Stability and degradation of hydroxysafflor yellow A and anhydrosafflor yellow B in the Safflower injection studied by HPLC-DAD-ESI-MS^n

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Abstract: Safflower is a popular Chinese medicinal plant and Safflower injection is extensively used for the clinical treatment of cerebrovascular and cardiovascular diseases. In this study, HPLC-DAD-ESI-MS^n was utilized to study the stability and degradation of the two major but chemically unstable bioactive compounds hydroxysafflor yellow A and anhydrosafflor yellow B, in Safflower injection. The impact of light irradiation, temperature, and pH on the stability of these two compounds were studied. The results showed that hydroxysafflor yellow A and anhydrosafflor yellow B could degrade at high temperature (>60 °C) or extreme pHs (pH≤3.0 or >7.0), but not under light irradiation. The common degradation product was *p*-coumaric acid. Chemical structures of the other degradation products were characterized by LC-MS. Hypothetical degradation pathways were proposed. In addition, ADP-induced platelet aggregation tests showed that the degradation of anhydrosafflor yellow B could reduce the anticoagulation activities of Safflower injection. Our results suggest that temperature and pH are critically important for the preparation and storage of Safflower injection.

Keywords: Hydroxysafflor yellow A; Anhydrosafflor yellow B; *p*-Coumaric acid; Stability; Safflower injection; HPLC-DAD-ESI-MS^n

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1. Introduction

Safflower, the dried floret of *Carthamus tinctorius*, is a well-known traditional Chinese medicine (TCM) for the treatment of hypertension, coronary heart disease, and cerebrovascular disease[1]. Previous work found that the water-soluble compounds in safflower such as hydroxysafflor yellow A (HSYA) were responsible for its therapeutic effects[2]. The State Food and Drug Administration of China approved the Safflower injection as a clinical therapeutic drug for the treatment of cerebral ischemia in 2000. Safflower injection possesses good curative effects as demonstrated by clinical data[3–5], and it becomes increasingly popular in the clinical use because of its minor side-effects.

In this study, HSYA and anhydrosafflor yellow B (AHSYB) are confirmed to be the main bioactive quinochalcones in water extract of safflower. Previous work reported the stability of these unstable quinochalcones, HSYA, safflower yellow B (SYB) and AHSYB, under various conditions such as high temperature, light irradiation, etc.[6–10]. Taniguchi and Ishihara studied the stability of HSYA in simulated intestinal fluid (pH 6.5 and 8.6) at 37 °C (incubation) for 72 h and found that it was partly degraded into *p*-coumaric acid[6]. The same changes took place under anaerobic incubation of SYB with human intestinal bacteria including *Peptostreptococcus anaerobius*[7]. Kazuma and Sato found that AHSYB could be transformed into carthamin in NMR solvent (methanol−pyridine, 95:5, v/v) at room temperature[8]. In traditional manufacturing procedure of Safflower Injection, the extraction temperature was at 100 °C, high-pressure sterilization was often used and the pH of the injection was adjusted to

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about 7.5. Thus, it was often observed that the two main quinochalcones may degrade into other compounds with potential bioactivities during the process. However, there is no report on the degradation of those bioactive compounds in Safflower injection.

In this paper, stability studies under the simulated manufacturing conditions of Safflower injection were performed. Degradation of the main quinochalcones was monitored under various temperatures, different pH levels and light irradiation by employing HPLC-DAD-ESI-MS^n methods. We observed new degradation products with potential bioactivities and hypothetical degradation pathways of HSYA and AHSYB were proposed. This study addresses the degradation behaviors of quinochalcones in Safflower injection for the first time, and will be valuable for the quality control of Safflower Injection.

2. Experimental

2.1. Chemicals and materials

Acetonitrile is of HPLC grade (Fisher Scientific, Fairlawn, NJ). Trifluoroacetic acid (TFA) is of reagent grade from Nankai University's Chemical Plant. The deionized water was prepared by using Millipore purification system (Millipore, Milford, MA, USA) and filtered with 0.45 μm membranes. Buffered aqueous solutions at different pH values were prepared according to the Pharmacopoeia of China[11]. Hydroxysafflor yellow A (purity≥99%), anhydrosafflor yellow B (purity≥98%) and *p*-coumaric acid (purity≥98%) were isolated by the authors from the florets of *Carthamus tinctorius*. Their structures were confirmed by NMR and MS spectroscopy. The crude drug safflower was purchased from Xinjiang Autonomous Region, P.R. China. The freeze-dried powder of safflower water extract was prepared in our lab. The plant material was identified by Prof. De-An Guo, School of Pharmaceutical Sciences, Peking University Health Science Center. All the standards and materials were preserved in desiccators in School of Pharmaceutical Sciences, Peking University Health Science Center. Photostability study was performed under the UV lamp (UV-II, 15 w, 365 nm, Beijing Zhiyuan Biotechnology Institute). The thermal stability study was carried out using water bath (Digital water bath SB-1000, Eyela) equipped with a thermostat controller. The pH was measured by Hanna pocket acidometer (Hanna Instruments, Asia Pacific, PTE Ltd.). HZQ-F constant temperature incubator (Donglian Technology) was used for the incubation experiments. Aggregation experiments were performed using an aggregometer (Model TYXN-96, TongYong Co.) based on the method of Born[12].

2.2. Animals

Male Spraque-Dawley rats weighing (250±20) g were supplied by the Lab Animal Center of Peking University Health Science Center (Grade II). Animal experimental procedures were carried out strictly in accordance with the guidelines for the care and use of laboratory animals (The Ministry of Science and Technology of China, 2006).

2.3. HPLC conditions

The analysis was performed on an Agilent 1100 liquid chromatography system (Agilent, Waldbronn, Germany) equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. Separation was achieved on an Alltech Alltima-C_{18} column (250 mm×4.6 mm, 5 μm) coupled with an Alltech Alltima-C_{18} guard column (12.5 mm×4.6 mm, 5 μm). The mobile phases consisted of acetonitrile (A) and 0.01% aqueous TFA (B), which were run with the gradient as follows: 0–18 min, linear gradient from A–B (2:98, v/v) to A–B (10:90, v/v); 18–50 min, linear gradient to A–B (30:70, v/v). Each run was followed by equilibration time of 8 min. The flow rate was 1.0 mL/min. The column temperature was set at 30 °C. Ultraviolet (UV) absorption was monitored at 280 nm.

2.4. HPLC-ESI-MS conditions

Experiments were performed using Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) with an electrospray ion source in negative mode. The HPLC conditions for the HPLC-ESI-MS were the same as in the HPLC-DAD analysis except that the eluent B was replaced by 0.5% acetic acid. The optimized parameters were as follows: ion spray voltage, 4.5 kV; sheath gas (N_2), 50 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 350 °C; capillary voltage, –4 V; tube lens offset voltage, –25 V. Full scan spectra were recorded in the range *m/z* 100–1500. The collision-induced dissociation (CID) energy was adjusted to 40% and the isolation width of precursor ions was 3.0 mass units.

2.5. Assay validation

The mixed stock solutions of HSYA and AHSYB were prepared by dissolving an appropriate amount in deionized water. Precision of the method was evaluated by analyzing the standard solutions consisting of HSYA and AHSYB at three different concentration levels (high, medium and low). The experiment was repeated six times on the same day and additionally on three consecutive days in triplicate to determine intra- and inter-day precision, respectively. Six samples from the same origin were analyzed under the same conditions. The relative standard deviation (RSD) value was calculated as a measurement of repeatability.

2.6. Stability of HSYA and AHSYB in aqueous safflower water extract

An aliquot of 1 g safflower water extract was dissolved in 50 mL deionized water in an ultrasonic water bath for several minutes and then filtered. The 2 mL filtrate solution at the concentration of 20 mg/mL was outlaid uniformly under the UV lamp (set at 365 nm) and sunlight in the laboratory (25 °C, 60% relative humidity). At the time period of 0, 1, 4, 6, 8 and 12 h under UV irradiation and 0, 12, 18, 24 and 48 h under sunlight irradiation, aliquots of the solutions (200 μL) were filtered through 0.45 μm membrane and 10 μL was directly injected into the HPLC system.

The thermal stability of the two compounds in safflower water extract solution was studied at 40 °C and 60 °C for 48 h and 100 °C for 12 h. An aliquot of 200 μL safflower water extract solution was taken at varying pre-determined intervals (40 °C and 60 °C: 0, 1, 2, 4, 8, 12, 24, 36 and 48 h; 100 °C: 0, 1, 2, 4, 8 and 12 h). All the sample solutions were filtrated through 0.45 μm micromembrane before being injected into HPLC system.

The filtrated safflower water extract solution at the concentration of 20 mg/mL was added to different buffers systems at pH 1.5, 3.0, 5.0, 7.0, 8.0 and 9.0 with a ratio 1:1 (v/v) and incubated for 24 h, respectively. The temperature was set at 25 °C and 37 °C. Aliquots of 200 μL were taken at different intervals (25 °C: 0, 1, 6, 12, 18 and 24 h; 37 °C: 0, 1, 4, 12, 18 and 24 h). Aliquot of each solution (200 μL) was immediately filtered through 0.45 μm membrane and 10 μL was injected for the HPLC-DAD analysis.

2.7. Sterilization stability studies of HSYA and AHSYB

In the procedure of sterilization, high-pressure sterilization was adopted. The pH before bottling was adjusted to 8.0. The safflower water extract solution was mixed with buffer solutions at pH 8.0 and boiled at 100 °C for 4 h to simulate the manufacturing process. Aliquots of 200 μL were taken at different intervals (0, 0.5, 1, 2 and 4 h). An aliquot of 10 μL filtrated solution was injected for LC and LC-MS analyses. In order to establish the possible degradation pathways and to identify the products, HSYA and AHSYB were studied separately under similar conditions.

2.8. Bioactivity assay

The bioactivities of major compounds involved in the degradation process were analyzed by assay of ADP-induced platelet aggregation of washed rat platelets. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood samples were taken from the ventral aorta and transferred into plastic tubes containing 3.8% trisodium citrate as anticoagulant in a volume ratio of 9:1. Platelet rich plasma (PRP) was obtained by centrifugation of blood at 200×g for 10 min at room temperature. Washed platelets were obtained by centrifugation of PRP at 1000×g for 10 min. Then the platelets were washed twice with Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 0.2 mM $MgSO_4$ and 5.0 mM glucose) containing 0.2 mM EGTA, pH 6.5. The washed platelets were then centrifuged again at 1000×g for 10 min and finally re-suspended in Tyrode buffer containing 1.8 mM $CaCl_2$, pH 7.4. Platelet number was counted and adjusted to 3×10^8 cells/mL. Washed platelets (200 μL) were incubated for 5 min with 10 μL AHSYB or *p*-coumaric acid at 100 μM and then aggregation was induced by the addition of 20 μM ADP (10 μL) while being stirred at 1000 r/min in a silicone-treated glass cuvette. The reaction was then allowed to proceed for 5 min, and the peak responses were measured. Aspirin (25 mg/L) was used as positive control.

3. Results and discussion

In this study, we showed that the HPLC method has good precision because RSD of peak area for each compound was not more than 2.24% in intra-day and inter-day precision tests. In repeatability test, RSD values of the two compounds were less than 1.65%, which showed high repeatability of the method (Table 1). Peak purity factors for HSYA and AHSYB were within the threshold as assessed by the Agilent Technologies spectral analysis software, indicating that no additional peaks were co-eluting with the two peaks. Photostability, thermal stability and pH stability were investigated to examine the most important factor in the Safflower injection preparation. Remaining percentage of each compound at different time was calculated as the ratio of the peak area to the initial peak area. The plot of remaining percentage versus time was obtained to describe the course of degradation process.

3.1. Photostability, thermal stability and stability at different pHs

The remaining percentage of HSYA and AHSYB in the photostability studies (Fig. 1) showed that light irradiation caused minor changes in the contents of the two compounds. Therefore, light irradiation is not a key factor in the degradation of HSYA and AHSYB.

In the thermal stability study (Fig. 2), HSYA was relatively stable at 40 °C and 60 °C, but degraded extensively at 100 °C in 12 h. The degree of thermal degradation of AHSYB was similar to that of HSYA. AHSYB was stable within the first 4 h at 60 °C, but about 45% of anhydrosafflor yellow B degraded at 24 h. About 32% of AHSYB degraded after exposure to boiling at 100 °C for 1 h, which suggested that the extraction temperature and extraction time should be set under 60 °C for less than 4 h.

Table 1. Intra-day, inter-day precision and repeatability for the assay of HSYA and AHSYB

Compound	Concentration (μg/mL)	Intra-day (n = 6)		Inter-day (n = 6)		Repeatability (n = 6)	
		Peak area	RSD (%)	Peak area	RSD (%)	Peak area	RSD (%)
HSYA	10.0	458.3±5.8	1.26	465.7±7.7	1.65		
	40.0	1817.9±3.0	0.17	1862.5±41.8	2.24	1621.5±26.8	1.65
	60.0	2778.4±4.4	0.16	2817.7±62.4	2.21		
AHSYB	62.8	207.9±0.2	0.10	208.8±1.0	0.48		
	251.2	843.8±2.3	0.28	848.0±8.0	0.94	903.8±12.0	1.32
	376.8	1292.9±1.3	0.10	1295.3±9.2	0.71		

RSD (%) = (SD/mean) × 100%.

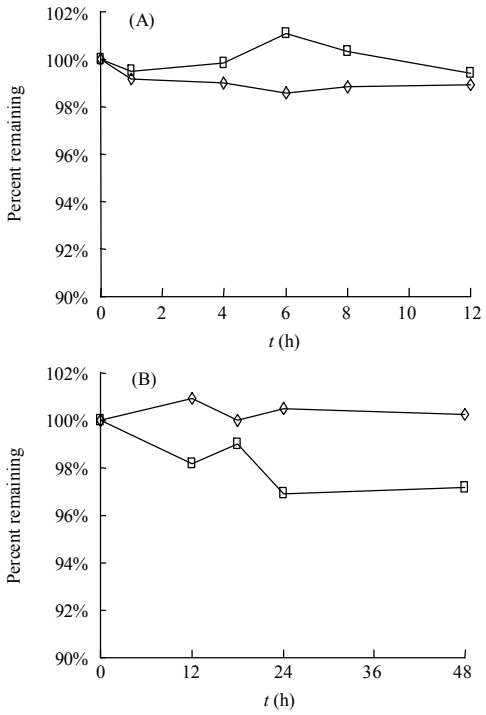


Figure 1. Photostability of HSYA and AHSYB in aqueous solution of safflower water extract. (A) light; (B) 365 nm. (—◇— HSYA, —□— AHSYB)

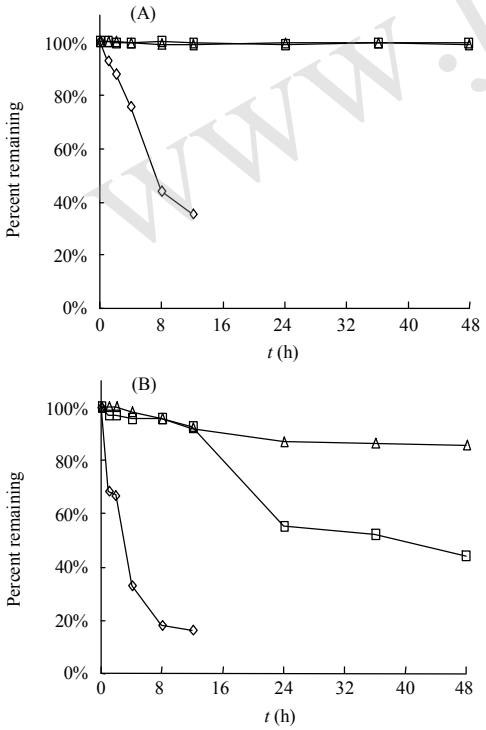


Figure 2. Thermal stability of HSYA and AHSYB in aqueous solution of safflower water extract at different temperatures (pH 7.0). (A) HSYA; (B) AHSYB. (—◇— 100 °C, —□— 60 °C, —△— 40 °C)

The results of the stability of HSYA and AHSYB in buffered solutions (at different pHs) at 25 °C and 37 °C (Fig. 3) showed that slow degradation of the two compounds were observed at various pHs because the temperature was low. HSYA was relatively unstable at pH 1.5. AHSYB was more unstable than hydroxysafflor yellow A in this test. Neither strong acidic condition (pH≤3.0) nor alkaline condition (pH>7.0) was suitable for AHSYB. Both of the compounds were stable at pH 5.0. The result indicated that the final pH for the injection should be adjusted to 5.0. When the final pH was set at 5.0, different temperatures such as 25, 37 and 100 °C were used to study the stability. Noticeable decreases in concentrations of the two compounds were observed at 100 °C (Fig. 4). It suggested that high-pressure sterilization was not suitable for Safflower injection. Therefore, these results demonstrated that temperature plays an important role in the degradation of HSYA and AHSYB in the injection preparation, pH is a less important factor, and light irradiation is the least important.

3.2. Sterilization stability studies

Decomposition curves of HSYA and AHSYB (Fig. 5) indicated their degradation process in the single compound solution and in the safflower water extract solution at pH 8.0 at 100 °C. The result showed that HSYA was relatively stable in the mixture solution. Furthermore, we found that about 80% of AHSYB degraded while only 2% of HSYA degraded within 1 h in the mixture solution at 100 °C. We consider that the main degradation products originate from AHSYB in the mixture solution and the degradation of AHSYB were temperature- and pH- dependent. Figure 6 illustrates the transformation process in the sterilization stability studies.

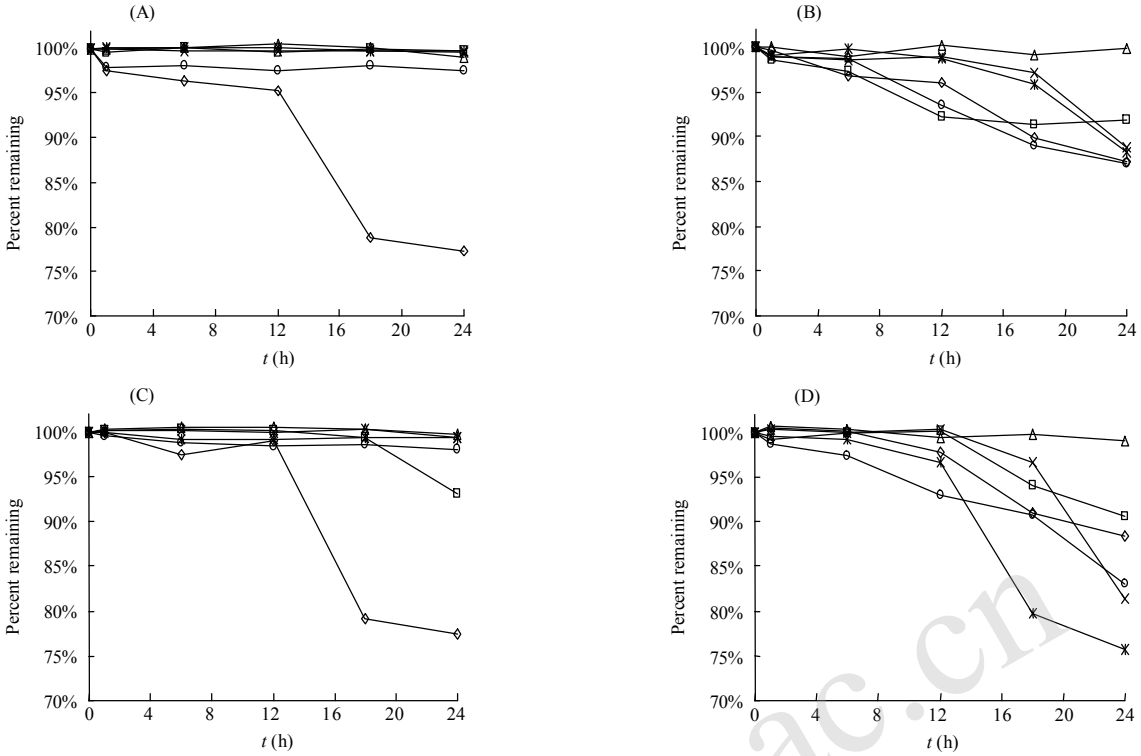


Figure 3. Stability of HSYA and AHSYB in buffer solutions of safflower water extract at different pHs at 25 °C and 37 °C. (A) HSYA at 25 °C; (B) AHSYB at 25 °C; (C) HSYA at 37 °C; (D) AHSYB at 37 °C. (pH 1.5, pH 3.0, pH 5.0, pH 7.0, pH 8.0, pH 9.0)

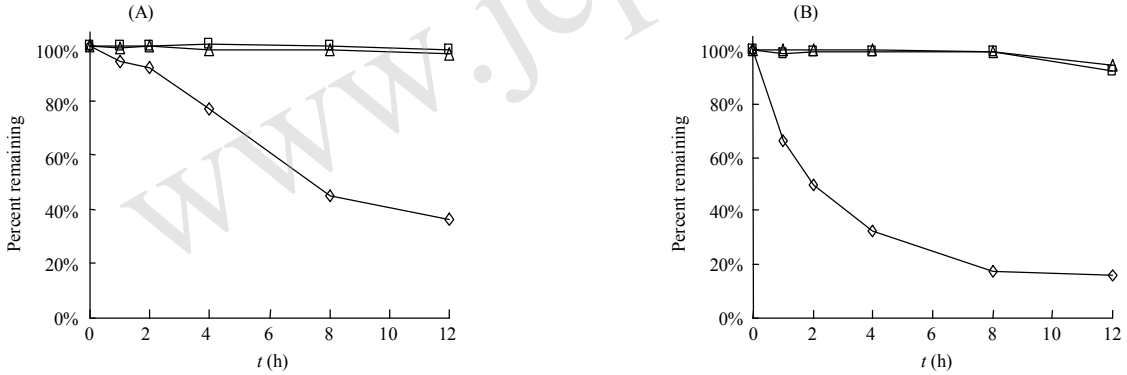


Figure 4. Stability of HSYA and AHSYB in buffer solutions of safflower water extract at pH 5.0 at different temperatures. (A) HSYA, (B) AHSYB. (100 °C, 37 °C, 25 °C)

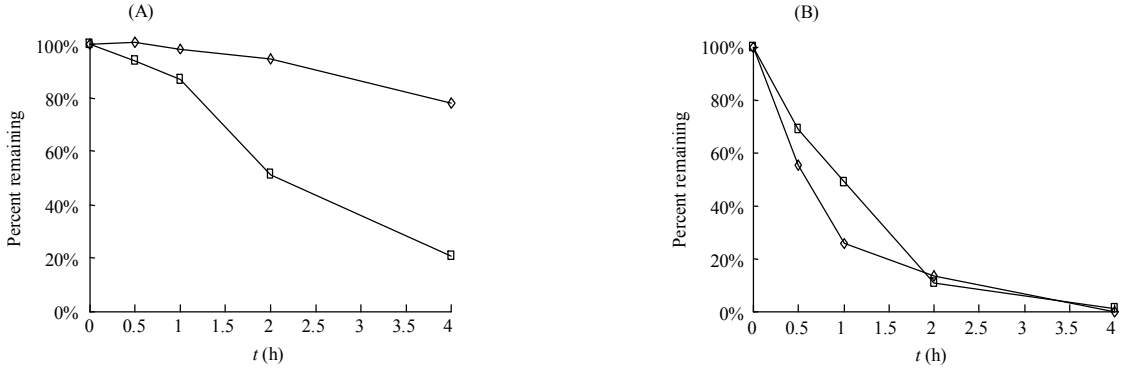


Figure 5. Sterilization stability studies of HSYA and AHSYB in buffer solution at pH 8.0 at 100 ℃ in single and mixture solution. (A) HSYA; (B) AHSYB. (Mixture, Single)

3.3. Degradation products and hypothetical degradation pathways

The degradation products in sterilization stability studies were analyzed by HPLC-DAD-ESI-MS^n method and confirmed by the comparison of LC behavior and MS^n data with reference standards. As shown in Figure 6, HSYA and AHSYB were shown to be the two major compounds (Fig. 6A); in the single compound reaction, HSYA (peak 1, *m*/*z* 612) could be turned into Product 1 (peak 4, *m*/*z* 626), Product 2 (peak 5, *m*/*z* 612) and *p*-coumaric acid (peak 3, *m*/*z* 164) (Fig. 6D); AHSYB (peak 2, *m*/*z* 1044) was transformed into Product 1, Product 3 (peak 6, *m*/*z* 1058), *p*-coumaric acid and HSYA (Fig. 6F). HSYA and AHSYB produced a common decomposition product, which was identified as *p*-coumaric acid, as suggested by Taniguchi et al[6]. In the mixture reaction, *p*-coumaric acid was the main degradation product (Fig. 6B). The ESI-MS data and fragmentations of those related compounds were listed in Table 2. All of them produced abundant $[M-H]^-$ ions. Sterilization stability studies of HSYA and AHSYB by LC-ESI-MS^n were presented in Figure 7. The UV spectrum of Product 1 showed that it might be a flavonol compound with λ_{max} 376 nm. The full-scan mass spectrum of Product 1 gave a significant $[M-H]^-$ ion at *m*/*z* 625, which was 14 units more than HSYA. The $[M-H]^-$ ion at *m*/*z* 611 and similar fragments to HSYA indicated that Product 2 was an isomer of HSYA. However, Product 2 may be not a chalcone but a flavanone according to its UV spectrum. Tentative structures of the products and the hypothetic degradation pathways were proposed as shown in Schemes 1–2.

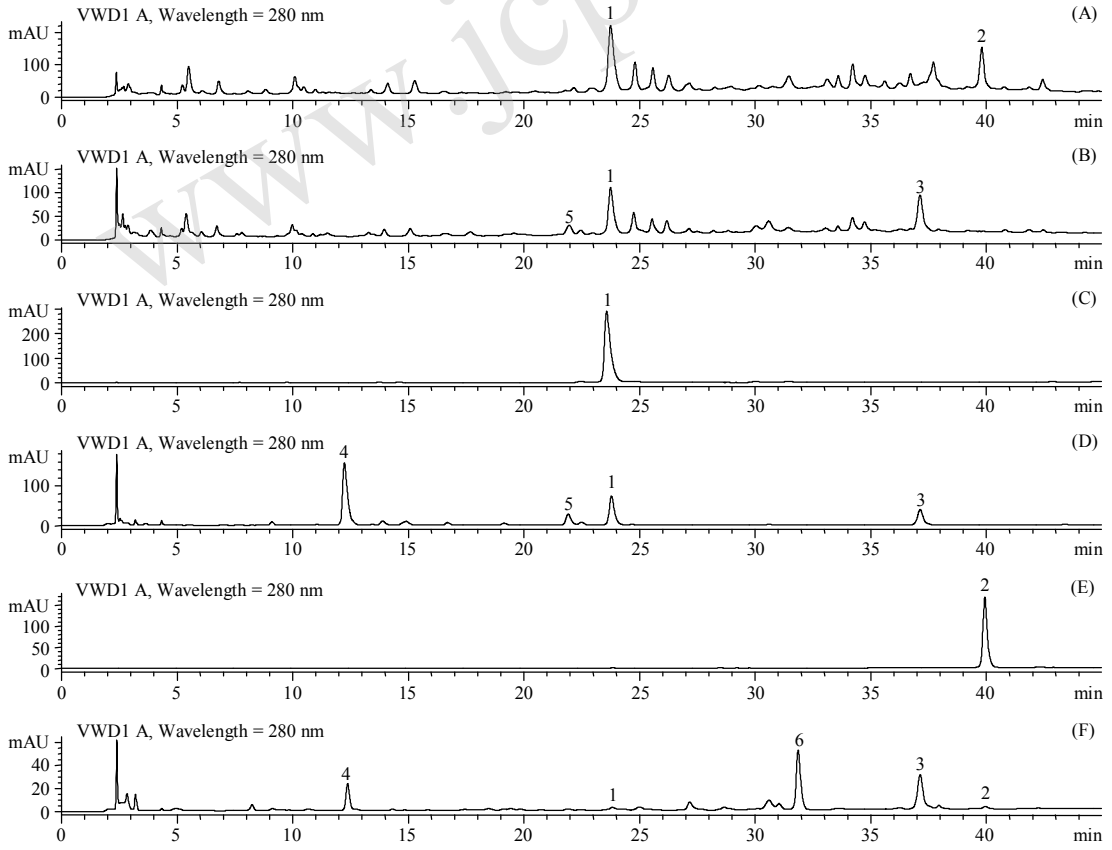


Figure 6. Representative transformation process in the sterilization stability studies by HPLC-DAD. (A) Aqueous solution of safflower water extract; (B) Safflower water extract in buffer solution at pH 8.0 at 100 ℃ for 4 h; (C) HSYA in buffer solution at pH 8.0 at 25 ℃; (D) HSYA in buffer solution at pH 8.0 at 100 ℃ for 4 h; (E) AHSYB in buffer solution at pH 8.0 at 25 ℃; (F) AHSYB in buffer solution at pH 8.0 at 100 ℃ for 2 h. (1) hydroxysafflor yellow A; (2) anhydrosafflor yellow B; (3) *p*-coumaric acid; (4) Product 1; (5) Product 2; (6) Product 3.

Table 2. MS^n data of degradation products formed during sterilization

Peak No.	Retention time (min)	UV (λ_{max}, nm)	$[M-H]^-$ (m/z)	HPLC-ESI-MS^n (m/z, % base peak)	Compounds
1*	24.74	228, 404	611	MS^2 [611]: 491 (100), 473 (18), 403 (28), 325 (23) MS^3 [491]: 473 (100), 456 (7), 354 (9), 353 (18), 327 (25), 323 (36), 301 (18), 283 (33)	Hydrosafflor yellow A
2*	39.47	246, 412	1043	MS^2 [1043]: 1025 (100), 923 (24), 593 (6), 449 (19) MS^3 [1025]: 1007 (28), 905 (13), 879 (40), 863 (100), 846 (17), 772 (16), 618 (13), 575 (9), 449 (12), 299 (6)	Anhydrosafflor yellow B
3*	36.96	234, 310	163	MS^2 [163]: 119 (100)	*p*-Coumaric acid
4	14.38	224, 294, 376	625	MS^2 [626]: 505 (100), 487 (3) MS^3 [505]: 487 (13), 427 (12), 355 (100), 342 (32) MS^4 [355]: 327 (100), 234 (12), 218 (8), 206 (18)	Product 1
5	23.49	226, 266, 312	611	MS^2 [612]: 491 (100), 473 (45), 465 (8), 345 (13) MS^3 [491]: 473 (94), 449 (9), 431 (12), 407 (26), 345 (100), 327 (18), 309 (15), 295 (12), 283 (10)	Product 2
6	33.61	240, 298, 398	1057	MS^2 [1057]: 1039 (41), 937 (100), 877 (9), 589 (41), 463 (8), 449 (14) MS^3 [937]: 919 (68), 893 (13), 775 (54), 774 (100), 757 (28), 756 (11), 729 (23), 489 (19), 327 (7)	Product 3

*Structures confirmed by comparison with the reference standards.

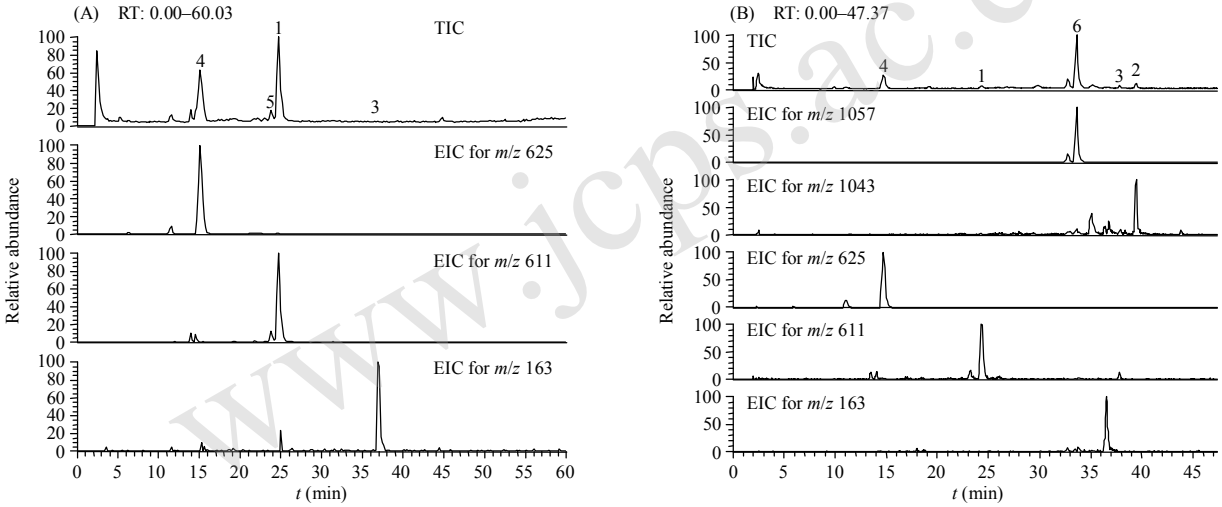
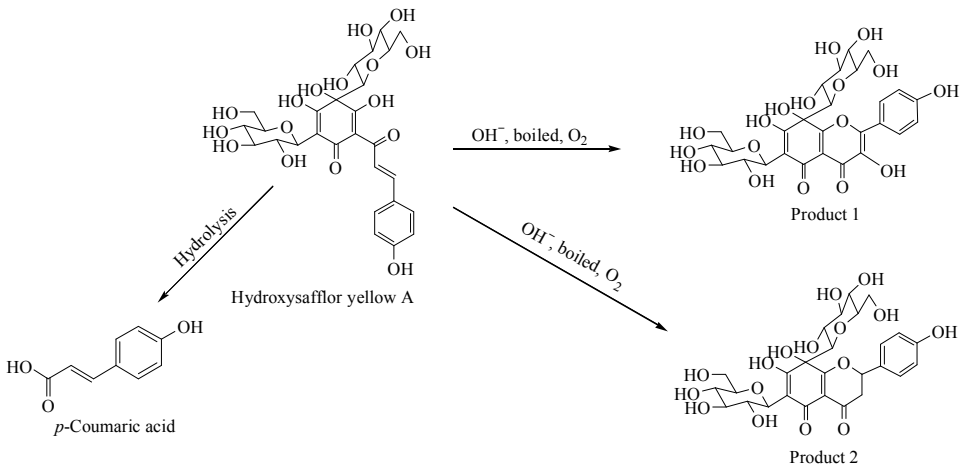
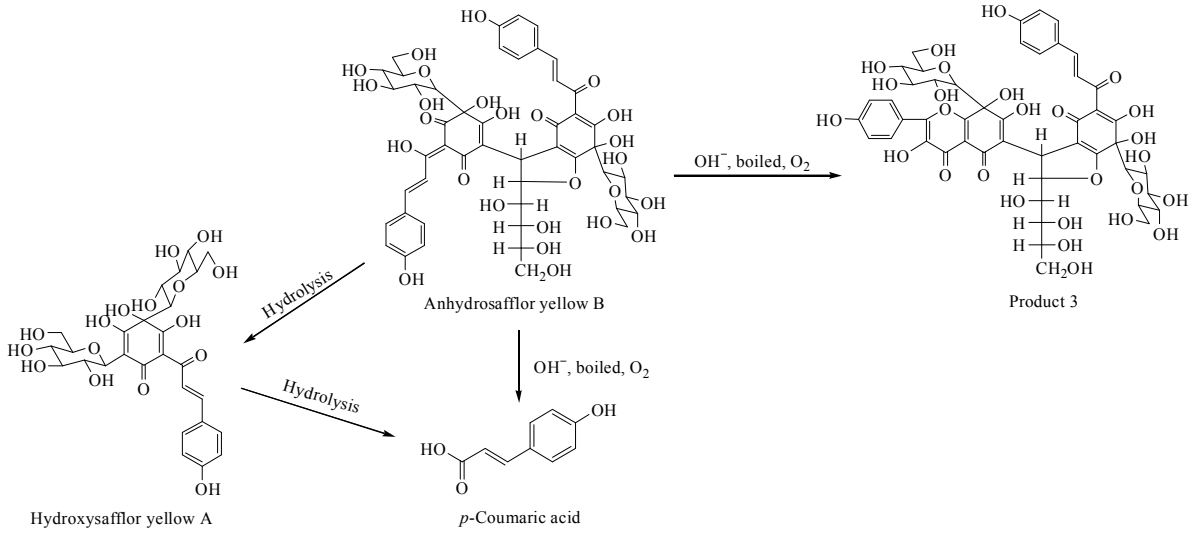


Figure 7. Sterilization stability studies of hydroxysafflor yellow A and anhydrosafflor yellow B by HPLC-ESI-MS^n. (A) hydroxysafflor yellow A; (B) anhydrosafflor yellow B.



Scheme 1. Proposed degradation pathways of hydroxysafflor yellow A in the buffer solution at pH 8.0 at 100 °C.



Scheme 2. Proposed degradation pathways of anhydrosafflor yellow B in the buffer solution at pH 8.0 at 100 ℃.

3.4. Inhibitory effects of the major compounds generated in the sterilization studies on ADP-induced platelet aggregation of washed rat platelets

As mentioned above, *p*-coumaric acid was formed mostly from AHSYB in the solution of safflower water extract at pH 8.0. We compared *p*-coumaric acid with AHSYB on their inhibitory activity by assay of ADP-induced platelet aggregation. The results showed that the inhibitory rate of AHSYB (62.68%) was twice as much as *p*-coumaric acid (31.88%), while the positive control of aspirin was 42.17%. This result indicated that degradation in pasteurization could decrease the activity of the injection.

4. Conclusion

In conclusion, HPLC-DAD-ESI-MS analytical technique was employed for the stability evaluation of water-soluble quinochalcones in the Safflower injection preparation. Possible structures of degradation products and hypothetic degradation pathways were proposed. The degradation of AHSYB evaluated in this study could explain the potential instability and weakened activity of Safflower injection by the existing preparation method. Based upon these results, we concluded that high temperature, strong acidic and alkaline conditions should be avoided in the preparation and storage of Safflower injection in order to avoid degradation and to maintain its biological activities.

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HPLC-DAD-ESI-MS^{n}法研究红花注射液中羟基红花黄色素A和脱水红花黄色素B的稳定性及降解过程

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摘要: 红花是一种常用中药, 红花注射液广泛地应用于临床治疗心脑血管疾病。本研究应用HPLC-DAD-ESI-MS^{n}方法研究红花注射液中两种主要物质, 羟基红花黄色素A和脱水红花黄色素B的稳定性及降解过程。考察了光照、温度、pH值对二者稳定性的影响。结果表明, 羟基红花黄色素A和脱水红花黄色素B在温度大于60 ℃, pH≤3.0 或 >7.0时易发生降解且最终降解产物为对羟基桂皮酸, 而光照对其影响不大。本研究应用LC-MS对其降解产物及降解过程进行推导, 首次描述了两种物质的降解途径。此外ADP诱导的血小板凝集实验结果显示脱水红花黄色素B降解以后会大大降低红花注射液的抗凝活性。建议红花注射液生产及储存过程中应严格控制温度和pH值。

关键词: 羟基红花黄色素A; 脱水红花黄色素B; 对羟基桂皮酸; 稳定性; 红花注射液; HPLC-DAD-ESI-MS^n