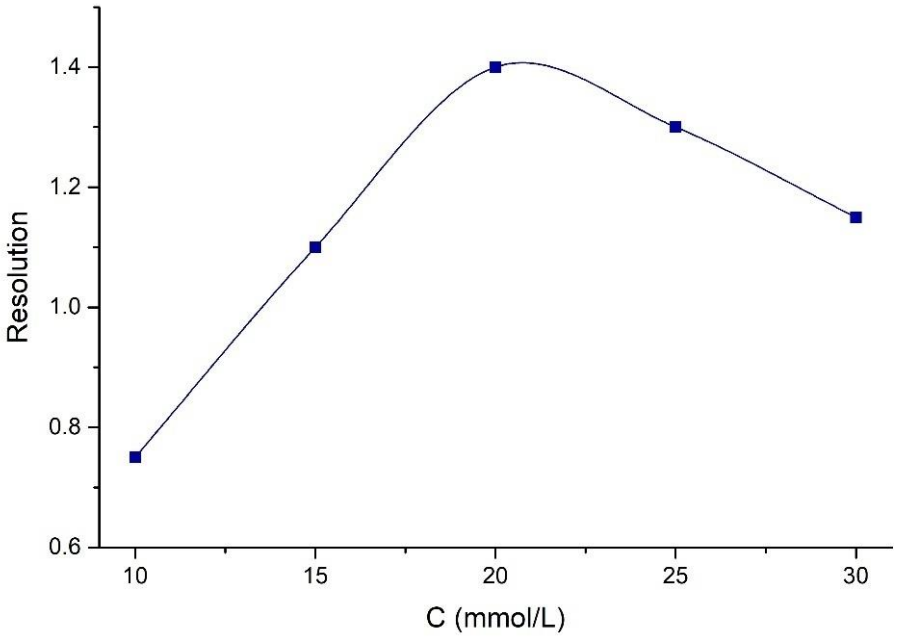


Figure 3. Concentration of separation PBS on the resolution of PMZ and DMPMZ.

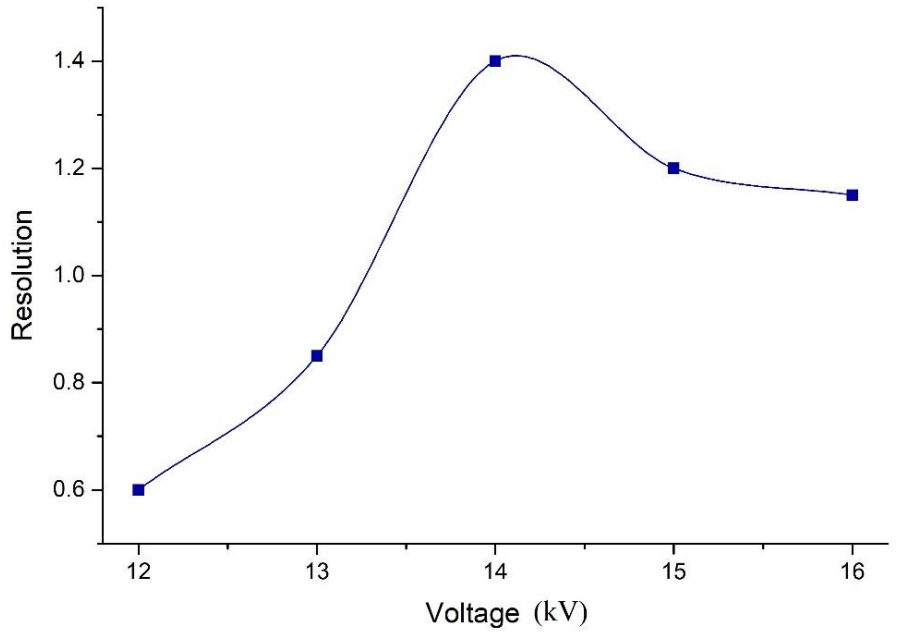


Figure 4. Effects of separation voltage on the resolution of PMZ and DMPMZ.

Therefore, the CE parameters selected by single factor experiments are 20 mmol/L PBS (pH 5.5) and separation voltage 14 kV.

3.2. Optimization of capillary electrophoresis parameters by RSM

3.2.1 Box-Behnken test and experimental results

The pH of PBS, concentration of PBS and separation voltage were used as research factors. The resolution was used as the response value. According to the results of single factor experiments, three factors and three levels (see table 1) were used to carry out the Box-Behnken test design [42-45].

Table 1. Factors and levels of the Box-Behnken test design.

Levels\Factors	pH of PBS	Concentration of PBS (mmoL/L)	Separation voltage (kV)
-1	5.0	15	13
0	5.5	20	14
1	6.0	25	15

The results of 17 response surface design trials (12 edge points plus 5 center points in Box-Behnken test design) are shown in Table 2.

Table 2. Response surface design and experimental results.

Number	pH of PBS	Concentration of PBS (mmol/L)	Separation voltage (V)	Resolution
1	6	15	14	0.75
2	5	20	13	0.30
3	5	25	14	0.70
4	5.5	25	15	1.20
5	6	20	13	0.36
6	6	20	15	1.14
7	5.5	20	14	1.41
8	5.5	15	15	0.90
9	5.5	15	13	0.32
10	5	20	15	0.70
11	5	15	14	0.46
12	5.5	20	14	1.39
13	5.5	20	14	1.42
14	5.5	20	14	1.39
15	5.5	20	14	1.40
16	6	25	14	1.23
17	5.5	25	13	0.57

3.2.2 Interaction among factors

The 3D surfaces and contours are plotted by Design Expert software, as shown in figure 5-figure 7. Each figure represents the influence of the interaction of two independent variables on resolution.

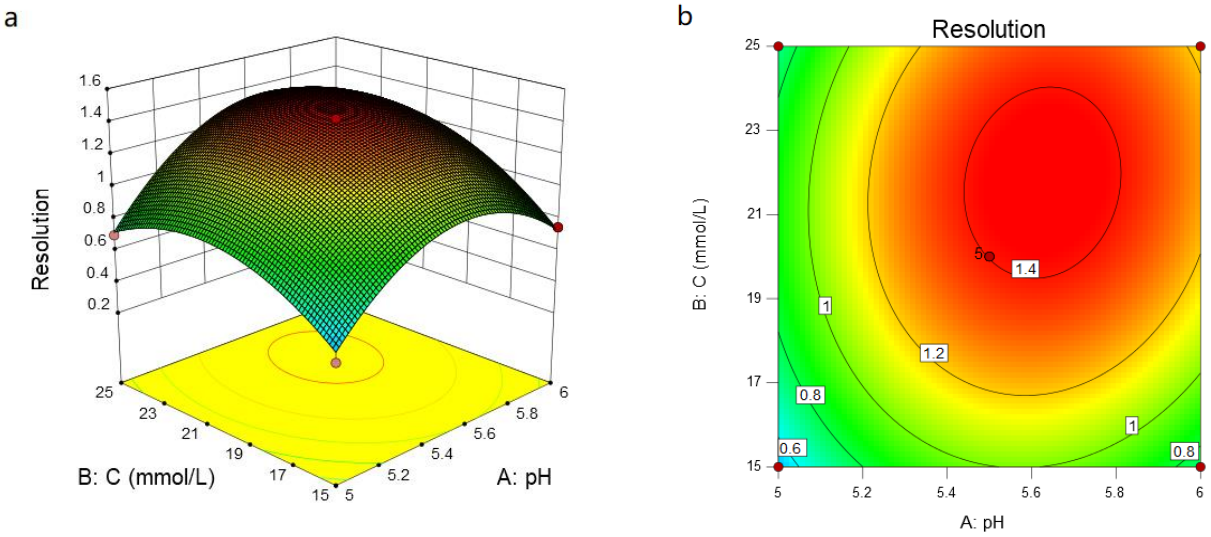


Figure 5. Effect of interaction of pH and concentration of PBS on resolutions.

Figure 5 is 3D surface (a) and contour (b) of the effects of the interaction of pH of PBS and concentration of PBS on resolutions. With the increase of pH of PBS and concentration of PBS on resolutions, the resolution increases. When the pH of PBS reaches 5.653 and the concentration of PBS reaches 21.846 mmol/L, the resolution reaches its maximum. When the pH of PBS and concentration of PBS continue to increase, the resolution begins to decrease.

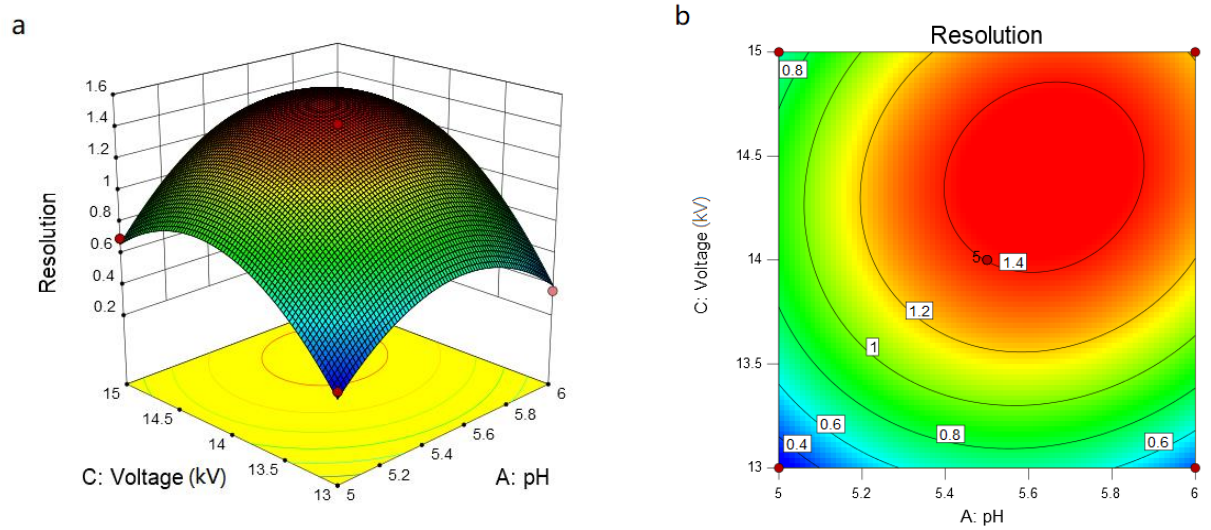


Figure 6. Effect of interaction of pH of PBS and separation voltage on resolutions.

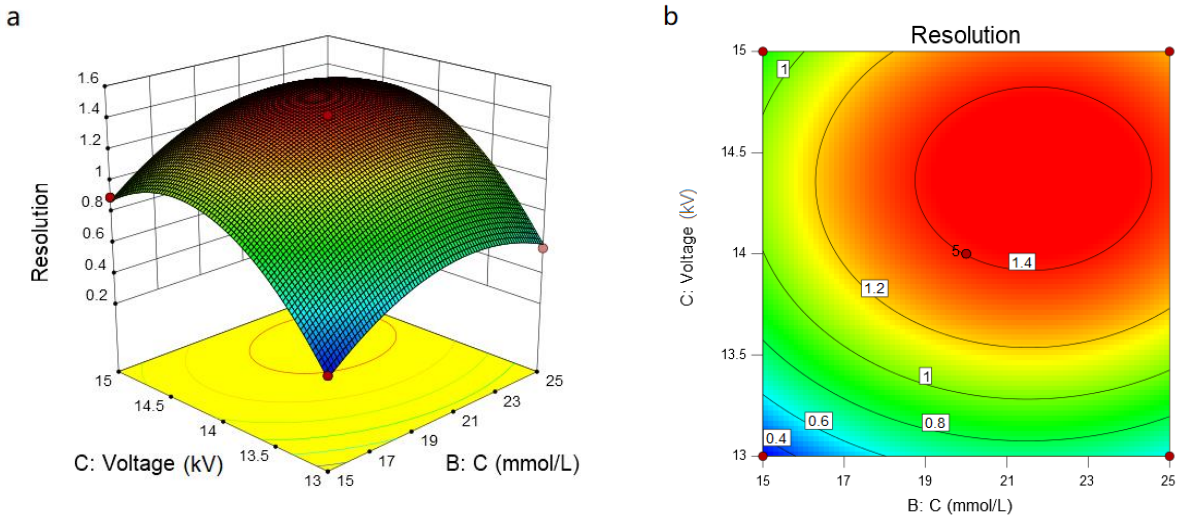


Figure 7. Effect of interaction of concentration of PBS and separation voltage on resolutions.

Figure 6 is 3D surface (a) and contour (b) of the effects of the interaction of pH of PBS and separation voltage on resolutions. With the increase of pH of PBS and separation voltage, the resolution increases. When the pH of PBS reaches 5.653 and the separation voltage reaches 14.408 kV, the resolution reaches its maximum. When the pH of PBS and separation voltage continue to increase, the resolution begins to decrease.

Figure 7 is 3D surface (a) and contour (b) of the effects of the interaction of concentration of PBS and separation voltage on resolutions. With the increase of concentration of PBS and separation voltage, the resolution increases. When the concentration of PBS reaches 21.846 mmol/L and the separation voltage reaches 14.408 kV, the resolution reaches its maximum. When the concentration of PBS and separation voltage continue to increase, the resolution begins to decrease.

Based on the above experimental results, it can be found that the optimum values of pH of PBS, concentration of PBS and separation voltage were 5.653, 21.846 mmol/L and 14.408 kV, respectively, when the maximum resolution is obtained. It is almost impossible to obtain the optimal CE conditions through single factor experiments, because the number of experiments needed is very large. According to the model, the maximum value of resolution was 1.517. This is 8.4% higher than that selected from single factor experiments. The key point is that the resolution is greater than 1.5, which can ensure the two components completely separated. In the literature, signal size, peak area or luminous intensity are usually used as response values to optimize multi parameter conditions [41-46]. There is no optimization work with resolution as response value. The resolution is the ratio of the difference of retention time between two adjacent components and the average width of their peak base. It can only be obtained by calculation using the measured data of electrophoretogram, which is really troublesome.

3.3Separation performance

The experiment was carried out under the separation conditions selected by single factor experiments, i.e. 20 mmol/L PBS (pH 5.5) and separation voltage14 kV. The separation of PMZ and DMPMZ is shown in Figure 8. At this time, the resolution is less than 1.5.

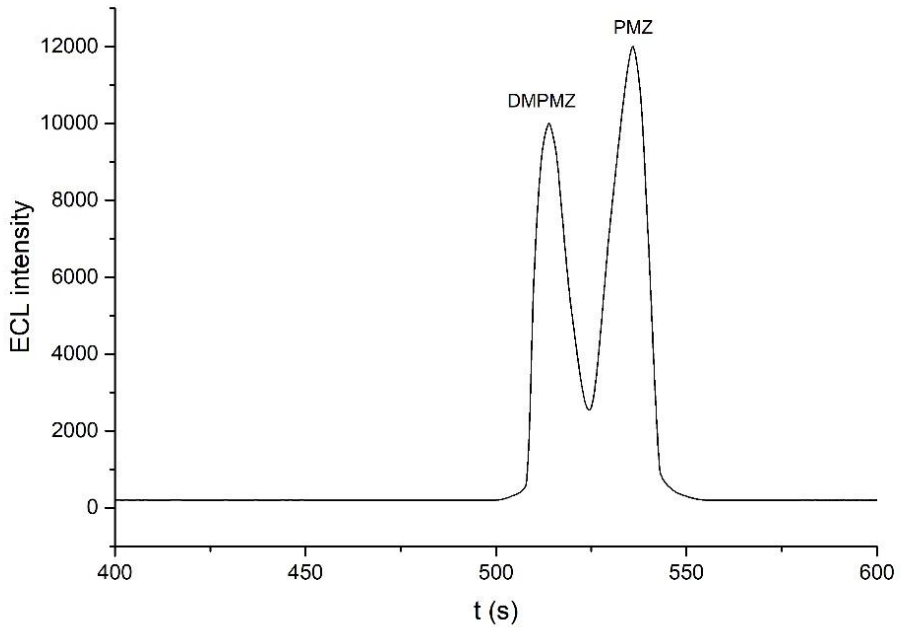


Figure 8. The separation of PMZ and DMPMZ under single factor experimental conditions.

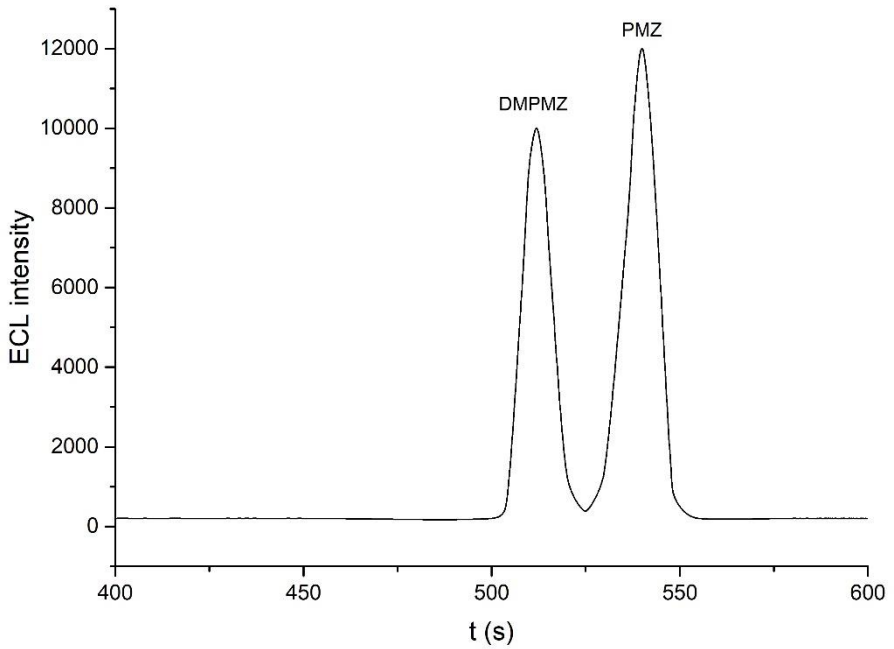


Figure 9. The separation of PMZ and DMPMZ under the separation conditions optimized by RSM.

The experiment was carried out under the separation conditions optimized by RSM, i.e. 21.846 mmol/L PBS (pH 5.653) and separation voltage 14.408 kV. The separation of PMZ and DMPMZ is shown in Figure 9. In this figure, the resolution is greater than 1.5.

The improvement of separation effect after optimization is obvious. As you can see, PMZ and DMPMZ can be separated well in CE process without any additives under the separation conditions optimized by RSM.

4. CONCLUSION

On the basis of single factor experimental conditions, the interactions of different factors on resolution were investigated by response surface methodology, which can improve the resolution of PMZ and DMPMZ. Finally, a rapid and simple CE-ECL method for separation and analysis PMZ and its metabolites was established. PMZ and DMPMZ can be separated well in CE process without any additives.

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