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A novel, rapid, and high efficiency method to purify natural punicalagin by preparative HPLC is described. The optimum purification condition for punicalagin purification was 14% methanol in 0.1% trifluoroacetic acid solution as the mobile phase. The flow rate was 12 ml/min. 81.7 mg punicalagin at 98.05% purity was obtained from 300 mg crude extract containing 30% punicalagin. The stability of purified punicalagin was also studied. Heating, solar radiation, the pH value, and a strong oxidizer decreased its stability in water. Fe^{3+} and Cu^{2+} were complexed by punicalagin. Punicalagin was stable with ultrasonication, ultraviolet radiation, and commonly used food and cosmetic additives.

Keywords preparative HPLC; punicalagin; purification; stability

INTRODUCTION

Pomegranate (Punica granatum L.) husk was used as traditional Chinese medicine (1,2). Punicalagin, the main ingredient of pomegranate husk, is a high molecular weight polyphenolic compound. Most studies investigated the remarkable pharmacological activities of punicalagin in vitro and in animals (3–7).

The conflicting reports regarding the toxicity of punicalagin (8–10) were not verified. Present studies showed that repeated oral administration of high doses of the punicalagin to rats for 37 days is not toxic (11). In vitro cytotoxic studies against three cell lines showed that punicalagin is toxic only at higher concentration (3). The stabilities of punicalagin under different physical and chemical conditions were not reported either. It influences the separation and purification procedure of punicalagin and the preservation of punicalagin. The effective application of punicalagin also needs the control of its stability.

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In order to perform further studies evaluating the biological effects of punicalagin in animal or clinical trials, it is necessary to obtain reasonably large quantities of highly purified compounds for experimental purposes. In studies to date, methods to isolate punicalagin included labor intensive and expensive solid phase extractions by column chromatography (C_{18}, polyamides, cellulose, Sephadex Lipophilic LH-20, Diaion HP20, siliga gel) (11–13). The yield of punicalagin by these methods was very low. High speed counter-current chromatography (HSCCC) was also used for punicalagin separation and purification, but the purity of the obtained punicalagin has been inadequate for clinical trials (14). Moreover, both solvent and time consumption by HSCCC are significantly high. Preparative chromatography is an important industrially applied separation process for the isolation and purification of food and other value products. Therefore, preparative HPLC is a valuable method for satisfying the demands of large amounts of highly purified punicalagin.

A novel method for punicalagin purification is described in this article. The method is rapid, has low cost, and high efficiency. The stabilities of purified punicalagin under different conditions were also studied. The results provide the basic data for the effective separation, purification, and preservation of punicalagin and further pharmacological studies on punicalagin.

MATERIALS AND METHODS

Plant Material and Agents

Pomegranate husk (Bozhou, Anhui province) was bought from Guo Yi Tang drugstore. Trifluoroacetic acid (TFA) (Merck, Germany) and methanol (Dima, Beijing, China) were HPLC grade. Other agents and commonly used food and cosmetic additives were of analytical grade. The standard punicalagin was obtained as reported in reference (14). The purity was calculated by external reference method.

Extraction and Purification

Fifty gram dried pomegranate husk was ground to powder. Twenty g amount of this powder was extracted by refluxing with 500 ml 50% acetone at 50°C for 3 hours. The mixture was filtered and the insoluble residue was extracted under the same condition twice. The supernatant fluid was mixed and concentrated to dry under reduced pressure.

The preparative liquid chromatography system equipment used was a Waters 4000 Prep LC controller, a 2487 Dual λ absorbance detector, an Empower workstation (Waters, USA) and a reversed phase C_{18} column (19×300 mm, 7 μm, Symmetry Prep^{TM}). In order to obtain optimum conditions including the solvent system, flow rate, and sample amount, an orthogonal experiment L_{16} (4^5) was designed. The solvent system consisted of MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). The flow rates were from 6.0 ml/min to 12 ml/min. 200∼500 mg crude extract were dissolved ultrasonically in 11 ml mobile phase followed by centrifugation at 4000 rpm for 15 min. Ten ml supernatant was injected into the column. Punicalagin was detected by absorbance at 378 nm (13).

The recovery of punicalagin was calculated as the crude punicalagin injected divided by the pure punicalagin obtained.

HPLC Analyses

The analytical HPLC equipment used was a Shimadzu LC-20AVP system with two LC-20AT solvent delivery units, an SPD-20A UV/VIS detector, a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan), a T2000P workstation (Beijing, China) and a reversed phase C_{18} column (4.6×250 mm, 5 μm, Cosmosil^{TM}, Japan). The solvent system consisted of MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). Gradient conditions: 0–10 min, 5%–20% A in B; 10–20 min, 20–40% A in B; 20–26 min, 70% A in B. This was followed by a 10 min re-equilibration. The column oven temperature was set at 30°C. The flow rate was 1.0 ml/min, and 10 μl portions were injected into the column. Punicalagin was detected by absorbance at 378 nm (13,15).

MS and NMR Analyses

A micromass 70-VSE mass spectrometer was used with an ion source temperature of 110°C and a probe temperature of 25°C. The spectrum was scanned at 30 eV from m/z 200 to 1200. NMR spectra were performed in acetone using a Bruker high-resolution AV600 NMR spectrometer at 600 MHz (Bruker Biospin Corporation, USA). MS and NMR spectra were obtained by analysts at the Center of Analysis, Beijing University of Chemical Technology (Prof. Du Zhenxia & Dr. Hu Gaofei).

Preparation of the Standard Curve

First, punicalagin (obtained by preparative HPLC) with 95% purity was dissolved in de-ionized water to obtain punicalagin solution at certain concentration. The solution was scanned by an ultraviolet and visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China) from 190 nm to 800 nm by the complete absorption spectrum.

Second, a stock solution was prepared with 10.0 mg of 95% punicalagin, which was dissolved and diluted to 10 ml with de-ionized water. Aliquots of the standard stock solution of punicalagin were pipetted into different 25 ml volumetric flasks and diluted to mark with de-ionized water. The final concentrations of punicalagin were in the range 4.0–20.5 μg/ml. Each solution was scanned at 260 nm in duplicate. The absorbance was recorded for all the solutions. Therefore, the appropriate range of punicalagin concentration was obtained and the appropriate concentration of punicalagin used for stability studies could be chosen referring to the result.

Stability Studies

Ten mg punicalagin with 95% purity was dissolved and diluted to 10 ml in volumetric flask with de-ionized water. Aliquots of punicalagin solution were pipetted into different 100 ml volumetric flasks and diluted to mark with de-ionized water. The final concentration of punicalagin was 10 μg/ml and 20 μg/ml.

Five ml diluted solution (10 μg/ml) was pipetted into a 10 ml test tube. Five test tubes were prepared for the next step. Each sample was heated in a water bath at 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C for an hour, respectively. After cooling in a water bath at 25°C for half an hour, the treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (10 μg/ml) was pipetted into a 10 ml test tube. Five test tubes were prepared for the next step. Each sample was treated by ultrasonication (Kunshan leo-sonic Instrument Co., Ltd., China) at 100 w 40 kHz for 10 min, 20 min, 30 min, 45 min, and 60 min. The treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (10 μg/ml) was pipetted into a 10 ml beaker. Four beakers were prepared for the next step. Each sample received solar radiation directly for 30 min, 60 min, 100 min, and 250 min, respectively. The treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (10 μg/ml) was pipetted into a 10 ml beaker. Five beakers were prepared for the next step. Each sample was treated by ultraviolet radiation at 254 nm for 10 min, 20 min, 30 min, 45 min, and 60 min. The treated punicalagin solutions were detected directly without delay

by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (10 μg/ml) was pipetted into a 10 ml test tube. Ten test tubes were prepared for the next step. 0.1% HCl or 0.1% NaOH was added into punicalagin solution to obtain different pH values from 1.5 to 10.0. The treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (20 μg/ml) was pipetted into a 50 ml conical beaker. Ten conical beakers were prepared for the next step. Five ml 0.02%, 0.04%, 0.06%, 0.08%, 0.1% Na_2SO_3, and 5 ml 0.02%, 0.04%, 0.06%, 0.08%, 0.1% H_2O_2 solution were added into each sample solution, respectively. The well-distributed punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (10 μg/ml) was pipetted into a 50 ml conical beaker. Nine conical beakers were prepared for the next step. Eight kinds of metal ion solution (0.01 mg/ml, 5 ml) including Na^+(NaCl), K^+(KCl), Ca^{2+}($CaCl_2$), Cu^{2+}($CuCl_2$), Zn^{2+}($ZnCl_2$), Mg^{2+}($MgCl_2$), Fe^{3+}($FeCl_3$), and Al^{3+}($AlCl_3$) were added into punicalagin solutions and well-distributed. Five ml de-ionized water was added into the last conical beaker as reference substance. The treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

Five ml diluted solution (20 μg/ml) was pipetted into a 10 ml conical beaker. Fourteen conical beakers were prepared for the next step. Thirteen kinds of commonly used food and cosmetic additive solutions (0.02 mg/ml, 5 ml) including glucose, amylin, saccharose, sodium alginate, sodium citrate, citric acid, sorbic alcohol, 2-tert-butylhydroquinen (TBHQ), carboxymethyl cellulose (CMC), hyaluronic acid (HA), vitamin C (Vc), vitamin B_3 (Vb_3), and vitamin B_5 (Vb_5) were added into 5 ml punicalagin solutions and mixed well. Five ml de-ionized water was added into the last conical beaker as reference substance. The treated punicalagin solutions were detected directly without delay by an ultraviolet and visible spectrophotometer at 260 nm in duplicate, respectively.

The experiments designed were referred to in some articles (16–18) that involved stability studies on natural products. The absorbance of punicalagin reflected the variation of the concentration or the alteration of the structure of punicalagin in different physical and chemical conditions.

RESULTS AND DISCUSSION

Extraction and Purification

The crude extract from pomegranate husk was 13.6 g. The punicalagin purity was 28.9% determined by HPLC.

Three main factors (flow rate, ratio of eluent A and B, the concentration of crude extract solution) influenced the purity and recovery of punicalagin. The orthogonal experiment $L_{16}(4^5)$ was carried out to achieve the optimized result.

The results of the orthogonal experiment were listed in Table 1. According to the range in the direct analysis in Table 2, the ratio of eluent A and B had the significant interaction of both purity and recovery of punicalagin. The significance in the variance analysis (Table 3)

TABLE 1
Results of the orthogonal experiment L_{16} (4^5) for punicalagin purification

Ratio of eluent A and B	Flow rate (ml/min)	Weight of crude extract (mg)	Weight of purified product (mg)	Purity of punicalagin (%)	Recovery (%)
10:90	8	300	52.12	97.81	66.71
	6	500	93.07	96.31	71.21
	12	200	43.17	97.58	82.47
	10	400	70.20	95.49	70.77
12:88	8	500	114.52	96.62	88.05
	6	300	66.08	94.36	84.17
	12	400	88.18	95.14	83.45
	10	200	36.78	96.11	69.67
14:86	8	200	43.23	97.44	81.17
	6	400	98.45	97.87	94.69
	12	300	81.70	98.05	98.80
	10	500	122.65	96.12	94.48
16:84	8	400	105.61	94.87	101.20
	6	200	58.44	93.92	105.49
	12	500	128.70	95.12	99.30
	10	300	73.82	95.08	94.84

TABLE 2
Direct analysis of the purity and recovery of punicalagin

Factor	Ratio of eluent A and B	Flow rate (ml/min)	Weight of crude extract (mg)	X	Y
		Purity			
K1	96.797	96.685	96.325	95.748	96.728
K2	95.558	95.615	96.043	96.335	95.992
K3	97.370	96.472	96.263	96.787	95.733
K4	94.748	95.700	95.843	95.602	96.020
R	2.622	1.070	0.482	1.185	0.995
		Recovery			
K1	72.790	84.282	86.130	87.532	82.593
K2	81.335	88.890	88.260	85.220	82.667
K3	92.285	91.005	84.700	90.012	90.580
K4	100.208	82.440	87.528	83.853	90.778
R	27.418	8.565	3.560	6.159	8.185

K—average; R—range.

confirmed the direct analysis. According to the K value of each factor; we can find the optimum combination of factor and level for the highest purity and highest yield of punicalagin, respectively.

Figure 1 showed three representative preparative HPLC chromatograms of different conditions for punicalagin purification. The first peak in each chromatogram was indentified as impurity after HPLC detection. The second and third peaks were identified as α- and β-punicalagin isomer, respectively, by HPLC. They quickly changed to each other and then balanced.

When the ratio of eluent A and B was 10:90, the whole period of purification lasted for over 50 min (Fig. 1(a)). This meant more solvent and time consumption, which was not good for industrial application after scaled up. The peak area of punicalagin in Fig. 1(a) was smaller than that in Fig. 1(b) and 1(c), which was in accordance with the low recovery of punicalagin listed in Table 1. When the ratio of eluent A and B was 16:84, the separating degree between punicalagin and the impurity decreased (Fig. 1(c)). This was in accordance with the decreased purity of punicalagin in Table 1. Thus the optimum conditions for punicalagin purification were: the ratio of eluent A and B was 14:86, the flow rate was 12 ml/min. 81.7 mg punicalagin at 98.05% purity was obtained from 300 mg crude extract containing 28.9%

TABLE 3
Variance analysis of the purity and recovery of punicalagin

	Ratio of eluent A and B	Flow rate	Weight of crude extract	Error
		Purity		
SSd	16.887	3.500	0.581	5.78
df	3	3	3	6
F	5.848	1.212	0.201	
F_α (0.05)	4.760	4.760	4.760	
significance	*			
		Recovery		
SS_d	1746.963	190.520	30.236	346.75
d_f	3	3	3	6
F	10.076	1.099	0.174	
F_α (0.05)	4.760	4.760	4.760	
significance	*			

SS_d—deviance; d_f—degree of freedom; F—F ratio; F_α —critical value.

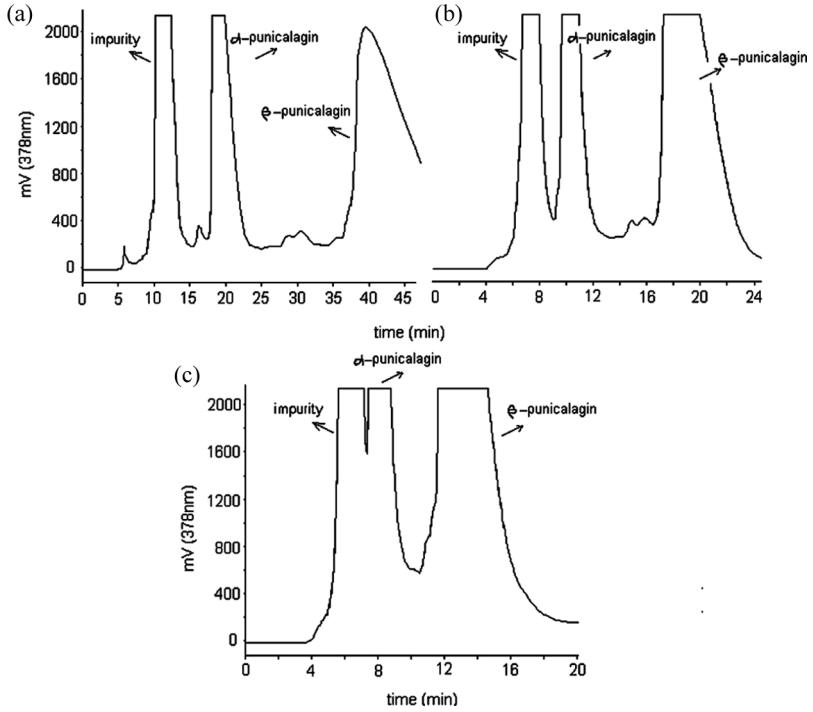

FIG. 1. Preparative HPLC chromatograms of punicalagin purification. Conditions: reversed phase C_{18} column (19×300 mm, 7 μm, Symmetry Prep^{TM}). Mobile phase: MeOH: 0.1% TFA = 10:90 (a), 14:86 (b), 16:84 (c). Flow rates: 12 ml/min, monitored at 378 nm.

punicalagin. The whole procedure lasted only 25 min (Fig. 1(b)).

The HPLC chromatograms of the crude extract and the purified product were shown in Fig. 2.

MS and NMR Analyses

The compound identity of punicalagin was confirmed by MS analyses where the ion at M−H m/z 1083 accounted for punicalagin as previously reported (13). The data in Table 4 were similar to those in reference (9,19).

Preparation of the Standard Curve

Two absorption maxima at 260 nm and 375 nm were obtained. 260 nm was chosen for further study because the absorption of punicalagin solution at 260 nm is stronger. Linear regression analysis of the absorbance (y) versus the theoretical concentration (x) gave the following equation: $y = 0.0203 + 49.592x$, $r^2 = 0.9997$. The correlation coefficient demonstrated linearity of the method over the concentration range analyzed.

Stability Studies

Figures 3(a) and 3(c) show that the absorbance of punicalagin decreased, which meant heating and solar radiation severely decreased the stability of punicalagin. More than 8.6% punicalagin broke down after heating for only one hour at 80°C. But nearly 100% punicalagin was stable

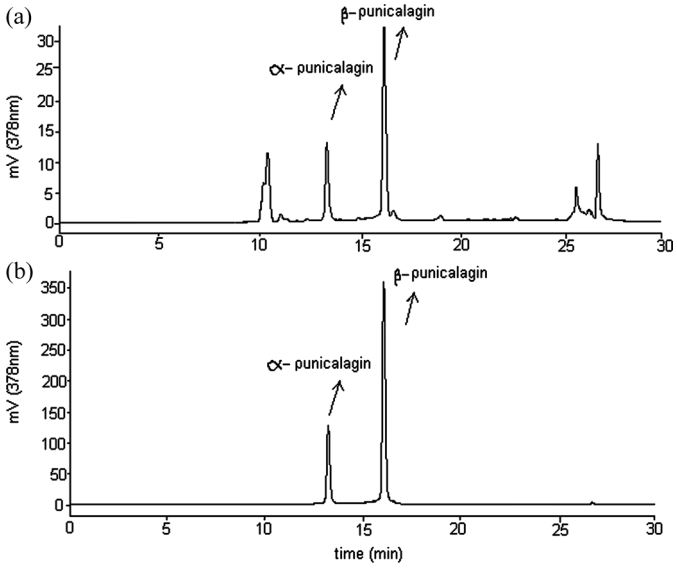

FIG. 2. HPLC analyses of crude extract (a) and purified punicalagin (b). HPLC conditions: reversed phase C_{18} column (250×4.6 mm, 5 μm, Cosmosil^{TM}). Column temperature: 30°C. Mobile phase: MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). Gradient conditions: 0–10 min, 5%–20% A in B; 10–20 min, 20–40% A in B; 20–26 min, 70% A in B. This was followed by a 10 min re-equilibration. Flow rate: 1.0 ml/min, monitored at 378 nm.

TABLE 4
The NMR analyses of punicalagin

^{1}H NMR (600 MHz, acetone d_6)	δ (ppm)	^{13}C NMR (600 MHz, acetone d_6)	δ (ppm)
2.10	dd, H-5	62.9, 64.2, 70.4, 70.7, 71.8, 73.9, 76.7, 77.0, 89.5	α-C_1
3.29	td, H-6	91.4	β-C_1
4.20	t, H-6	155.6, 156.2	δ-lactone
4.76	t, H-4	165.1, 165.5, 166.0, 166.4	CO_2
4.90	dd, H-2		
5.13	t, H-1a		
6.52, 6.62, 6.65, 7.01	s, aromatic H		

when it was kept under 30°C. When punicalagin is extracted, the temperature should not be over 30°C. Punicalagin was not stable under the condition of solar radiation either. Thirty minutes of direct irradiation made 16.4% punicalagin broken down. Long time irradiation caused great punicalagin damage. Therefore protection of punicalagin from sunlight and cold age is required.

Figure 3(b) shows that the absorbance of punicalagin changed slightly. With an increase in ultrasonication time, the water temperature in the ultrasonic generator increased. This caused the degradation of punicalagin. Figure 3d shows that the absorbance of punicalagin did not vary much from the beginning to the 60th min. Punicalagin was stable under the condition of ultraviolet radiation at 254 nm.

Figure 4(a) shows that the punicalagin had the highest absorbance at pH 3.0. Punicalagin was comparatively stable from pH 4.0 to pH 9.0 according to the slowly decreased absorbance of punicalagin. At $pH < 3.0$ or $pH > 9.0$, the absorbance of punicalagin decreased rapidly. This meant the stability of punicalagin decreased sharply. The mechanism of how punicalagin varies at different pH values needs further study.

Figure 4(b) shows that high H_2O_2 concentrations also influenced the stability of punicalagin. When the concentration of H_2O_2 was over 0.8%, about 3.8% punicalagin was broken down by H_2O_2. Punicalagin was oxidized by H_2O_2 and its absorbance decreased. Low concentration of Na_2SO_3 caused little damage of punicalagin. With increasing Na_2SO_3 concentration, the color of the solution became more intense. So the absorbance of punicalagin solution increased slowly.

The absorption maxima at 370 nm of the punicalagin solution, when it was treated with Fe^{3+} and Cu^{2+}, disappeared. The results show that punicalagin was complexed with Cu^{2+} and Fe^{3+} and the molecular structure of punicalagin changed. Figure 4(c) shows that the absorbance of punicalagin at 260 nm had a remarkable increase. Other metal ions did not react with punicalagin and the absorbance of punicalagin samples with other metal ions were

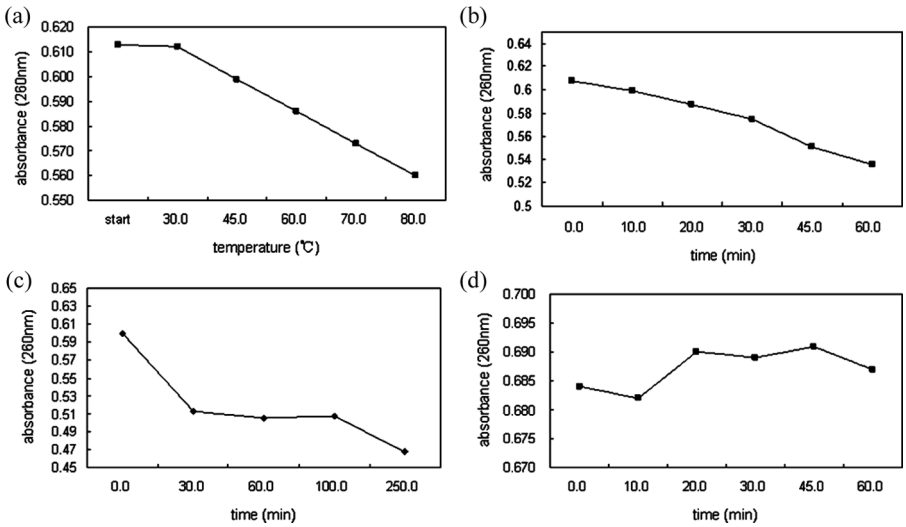

FIG. 3. The effects of physical factors on punicalagin stability. (a) heated in water bath for 1 h; (b) ultrasonication; (c) solar radiation; (d) ultraviolet radiation at 254 nm.

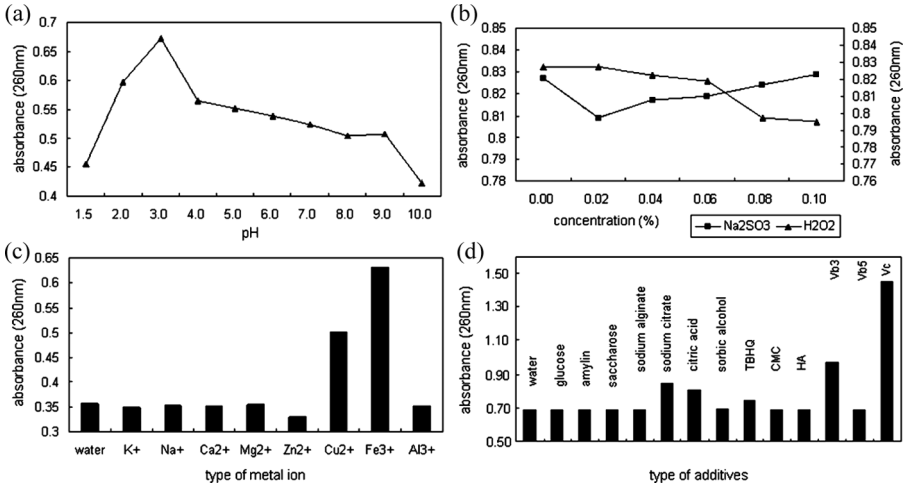

FIG. 4. The effects of chemical factors on punicalagin stability. (a) at pH 1.5 to 10.0; (b) with reductant-oxidant; (c) with different metal ions; (d) with different additives. (all samples were detected directly without delay at 260 nm).

nearly the same as that of punicalagin solution. So when punicalagin is extracted or separated, ironworks and copper vessels should not be used.

Figure 4(d) shows that the stability of punicalagin was not affected when most commonly used food and cosmetic additives were added except vitamin C. The absorbance of punicalagin with Vc was about two times as much as those of punicalagin with other additives. The absorption maximum of Vc is 245 nm and the absorbance of punicalagin increased obviously because of the existing of Vc. The presence of sod citrate changed the pH value of the punicalagin solution, so the absorption of punicalagin solution increased. The stability of punicalagin with commonly used food and cosmetic additives made it possible to become the ingredient of new functional food and cosmetic. The errors in these measurements were ranged from 3% to 8%.

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