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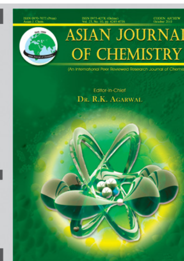

Separation and Determination of Salidroside, Caffeic Acid and Gallic Acid in *Rhodiola* L. by Large-Volume Sample Stacking-Sweeping-Micellar Electrokinetic Chromatography

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In this study, a new method for simultaneous determination of salidroside, caffeic acid and gallic acid in *Rhodiola* L. by large volume sample stacking-Sweeping micellar electrokinetic chromatography (LVSS-sweeping-MEKC) has been proposed. The determination conditions containing pH in background solution, concentrations of $Na_2B_4O_7$, sodium dodecyl sulfate (SDS) and acetonitrile, injection time, negative voltage and separation voltage were optimized. The operating buffer was composed of 20 mmol/L $Na_2B_4O_7$, 80 mmol/L sodium dodecyl sulfate and 10 % (by volume) acetonitrile at pH 9.7 with a constant temperature of 25 ºC. The UV detection wavelength was 213 nm, the reverse voltage was -8 kV, separation voltage was 20 kV and injection time was 120 s. The linear ranges of the method were 13.1-49.6 µg/L for salidroside, 1.0-15.7 µg/L for caffeic acid and 1.1-18.2 µg/L for gallic acid. The recoveries were 95-106, 98-112 and 91-108 %, respectively. The RSDs of peak area were less than 4 % and detection limits (S/N = 3) were 194, 28 and 29 µg/L for salidroside, caffeic acid and gallic acid respectively.

Key Words: Micellar electrokinetic chromatography, Electrostacking, Large volume sample stacking, Sweeping, Salidroside, Caffeic acid, Gallic acid.

INTRODUCTION

Members of the genus *Rhodiola* L. are perennial herbaceous plants indigenous to high altitudes in the Arctic and mountainous regions throughout Europe and Asia. There are about 90 species recorded worldwide and above 70 species found in China. *Rhodiola* L. has a long history as traditional Chinese medicine (TCM) salidroside, caffeic acid and gallic acid are the three important compounds in *Rhodiola* L and their structures are shown in Fig. 1. This traditional Chinese medicine has been used to treat antihypoxia, antimicrowave radiation, antifatigue and extend human life. Also, it has other biological activities, such as visceral organ protection, antioxident activity, antidiabetic activity, anticancer and enhancement of learning and memory[1].

The main active compounds of *Rhodiola* L. were previously identified and determined by different methods such as HPLC[2], HPCE[3] and so on. Capillary electrophoresis (CE) is a developed analytical separation technique, noted for rapid analysis, high efficiency and low sample consumption. These benefits make capillary electrophoresis complementary to HPLC and other analytical separation techniques. However, capillary electrophoresis has a few limitations, mainly poor

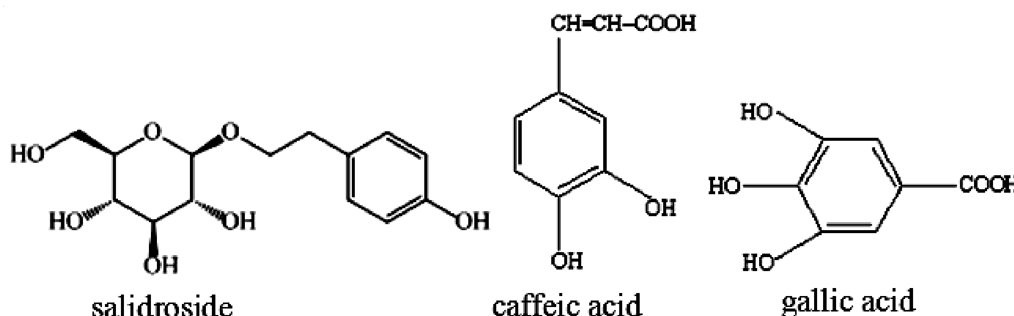


Fig. 1. Chemical structures of three active compounds

detection limits and precision in the case of UV-detection. Fortunately, this problem can be easily solved by the methods of preconcentration and stacking. Large volume sample stacking[4-7] is a preconcentration technology that is the volume of sample injection is more than the normal stacking mode, with sample zone up to 1/3-1/2 of the capillary capacity. With large volume sample injected, there also bring some shortcomings such as sample zone becomes wide, column efficiency and resolution degree declines. In order to improve resolution, sample matrix should be moved and exited before sample separated. It could be achieved by changing the direction of electroosmotic flow (EOF)[8-11] and electrode polarity switching is an effective method to change the direction of electroosmotic flow.

The aim of this study is to develop a capillary electrophoresis method with large volume sample stacking and apply it to separate and determine the active compounds in TCM. In this study, electrode polarity was switched so as to acquire a reversed electroosmotic flow. Basic schematic diagrams are illustrated in Fig. 2. In the initial step, the capillary was filled with background solution (BGS). The sample solution was injected into capillary with a certain length and then, following the sample solution, a certain length of high-conductivity salt (NaCl) was injected into capillary. After that, a reversed voltage (negative polarity) was applied. At this moment, the direction of the electroosmotic flow was point to the inlet and the sample was pushed into the high-conductivity salt (NaCl) zone with sample preconcentration. Meanwhile, sample matrix was gradually moved and exited the capillary from the injection end (inlet). Finally, a positive polarity was applied, the separation and determination of the sample was finished by micellar sweeping. In this paper, the above method was applied to the analysis of salidroside, caffeic acid and gallic acid in *Rhodiola* L. with a satisfying result.

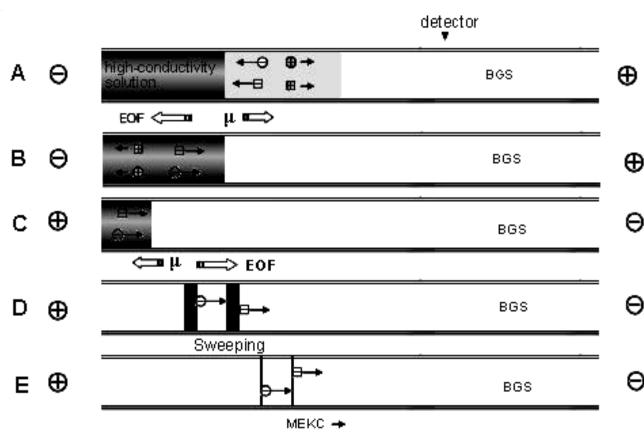


Fig. 2. Basic schematic diagrams

EXPERIMENTAL

Salidroside, caffeic acid and gallic acid were of analytical standard purity, which were purchased from the Beijing Medicine Inspection Institute, China. *Rhodiola* L. was purchased from Wuhan Mayinglong Pharmaceutical Company, Wuhan, China. Sodium borate, Sodium hydroxide and sodium dodecyl sulfate (SDS) were of analytical grade. Acetonitrile and methanol were LC grade solvents. All solutions and samples were filtrated and degassed before analysis by capillary electrophoresis.

All analyses were carried out on a ACS2000 capillary electrophoresis system (Beijing Cailu Insrumental Co., Beijing, China). The apparatus was equipped with a power supply (up to constant voltage 30kV), a HW-2000 chromatography workstation and a UV-VIS detector (double light beams, λ = 190-740 nm, set at 213 nm). A fused-silica capillary (Factory of Yongnian Optical Fiber, Hebei, China) was used, with total length 60 cm, effective length 47 cm, ID50 μm. The runs were carried out under 25 ºC cooling air. Before runs, the new capillary was conditioned by rinsing with 1.0M NaOH for 15 min, redistilled water for 15 min and running buffer for 0.5 h, in turn. A UV-2102PC UV-visible spectrophotometer from UNICO instruments Co. Ltd. was used. DL-60D ultrasonic bath from Letter to Shanghai Instrument Co. Ltd. was used to extract sample and degass solutions. Analytical balance form Austrian House (Shanghai) Co. Ltd. was used. The pH of the solutions was measured by employing a model EF20 Laboratory pH meter Mettler-Toledo Instruments (Shanghai).

Preparation of standard and sample solutions: The standard stock solutions of salidroside (456 mg/L), caffeic acid (392 mg/L) and gallic acid (620 mg/L) were prepared by dissolving weighed amount of the three standards in volumetric flask with 25 mL methanol, respectively. Calibration standard was prepared by diluting calculated volume of the stock solution with redistilled water.

Rhodiola L. was finely powdered in a mortar. An appropriate amount of *Rhodiola* L. powder was extracted with methanol for 1 h in an ultrasonic bath and then centrifuged at 2500 rpm min^{-1} for 10 min. The extraction was repeated three times. The extracts were combined and filtered through filter paper and then diluted to 10 mL in a volumetric flask with methanol. The solutions were used in capillary electrophoresis analysis after filtration from a 0.45 μm membrane filter and degassing in an ultrasonic bath. All the solutions were stored in a refrigerator at 4 ºC.

Chromatography condition: The operating buffer was composed of 20 mmol/L $Na_2B_4O_7$, 80 mmol/L SDS and 10 % (by volume) acetonitrile at pH 9.7 with a constant temperature of 25 ºC. The UV detection wavelength was 213 nm, the reverse voltage was -8 kV, separation voltage was 20 kV and injection time was 120 s.

RESULTS AND DISCUSSION

Effect of borate concentration: Borate buffer is often employed in capillary electrophoresis analysis of naturally occurring polyphenols due to its possibility to form anionic complexes with compounds possessing *vicinal* -OH groups[12]. The effect of borate concentration was investigated using 10, 15, 20, 25 and 30 mmol/L sodium tetraborate. With the increase of borate concentration, the quality of separation was improved and the buffer pH was increased, but the analysis time was prolonged. However, when the concentration of sodium tetraborate was above 25 mmol/L, the baseline noise was increased. Hence, 20 mmol/L sodium tetraborate was chosen as the optimum concentration.

Effect of sodium dodecyl sulfate (SDS) concentration: The effect of SDS concentration (40-120 mM) on the resolution of the three compounds was investigated and its relationship

was shown in Fig. 3. As we can see, with the increase of SDS concentration, the peak height increased in low SDS concentrations, it was indicated that the increasing of SDS concentrations could make better sweeping effect. However, the peak area decreased and the migration time was shortened with the SDS concentration increased. When SDS concentration was above 80 mM, the increasing of SDS concentration would bring high ionic strength and high current, which result the peak area decreased and the baseline noise increased. Therefore, 80 mM SDS was selected as the optimal SDS concentration.

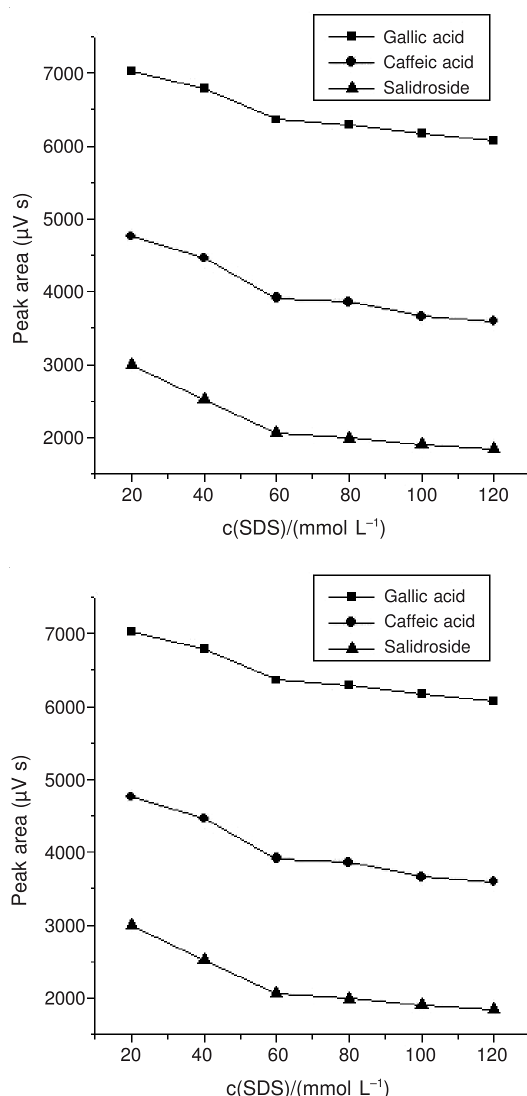


Fig. 3. Effect of SDS concentration on peak height and peak area

Effect of the type of high-conductivity solution system: At first, sodium tetraborate with different concentration was tried to employ as high-conductivity solution for sample stacking. However, due to the high pH of sodium tetraborate solution, it had not obvious and satisfied effect with the employment of sodium tetraborate for sample stacking. When sodium chloride was chosen as high-conductivity solution, there was obvious efficiency of sample stacking with 1 mol/L sodium chloride within 2 min sample injection. Meanwhile, superior peak shape and low baseline noise were achieved. Hence, the sodium chloride was selected as the high-conductivity solution.

Optimization of the ratio of injection time of sample to high-conductivity solution: After a much larger sample injection, a negative voltage was used to gradually move and exit the matrix in sample. And the volume ratio of sample injection to high-conductivity solution (NaCl) had an important effect on the exit of sample matrix and enrichment multiple. As the volume ratio of sample injection to high-conductivity solution was 1:1, it could be obtained better peak shape and superior enrichment efficiency. And when the injection time of sample was 120 s, the better column efficiency and theoretical plate number were obtained. Therefore, the injection time of sample was selected as 120 s and the volume ratio of sample injection to high-conductivity solution was 1:1.

Optimization of the reverse voltage: A significant parameter in LVSS was the reverse voltage. Due to the reverse voltage had an effect on the transfer speed of sample matrix and analytes, a higher voltage was necessary for rapid capillary electrophoresis analysis. It was found that sample matrix was removed more quickly when the applied voltage ranged from -6 to -12. But the enrichment efficiency of sample became poor. In order to achieve better theoretical plate number and column efficiency, -8 kV was selected as the optimum reverse voltage in this paper.

Validation of the method

Linearity: Under the optimized buffer condition, when the sample injection time within 1 min, LVSS-sweeping-MEKC and sweeping-MEKC showed no evident differences in sensitivity and enrichment multiple. However, as the sample injection time surpass 1 min, the column efficiency and separation efficiency of sweeping-MEKC declined. While the peak area and injection time had a good linear relationship using LVSS-sweeping-MEKC with the sample injection time within 2 min. The repeatability of the results expressed as the relative standard deviation can be considered satisfactory since the RSD values of peak areas did not exceed 4 %. The LODs based on three times the signal-to-noise (S/N = 3) were to be 194 µg/L for salidroside, 28 µg/L for caffeic acid and 29 µg/L for gallic acid. These validation data are given in Table-1 (Fig. 4).

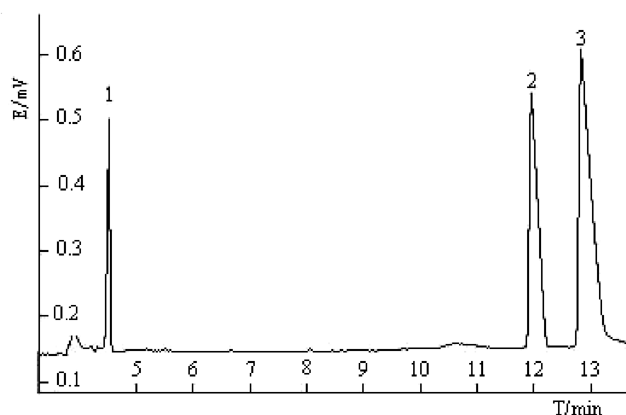


Fig. 4. Electrophoretogram of the three compounds by LVSS-sweeping-micellar electrokinetic chromatography buffer solution: 80 mmol/L SDS + 20 mmol/L $Na_2B_4O_7$(pH 9.7) + 10 % acetonitrile (v/v); reverse voltage: -8 kV; separation voltage: 20 kV; injection time: 120 s; concentration of three compounds in standard: 49.6 mg/L, 15.68 mg/L, 18.24 mg/L; 1. Salidroside, 2. caffeic acid, 3. gallic acid

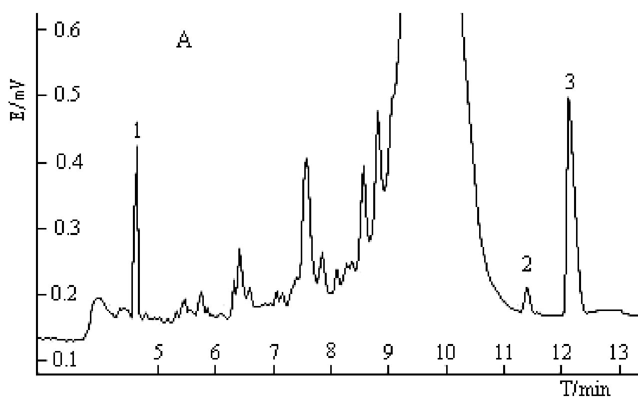
TABLE-1
LINEARITY, REGRESSION EQUATION, LODS BY LVSS-SWEEPING-MEKC

Compound	Linear range (mg/L)	Regression equation	Correlation coefficient	LOD (µg/L)	RSD (%) (n=5)
Salidroside	3.1-49.6	y = 40.2x – 11.1	0.9998	194	2.6
Caffeic acid	1.0-15.7	y = 244.8x + 12.4	0.9997	28	2.3
Gallic acid	1.1-18.2	y = 351.9x – 97.7	0.9996	29	3.4

Analysis of sample and recoveries: The recoveries of salidroside, caffeic acid and gallic acid from the extracts of *Rhodiola* L. Samples were determined by the method of standard addition. For each compound, three concentration levels were tested. Each sample was analyzed in triplicate. The recovery values were obtained by comparing the results from samples and fortified samples. As a result, the recoveries were satisfactory between 91-112 % in all tests. The results are listed in Table-2. This new method was utilized to separate and determine the three active constituents in the extracted sample of *Rhodiola* L. The extracted samples were injected directly and analyzed and the results were as good as those obtained with pure standard substances with pure standard substances without any interference and the electrophoretograms were shown in Fig. 5.

TABLE-2
RESULTS OF RECOVERY TEST

Compound	Added (µg/L)	Found (µg/L)	Recovery (%)
Salidroside	3.10	2.93	95
	6.20	5.98	96
	12.40	13.17	106
Caffeic acid	0.98	1.02	104
	1.96	2.19	112
	3.92	3.84	98
Gallic acid	1.14	1.23	108
	2.28	2.38	104
	4.56	4.14	91



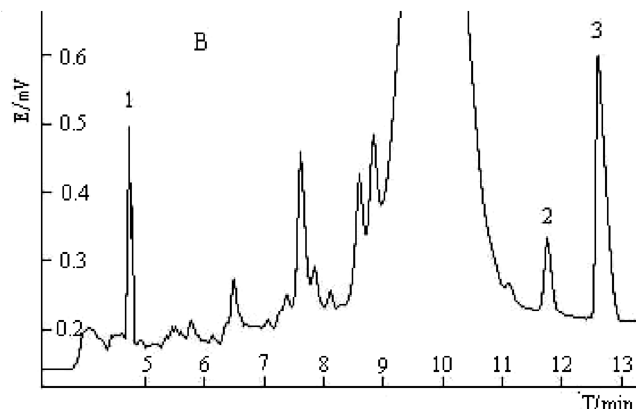


Fig. 5. LVSS-Sweeping-MEKC electrophoretograms of real sample (A) and real sample spiked standard (B) 1. Salidroside; 2. Caffeic acid; 3. Gallic acid

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