

Determination of Estrogen Residues in Milk Powder by Accelerated Solvent Extraction and Capillary Electrophoresis

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A novel method which based on accelerated solvent extraction (ASE) and capillary electrophoresis (CE) was established for simultaneous determination of 7 estrogen residues in milk powders. During the ASE, the effects of extractant, extraction temperature and extraction time on extraction efficiency of 7 estrogens were studied. The optimized ASE process is as follows: mixing 1 g milk powder sample and 2 g diatomite in extraction tank, add 10 ml ethanol-acetonitrile (1:1, v/v), perform static extraction for 210 s at an extraction temperature of 120 °C, an extraction pressure of 10.0 MPa and cycle 3 times. During the CE separation, the effects of pH and ionic strength of the mobile phase, and separation voltage on the separation efficiency of 6 pair estrogens were investigated. The selected CE mobile phase is 50 mmol/L phosphate buffer solution (PBS) (pH=6.5) containing 25 mmol/L NaCl, and separation voltage is 15 kV. All of 7 estrogens have good linear relationship. Their detection limits were 0.5 - 0.8 µg/kg. Their recoveries in 3 milk powder samples were 84.7% - 114.8%.

Keywords: Estrogen, Milk powder, Accelerated solvent extraction, Capillary electrophoresis

1. INTRODUCTION

Milk powder is favored by people because it is rich in nutrients such as protein, vitamins, and calcium. However, in recent years, it has been found that the content of estrogen in milk powder has reached a level that cannot be ignored [1]. Estrogen is a class of steroid hormones with similar chemical structures that can directly affect animal metabolism and reproduction. The main ones used as growth promoters in livestock production include natural estrogens (such as beta-estradiol, estriol and estrone) and synthetic estrogens (such as diethylstilbestrol, dihydrodiethylstilbestrol, ethinyl estradiol and ethinyl estradiol methyl ether). When estrogen enters the human body through the food chain, it will interfere with endocrine function and cause various health problems [2, 3]. Therefore, the analysis and detection of residual estrogen in milk powder has important practical significance.

At present, common methods for determining hormonal drug residues have been reported, such as high performance liquid chromatography (HPLC) [4-8], fluorescence polarization (FP) [9], enzyme-linked immunosorbent assay (ELISA) [10], HPLC-mass spectrometry (HPLC-MS) [11-15], gas chromatography-MS (GC-MS) [16], stable isotope labeling technique [17] and so on.

Due to the complex matrix of milk powder, the application of pretreatment techniques such as enrichment and extraction of target analytes is necessary [5-7, 11, 13, 14, 16, 18, 19]. Accelerated solvent extraction (ASE) is an extraction technique that extracts solids or semi-solids with organic solvents at higher temperatures and pressures [20, 21]. Higher temperatures greatly weaken the interaction forces caused by van der Waals forces, hydrogen bonding, and dipole attraction between target molecules and active sites in the sample matrix. Increasing the pressure in the extraction cell enables the solvent to be used at temperatures above its atmospheric boiling point. Since the dissolving ability of liquid is much greater than that of gas, the extraction ability of this method will be greatly improved. The advantages of this method are the low amount of organic solvent, fast extraction, little influence of matrix, high recovery and good reproducibility. Accelerated solvent extraction technology has been widely used in the environmental [22, 23], pharmaceutical [24, 25], food [26, 27] and polymer [28].

Capillary electrophoresis (CE) is a liquid-phase separation technology with capillary as the separation channel and high-voltage direct current electric field as the driving force [29]. It enables analytical chemistry from the microliter level to the more economical and efficient nanoliter level. Usually, samples that can be made into solutions or suspensions can be separated and analyzed by CE, so it is widely used [30-35].

In this study, ASE was used to enrich and purify estrogens in the samples, and they were separated by CE and detected by ultraviolet (UV) absorption. A method based on ASE-CE for simultaneous determination of 7 estrogen residues in milk powder was established for the first time. The results show that the method has the advantages of simple pretreatment, high sensitivity and accurate detection results.

2. EXPERIMENTAL

2.1. Materials and drugs

Standard substances of beta-estradiol (BE), estriol (EI), estrone (EO), diethylstilbestrol (DS), dihydrodiethylstilbestrol (DDS), ethinyl estradiol (EE) and ethinyl estradiol methyl ether (EEME) were purchased from National Institutes for Food and Drug Control (Beijing, China). Ethanol, acetonitrile, hexane, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and sodium chloride (NaCl) were all of analytical reagent grade and were purchased from Beijing Chemical Factory (Beijing, China).

2.2. Accelerated solvent extraction

Accurately weigh 1 g of milk powder sample and 2 g of diatomite, and put them into the extraction tank after mixing. Add 10 ml of extractant, perform static extraction for 120 s to 270 s at an extraction temperature from 110 °C to 135 °C, an extraction pressure of 10.0 MPa and cycle 3 times.

The collected extract was evaporated to dryness. It was completely dissolved with 2.0 mL of mobile phase, and was filtered with a 0.22 μm filter membrane and retained for subsequent analysis.

2.3. Apparatus and conditions

CE-UV was performed on a MPI - B multi-parameter chemiluminescence analysis test system (Xi'an Remex analytical instruments Co., Ltd., Xi'an, China). Capillary (25 μm x 40 cm) 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. ASE350 rapid solvent extractor (Thermo Fisher Scientific Co., Ltd., USA).

CE conditions: The mobile phase consisted of phosphate buffer solution (PBS) (50 mmol/L and pH 6.5) containing 25 mmol/L NaCl. PBS is prepared from Na_2HPO_4 , NaH_2PO_4 and deionized water. Separation voltage is 15 kV.

UV conditions: Detection wavelength is 283 nm.

3. RESULTS AND DISCUSSION

3.1. Optimization of ASE conditions

In the ASE method, the type of extractant, the amount of extractant, the extraction temperature, the extraction pressure, the extraction time and the extraction times all have a certain influence on the extraction rate [20]. Here, the recovery rate of 7 estrogens will be used as the test index to study the influence of three main factors: the type of extraction agent, extraction temperature and extraction time.

3.1.1 Extraction agent

Estrogen is easily soluble in organic solvents but not in aqueous solutions. Therefore, ethanol, acetonitrile, hexane, ethanol-acetonitrile (1:1, v/v), ethanol-hexane (1:1, v/v), and hexane-acetonitrile (1:1, v/v) were selected as extractants for comparison in our experiments. At the extraction temperature of 120 $^\circ\text{C}$, the extraction time of 210 s, the extraction pressure of 10 MPa and cycle 3 times, the spiked recovery of each component was calculated. The results are shown in Figure 1.

As you can see, the extraction effect of mixed extractants was generally better than that of single extractants, which was consistent with the results in the literature [21-24, 26, 27]. Among the six selected extractants, the comprehensive extraction effect of ethanol-acetonitrile on seven estrogens was better than that of the other five extractants. Therefore, ethanol-acetonitrile (1:1, v/v) mixed extractant was selected in this experiment.

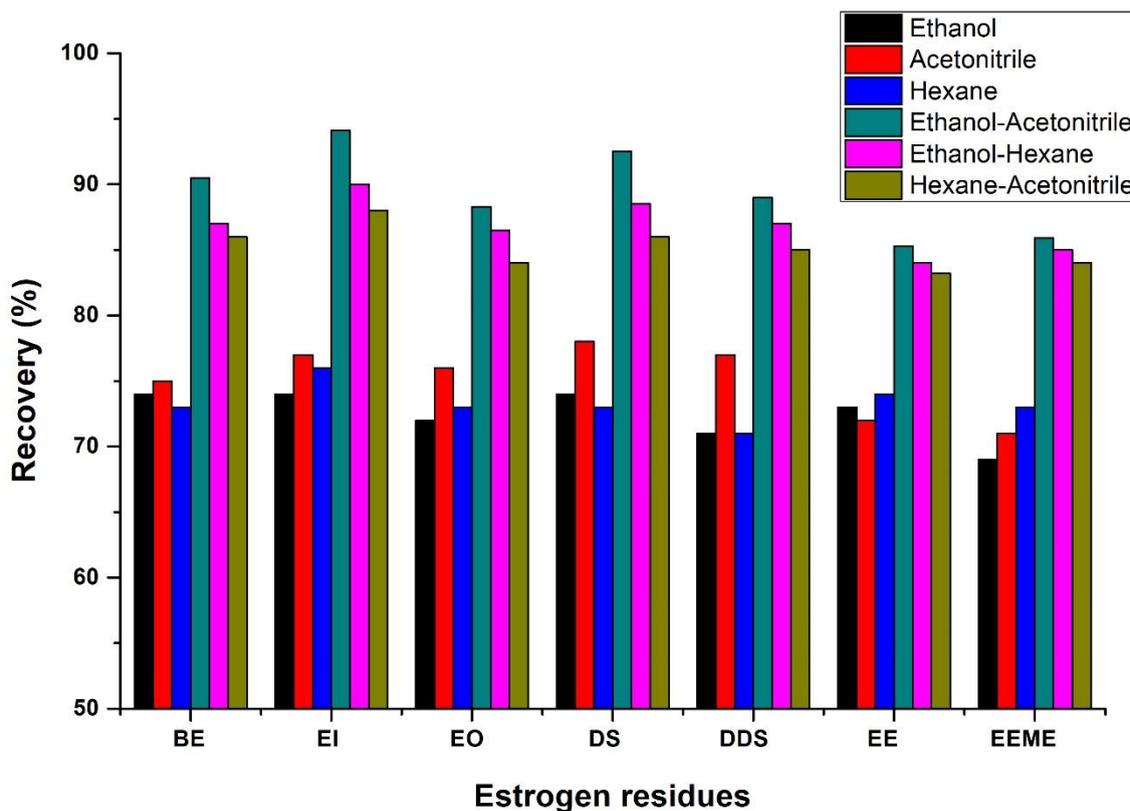


Figure 1. Effects of extraction agent on the recoveries of 7 estrogens under extraction temperature of 120°C, extraction time of 210 s, extraction pressure of 10 MPa and cycle 3 times.

3.1.2 Extraction temperature

Extraction temperature is an important parameter of ASE. High temperature is beneficial to the improvement of extraction efficiency [21]. Generally, as the temperature increases, the viscosity of the solvent decreases, the ability of the solvent to wet the matrix increases, and the ability of the solvent to dissolve the analyte increases. In addition, high temperature can accelerate the diffusion of analytical components to the matrix surface, thereby improving the extraction efficiency. In this experiment, ethanol-acetonitrile (1:1, v/v) was used as the ASE solvent, and the extraction temperature was 110°C, 115°C, 120°C, 125°C, 130°C and 135°C, and the static extraction was performed for 210 s at an extraction pressure of 10.0 MPa and cycle 3 times to investigate the effect of extraction temperature on the recoveries of various components, and the results are shown in Figure 2.

It can be seen from the figure that when the extraction temperature is lower than 120 °C, the extraction amount of each component increases with the increase of temperature. When the extraction temperature was 120 °C, the recoveries of various components were more than 85%. When the extraction temperature continued to increase, the recoveries of various components began to decline. The reason for this phenomenon may be due to the excessive temperature, the evaporation of the hot solvent leads to a decrease in the amount of extraction [23, 27]. Comprehensive consideration, choose 120 °C as the ASE temperature in this experiment.

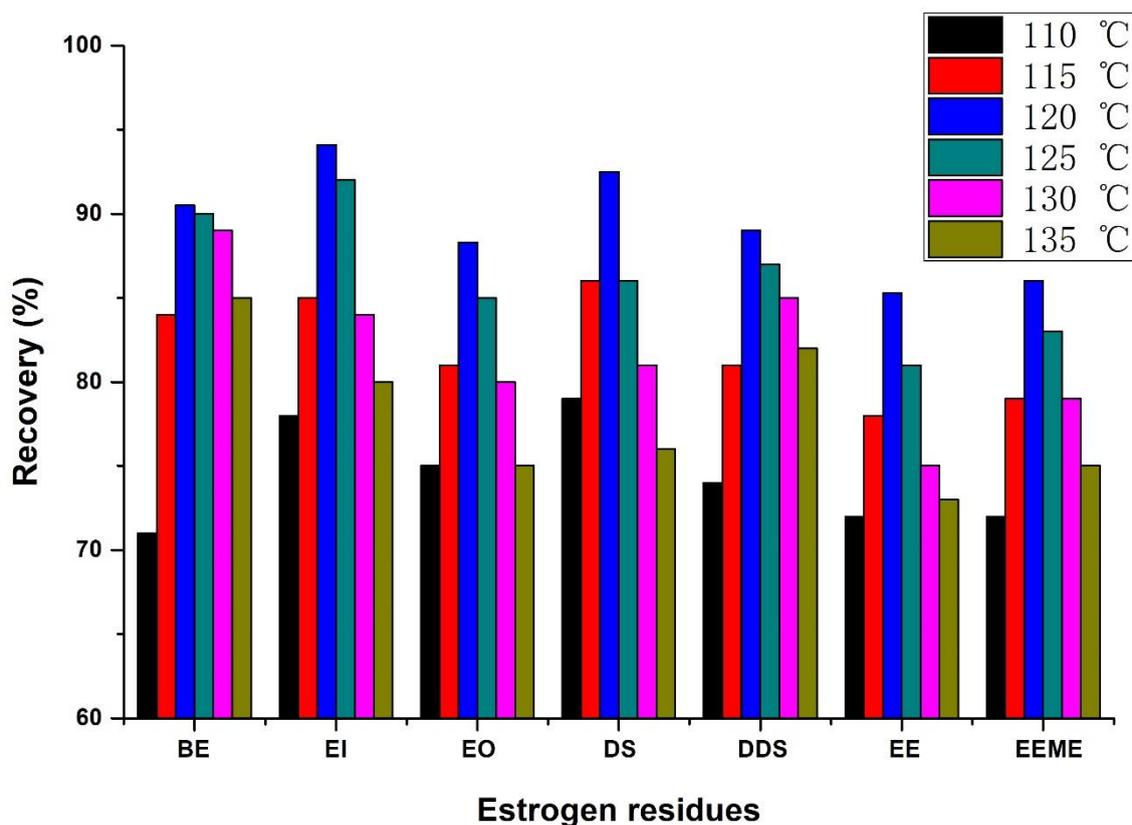


Figure 2. Effects of extraction temperature on the recoveries of 7 estrogens under extraction time of 210 s in ethanol-acetonitrile, extraction pressure of 10 MPa and cycle 3 times.

3.1.3 Extraction time

The length of extraction time is related to the degree of diffusion of the components to be extracted in the sample and the solvent. Increasing the extraction time facilitates the diffusion of the substance to be extracted from the sample matrix into the extraction solvent [24, 26]. In this experiment, ethanol-acetonitrile (1:1, v/v) was used as the ASE solvent, the extraction temperature was 120°C, and the static extraction was performed for 120s, 150s, 180s, 210s, 240s and 270s, respectively, at extraction pressure of 10 MPa and cycle 3 times, to investigate the effect of extraction time on the recoveries of various components. The results are shown in Figure 3.

It can be seen from the figure that when the extraction time is in the range of 120-210s, the extraction amount of each component shows an increasing trend with the extension of the extraction time. When the extraction time reached 210s, the recovery of each component was the highest. Continue to prolong the time, the extraction amount of each component is basically unchanged. Therefore, 210 s was chosen as the ASE time.

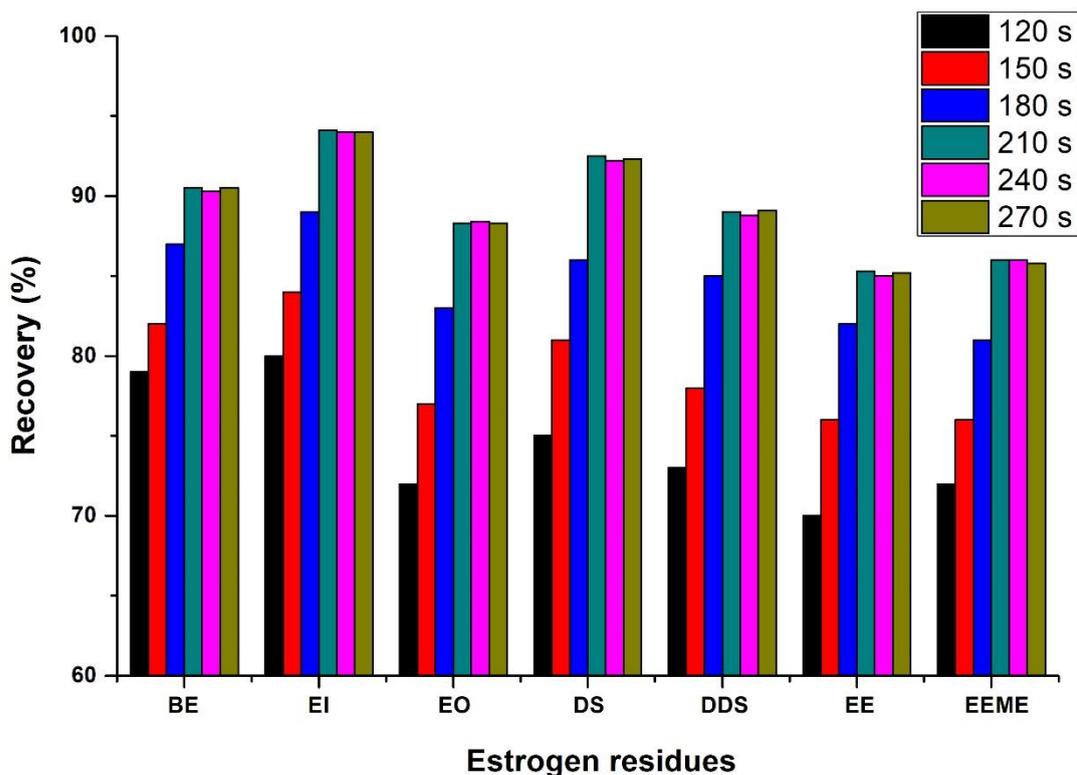


Figure 3. Effects of extraction time in ethanol-acetonitrile on the recoveries of 7 estrogens under extraction temperature of 120 °C, extraction pressure of 10 MPa and cycle 3 times.

3.2. Optimization of CE conditions

In the process of CE separation, mobile phase type, mobile phase concentration, mobile phase acidity, mobile phase ionic strength and separation voltage all have a certain influence on the separation effect [29-33]. The separation effect is usually quantified by the resolution (R). R is an indicator of the degree of separation of the two components. It is defined as: the ratio of the difference of two components retention time (t) and the average width of peak base (Y). Expressed mathematically as:

$$R = \frac{t_2 - t_1}{(Y_1 + Y_2) / 2}$$

When the resolution of the two components is greater than 1.5, they can be completely separated. See our previous work for detailed calculation [36]. Here, the resolution of 7 estrogens in the electropherogram will be used as the test index to study the influence of three main factors, namely, the acidity of the mobile phase, the ionic strength of the mobile phase and the separation voltage.

3.2.1 Acidity of the mobile phase

The acidity of the mobile phase is an important factor affecting the separation by capillary

electrophoresis. Changes in mobile phase acidity can affect electroosmotic flow, resulting in changes in peak shape and resolution [31, 34]. Typically, buffer solutions are used to keep the acidity of the mobile phase relatively stable during capillary electrophoresis separations [29-33]. In this paper, the effects of 50 mmol/L PBS of pH 5.5, pH 6.0, pH 6.5, pH 7.0, pH 7.5 or pH 8.0 containing 25 mmol/L NaCl at separation voltage 15 kV on the resolutions of 6 pairs of estrogens were investigated. The results show that when the PBS of pH 6.5 is selected, better peak shape, moderate analysis time and greater resolution can be obtained. Therefore, PBS with pH 6.5 containing 25 mmol/L NaCl was selected as the mobile phase for separation.

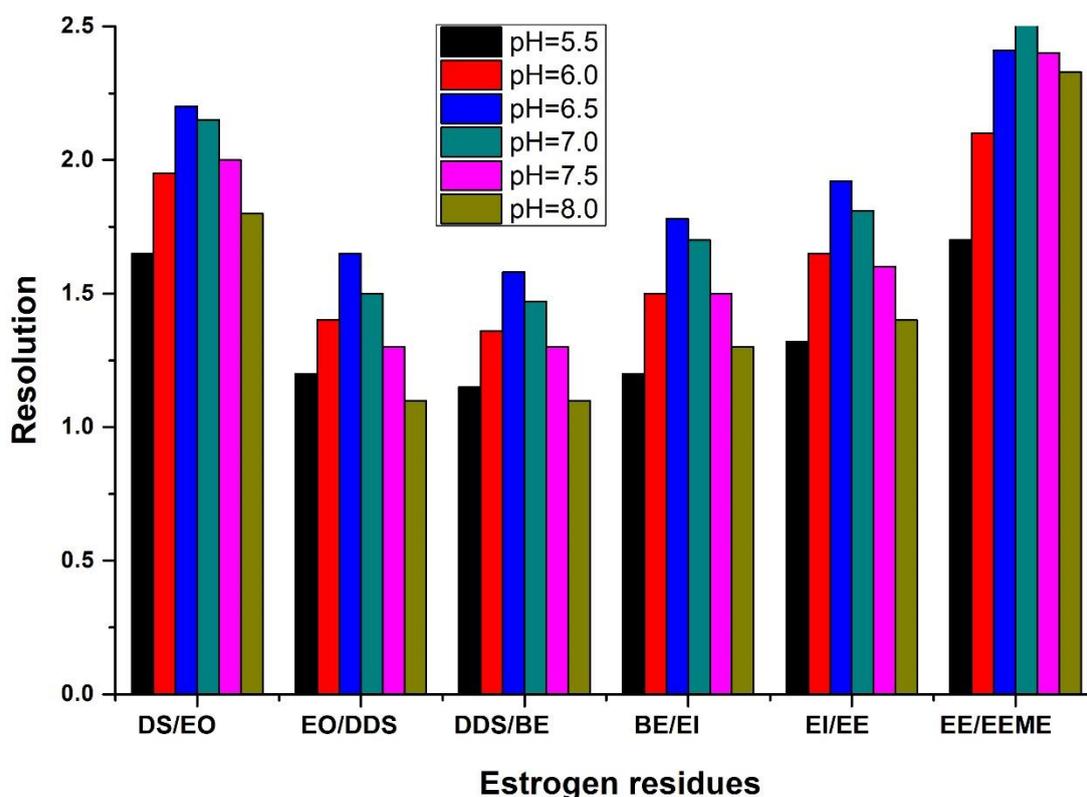


Figure 4. Effects of pH of mobile phase on the resolutions of 6 pairs of estrogens in 50 mmol/L PBS containing 25 mmol/L NaCl at separation voltage 15 kV.

3.2.2 Ionic strength of the mobile phase

Changes in the ionic strength of the mobile phase can change the structure of the electric double layer, thereby affecting the electroosmotic flow, and changing the migration time and degree of separation of the separated components [32-34]. Adjusting the concentration of the buffer solution can change the ionic strength of the mobile phase. However, the ionic strength of the mobile phase can be adjusted more economically and efficiently by adding neutral salts [36]. In this paper, the effects of mobile phases containing different concentrations of NaCl on the resolutions of 7 estrogens in 50 mmol/L PBS (pH 6.5) at separation voltage 15 kV were investigated. The results showed that the

migration time was prolonged with the increase of NaCl concentration, and the separation of adjacent components was improved. However, when the concentration of NaCl is large, it is affected by the Joule heating effect, which causes the band broadening, thereby reducing their separation [35]. When the NaCl concentration is 25 mmol·L⁻¹, the components can be well separated.

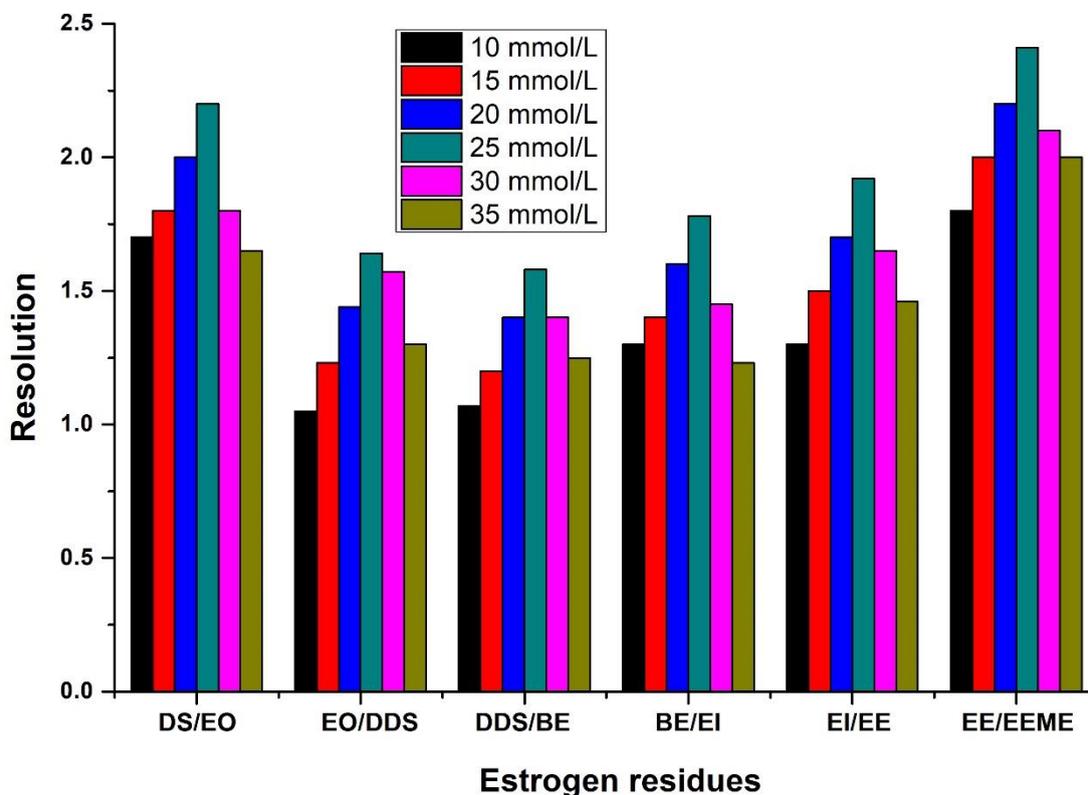


Figure 5. Effects of ionic strength of mobile phase on the resolutions of 6 pairs of estrogens in 50 mmol/L PBS (pH 6.5) at separation voltage 15 kV.

3.2.4 Separation voltage

The magnitude of the separation voltage affects the migration time and resolution of the components. The higher the separation voltage, the shorter the migration time of the analyte. However, when the separation voltage is large, the migration current increases, resulting in an increase in Joule heating. When the CE system cannot effectively dissipate the generated Joule heat in a timely manner, the column temperature will increase, and the column efficiency and resolution will decrease [30, 32-35]. In this paper, the effects of the separation voltages of 13 kV, 14 kV, 15 kV, 16 kV, 17 kV and 18 kV on the resolutions of 7 estrogens in 50 mmol/L PBS (pH 6.5) containing 25 mmol/L NaCl were investigated. The results show that the migration time of each component is longer when it is lower than 15 kV. When the temperature is higher than 16 kV, the migration time of each component is too short, and the adjacent components cannot be well separated. When the temperature is 15 kV, the migration time of each component is moderate and the separation effect is good. Therefore, the separation voltage

of 15 kV was selected to complete the subsequent experiments.

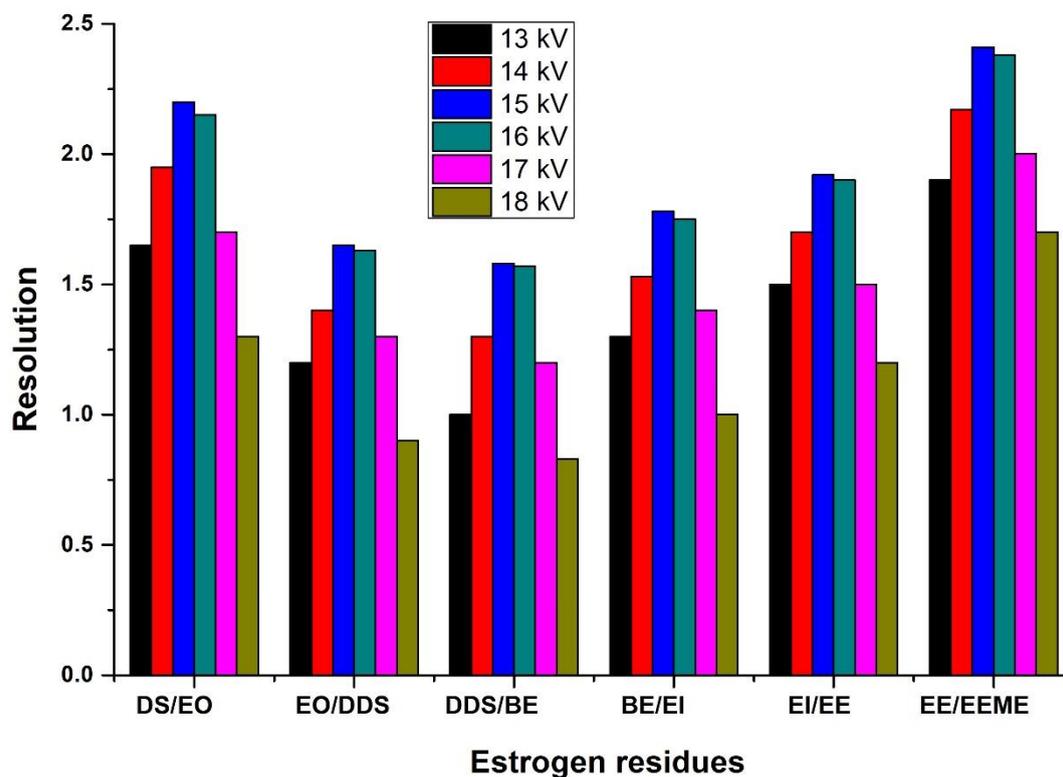


Figure 6. Effects of separation voltage on the resolutions of 6 pairs of estrogens in 50 mmol/L PBS (pH 6.5) containing 25 mmol/L NaCl.

3.3 Methodology

According to the optimization results of ASE conditions in 3.1, we determined the extraction method of ASE. Accurately weigh 1 g of milk powder sample and 2 g of diatomite, and put them into the extraction tank after mixing. Add 10 ml ethanol-acetonitrile (1:1, v/v), perform static extraction for 210 s at an extraction temperature of 120 °C, an extraction pressure of 10.0 MPa and cycle 3 times. The collected extract was evaporated to dryness. It was completely dissolved with 2.0 mL 50 mmol/L PBS (pH 6.5) containing 25 mmol/L NaCl, and was filtered with a 0.22 μm filter membrane and retained for analysis.

According to the optimization results of CE conditions in 3.2, we determined the CE method. The mobile phase consisted of 50 mmol/L PBS (pH 6.5) containing 25 mmol/L NaCl. Separation voltage is 15 kV.

The UV absorbance of the capillary electrophoresis effluent measured at a wavelength of 283 nm was used as a quantitative basis for various estrogens.

A series of estrogen standard solutions were prepared and determined according to the experimental method. The linear relationship, linear range and detection limit of the method were

investigated. The results were summarized in Table 1. Their detection limits are 0.5-0.8 µg/kg. This shows that the method is sensitive.

Table 1. Regression equation, linear range and detection limit of 7 estrogens.

Number	Estrogen	Regression Equation	Linear Range/(µg/kg)	Detection Limit/(µg/kg)
1	BE	$I = 11.7C + 2.5$	1.0-500	0.8
2	EI	$I = 24.2C + 10.4$	1.0-1000	0.8
3	EO	$I = 8.1C + 6.7$	1.0-1000	0.7
4	DS	$I = 4.6C + 3.0$	0.8-500	0.5
5	DDS	$I = 13.8C + 0.6$	0.8-500	0.5
6	EE	$I = 31.4C + 4.6$	0.8-800	0.5
7	EEME	$I = 22.4C + 13.2$	0.8-800	0.5

3.4 Sample analysis

We analyzed the milk powder samples using the ASE-CE-UV method established in 3.3. The residue and recovery of 7 estrogens in 3 samples were studied. The recoveries of 7 estrogens in them are 85.7% - 114.8%. Residual 7 estrogens were detected in all 3 milk powder samples, and the results are shown in Table 2.

Table 2. Analysis results of actual samples.

estrogens	Measured value (µg/kg)			Added value (µg/kg)	Recovery (% , n=9)		
	Sample1	Sample2	Sample3		Sample1	Sample2	Sample3
BE	2.4	ND*	6.5	20	92.7	100.9	99.2
EI	7.1	3.0	ND	20	106.5	114.8	92.5
EO	ND	ND	2.7	20	93.6	91.3	107.5
DS	1.9	4.4	ND	20	103.5	89.9	100.3
DDS	3.1	ND	ND	20	85.7	108.4	87.2
EE	ND	ND	1.6	20	94.5	88.9	113.2
EEME	5.2	4.7	ND	20	111.3	92.1	100.5

*Not detected

4. CONCLUSION

This work demonstrated a new analytical procedure for simultaneous determination of 7 estrogen residues in milk powder samples by ASE-CE-UV. The 7 estrogens could be well separated and analyzed with high sensitivity, wide linear range and good reproducibility.

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