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Investigation on the origin of 5-HMF in Shengmaiyin decoction by RP-HPLC method*

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Abstract: The origin of 5-HMF (5-hydroxymethyl-2-furaldehyde) in a Shengmaiyin decoction was investigated by the RP-HPLC method below. A C_{18} column (250 mm×4.6 mm, i.d. 5 μm) with a column temperature of 25 °C was used. The mobile phase was a mixture of ultra-pure water-acetonitrile (95:5, *V*/*V*) and the flow rate was 1.0 ml/min. The detection wavelength was 280 nm. The injection volume was 1 μl and the running time was about 20 min. The addition of *Schisandra* was regulated to assess the contribution of an acid environment to the production of 5-HMF. In order to confirm the role of saccharides in the production of 5-HMF, different amount of fructose was used. The 5-HMF level in decoctions of processed and unprocessed *Schisandra* was investigated in order to determine the origin of 5-HMF. The results showed that 5-HMF was derived mainly from the decoction of *Schisandra* only and not the mixed decoction of *Ophionpogon* and *Schisandra*. The appearance of 5-HMF is not simply the result of the decomposition of saccharides under the acid environment created by *Schisandra*, but the processing procedure plays an important role in the production of 5-HMF.

Key words: Shengmaiyin, 5-HMF, Origin, *Schisandra*, Process
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INTRODUCTION

Shengmaiyin or Shengmaisan, one of the famous herbal recipes in traditional Chinese medicine, is made from Radix ginseng, *Ophiopogon* and *Schisandra*. It is used to treat hypotension (Chen *et al*., 2000; Gui, 2001), cardiovascular system diseases (Qian *et al*., 2004; Zeng and Zeng, 2004), to increase immunity (Wu *et al*., 1997; Guo *et al*., 2004) and cure other diseases (Li *et al*., 1996; Liu *et al*., 1998; Fang *et al*., 2000; Yi *et al*., 2003; Xu *et al*., 2003; Wang *et al*., 2004a; Wang *et al*., 2004b). Zhu *et al*.(1998a) found a new component, 5-hydroxymethyl-2-furaldehyde (5-HMF) in the decoction of Shengmaiyin and believed that 5-HMF derives from the decoction of *Ophiopogon* and *Schisandra* in the decomposition of saccharide under the acid environment created by *Schisandra* (Xia *et al*., 1998). The contribution of 5-HMF to the cure of cardiovascular disease (Zhu *et al*., 1998b) was also found in many other herbs (Li *et al*., 1999; Zhang *et al*., 2001; Meng *et al*., 2002; Wang *et al*., 2003; Dong and Wang, 2004; Yuan *et al*., 2004; Xu *et al*., 2004).

Normally, 5-HMF is poisonous to the nervous system (Chi *et al*., 1998), by combining to protein and thus causing the accumulation of poisons in the body; and has potent reproductive toxicity (Zdzienicka *et al*., 1978; Wang *et al*., 1994; Qasim *et al*., 1995), and also damages striated muscles and viscera (Reinald *et al*., 1995). Many countries strictly limit the content of 5-HMF in honey, beer and glucose injections (Cohen *et al*., 1994; Lo *et al*., 1995; Zeng *et al*., 2002; Li *et al*., 2004).

It is very important to control the level of 5-HMF for bio-activity and side effects of the herbs. The origin of 5-HMF in the Shengmaiyin was investigated in order to optimize Shengmaiyin production to acheieve quality control and high curative effect with minimal side-effects, and to contribute to better understanding of traditional Chinese medicine.

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MATERIALS AND INSTRUMENTS

Materials and reagents

Unprocessed *Schisandra* is the dry, ripe fruit of *Schisandra chinensis* (Turcz.) Baill, but medicinal *Schisandra* is made from both *Schisandra chinensis* (Turcz.) Baill and *Schisandra sphenanthera* Rehd. et Wils. by different processes. *Ophiopogon* is the dry root of *Ophiopogon japonicus* (Thunb.) Ker-Gawl. All crude herbal and medicinal medicines were authenticated by Professor Chen Kong-rong, Zhejiang College of Traditional Chinese Medicine and met the standards of the Pharmacopoeia of PRC 2005 Edition. Chemicals used included HPLC grade acetonitrile (Merck, Germany), ultra-pure water (milli-Q) and 5-HMF (Sigma, USA).

Instrumentation

PHSJ-4A pH meter and Agilent HP 1100 HPLC systems equipped with a G1322A solvent degasser, a G1354A quaternary gradient solvent pump, a G1313A multiple auto-sampler, a G1316A thermostat column compartment, a G1314A UV-Vis detector and Agilent HP 1100 chromatography workstation were used for analyses.

METHODS

Analytical methods

The standards and samples of 5-HMF were all analyzed by RP-HPLC. Chromatographic conditions were as follow: Chromatographic separation was performed on a Diamonsil C_{18} column (250 mm×4.6 mm i.d., 5 μm; Dikma) with a guard column (4 mm×3 mm i.d., 5 μm; Phenomenex) packed with the same material at 25 °C. The mobile phase was a mixture of ultra-pure water-acetonitrile (95:5, *V*/*V*) filtered through a 0.45 μm filter and delivered at a flow rate of 1.0 ml/min and detection wavelength of 280 nm, injection volume was 1 μl and running time was about 20 min.

Preparation of calibration curve

The stock solution of 5-HMF was prepared by dissolving 13.62 mg 5-HMF in a 50 ml volumetric flask with ultra-pure water and stored at 4 °C. Seven concentrations of 5-HMF standard solutions (2.724, 5.448, 8.172, 10.896, 13.620, 16.344, 27.240 mg/100 ml) were obtained by appropriately diluting the stock solution with mobile phase and prepared for evaluating the linearity by HPLC. The injection volume was 1 μl. The calibration curve was obtained by plotting the peak areas against the concentrations of 5-HMF standard solutions.

Samples preparation

Schisandra and fructose samples: 12.5 g medicinal *Schisandra* and a series of different weights of fructose (0 g, 0.25 g, 0.5 g, 1.0 g, 1.5 g, 2.0 g) were soaked in 250 ml deionized water for 2 h and weighed, then decocted for 1 h at boiling temperature and reweighed. Deionized water was added to the decocted samples to make them reach the weight before decoction. The decoction was filtered and the filtrate was passed through a 0.45 μm filter for HPLC analysis.

Ophiopogon and acid samples: 25 g medicinal *Ophiopogon* were soaked in a series of different pH value acid solutions regulated by hydrochloric acid, sulfuric acid and acetic acid (HCl: 2.972, 3.142, 3.454; H_2SO_4: 2.987, 3.058, 3.401; HAc: 2.894, 3.012, 3.331) for 2 h and weighed, then decocted for 1 h at boiling temperature, reweighed, and then deionized water was added to the decocted samples to make them reach the weight of the sample before decoction. The decoction was filtered and the filtrate was passed through a 0.45 μm filter for HPLC analysis. *Ophiopogon* samples were prepared by soaking 25 g medicinal *Ophiopogon* in 250 ml deionized water for 2 h and then treated the same way as that for the *Ophiopogon* and acid samples.

Schisandra and *Ophiopogon* samples preparation: 25 g medicinal *Ophiopogon* and a series of different weights of medicinal or unprocessed *Schisandra* (6.25 g, 12.5 g, 18.75 g, 25 g, 50 g) were soaked in 250 ml deionized water for 2 h and weighed, then decocted for 1 h at boiling temperature, reweighed, and then deionized water was added to the decocted samples to make them reach the weight of the sample before decoction. The decoction was filtered and the filtrate was passed through a 0.45 μm filter for HPLC analysis.

Schisandra samples: different weights of medicinal or unprocessed *Schisandra* (6.25 g, 12.5 g, 18.75 g, 25 g, 50 g) were soaked in 250 ml deionized

water for 2 h and weighed, then decocted for 1 h at boiling temperature and reweighed, and then deionized water was added to the decocted samples to make them reach the weight before decoction. The decoction was filtered and the filtrate was passed through a 0.45 μm filter for HPLC analysis.

Precision

Both standard solutions and samples were injected at a volume of 1 μl six times repeatedly according to the analytical method mentioned above, and the relative standard deviations were calculated.

Stability

Both standard solutions and sample decoctions at 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h were analyzed according to the analytical method mentioned above, and the relative standard deviations were calculated.

Accuracy

In 250 ml deionized water 12.5 g processed medicinal *Schisandra* were soaked for 2 h and weighed, then decocted for 1 h at boiling temperature and reweighed, and then deionized water was added to the decocted samples to make them reach the weight before decoction. The decoction was filtered and the filtrate was passed through a 0.45 μm filter for HPLC analysis. Six *Schisandra* samples were prepared under the same conditions above, and the relative standard deviation of the six samples was calculated.

Recoveries

Different weight 5-HMF was added to the *Schisandra* decoction samples and the content of 5-HMF in the decoctions was analyzed by the method mentioned above. The recoveries were calculated by using the ratio of the detected content to added content of 5-HMF.

RESULTS AND DISCUSSION

Performance of HPLC system

The mobile phase containing different proportions of ultra-pure water and acetonitrile was tested to find the optimal combination. After several trials, a mobile phase consisting of ultra-pure water and acetonitrile (95:5, *V*/*V*) was used which could achieve effective separation and acceptable sensitivity, as well as symmetric peak shape and short analytical period. Representative chromatograms are shown in Fig.1. The retention time of 5-HMF was about 13 min, no interferences were observed.

System suitability

The number of theoretical plates of column (*N*) and the tailing factor (*T*) to analyze 5-HMF were about 14300 and 0.96, respectively. Both conformed to the Pharmacopoeia of PRC.

Linearity

The peak area (*Y*) and concentration (*X*) of 5-HMF standard solutions were subjected to regression analysis to calculate the calibration equation and correlation coefficient. The calibration equation was Y=7376.8X+6.8016, R^2=0.9999. The results showed excellent correlation between the peak area and the concentration of 5-HMF within the range of concentrations from 0.02724 to 0.2724 mg/ml.

Precision

The relative standard deviations of six injections for standard and samples were 0.14% and 0.71%, respectively. The system yielded excellent performance for analysis of 5-HMF content.

Stability

5-HMF in standard solution and in samples showed excellent stability according to the results. The relative standard deviations of standard solution and sample were 0.15% and 1.23%, respectively.

Repeatability

The content of 5-HMF in 6 *Schisandra* samples prepared under the same conditions showed no marked difference. The relative standard deviation of the six data was 1.58%.

Recoveries

The average extraction recovery of 5-HMF in decoction was 98.89% and the relative standard deviation was 2.78%. The average recoveries were sufficiently high to reach the normal analytical criterion. The extraction recovery data of 5-HMF are presented in Table 1.

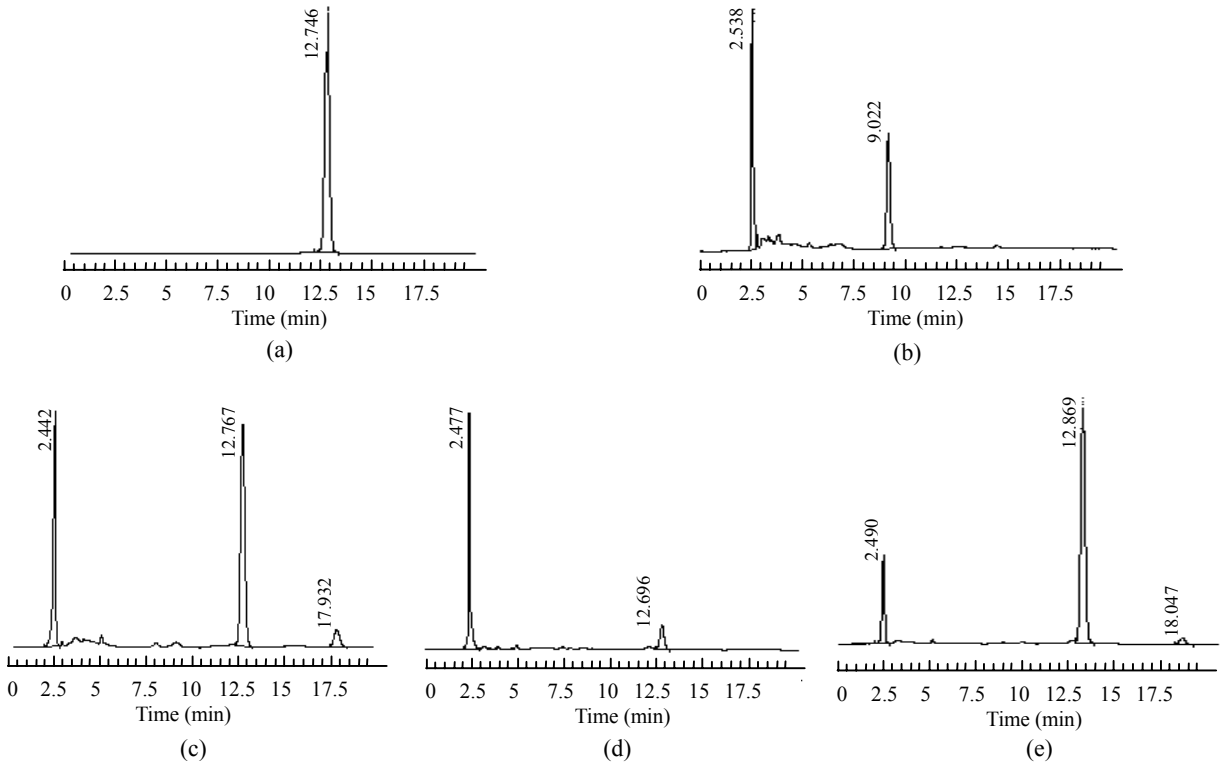

Fig.1 Chromatograms of standard and samples

(a) Chromatogram of 5-HMF standard solution; (b) Chromatogram of unprocessed *Schisandra* decoction from *Schisandra chinensis* (Turcz.) Baill; (c) Chromatogram of medicinal *Schisandra* decoction from *Schisandra chinensis* (Turcz.) Baill processed by factory A; (d) Chromatogram of medicinal *Schisandra* decoction from *Schisandra sphenanthera* Rehd. et Wils. processed by factory B; (e) Chromatogram of medicinal *Schisandra* decoction from *Schisandra sphenanthera* Rehd. et Wils. processed by factory C

Table 1 The extraction recovery data on 5-HMF

Sample	Found (mg)	Added (mg)	Extraction recovery (%)	Average (%)	*RSD* (%)
1	13.46	13.62	98.83	98.89	2.78
2	13.04	13.62	95.77		
3	13.64	13.62	100.12		
4	13.30	13.62	97.66		
5	13.25	13.62	97.31		
6	14.12	13.62	103.65		

The influence of fructose addition on the 5-HMF level

The effect of adding fructose is presented in Table 2. The results showed that additional fructose had no evident effects on the 5-HMF level of the decoctions (Table 2).

The influence of pH value variation on 5-HMF level

Although variation in the quantity of *Schisandra* had very little effect on the pH value, there was nonetheless evident effect on the 5-HMF level in the decoctions. The small change of pH value caused by different acids had no evident effect on the production of 5-HMF when compared to the effects produced by *Schisandra*. Table 3 shows the influence of the quantity of *Schisandra* on pH value and the pH value variation on 5-HMF level. The results showed that it was not simply the decomposition of saccharide under the acid environment created by *Schisandra* but

that *Ophiopogon* alone can also produce 5-HMF at a very low level when decocted without *Schisandra* or acid. Maybe 5-HMF originated mainly from *Schisandra*, not in combination with *Schisandra*/*Ophiopogon* decoction and there are other factors affecting the producing of 5-HMF besides the acid environment created by *Schisandra*. The result conflicts with Zhu *et al*.(1998b)'s report. Straightforward detection of 5-HMF in the sample without any treatment in this experiment makes the result more scientific. Some changes in *Schisandra* components, properties inevitably occur in the course of sample treatment.

The influence of different *Schisandra* on the 5-HMF level of *Ophiopogon* existence or inexistence

There were great differences in 5-HMF level among the three decoctions of medicinal or unprocessed *Schisandra* and *Ophiopogon*. The 5-HMF level between the decoctions of unprocessed *Schisandra* only and *Schisandra*/*Ophiopogon* group was different but there was no evident difference between the decoctions of medicinal *Schisandra* only and *Schisandra*/*Ophiopogon* group. 5-HMF content differed with the processing methods of *Schisandra*. The results showed that the presence of *Ophiopogon* had no evident effect on the production of 5-HMF when *Schisandra* was processed but that the processing method had great effect on the 5-HMF level in the decoctions (Table 4, Fig.2). The changing 5-HMF level in the decoction was mainly affected by *Schisandra* processing methods. The results was confirmed in the report that 5-HMF appeared in the course of *Ephedra sinica Stapf* processing (Xu *et al*., 2004). The processing time, processing temperature and herb species probably all affect 5-HMF level of medicinal *Schisandra* during the course of the processing.

Table 2 The influence of adding fructose on the 5-HMF level

Fructose (g)	5-HMF concentration (mg/100 ml)	Average (mg/100 ml)	*RSD* (%)
0	17.6		
0.2500	17.7		
0.7253	17.3		
1.0249	17.9	17.5	2.18
1.2650	17.8		
1.6482	16.9		
2.3189	17.3		

Table 3 The influence of *Schisandra* quantity on pH value and 5-HMF level

Acid environment creating material		*Ophiopogon* (g)	pH	5-HMF content (mg/100 ml)
Schisandra (g)	Acid			
6.25	−	25	3.094	8.70
12.50	−	25	3.046	16.40
18.75	−	25	3.024	24.20
25.00	−	25	2.993	31.60
50.00	−	25	2.930	59.90
−	HCl	25	2.972	0.39
−	HCl	25	3.142	0.46
−	HCl	25	3.454	0.46
−	H_2SO_4	25	2.987	0.44
−	H_2SO_4	25	3.058	0.50
−	H_2SO_4	25	3.401	0.40
−	HA_C	25	2.894	1.15
−	HA_C	25	3.012	0.67
−	HA_C	25	3.331	0.52
−	−	25	6.576	0.34

Table 4 The influence of the *Ophiopogon* and non-*Ophiopogon* existence on the level of 5-HMF in decoctions

No.	*Schisandra* content (g)	5-HMF content (mg/100 ml)					
		Ophiopogon+*Schisandra* group			*Schisandra* group		
		a1	b1	c1	a2	b2	c2
1	6.25	8.67	3.98	1.06	8.32	3.28	0.52
2	12.50	16.35	6.72	1.62	16.08	6.22	0.81
3	18.75	24.23	9.95	2.15	24.44	9.53	1.22
4	25.00	31.55	13.26	3.01	29.95	13.37	1.07
5	50.00	59.91	24.25	5.18	62.11	23.67	2.48

a1: Medicinal *Schisandra* (made by factory A)+*Ophiopogon*; a2: Medicinal *Schisandra* (made by factory A); b1: Medicinal *Schisandra* (made by factory B)+*Ophiopogon*; b2: Medicinal *Schisandra* (made by factory B); c1: Unprocessed *Schisandra*+*Ophiopogon*; c2: Unprocessed *Schisandra*

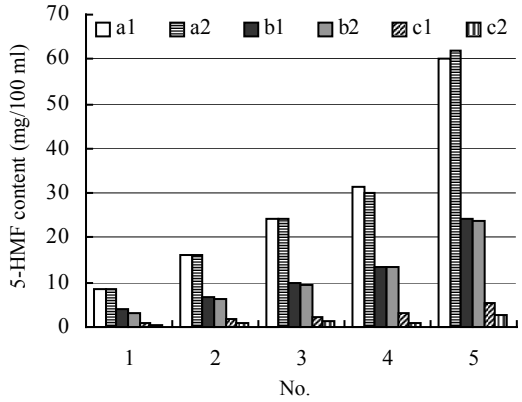

Fig.2 The influence of *Ophiopogon* addition on the production of 5-HMF

CONCLUSION

The composition of traditional Chinese medicine is very complex and every procedure of processing may change the results. The method of analysis established can reduce the operational error by a straight analysis of the components of herbal decoctions without extra treatment and provide more accurate results.

It was concluded that 5-HMF of Shengmaiyin was mainly derived from the decoction of *Schisandra* and not from the combined *Ophiopogon*/*Schisandra* decoction, because there was no evident difference in the 5-HMF level between the medicinal *Schisandra/Ophiopogon* group and *Schisandra* alone group and *Schisandra* content or *Schisandra* processed methods had great effect on the 5-HMF level. The factors affecting 5-HMF level in *Schisandra* will be reported in succession.

The addition of fructose has no effect on the production of 5-HMF in the decoction, showing that the appearance of 5-HMF is not simply the decomposition of saccharide under the acid environment created by *Schisandra*. The difference in 5-HMF content between the decoctions of unprocessed and processed *Schisandra* showed that the processing method played an important role in the production of 5-HMF. Because 5-HMF is found in many herbs, the physiological and biochemical roles of 5-HMF in the herbs deserved further investigation in the future.

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