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Effects of Genotype and Environment on Active Components of *Salviae miltiorrhizae* by HPLC

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Six cultivars and five experimental site were selected to study the effects of genotype and environment on the contents of fat-soluble components and water-soluble components in *Salviae miltiorrhizae*. The results showed that the contents of water-soluble components rosmarinic acid and salvianolic acid B in *Salvia miltiorrhiza* were significantly affected by genotype, environment and interaction effects of genotype environment and the effects of genotype were much more than the effects of environment and the interaction effects of genotype environment, which showed that water-soluble component content in *Salvia miltiorrhiza* was mainly affected by genetic effects. Fat-soluble components were different from water-soluble components, contents of tanshinone I and tanshinone II_A were affected by environmental effects, for fat-soluble component salvianolic acid B, genotype and experimental site are both important. Coefficient of variation indicated that fat-soluble component in salvia is affected by external factors more easily than water-soluble component.

Key Words: Fat-soluble components, Water-soluble components, *Salviae miltiorrhizae*, HPLC.

INTRODUCTION

Salvia (*Salvia miltiorrhiza* Bge.) belongs to Salvia of Lamiaceae family, whose root is a valuable medicine. Active ingredient of Salvia are plant secondary metabolites, including fat-soluble components tanshinone II_A, tanshinone I and cryptotanshinone, which are Salvia's active ingredient for anti-inflammatory, antibacterial and treating coronary heart disease; water-soluble composition are phenolic compounds, such as salvianolic acid A, B, C and tanshinol, protocatechualdehyde, which have the roles of antiplatelet aggregation and free radical scavenging.

Salvia is widely distributed in north, east and southwest provinces of China, including Sichuan province, Hebei province, Shandong province, Shanxi province and Anhui province. It is reported that active ingredient contents of Salvia from different regions are very different, fat-soluble components and water-soluble component content have several times or even ten times the difference[1,2]. Xiulan *et al.*[3] measured tanshinone II_A content in *Salvia miltiorrhiza* from 10 different production areas, the results indicated that *Salvia miltiorrhiza* produced from Ju County of Shandong province contain the highest levels of tanshinone II_A, followed by Henan, Hubei and Jiangsu at the middle level, Hebei and Liaoning are the lowest. The active ingredient of *Salvia miltiorrhiza* is still quite different if the same germplasm grown in different ecological environments[4]. In this study, we selected salvia lines whose genetic traits is relatively stable and consistent as the experimental materials to study the effects of genotype and origin on active ingredients of Salvia variation, which will provide the basis for the quality control of *Salvia miltiorrhiza*.

EXPERIMENTAL

Field experimental design

Test point set: Test points are located in the main producing areas of Salvia: Shangluo county of Shanxi province (latitude 34°, longitude 110°). Ju County of Shandong province (latitude 36°30', longitude 118°30'); (N36° 30', E118° 30'). Bozhou of Anhui province (latitude 34 °, longitude 116 °); (N34°, E11°) Zhongjiang of Sichuan province (latitude 31 °, longitude 105 °); (N31°, E105°) Beijing Haidian Medicinal Plant Research Institute (latitude 42°, longitude 116°). (N42°, E116°)

Material: Salvia cultivars: "99-2", "99-4", "99-3", "99-5", "DS-2000" and "shh".

Field experimental design: The above six salvia cultivars were grown in five different salvia origins. The length of experimental plot length is 4.5 m, width is 2.0 m; four rows were planted in each plot and plant spacing and row spacing are both 40 cm. A randomized block design of six salvia cultivars was used in each region, each cultivar has three replicates in each region.

Determination of the active ingredient of Salvia: Samples were dried at 60 ºC, crushed by high-speed grinder. Determined the active ingredient of Salvia according to Chinese Pharmacopoeia[5].

Instrument parameters: Waters 600 high pressure liquid chromatography, waters 2487 UV detector, Empower Chromatography workstation, Venusil ASB-C_{18} column (5 µm, 250 mm × 4.6 mm), flow speed: 1 mL/min, the injection volume is 10 µL.

Parameters of detector: Detection wavelength of fat-soluble components was 270 nm and mobile phase was methanol-water (75:25); detection wavelength of water-soluble components was 286 nm, mobile phase was acetonitrile-phosphoric acid, gradient elution, the gradients were as follows (Table-1).

TABLE-1
MOBILE PHASE GRADIENT CONCENTRATIONS

Time (s)	Volume	Phosphoric acid solution	Acetonitrile
0	1.00	85	15
7	1.00	65	35
12	1.00	80	20
17	1.00	80	20
20	1.00	85	15
25	1.00	85	15

Sandard curve: Weighed acurately 5 mL standard solutions of tanshinone II_A, 1 mL tanshinone I and 1.5 mL cryptotanshinone into 10 mL brown flask, respectively, constant volume with methanol, injection, 2, 4, 8, 10, 15 and 20 µL, respectively, determined peak area, draw standard curves, calculate the regression equation just as Table-2.

Weighed acurately 0.8 mL standard solutions of rosmarinic acid and 3 mL salvianolic acid B into 5 mL brown flask, respectively, constant volume with methanol, injection, 2, 5, 8, 10, 15 and 20 µL, respectively, determined peak area, draw standard curves, calculate the regression equation just as Table-2.

RESULTS AND DISCUSSION

Content of fat-soluble components in salvia root

Content of tanshinone I: The results showed that fat-soluble component tanshinone I in salvia was significantly affected by genotype and environment ($p < 0.01$) (Table-3) and the interaction effect of genotype and environment was not significant, in which the role of the environment is larger than that of genotype effect.

Contents of tanshinone I of six salvia species grown in five experimental sites had significant differences between each other ($p < 0\ 01$) and content in "99-3" grown at Bozhou of Anhui province was the highest, which is 0.1289 %; the lowest was species "shh" grown at Zhongjiang of Sichuan province, 0.0013 %, the difference is nearly 100 times. The coefficients of variation of Haidian of Beijing, Bozhou of Anhui province, Ju county of Shandong province, Shangluo of Shanxi province and Zhongjiang of Sichuan province were 71.21, 62.33, 53.36, 41.78 and 61.16 %, respectively, which showed that tanshinone I level has the greatest change in Beijing's Haidian and the least in Shangluo of Shanxi province (Table-3).

According to the genotype, the cultivar "99-3" contained the highest levels of tanshinone I, the average content of five origin was 0.1050 %, content of "shh" was the lowest, which was 0.0050 %; according to place of origin, content of tanshinone I in salvia grown at Shangluo of Shanxi province was the highest, whose average content of six cultivars was 0.0955 %, the content from Ju county of Shandong province was the lowest (0.0460 %) (Table-4).

Cryptotanshinone content in *Salvia miltiorrhiza*: Just as tanshinone I, contents of cryptotanshinone of six salvia species grown in five experimental sites have significant differences between each other ($p < 0.01$) (Table-3), in which the role of the main effects of genotype (F value) was maximum, secondly was the main effect of the environment, the interaction effect of genotype and environment was the least.

Contents of cryptotanshinone had significant differences between the six Salvia species at all five test point ($p < 0.01$) and "shh" grown at Shangluo of Shanxi province contained the highest cryptotanshinone (0.9905 %) and "99-2" at Bozhou city of Anhui province the lowest (0.0823 %). The coefficients of variation of Haidian of Beijing, Bozhou of Anhui province, Ju county of Shandong province, Shangluo of Shanxi province and Zhongjiang of Sichuan province were 47.59, 72.41, 22.86, 62.77 and 83.9 %, respectively, which showed that cryptotanshinone level has the greatest change at Zhongjiang of Sichuan province and the least at Ju county of Shandong province (Table-3).

In all cultivars, the cultivar "shh" contained the highest levels of cryptotanshinone, the average content of five origin was 0.4835 %, content of "99-2" was the lowest, which was 0.1406 %; for the five places of origin, content of cryptotanshinone in salvia grown at Shangluo of Shanxi province was the highest, whose average content of six cultivars was 0.4433 %, the content from Ju county of Shandong province was the lowest (0.1025 %) (Table-4).

Tanshinone II_A content in *Salvia miltiorrhiza*: It could be concluded that contents of tanshinone II_A of six salvia species grown in five experimental sites had significant differences

TABLE-2
STANDARD CURVE REGRESSION EQUATION OF REFERENCE SUBSTANCE

Standard samples	Regression equation	r	Concentration range (µg mL^{-1})
Tanshinone I	$Y = 3.43 \times 10^6X - 3.51 \times 10^3$	0.9994	5.05-50.51
Cryptotanshinone	$Y = 4.62 \times 10^6X - 9.37 \times 10^3$	0.9994	4.28-42.80
Tanshinone II_A	$Y = 6.07 \times 10^6X - 2.47 \times 10^4$	0.9993	4.16-41.60
Rosmarinic acid	$Y = 1.68 \times 10^6X + 11.10 \times 10^4$	0.9991	3.26-32.64
Salvianolic acid B	$Y = 1.05 \times 10^6X + 5.99 \times 10^4$	0.9999	75.12-751.20

TABLE-3
MEASUREMENT RESULTS OF EFFICIENCY COMPONENT IN DIFFERENT SALVIA VARIETIES GROWN AT DIFFERENT EXPERIMENTAL SITES

Experimental sites	Cultivars	Tanshinone I	Cryptotanshinone	Tanshinone II_A	Rosmarinic acid	Salvianolic acid B
Haidian district Beijing city	DS-2000	0.0549 ± 0.003	0.1339 ± 0.002	0.1741 ± 0.006	0.1998 ± 0.013	6.2770 ± 0.313
	99-2	0.0341 ± 0.001	0.0864 ± 0.001	0.1149 ± 0.002	0.3238 ± 0.005	7.0992 ± 0.019
	99-3	0.1017 ± 0.009	0.2331 ± 0.004	0.3207 ± 0.015	0.2409 ± 0.019	6.5376 ± 0.031
	99-4	0.0366 ± 0.004	0.1134 ± 0.008	0.1467 ± 0.010	0.3360 ± 0.037	6.2560 ± 0.260
	99-5	0.0547 ± 0.012	0.1608 ± 0.020	0.1899 ± 0.024	0.2785 ± 0.008	5.7035 ± 0.246
	shh	0.0014 ± 0.001	0.3039 ± 0.034	0.0216 ± 0.002	0.2518 ± 0.035	6.7539 ± 0.493
	Amplitude	0.0014-0.1017	0.0864-0.3039	0.0216-0.3207	0.1998-0.3360	5.7035-7.0992
	Average	0.0470 ± 0.0335	0.1719 ± 0.0818	0.1613 ± 0.0982	0.2718 ± 0.0518	6.4378 ± 0.4786
	Coefficient of variation	0.7121	0.4759	0.6091	0.1905	0.0743
	F value	115.99	104.36	91.84	14.74	15.99
Bozhou city Anhui province	DS-2000	0.0703 ± 0.006	0.1586 ± 0.005	0.2547 ± 0.016	0.1748 ± 0.006	8.5859 ± 0.380
	99-2	0.0526 ± 0.001	0.0823 ± 0.002	0.1646 ± 0.002	0.2846 ± 0.021	7.8095 ± 0.298
	99-3	0.1289 ± 0.001	0.2553 ± 0.002	0.3392 ± 0.004	0.1127 ± 0.003	6.6446 ± 0.253
	99-4	0.0746 ± 0.001	0.1793 ± 0.005	0.2202 ± 0.067	0.3550 ± 0.066	6.7750 ± 0.217
	99-5	0.0485 ± 0.005	0.0858 ± 0.002	0.2099 ± 0.019	0.2214 ± 0.017	5.6302 ± 1.541
	shh	0.0078 ± 0.002	0.5861 ± 0.001	0.0285 ± 0.000	0.3603 ± 0.124	8.3106 ± 0.971
	Amplitude	0.0078-0.1289	0.0823-0.5861	0.0285-0.3392	0.1127-0.3603	5.6302-8.5859
	Average	0.0638 ± 0.0397	0.2246 ± 0.1884	0.2028 ± 0.1034	0.2515 ± 0.0997	7.2926 ± 1.1338
	Coefficient of variation	0.6233	0.8390	0.5098	0.3965	0.1555
	F value	473.58	102.25	39.7	13.33	17.38
Ju county Sahndong province	DS-2000	0.0560 ± 0.008	0.1056 ± 0.010	0.1916 ± 0.031	0.3550 ± 0.026	8.5023 ± 0.893
	99-2	0.0452 ± 0.006	0.0827 ± 0.071	0.1437 ± 0.001	0.2103 ± 0.011	6.3870 ± 0.096
	99-3	0.0729 ± 0.001	0.1433 ± 0.001	0.1890 ± 0.014	0.3216 ± 0.011	7.5748 ± 0.239
	99-4	0.0475 ± 0.002	0.0877 ± 0.002	0.1565 ± 0.006	0.3521 ± 0.046	7.5503 ± 0.439
	99-5	0.0546 ± 0.002	0.1125 ± 0.002	0.1613 ± 0.002	0.3933 ± 0.015	8.5695 ± 0.093
	Shh	0.0013 ± 0.000	0.0834 ± 0.005	0.0155 ± 0.005	0.4117 ± 0.016	9.2424 ± 0.405
	Amplitude	0.0013-0.0729	0.0827-0.1433	0.0155-0.1916	0.2103-0.4117	6.3870-9.2424
	Average	0.0460 ± 0.0246	0.1025 ± 0.0234	0.1429 ± 0.0652	0.3407 ± 0.0714	7.9710 ± 1.0100
	Coefficient of variation	0.5336	0.2286	0.4560	0.2096	0.1267
	F value	232.22	337.16	199.31	70.38	26.86
Shangluo Shanxi province	DS-2000	0.0765 ± 0.007	0.2315 ± 0.047	0.2437 ± 0.021	0.3297 ± 0.017	9.4469 ± 0.454
	99-2	0.0837 ± 0.089	0.3594 ± 0.006	0.2601 ± 0.029	0.3465 ± 0.028	7.3928 ± 0.722
	99-3	0.1126 ± 0.001	0.3031 ± 0.003	0.3519 ± 0.005	0.3615 ± 0.003	8.3719 ± 0.115
	99-4	0.0887 ± 0.003	0.4581 ± 0.017	0.3349 ± 0.008	0.6431 ± 0.005	7.5972 ± 0.054
	99-5	0.0807 ± 0.007	0.3172 ± 0.002	0.3092 ± 0.005	0.4102 ± 0.005	6.7924 ± 0.225
	shh	0.0166 ± 0.001	0.9905 ± 0.029	0.0552 ± 0.002	0.3192 ± 0.031	7.8484 ± 0.265
	Amplitude	0.0166-0.1126	0.2315-0.9905	0.0552-0.3519	0.3192-0.6431	6.7924-9.4469
	Average	0.0765 ± 0.0320	0.4433 ± 0.2782	0.2592 ± 0.1083	0.4017 ± 0.1225	7.9083 ± 0.9158
	Coefficient of variation	0.4178	0.6277	0.4180	0.3049	0.1158
	F value	116.7	449.95	420.99	153.93	39.69
Zhongjiang Sichuan province	DS-2000	0.0566 ± 0.007	0.1101 ± 0.005	0.1566 ± 0.016	0.2297 ± 0.029	6.7412 ± 0.049
	99-2	0.0577 ± 0.005	0.0921 ± 0.014	0.1686 ± 0.012	0.1744 ± 0.046	5.7046 ± 0.165
	99-3	0.1076 ± 0.007	0.2293 ± 0.007	0.2562 ± 0.013	0.0882 ± 0.017	5.3237 ± 0.346
	99-4	0.0566 ± 0.002	0.1458 ± 0.008	0.2187 ± 0.014	0.2114 ± 0.001	4.8010 ± 0.073
	99-5	0.0557 ± 0.006	0.1115 ± 0.018	0.2057 ± 0.010	0.2961 ± 0.046	5.7594 ± 0.318
	shh	0.0013 ± 0.000	0.4534 ± 0.052	0.0230 ± 0.001	0.1180 ± 0.011	5.0662 ± 0.100
	Amplitude	0.0013-0.1076	0.0921-0.4534	0.0230-0.2562	0.0882-0.2961	4.8010-6.7412
	Average	0.0557 ± 0.0341	0.1904 ± 0.1378	0.1715 ± 0.0811	0.1863 ± 0.0762	5.5660 ± 0.6828
	Coefficient of variation	0.6116	0.7241	0.4727	0.4088	0.1227
	F value	351.99	118.84	200.35	13.33	17.38
F value	F(g)	5.92**	643.95**	114.13**	104.21**	119.94**
	F(e)	12.96**	549.04**	349.28**	36.89**	24.05**
	F(g × e)	1.57	86.81**	11.00**	15.01**	7.62**

**Shows the difference reached a significant level ($p < 0.01$).

TABLE-4
AVERAGE CONTENT OF ACTIVE INGREDIENTS OF DIFFERENT SALVIA CULTIVARS AND DIFFERENT EXPERIMENTAL SITE (%)

Cultivars	Tanshinone I	Cryptotanshinone	Tanshinone II_A	Rosmarinic acid	Salvianolic acid B
DS-2000	0.0630b	0.1480b	0.2042b	0.2578b	7.9107a
99-2	0.0772ab	0.1406b	0.1704b	0.2679b	6.8786bc
99-3	0.1050a	0.2328b	0.2914a	0.2250b	6.8905bc
99-4	0.0610b	0.1969b	0.2154b	0.3795a	6.5959c
99-5	0.0590b	0.1576b	0.2152b	0.3199ab	6.4910c
shh	0.0050c	0.4835a	0.0288c	0.2922ab	7.4443bc
Amplitude	0.0050-0.1050	0.1406-0.4835	0.0288-0.2914	0.2250-0.3795	6.4910-7.9107
Average	0.0617 ± 0.0327	0.2266 ± 0.1306	0.1876 ± 0.0873	0.2904 ± 0.0541	7.0352 ± 0.5419
Coefficient of variation	0.5296	0.5763	0.4654	0.1865	0.0770
Site	Tanshinone I	Cryptotanshinone	Tanshinone II_A	Rosmarinic acid	Salvianolic acid B
HD	0.0472b	0.1719d	0.1613c	0.2718c	6.4378c
BZ	0.0638b	0.2246b	0.2028b	0.2515c	7.2926b
JC	0.0460b	0.1025e	0.1429d	0.3407b	7.9711a
SL	0.0955a	0.4433a	0.2592a	0.4017a	7.9083a
ZJ	0.0560b	0.1904c	0.1715c	0.1863d	5.5660d
Amplitude	0.0472-0.0955	0.1025-0.4433	0.1429-0.2592	0.1863-0.4017	5.5660-7.9711
Average	0.0617 ± 0.0202	0.2264 ± 0.1291	0.1877 ± 0.0455	0.2893 ± 0.0824	7.0351 ± 1.0271
Coefficient of variation	0.3279	0.5700	0.2422	0.2846	0.1460

Note: Different letters shows the difference reached a significant level ($p < 0.01$).

between each other (p < 0 01) (Table-3), in which the role of the main effects of environment (F value) was maximum, secondly was the main effect of the genotype and the interaction effect of genotype and environment had the least effect.

Contents of tanshinone II_A had significant differences between the six Salvia species at all five experimental sites ($p < 0.01$) and "99-3" grown at Shangluo of Shanxi province contained the highest tanshinone II_A (0.3519 %) and "shh" at Ju county of Shandong province the lowest (0.0155 %), which had 23 times difference. The coefficients of variation of Haidian of Beijing, Bozhou of Anhui province, Ju county of Shandong province, Shangluo of Shanxi province and Zhongjiang of Sichuan province were 60.91, 50.98, 47.27, 41.8 and 45.6 %, respectively, which showed that tanshinone II_A level had the greatest change at Haidian of Beijing and the least at Shangluo of Shanxi province (Table-3).

According to the genotype, the cultivar "99-3" contained the highest levels of tanshinone II_A, the average content of five origin was 0.2914 %, content of "shh" was the lowest, which was 0.0288 %; according to place of origin, content of tanshinone II_A in salvia grown at Shangluo of Shanxi province was the highest (0.2592 %), the content from Haidian of Beijing was the lowest (0.1613 %) (Table-4).

Content of water-soluble components in salvia root

Rosmarinic acid content in *Salvia miltiorrhiza*: The results showed that content of rosmarinic acid in *Salvia miltiorrhiza* was significantly affected by genotype, environment and the interaction effect of genotype and environment ($p < 0.01$) (Table-3), in which the role of the main effects of genotype (F value) was maximum, secondly was the main effect of the environment and the interaction effect of genotype and environment had the least effect.

Contents of rosmarinic acid had significant differences between the six Salvia species at all five experimental sites ($p < 0.01$) and cultivars "99-5" grown at Zhongjiang of Sichuan province contained the highest rosmarinic acid (0.6431 %) and "99-3" at Shangluo of Shanxi province the lowest (0.0882 %), which had 7 times difference. The coefficients of variation of Haidian of Beijing, Bozhou of Anhui province, Ju county of Shandong province, Shangluo of Shanxi province and Zhongjiang of Sichuan province were 19.05, 30.49, 70.38, 40.88 and 39.65 %, respectively, which showed that rosmarinic acid level had the greatest change at Ju county of Shandong province and the least at Haidian of Beijing (Table-3).

According to the genotype, the cultivar "99-4" contained the highest levels of rosmarinic acid, the average content of five origin was 0.3795 %, content of "99-3" was the lowest, which was 0.2250 %; according to place of origin, content of rosmarinic acid in salvia grown at Shangluo of Shanxi province was the highest, the average content of the six cultivars was 0.4017 %, the content from Zhongjiang of Sichuan province was the lowest (0.1863 %) (Table-4).

Salvianolic acid B content in *Salvia miltiorrhiza*: Table-3 showed that content of salvianolic acid B in *Salvia miltiorrhiza* was significantly affected by genotype, environment and the interaction effect of genotype and environment ($p < 0.01$), in which the role of the main effects of genotype (F value) was maximum, secondly was the main effect of the environment and the interaction effect of genotype and environment had the least effect.

Contents of salvianolic acid B had significant differences between the six salvia cultivars at all five experimental sites ($p < 0.01$) and cultivars "shh" grown at Shangluo of Shanxi province contained the highest salvianolic acid B (9.4469 %) and "99-4" at Zhongjiang of Sichuan province the lowest (4.8010 %). The coefficients of variation of Haidian of Beijing, Bozhou of Anhui province, Ju county of Shandong province, Shangluo of Shanxi province and Zhongjiang of Sichuan province were 7.43, 12.27, 26.86, 11.58 and 15.55 %, respectively, which showed that Salvianolic acid B level had the greatest change at Ju county of Shandong province and the least at Haidian of Beijing just the same as rosmarinic acid (Table-3).

In the six cultivars, the cultivar "DS-2000" contained the highest levels of salvianolic acid B, the average content of five origin was 7.9107 %, content of "99-5" was the lowest, which was 6.4910 %; in the five place of origin, content of salvianolic acid B in salvia grown at Ju county of Shandong province was the highest, the average content of the six cultivars was 7.9711 %, the average content from Zhongjiang of Sichuan province was the lowest (5.5660 %) (Table-4).

The contents of water-soluble components rosmarinic acid and Salvianolic acid B in *Salvia miltiorrhiza* were significantly affected by genotype, environment and interaction effects of genotype environment and the effects of genotype were much more than the effects of environment and the interaction effects of genotype environment, which showed that water-soluble component content in *Salvia miltiorrhiza* was mainly affected by genetic effects. So it is the most important to breed new cultivars that contain more water-soluble components if we want to produce more rosmarinic acid and salvianolic acid B, followed by the appropriate choice of origin to plant. Fat-soluble components were different from water-soluble components, contents of tanshinone I and tanshinone II_A were affected by genotype and the interaction of genotype and environment, so if we want to increase their contents the most important thing is to select the appropriate choice of origin. But for fat-soluble component salvianolic acid B, breeding good cultivars containing more salvianolic acid B and selecting the appropriate choice of origin are both important.

Coefficient of variation of fat-soluble component content was much greater than the water-soluble components, which indicated that fat-soluble component in Salvia is affected by external factors more easily than water-soluble component.

Many factors can affect the formation of terpenoids in plants, two important factors are genetic and ecological environment factors[6,7]. The results of this study were consistent with previous studies.

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