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Determination of Three Preservatives in Food by Ultrahigh Conductivity Zone-Low Temperature Zone/Sweeping-Micellar Electrokinetic Chromatography in Non-Aqueous Capillary Electrophoresis

WEN-YI HUANG[1], KE SUN[1,2], LI-JUN LI[1,*], RUI-RUI ZHANG[1], YING LUO[1], DA-CHUN HU[1], CHUN-YAN DENG[1] and YAN-QING LI[1]

[1]College of Biological and Chemical Engineering, Guangxi Key Laboratory of Green Processing of Sugar Resources, Key Laboratory for Processing of Sugar Resources of Guangxi Higher Education Institutes, Guangxi University of Science and Technology, Liuzhou 545006, Guangxi, P.R. China
[2]Department of Biochemistry and Molecular Biology, Youjiang Medical University For Nationalities, Baise 533000, Guangxi, P.R. China

*Corresponding author: Tel/Fax: Tel: +86 77 22685028; E-mail: lilijun0562@sina.com

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An ultrahigh conductivity zone-low temperature zone (UHCZ-LTZ)/sweeping-micellar electrokinetic chromatography (MEKC) method was used for the simultaneous separation and determination of three preservatives *i.e.*, benzoic acid, sorbic acid and dehydroacetic acid in food. The result indicated that a best separation of the three preservatives was obtained on a uncoated fused silica capillary column (50 cm × 50 μm, effective length 36 cm) by using the background electrolyte containing 50 mmol/L ammonium acetate (CH_3COONH_4), 6 mmol/L sodium hydroxide (NaOH), 6 mmol/L cetyltrimethyl ammonium bromide (CTAB) and 10 % acetonitrile (CH_3CN) (by volume) in methanol. The detection wavelength was set at 230 nm, separation voltage was -22 kV, sample injection voltage was -20 kV and injection time was 8 s, injection time of high-conductivity zone was 15 s. Under the optimum conditions, the three preservatives was performed within 20 min with relative standard deviation (RSDs) of peak area less than 5 %. The detection limits (S/N = 3) of benzoic acid, sorbic acid and dehydroacetic acid were 0.61, 0.361 and 0.999 ng/mL, respectively.

Keywords: Micellar electrokinetic chromatography-Non-aqueous capillary electrophoresis, Preservatives, High conductivity.

INTRODUCTION

Preservatives owning antimicrobial properties are permitted food additives in various food products to preserve the growth of yeasts, moulds and bacteria in food and beverages. Benzoic acid, sorbic acid and dehydroacetic acid (Fig. 1) are important chemical preservatives used widely in pharmaceutical, cosmetic and food industry. Benzoic acid inhibits bacterial development. sorbic acid is an antifungal preservative against molds and yeasts[1,2]. These preservatives are allowed by legislation that establishes the maximum permitted concentrations in each type of food. However, the presence of these preservatives at higher than permitted safety levels, can be harmful to human health. The maximum permitted concentrations of preservatives in each type of food are controlled by legislation[3]. The analyses of these compounds in food samples of differing matrices had been carried out by spectrophotometry[4], dispersive solid phase extraction[5], high-performance liquid chromatography[6-8], gas chromatography-mass spectrometry[9] and capillary electrophoresis[10-13].

Capillary electrophoresis is a versatile separation technique for efficient analysis of various chemical species such as inorganic and organic ions, neutral compounds and macromolecules. Among the different strategies to overcome the lack of sensitivity inherent to capillary electrophoresis, on-line preconcentration techniques allow to increase analyze mass loading, with the subsequent decreasing of detections limits. Among these pre-concentration methods such as micellar electrokinetic chromatography (MEKC)[14,15], field amplified sample injection[16-18], large volume sample stacking[19-20], ultrahigh conductivity zone-low temperature zone (UHCZ-LTZ)[21,27], sample stacking is an example of efficient method and it has been widely explained in the literature.

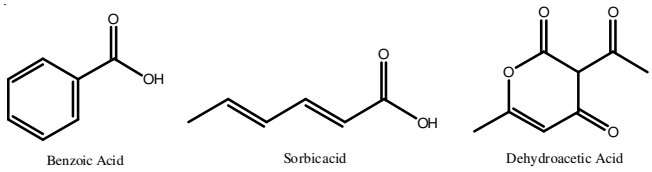

Fig. 1. Molecular structure of benzoic acid, sorbic acid and dehydroacetic acid

Non-aqueous capillary electrophoresis (NACE) had been introduced in 1984 by Walbrohel and Jorgenson[22], was based on the use of electrolyte solutions prepared in pure organic solvents and offered a number of attractive features such as improved selectivity by changing the solvent or solvent mixture, extended application scope with a better solubility for hydrophobic compounds, reduced electrophoretic currents, less joule

heating and better detection[23,24]. For these reasons, NACE had drawn much attention in recent years[25-26] including the implementation of on-line pre-concentration methods such as FASS, LVSEP-ASEI and LVSEP[27-29]. In this paper, benzoic acid, sorbic acid and dehydroacetic acid were difficult to dissolve in water; we found the separation resolution increased by using non-aqueous capillary electrophoresis (NACE).

The aim of this work was to develop and optimize a CE-UV method for the analysis of benzoic acid, sorbic acid and dehydroacetic acid. In order to improve detection limits, the use of UHCZ-LTZ/sweeping-MEKC in reversed polarity was evaluated. The proposed methodology has been validated in terms of linearity, limits of detection and limits of quantification, repeatability and intermediate precision and applied to the analysis of preservatives in bread, pickles and fruit juice samples at the ng/mL level.

EXPERIMENTAL

All analyses were carried out on an ACS2000 capillary electrophoresis system (Beijing Cailu Instrumental Co., Beijing, China). The apparatus was equipped with a power supply (up to constant voltage 30 kV), a HW-2000 chromatography workstation and a UV-visible detector (double light beams, λ = 190-740 nm, set at 230 nm), Data acquisition was carried out with a maxima 820 chromatography workstation. A fused silica capillary (Factory of Yongnian Optical Fiber, Hebei, China) were used, with total length 60 cm, effective length 36 cm, I.D.50 μm. The runs were carried out under 25 °C cooling air. A UV-2102PC UV-visible spectrophotometer (UNICO instruments CO., Ltd) was used to detect ultraviolet visible spectrum;DL-60D ultrasonic bath (Letter to Shanghai Instrument Co., Ltd.)was used to extract sample and degas solutions; The standard solid sample was weighed by Analytical balance (Austrian House Shanghai Co., Ltd.). The pH of the solutions was measured by employing a model EF20 Laboratory pH meter Mettler-Toledo Instruments (Shanghai). A CW-2000 ultrasonic-microwave synergistic extraction apparatus from Shanghai XinTuo Microwave sample-dispelling test CO., Ltd was used to extract analytic components from food sample.

Standards of benzoic acid, sorbic acid and dehydroacetic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Food was purchased from local supermarket (Liuzhou, China). Cetyltrimethyl ammonium bromide was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Other reagents were purchased from Tianjin Chemical Reagent Factory (Tianjin, China). All chemicals and solvents were of analytical reagent grade and were used without further purification. The water used for experiment was double distilled water.

Solutions and samples preparation: Sample handling method as follows: Stock standard solutions were prepared in volumetric flask with 25 mL MeOH to give the concentration of three preservatives with 448 μg/mL for benzoic acid, 400 μg/mL for sorbic acid and 312 μg/mL for dehydroacetic acid. The working standard solutions were prepared daily by diluting the stock standard solution with MeOH.

The bread, pickles and fruit juice were precision weighed to 2.0735, 3.7230 and 7.8718 g, respectively. Then they were pulverized and soaked with 25 mL MeOH for 12 h and extracted with methanol for 200 S in an ultrasonic bath, respectively. The extraction was repeated 3 times. The extracts were combined and filtered through filter paper and then diluted to 25 mL in a volumetric flask with methanol. All the solutions were stored in a refrigerator at 3 °C. Every day all solutions were filtered through a 0.22 μm syringe filter before use.

The capillary was conditioned prior before experiment by consecutively flushing with 1 mol/L HCl for 15 min, 1 mol/L NaOH for 15 min, double distilled water for 15 min, MeOH for 15 min and electrophoresis buffer for 20 min. After each run, the capillary was rinsed with double distilled water for 5 min, follow by MeOH for 10 min, the electrophoresis buffer for 10 min. The buffer was renewed after every three runs for good reproducibility. The capillary was rinsed with double distilled water after experiment everyday.

Methodology

Sweeping-micellar electrokinetic chromatography: Sweeping have rapidly grown in popularity over the past few years[30-32]. The MEKC had been introduced in 1984 by Terabe *et al.*[33] and proved to be not only the method of choice in analysis of neutral compounds but also one of the most versatile separation approaches among the electro-migration methods. In MEKC, a surfactant was introduced into the background electrolyte at the concentration above the critical micelle concentration in order to generate a micellar pseudo-stationary phase. Separation mechanism was a combination of chromatographic partitioning of solutes between pseudo-stationary phase and continuous phase and the electrophoretic mechanism. The separation selectivity of MEKC could be modulated not only by variation of background electrolyte type, pH and concentration, but also by profit of the proper selection of the surfactant as well as by optimizing its concentration.

UHCZ/Sweeping-MEKC: Ultra high conductivity zone method was based on in a mismatch between the electric conductivity of the sample and that of the running buffer and it was achieved by injecting the sample diluted in a solvent of higher conductivity than that of the carrier electrolyte. This difference in buffer conductivity results in sample stacking at the beginning of the separation[34], which improves separation efficiency. In this mode, a UHCZ was inserted between the sample zone and BGS to build a conductivity gradient. The sample was stacked along the capillary axis, to then reduce its speed and to be accumulated near the junction because of the sudden increase in conductivity. Once in the separation buffer, the injected components of the sample migrate in different zones according to their charge/mass characteristics. Ultra high conductivity zone has been successfully applied to the analysis of compounds such as phenolic acids[21]. Compared to a normal MEKC stacking method, this method permitted a higher efficiency for sample stacking.

UHCZ-LTZ/Sweeping-MEKC: Low temperature zone was another method to build a conductivity gradient without inserting an ultra-high conductivity zone between the sample zone and background solution. In this mode, a short of low temperature bath was in place of the ultra-high conductivity zone to produce the high-electric field zone. The low temperature bath served as a "pseudo-high-conductivity zone" to

the fact that the conductivity would increase when the temperature was decreased[21,27]. Then the sample also reduced its speed and accumulated near the junction.

Fig. 2 shows schematic diagrams of sweeping-MEKC (a), UHCZ/sweeping-MEKC (b) and UHCZ-LTZ/sweeping-MEKC (c) methods, respectively.

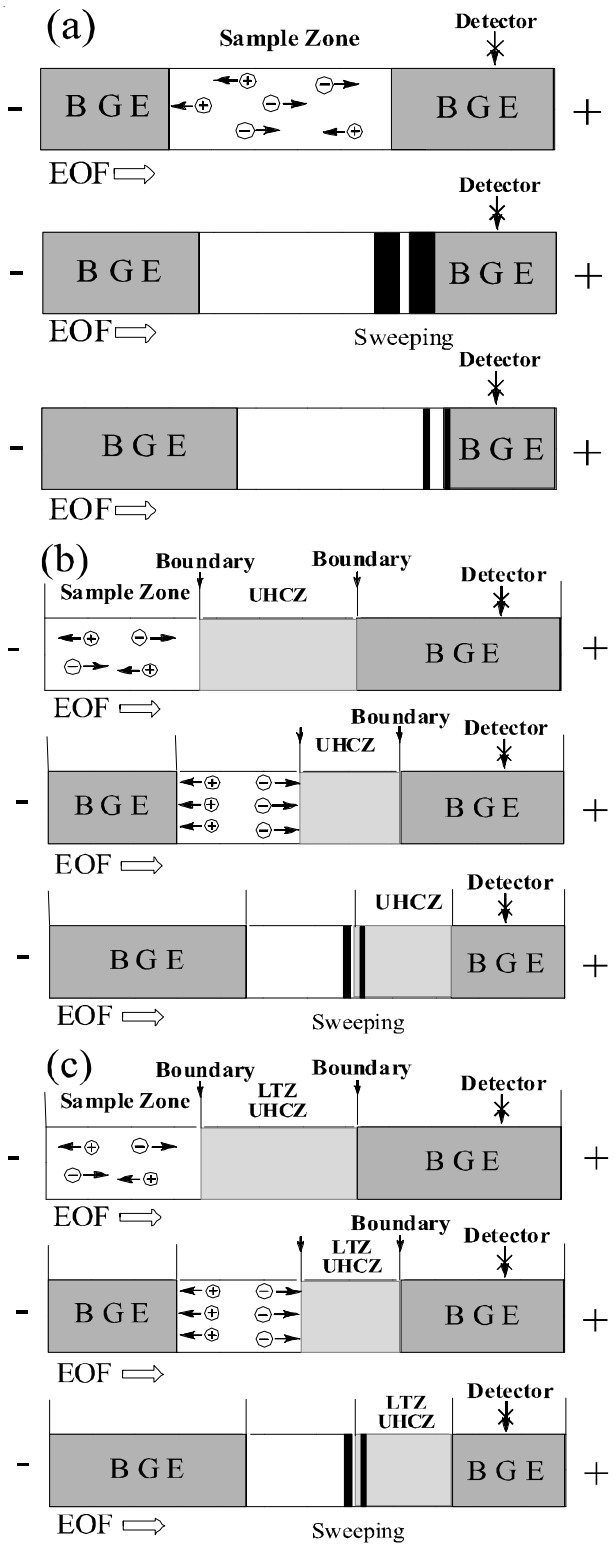

Fig. 2. Schematic diagram of sweeping-MEKC (a), UHCZ/sweeping-MEKC (b) and UHCZ -LTZ/sweeping-MEKC (c) methods. BGS: background solution; LTZ: low temperature zone; EOF: electro-osmotic flow

RESULTS AND DISCUSSION

Optimization of non-aqueous capillary electrophoresis conditions: In the selection of background electrolyte, the main consideration is the ionization characteristic of the analytes. The pKa values for benzoic acid and sorbic acid are 4.20, 4.76, respectively. When pH > 4.5 the values indicated that the analytes have a net negative charge. Therefore, in present study, negative polarity separation voltage was used[35]. Several electrolytes utilized as the buffer solution were tested, including sodium borate-sodium hydroxide, ammonium acetate-sodium hydroxide and sodium borate-boric acid. Finally, ammonium acetate-sodium hydroxide was selected as background electrolyte, because the best resolution was obtained with this buffer. Initially, an electrolyte containing 50 mmol/L ammonium acetate and 4mmol/L sodium hydroxide in methanol was used as buffer.

In this paper, the pH of background electrolyte was examined by the concentration of NaOH because the pH could not be exactly determined in NACE. To verify the effect of pH on migration behaviour, experiments were performed with the concentration of NaOH varying from 2 to 10 mmol/L. We found that the separation resolution increased with the NaOH concentration increasing. However, analysis time was prolonged at higher concentration of NaOH, which made it less suitable for routine analyses. In order to obtain the best separation efficiency and analytical time, a concentration of 6 mmol/L was chosen as the suitable buffer acidity.

The effect of CH_3CN on the electrophoretic behaviour of analytes was studied by adding different volume fractions of CH_3CN (5, 10, 15 and 20 %) to the electrolyte. With the increasing of CH_3CN volume fraction, the migration time of the analytes was shortened and UV response of the analytes enhanced, but the resolution among the three analytes worsened. Finally, 10 % CH_3CN was selected as optimum as this value permitted a good compromise between migrate time and separated degree.

The injection time of sweeping-MEKC was varied between 40 s and 120 s in this paper. When the injection time prolonged the peak area of all compounds also increased, but the theoretical plate number would reduced. And when the injection times longer than 100 s, a loss of resolution between peaks was observed. For this reason, 100 s of injection time was chosen as optimal value.

The effect of separation voltage on the migration time and peak area was also studied in UHCZ-LTZ/sweeping-MEKC. The results illustrated that the high separation voltage gave shorter migration time and less peak area for all analytes and it produced more Joule heating and decreased the resolution. Thus, -22 kV was chosen as the optimal separation voltage in considering the analytical time and resolution. The injection plug of sample was optimized by varying the injection time at a fixed injection voltage of -20 kV in our experiment, the peak-area signals were observed when the injection time was from 4 to 12 s. When the injection time was longer than 10 s, peak areas were no longer increased obviously when the complete sample plug was injected. The greatest enhancement of the total peak area was observed when the injection time was 8 s. Hence, 8 s was chosen as the injection time for UHCZ-LTZ/Sweeping-MEKC.

Effect of cetyltrimethyl ammonium bromide: In this paper, a negative polarity separation voltage mode was used, the direction of the EOF was opposite to that of anion electromigration resulted in very poor resolution and detection capability when pH > 4.5, it is favorable to use the EOF modifier to suppress or reverse EOF direction[35]. In our study, CTAB was used as the EOF modifier. The effect of CTAB concentrations (3-7 mmol/L) on the resolution of the three compounds was investigated. The results were shown in Fig. 3. It was found that the peak area and the peak height decreased with the increasing of CTAB concentrations. While the CTAB concentrations up to 6 mmol/L, the further increasing of CTAB concentrations would decreased the peak area and the peak height and shortened the migration time. It may be explained that the increasing concentrations could make better sweeping efficiency, while it also bring high ionic strength and high current, which would led to the worse baseline and the decrease of peak area and peak height. Therefore, 6 mol/L CTAB was selected as the optimal CTAB concentration since this provided the shortest analysis time and also had better separation of all three compounds.

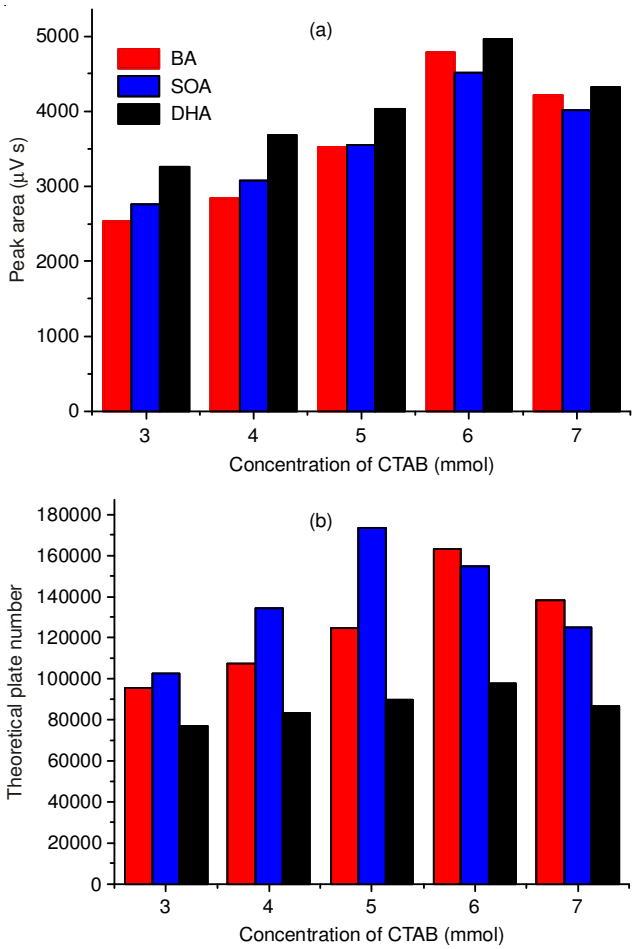

Fig. 3. Effect of CTAB concentration on peak area (a) and theoretical plate number (b)

Effect of ultra high conductivity zone: In order to achieve maximum concentration factors, different parameters that affected the stacking effect of those methods were optimized. First, different concentrations of ammonium acetate were investigated for UHCZ-LTZ/sweeping-MEKC under the temperature of UHCZ was at 3 °C. In Fig. 4, electropherograms (a-c) show the results obtained from different test concentrations of UHCZ (a-c: ammonium acetate concentration, 200, 150 and 100 mM). The findings showed that the peak height had not so much relationship with the ammonium acetate concentration. However, the peak shape was intensively influenced with the concentration, as shown in Fig. 4. When the concentration of UHCZ was from 200 to 100 mM, the peak became sharper, meanwhile, the migration time was shorter. Therefore, 150 mM ammonium acetate was selected as the optimal concentration in considering the analytical time and resolution for further investigation. Second, the injection time of ammonium acetate was studied in this paper. We found that migration time, peak area and theoretical plate number was changed little when the injection time was from 5 to 25s. Finally, 15 s was chosen as the injection time of ammonium acetate.

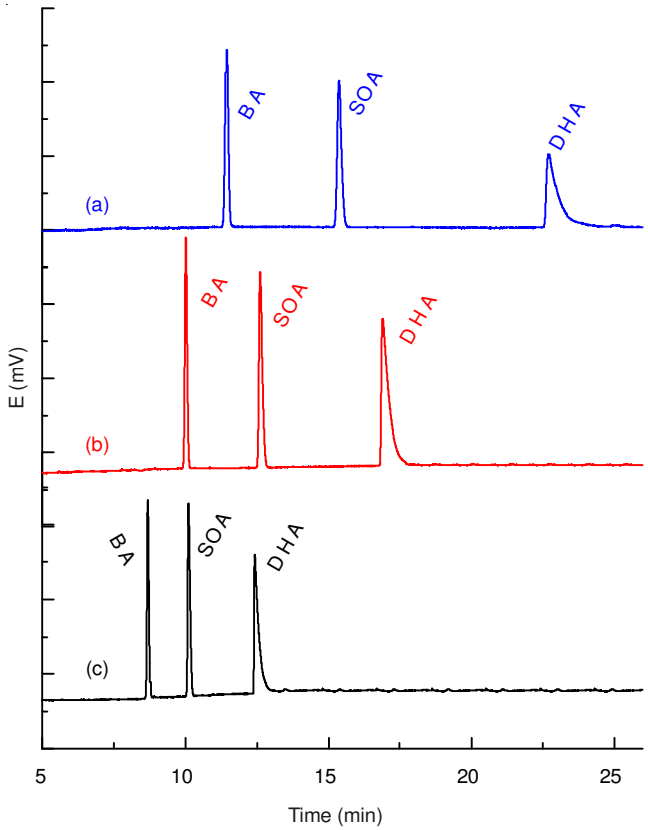

Fig. 4. Concentrations of ammonium acetate by UHCZ-LTZ/sweeping-MEKC in Fig. 4a-c were 200, 150 and 100 mmol/L, respectively. buffer solution: 50 mmol/L CH_3COONH_4-6 mmol/L NaOH-6 mmol/L CTAB-5 % CH_3CN (v/v); separation voltage: -22 kV; injection voltage: -20 kV; injection time: 8s; injection time of UHCZ: 10s, 3 °C

Method evaluation: Standard solutions were preconcentrated by each stacking methods under the optimal conditions described above to determination of the regression equation of each analyte and linear range and the limit of detection (LOD) values. The electrophoretograms of sweeping-MEKC, UHCZ/Sweeping-MEKC and UHCZ-LTZ/sweeping-MEKC methods were shown in Fig. 5a-c. These results were summarized in Table-1. The results indicated that the calibration curves

TABLE-1
LINEAR RANGE, REGRESSION EQUATION, LODs, RSD BY SWEEPING-MEKC (A), UHCZ/SWEEPING-MEKC (B) AND UHCZ-LTZ/SWEEPING-MEKC (C) METHODS

Methods	Compound	Benzoic acid	Sorbic acid	Dehydroacetic acid
(A) Sweeping-MEKC	Linear range (μg/mL)	1.12-17.92	0.75-12	1.37-21.84
	Regression equation	y =140.48x + 48.875	y = 377.44x - 91.083	y = 356.83x + 107.56
	R^2	0.999	0.9996	0.9995
	LOD (ng/mL)	64.9	25	33
	RSD (%) (n=5)	3.03	2.24	4.03
(B) UHCZ/sweeping-MEKC	Linear range (μg/mL)	56-896	50-800	117-872
	Regression equation	y = 2.931x - 0.708	y = 3.7574x - 55.583	y = 3.124x + 45.458
	R^2	0.9994	0.9991	0.9993
	LOD (ng/mL)	2.08	2.49	2.78
	RSD (%)(n=5)	2.04	3.12	2.9
(C) UHCZ -LTZ/sweeping-MEKC	Linear range (μg/mL)	14-224	12.5-200	29.3-468
	Regression equation	y = 9.1046x - 14.083	y = 10.956x - 18.083	y = 6.274x - 13.048
	R^2	0.9993	0.9994	0.9997
	LOD (ng/mL)	0.61	0.361	0.999
	RSD (%) (n=5)	1.84	1.32	2.09

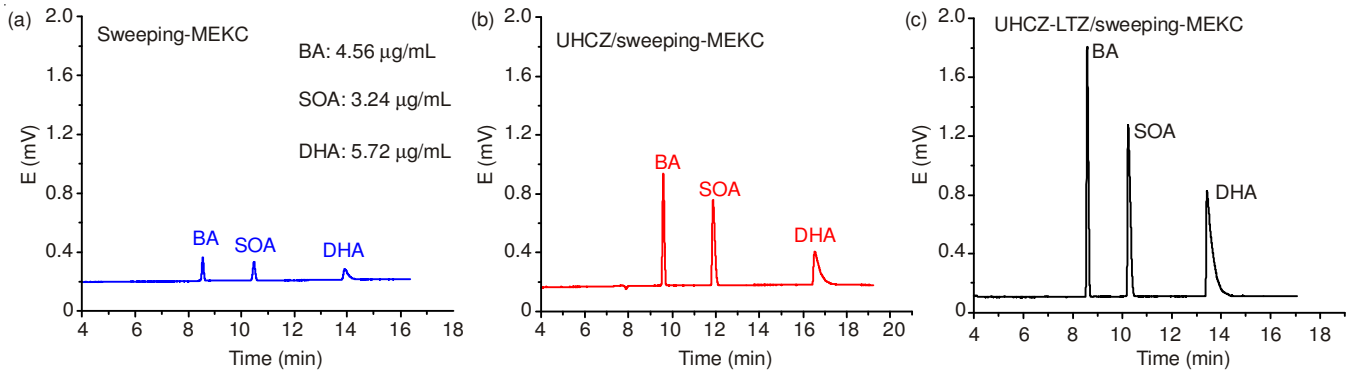

Fig. 5. Electropherogram of four materials by sweeping-MEKC (a) ,UHCZ/sweeping-MEKC (b) and UHCZ -LTZ/sweeping-MEKC (c) Buffer solution: 50 mmol/L CH_3COONH_4-6 mmol/L NaOH-6 mmol/L CTAB-10 % CH_3CN (v/v); (a) separation voltage: -22 kV; injection time:100 s; (b) separation voltage: -22 kV; injection voltage: -20 kV; injection time: 8s; injection time of UHCZ:15 s; (c) separation voltage: -22 kV; injection voltage: -20 kV; injection time: 8s; injection time of UHCZ:15 s, 3 °C

were linear over the studied concentration ranges for those stacking methods. The detection limits (S/N = 3) of benzoic acid, sorbic acid and dehydroacetic acid were 0.61, 0.361 and 0.999 ng/mL, respectively.

For convenient comparing of these stacking modes, the stacking factors evaluated with peak height and peak area were shown in Table-2. The ratio of stacking factors of UHCZ/sweeping-MEKC (N_1), UHCZ-LTZ/sweeping-MEKC stacking (N_2) and to sweeping-MEKC (N_0) was also shown in Table-2. From the results were shown in Tables-1 and 2, it could be seen that with the aid of LTZ and UHCZ, the LOD could be further improved to ng/mL level compared with MEKC stacking. The peak shape was well preserved even much longer sample zone was injected. The peak height and peak area can be further increased 3.6-8.1 fold and 3.5-8.2 fold, respectively, for UHCZ/sweeping-MEKC method compared to sweeping-MEKC method. For UHCZ-LTZ/sweeping-MEKC stacking method, the data were 7.5-12.7 fold and 8.3-13.2 fold, respectively. Comparing the values of N_1/N_0 and N_2/N_0, it could be seen that UHCZ/Sweeping-MEKC and UHCZ-LTZ/sweeping-MEKC had comparable detection sensitivity improvement capability evaluated by peak height and peak area. The conductivity difference between the sample zone and the BGS was increased by using the UHCZ and the LTZ together on one capillary, which may lead to the synergism effect for analytes[21].

Analysis of sample and recoveries: The recoveries of benzoic acid, sorbic acid and dehydroacetic acid from the extracts of three samples were determined by the method of UHCZ-LTZ/sweeping-MEKC. For each compound, three concentration levels were tested. Each sample was analyzed in triplicate. The results are listed in Table-3. The sample electrophoretograms of bread, pickles and fruit juice were shown in Fig. 6a-c, respectively. In Table-3, we also could find the results confirmed that bread contain benzoic acid and sorbic acid only, while benzoic acid, sorbic acid and dehydroacetic acid were found in pickles and fruit juice, which were in accord with the label ingredients. The determination results of benzoic acid, sorbic acid and dehydroacetic acid in three samples were lower than the maximum addition levels established by China[3].

Recovery experiments were performed by adding accurate amounts of benzoic acid, sorbic acid and dehydroacetic acid to the three samples, respectively. The resulting recovery values

TABLE-2
COMPARISON OF METHODS

Analytes	Stacking factor	Sweeping-MEKC (N_0)	UHCZ /Sweeping-MEKC (N_1)	N_1/N_0	UHCZ -LTZ/Sweeping-MEKC (N_2)	N_2/N_0
Benzoic acid	SE_A	395	3220	8.2	5220	13.2
	SE_H	84	684	8.1	1068	12.7
Sorbic acid	SE_A	742	2963	4.0	6169	8.3
	SE_H	117	416	3.6	873	7.5
Dehydroacetic acid	SE_A	715	2518	3.5	8467	11.8
	SE_H	51	218	4.3	504	9.9

TABLE-3
RESULTS OF RECOVERY TEST AND DETERMINATION

Compound		Added (ng/mL)	Found (ng/mL)	Recovery (%)	Determination (g/kg)	Standard (g/kg)
Bread	Benzoic acid	28	26.84	95.86	NG	0
		56	59.32	105.93		
		112	107.5	95.98		
	Sorbic acid	25	27.32	109.28	0.7532	1.0
		50	45.67	91.34		
		100	102.44	102.44		
	Dehydroacetic acid	58.5	59.64	101.95	0.3825	0.5
		117	116.35	99.44		
		234	237.4	101.45		
Pickles	Benzoic acid	28	30.21	107.89	0.3129	0.5
		56	58.5	104.46		
		112	109.36	97.64		
	Sorbic acid	25	23.47	93.88	0.2843	0.5
		50	46.59	93.18		
		100	96.51	96.51		
	Dehydroacetic acid	58.5	59.04	100.92	0.1928	0.3
		117	119.08	101.78		
		234	232.57	99.39		
Fruit juice	Benzoic acid	28	25.69	91.75	0.9858	2.0
		56	58.75	104.91		
		112	113.64	101.46		
	Sorbic acid	25	24.12	96.48	0.3942	0.5
		50	48.64	97.28		
		100	103.21	103.21		
	Dehydroacetic acid	58.5	56.67	96.87	0.1699	0.3
		117	118.31	101.12		
		234	233.16	99.64		

NG: Not detected

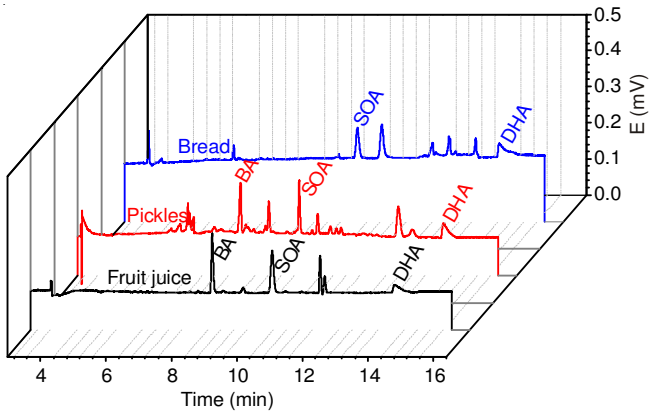

Fig. 6. UHCZ-LTZ/sweeping-MEKC electrophorograms of the three samples. Buffer solution: 50 mmol/L CH_3COONH_4-6 mmol/L NaOH-6 mmol/L CTAB-10 % ACN (V/V); separation voltage: -22 kV; injection voltage: -20 kV; injection time: 8 s; injection time of UHCZ:15 s, 3 °C

were summarized in Table-3 indicated that the recoveries were satisfactory between 91.34-109.28 % in all tests. Satisfactory spiked recoveries show that the proposed UHCZ-LTZ/Sweeping-MEKC method was suitable to be utilized for the determination of benzoic acid, sorbic acid and dehydroacetic acid in bread, pickles and fruit juice.

Conclusion

A sensitive method for the simultaneous determination of benzoic acid, sorbic acid and dehydroacetic acid in bread, pickles and fruit juice by UHCZ-LTZ/Sweeping-MEKC has been developed. For the first time, to our knowledge, UHCZ-LTZ/sweeping-MEKC coupling a sample clean-up method for the analysis of benzoic acid, sorbic acid and dehydroacetic acid had been optimized. The limits of detection of benzoic acid, sorbic acid and dehydroacetic acid were 0.61, 0.361 and 0.999 ng/mL, respectively and they were 10-30 fold lower than FESI-CE-C_4D method[35]. The UHCZ-LTZ/sweeping-MEKC technique was advantageous in terms of simplicity, excellent repeatability, high sensitivity, as well as cost effectiveness. It can be appreciated that the analysis of food and drug. It also can be concluded that the non-aqueous capillary electrophoresis method is appropriate for the determination

of preservatives in food samples. The current results provided a great incentive to further investigate the applicability of UHCZ-LTZ/Sweeping-MEKC method in order to achieve detection limits below the legislated maximum concentration limits.

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