Degradation kinetics of fisetin and quercetin in solutions as effected by pH, temperature and coexisted proteins

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Running title: DEGRADATION KINETICS OF TWO FLAVONOIDS

Abstract

Impacts of pH, temperature and coexisted proteins on degradation kinetics of two flavonoids fisetin and quercetin were assessed by spectroscopic method in the present study. Based on their degradation rate constants (*k*), fisetin was more stable than quercetin. Increasing medium pH from 6.0 to 7.5 at 37 °C would enhance respective *k* values of fisetin and quercetin from 8.30×10^{-3} and 2.81×10^{-2} to 0.202 and 0.375 h^{-1} ($P<0.05$). In comparison with their degradation at 37 °C, fisetin and quercetin at higher temperature showed larger respective *k* values (0.124 and 0.245 h^{-1} at 50 °C, or 0.490 and 1.42 h^{-1} at 65 °C). Four protein products in medium provided stabilization to both fisetin and quercetin ($P<0.05$), as protein addition at 0.10 g L^{-1} could decrease respective *k* values to 2.28×10^{-2}–2.98×10^{-2} and 4.37×10^{-2}–5.97×10^{-2} h^{-1}. Hydrophobic interaction between the proteins and the two flavonoids was evidenced responsible for the stabilization mostly, as sodium dodecyl sulfate could destroy the stabilization significantly ($P<0.05$). Both casein and soybean protein products provided greater stabilization than whey protein isolate. It thus concluded that higher temperature and alkaline pH will bring about greater flavonoid loss, but proteins can inhibit flavonoid degradation.

***Keywords**:* fisetin; quercetin; degradation kinetics; temperature; pH; proteins

INTRODUCTION

Flavonoids are plant-derived polyphenolic compounds with a chemical structure characterized by a C6-C3-C6 backbone,[1] and are categorized mainly into six groups as flavones, flavonols, flavanols, flavanones, flavanonols, and isoflavones. Flavonoids are usually abundant in plant-based foods and beverages such as onions, apples, berries, tea, and red wine, and are the most important components of the well-known phytochemicals. Many researchers have focused on their attention on the health benefits of flavonoid to the body, for example, their antioxidant and especially anti-cancer properties.[2] Flavonoid compounds such as myricetin, quercetin, and rutin have been reported to have activity to protect DNA damage in both Caco-2 and Hep G2 cells induced by H_2O_2,[3] while those from cocoa are able to prevent high glucose-induced oxidative stress on HepG2 cells.[4] Anticancer properties of flavonoids are among the most studied topics, for example, both flavones and flavonols have been observed to have *in vitro* cytotoxicity to human oesophageal adenocarcinoma cell line (OE33) and human esophageal squamous cell carcinoma cell line (KYSE-510).[5,6] In total, potential health benefits of flavonoids have been widely studied and clarified. However, most of the past studies essentially focused on *in vitro* bioactivities of the flavonoids. Whether food processing and coexisted food components (*e.g.* proteins, carbohydrates and others) have impacts on bioactivity and stability of flavonoids, are not well-studied so far.

Flavonoids contain sensitive chemical groups and structural element in their molecules, and thus are susceptible to degradation accelerated by many factors. The chemical instability of flavonoids mainly comes from the hydroxyl groups and instable pyrone structure (*i.e.* the second ring).[7,8] It is observed that hydroxylation degrees of flavones and flavonols exert significantly influence on their stability, in a decreased order of resorcinol-type, catechol-type, and pyrogallol-type; however, glycosylation of those hydroxyl groups in the flavonoids obviously results in enhanced stability.[8] It has been found in the recent that flavonoids can interact with some food components, especially with those macromolecular materials.[1] Polyphenol-protein interaction can affect tea and coffee taste, antioxidant properties, and protein digestibility.[9] Complexation of tea polyphenols with milk proteins results in alteration on antioxidant activity of the polyphenols and secondary structure of the proteins.[10]

Interaction of starch with tannins and other phenolic compounds is also clarified adverse to starch digestibility.[11] And more, increasing protein level of the milk would decrease degradation of a flavonoid compound epigallocatechin gallate at two temperatures.[12]

Plant foods usually undergo necessary thermal processing and storage, during which flavonoids degradation occurs. The environmental conditions and the coexisted compounds or components might have influence on flavonoids degradation, consequentially, on flavonoids' bioactivities. Thereof, these factors should be assessed. Two flavonoid fisetin and quercetin are rich in strawberry and onion.[13] In the present study, their degradation kinetics in solutions was investigated via spectroscopic method. Impacts of pH, and temperature of the medium as well as some widely used protein ingredients on their degradation were assessed. The aim was to reveal chemical stability of two flavonoids and proteins stabilization towards them.

EXPERIMENTAL

Materials and chemicals

Fisetin and quercetin with purity larger than 98% were purchased from Shanghai Yousi Biotechnology Co. Ltd. (Shanghai, China) and Dalian Meilun Biotechnology Co. Ltd. (Dalian, China), respectively. Dimethyl sulfoxide (DMSO) was obtained from Solarbio Science & Technology Co. Ltd. (Beijing, China). Casein (protein content of 88.93% on dry basis) was purchased from Beijing Aoboxing Bio-Tech Co. Ltd. (Beijing, China). Defatted soybean flour was purchased from Harbin Hi-tech Soybean Food Co. Ltd. (Harbin, Heilongjiang, China). Whey protein isolate (WPI) (protein content of 87.95% on dry basis) was purchased from Brewster Dairy (Brewster, OH, USA). All other chemicals used were of analytical grade.

Preparation of protein samples

Defatted soybean flour was extracted at ambient temperature with 75% ethanol (v/v) by stirring the suspension for 12 h, using a defatted soybean flour-to-solvent ratio of 1:10 (w/v). After the extraction, the precipitate was collected and re-extracted again by the same ethanol solution until the separated ethanol solution no longer showed a yellow color upon addition of NaOH solution (100 mmol L^{-1}). The obtained precipitate *(i.e.* defatted and dephenolized

soybean flour) was dried at ambient temperature overnight, and used to prepare soybean protein isolate (SPI) as per the method of Petruccelli and Añón.[14] The SPI thus prepared was dephenolized SPI (assigned as DSPI), which was adjusted into pH 7.0, and freeze-dried to obtain powder sample. The DSPI solution prior to freeze-drying was heated at 85 °C for 20 min, cooled, freeze-dried to obtain thermal denatured DSPI, and assigned as DDSPI.

Protein samples prepared were detected for their protein content by the classic Kjeldahl method,[15] using a conversion factor of 6.25.

Spectroscopic analysis of fisetin and quercetin solutions during storage

Both fisetin and quercetin were dissolved in DMSO to prepare stock solutions of 300 mmol L^{-1}, and then were stored at 4 °C before using. The stock solutions were diluted to 3 mmol L^{-1} with ethanol, and then further diluted by a phosphate buffer (100 mmol L^{-1}, pH 6.8) to 30 μmol L^{-1}. The diluted two solutions were incubated in a water bath of 37 °C. After the incubation times of 1–6 hours, their spectra (200–600 nm) were recorded at a UV-visible spectrophotometer (UV-2401 PC, Shimadzu Co. Kyoto, Japan), using the buffer as blank.

Assaying of degradation kinetics of fisetin and quercetin at different temperatures and pH values

The stock fisetin and quercetin solutions were diluted to 3 mmol L^{-1} with ethanol, and then diluted by three phosphate buffers (100 mmol L^{-1}, pH 6.0, 6.8 and 7.5), respectively, to a concentration near 30 μmol L^{-1}. The diluted solutions of pH 6.8 were incubated in a water bath at three respective temperatures (37, 50, and 65 °C). The diluted solutions of pH 6.0 and 7.5 were incubated in a water bath at 37 °C. At different incubation time intervals, the solutions were measured at 360 and 368 nm to detect residual fisetin and quercetin concentrations, respectively, by using the UV-visible spectrophotometer and the respective buffers as blanks. The fisetin and quercetin concentrations were calculated based on the standard curves generated prior to the measurement.

To ensure efficient degradation and precise spectroscopic analysis, the incubation time intervals used for fisetin and quercetin solutions were as following. At pH 6.8, the fisetin solutions were measured at every 60 (37 °C), 30 (50 °C), and 12 (65 °C) min, while the quercetin solutions were measured at every 30 (37 °C), 15 (50 °C), and 6 (65 °C) min, respectively. At pH 6.0 and 7.5, the fisetin solutions were measured at every 120 and 30 min,

while the quercetin solutions were measured at every 60 and 30 min, respectively.

Assaying of stabilization of coexisted proteins towards fisetin and quercetin

Four protein samples, including casein, DSPI, DDSPI and WPI, were all dispersed in a phosphate buffer (100 mmol L^{-1}, pH 6.8), respectively. The stock fisetin and quercetin solutions were also diluted as above by ethanol and the buffer, but merged with the prepared protein solutions, to give final fisetin and quercetin concentrations near 30 μmol L^{-1} as well as protein concentrations of 0.05–0.20 (casein) and 0.10 (other proteins) g L^{-1}, respectively. After that, the generated solutions were kept in a water bath of 37 °C, and measured for residual fisetin and quercetin as above. The time intervals used for fisetin and quercetin solutions were 60 and 30 min, respectively. Quantitative analysis of fisetin and quercetin were also based on the generated standard curves.

Statistical analyses and calculation of degradation rate constants

All experiments and analyses were performed in triplicate. Data were analyzed by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA), and expressed as means ± standard deviations. Degradation rate constants (k) of fisetin and quercetin were calculated by using a linear regression model of first-order reaction.[16]

RESULTS AND DISCUSSION

Degradation kinetics of fisetin and quercetin as well as the effects of pH and temperature

Both fisetin and quercetin exhibited chemical instability in solutions, reflected by the obtained spectra scanning at 200–600 nm (Fig. 1). The spectra demonstrate that fisetin and quercetin in the solutions gave stronger absorption around 360 and 368 nm, respectively. However, when be keeping at 37 °C for longer time, the two solutions showed decreased absorption intensities in the two regions, behaving a time-dependent manner (Fig. 1A&B). No new absorption peak was clearly observed in the measured wavelength range. These spectra validate that there occurred the degradation of fisetin and quercetin, and long-time resulted in them greater degradation.

<Insert Fig. 1 here>.

The data in Fig. 2 report the influences of two factors (pH and temperature) of the medium on the degradation of fisetin and quercetin, which demonstrate that both fisetin and

quercetin in the solutions behaved a decreasing trend in their contents during different time periods. By using the first-order reaction model, the respective degradation rate constants of fisetin and quercetin are thus calculated, and listed in Table I. Some results are therefore obtained, based on the changes of the rate constants under different pH values and temperatures. The first one is that fisetin was more stable than quercetin, because it gave smaller rate constants in all cases. The second one is that both fisetin and quercetin were sensitive to pH change, especially at an alkaline pH. Increasing pH value of the medium from 6.0 to 7.5, the *k* values of fisetin and quercetin were increased by 24- and 12-fold (from 8.30×10^{-3} and 2.81×10^{-2} to 0.202 and 0.375 h^{-1}, respectively). At the same time, if pH value of the medium was increased from 6.0 to 6.8, the respectively *k* values were 8.30×10^{-3} and 3.58×10^{-2} h^{-1}, respectively, yielding about 3- and 2-fold increased in rate constants only. The third one is that higher temperature conferred fisetin and quercetin with greater degradation, as both fisetin and quercetin showed larger rate constants at higher temperature. If fisetin and quercetin were kept at a temperature higher than 37 °C (*e.g.* 50 or 65 °C), the measured *k* value of fisetin was enhanced to 0.124 or 0.490 h^{-1}, while that of quercetin was enhanced to 0.245 or 1.42 h^{-1}.

<Insert Fig. 2 here>

<Insert Table I here>

Flavonoids in aqueous solutions show instability, resulting in loss of their contents (*i.e.* degradation). Stability of flavonoids depends on their chemical structures. For example, more hydroxyl groups in the molecule leads to lower stability.[8] Fisetin and quercetin has 4 and 5 hydroxyl groups, respectively; it is reasonable that quercetin is more liable than fisetin. The pH value of the medium has an important influence on the degradation of the flavonoids. A previous study had reported that plant phenolic compounds showed susceptibility to pH change.[17] When Buchner *et al.* assessed thermal degradation of quercetin and rutin at 100 °C in solutions from weaker acidic pH 5.0 to alkaline pH 8.0, they found two flavonoids were more instable in the alkaline pH.[7] Kırca *et al.* also studied the effect of pH on thermal stability of black carrot anthocyanins in the solutions of six pH values (2.5–7.0), and observed decreased anthocyanin stability at pH values larger than 5.0.[16] Tanchev and Joncheva had evaluated the degradation of cyanidin-3-rutinoside at 78 °C in citrate buffers of

pH 2.5, 3.5 and 4.5, respectively, and reported that the measured *k* values were 1.44×10^{-5}, 2.58×10^{-5}, and 2.80×10^{-5} sec^{-1};[18] that is, higher pH value brought about faster degradation. These mentioned studies shared similar conclusion to the present study, clarifying that fisetin and quercetin were more stable (but instable) in acidic (and alkaline) conditions.

Chemical reactions are accelerated by higher temperature. When black carrot was thermal treated at fixed pH 6.0, the *k* values of anthocyanins at a temperature range of 70–90 °C ranged from 4.15×10^{-2} to 0.138 h^{-1}.[16] If roselle anthocyanin in the solution was heated at five temperature levels of 60, 70, 80, 90 and 100 °C, the measured *k* values were 0.6×10^{-3}, 1.0×10^{-3}, 1.6×10^{-3}, 3.6×10^{-3}, and 7.9×10^{-3} min^{-1}, respectively.[19] De Paepe *et al*. studied the thermal treatment of apple juice samples over a temperature range of 80–145 °C, and also observed that the degradation of 39 phenolic compounds increased with temperature rise.[20] It is reasonable in the present study that higher temperature (50 or 65 °C) would result in both fisetin and quercetin larger rate constants.

Stabilization of coexisted proteins towards the degradation of fisetin and quercetin

When studying degradation of fisetin and quercetin in solutions of pH 6.8 at 37 °C, some proteins were added into medium as coexisted components, to clarify their effects on the degradation of fisetin and quercetin. Based on the measured data (Fig. 3), rate constants of fisetin and quercetin were calculated and given in Table II. These proteins effectively inhibited the degradation of fisetin and quercetin ($P<0.05$), as the *k* values were decreased in all cases. Rate constants of fisetin were decreased from 3.58×10^{-2} to 1.76×10^{-2}–2.98×10^{-2} h^{-1}, with decreasing levels about 17–51%. At the same time, *k* values of quercetin were decreased from 7.99×10^{-2} to 3.80×10^{-2}–5.97×10^{-2} h^{-1}, with decreasing levels about 25–52%. These coexisted proteins thus provided stabilization towards fisetin and quercetin in the solutions. It is speculated that potential interactions between these coexisted proteins and the two flavonoids were responsible for the stabilization.

<Insert Fig. 3 here>

<Insert Table II here>

Some interesting results can be obtained from the data in Table II. It is shown that casein at 0.20 g L^{-1} provided the greatest stabilization towards fisetin and quercetin, as their *k* values were the lowest (1.76×10^{-2} and 3.80×10^{-2} h^{-1}). Decreasing casein level brought about less

stabilization towards them, as the measured rate constants showed decreasing trends. Another milk protein product WPI (at 0.10 g L^{-1}) showed very weaker stabilization towards fisetin and quercetin than casein, as the measured rate constants become higher. Two prepared soybean protein products DSPI and DDSPI at 0.10 g L^{-1} exhibited the same stabilization towards fisetin than casein (k values 2.28×10^{-2} *versus* 2.32×10^{-2} h^{-1}), but provided slightly stronger stabilization towards quercetin than casein (k values 4.37×10^{-2} or 4.49×10^{-2} *versus* 5.36×10^{-2} h^{-1}). And more, thermal denaturation of soybean protein is observed to have unclear impact on the measured stabilization, because addition of DSPI and DDSPI in the medium resulted in fisetin and quercetin with similar rate constants. However, if SDS (anionic detergent) also existed in the medium, it diminished the stabilization of casein towards fisetin and quercetin clearly and effectively (P<0.05), as it could enhance respective k values of fisetin and quercetin from 2.37×10^{-2} and 5.36×10^{-2} to 2.73×10^{-2} and 7.21×10^{-2} h^{-1}. The respective k values of fisetin and quercetin without any coexisted proteins were 3.58×10^{-2} and 7.99×10^{-2} h^{-1} only. This fact points out that SDS was to efficient to diminish the interactions between the coexisted casein and the two flavonoids; consequentially, casein stabilization towards them did not existed any more.

Past studies have demonstrated that polyphenols are able to bind to proteins.[9, 10] Song *et al.* reported that green tea flavan-3-ols could complex with milk protein to enhance their stability.[12] Shpigelman *et al.* used native and thermally treated β-lactoglobulin to protect tea polyphenols, and found decreased degradation.[21] Xiao and Högger observed that myricetin had longer half period of life ($t_{1/2}$) in human plasma than in cell culture medium, suggesting that higher total protein concentration (61.7 *versus* 0.41 g L^{-1}) brought about greater myricetin stability.[8] The four protein products assessed in the present study were capable of decreasing degradation rate constants of the two flavonoids, especially casein and SPI. The present study shared the same conclusion to these mentioned studies, evidencing that these widely used protein products could provide beneficial effect on the stability of fisetin and quercetin. Based on literature data, both casein and soybean protein contain more hydrophobic amino acids than whey protein.[22] They thus could interact with the two flavonoids stronger than WPI. As the consequence, they at the same protein concentration could provide stronger stabilization than WPI. Hydrophobic interaction can be efficiently

destroyed by the detergents, for example, SDS. If SDS was added to fisetin and quercetin solutions together with casein, hydrophobic interaction between the two flavonoids and casein was destroyed mostly. Therefore, two flavonoids mostly were in free and therefore degraded quickly. It thus is concluded that hydrophobic interaction contributed the stabilization of casein mostly. However, stabilization of the proteins via hydrogen binding needs a quantitative assaying.

CONCLUSIONS

Stability and degradation kinetics of fisetin and quercetin in solutions are impacted by medium conditions including pH, temperature, and coexisted proteins. Quercetin with more hydroxyl groups is more instable than fisetin. Both fisetin and quercetin are more sensitive at alkaline pH and higher temperature, resulting larger rate constants. However, the coexisted proteins can provide stabilization to them mainly via hydrophobic interaction between the proteins and the flavonoids, resulting in decreased rate constants. Both casein and soybean protein products are more capable of providing greater stabilization than whey protein isolate.

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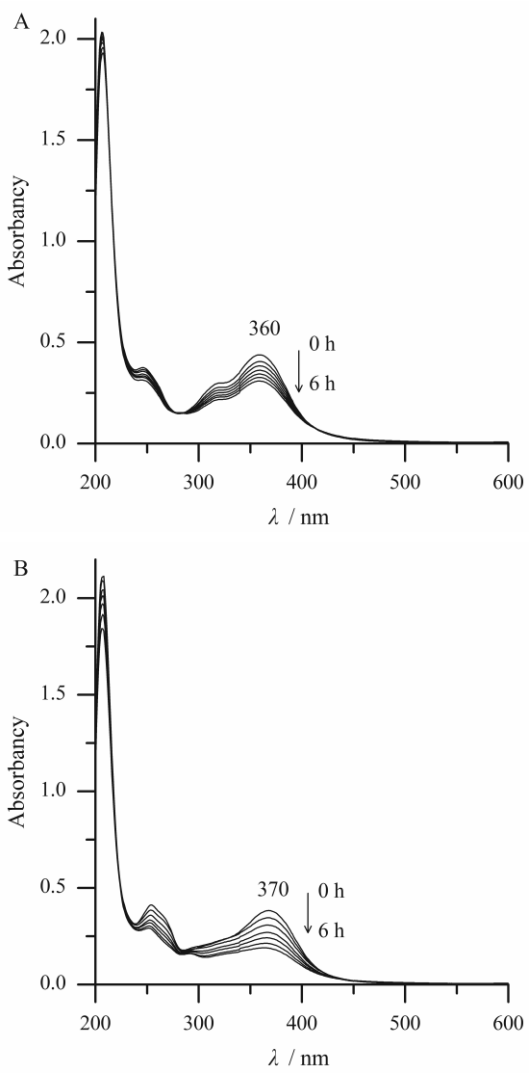
Figure Captions

Fig. 1 Measured absorption spectra of fisetin (A) and quercetin (B) solutions (30 μmol L^{-1}, in 100 mmol L^{-1} phosphate buffer, pH 6.8) kept at 37 °C for 0, 1, 2, 3, 4, 5, and 6 h, respectively.

Fig. 2 Detected residual concentrations of fisetin (A) and quercetin (B) in solutions subjected to different temperatures, pH values and time periods.

Fig. 3 The detected residual concentrations of fisetin (A) and quercetin (B) in solutions kept at 37 °C, pH 6.8 and different time periods, in the presence of the assessed coexisted components. DSPI, DDSPI, WPI, and SDS denote dephenolized soybean protein isolate, thermal denatured DSPI, whey protein isolate, and sodium dodecyl sulfate, respectively.



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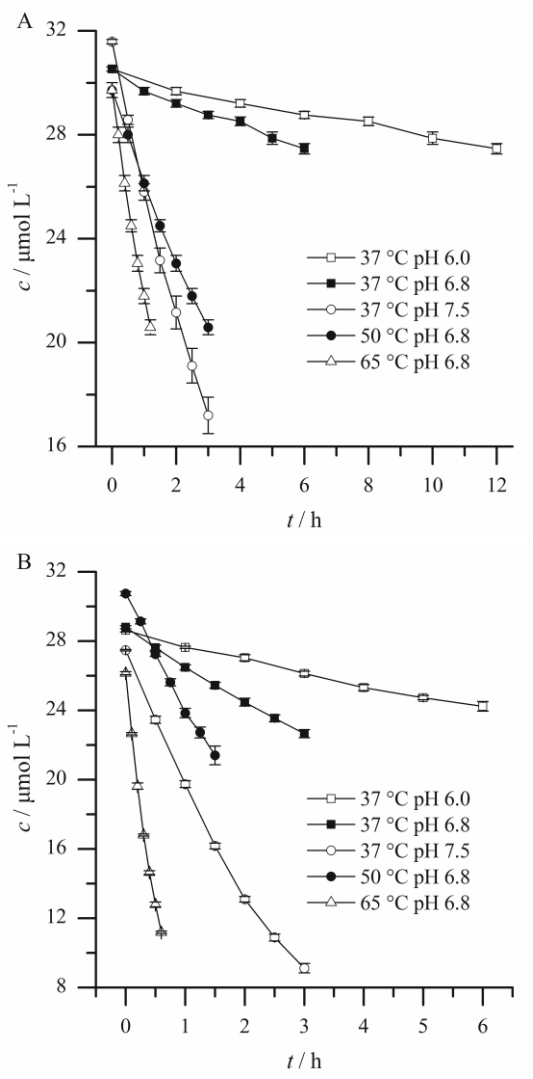

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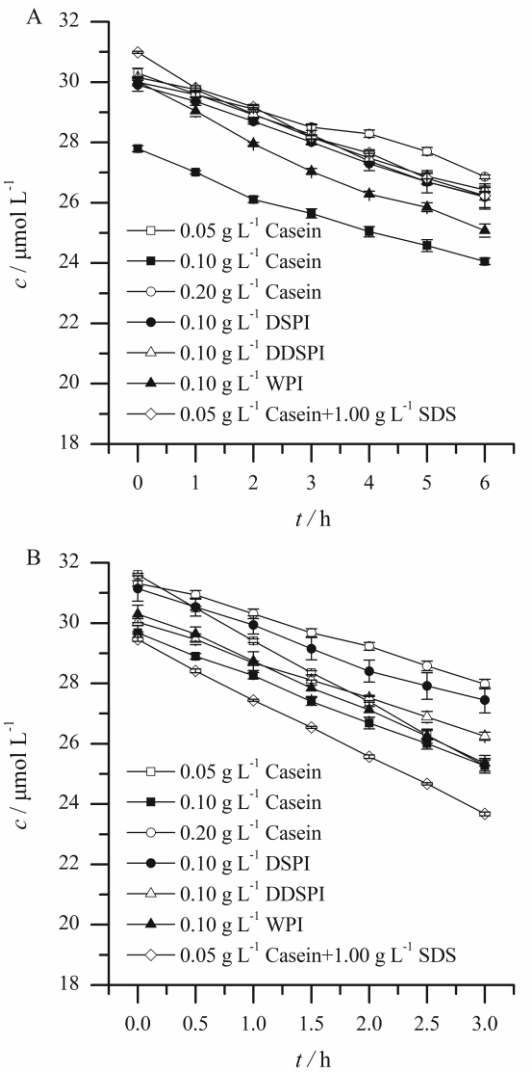

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Table Captions

TABLE I Impacts of temperatures and pH values of the medium on the degradation rate constants (*k*) of fisetin and quercetin in solutions

TABLE II Impacts of the coexisted components on the degradation rate constants (*k*) of fisetin and quercetin in solutions

TABLE I Impacts of temperature and pH values of the medium on the degradation rate constants (*k*) of fisetin and quercetin in solutions

Medium conditions		k / h^{-1}	
Temperatures, °C	pH values	Fisetin	Quercetin
37	6.0	$(8.30\pm0.45)\times10^{-3a}$	$(2.81\pm0.14)\times10^{-2a}$
37	6.8	$(3.58\pm0.11)\times10^{-2b}$	$(7.99\pm0.32)\times10^{-2b}$
37	7.5	0.202 ± 0.014^{c}	0.375 ± 0.008^{c}
50	6.8	0.124 ± 0.002^{d}	0.245 ± 0.011^{d}
65	6.8	0.490 ± 0.009^{e}	1.42 ± 0.10^{e}

Different lowercase letters after the mean values as the superscripts in same column indicate that one-way ANOVA of the mean values is significantly different ($P < 0.05$). Coefficient (R^2) for regression analysis of the rate constants ranged from 0.984 to 0.999.

TABLE II Impacts of the coexisted components on the degradation rate constants (*k*) of fisetin and quercetin in solutions

Coexisted components	Levels, g L^{-1}	k / h^{-1}	
		Fisetin	Quercetin
No	0	$(3.58\pm0.11)\times10^{-2e}$	$(7.99\pm0.32)\times10^{-2f}$
Casein	0.05	$(2.49\pm0.20)\times10^{-2b}$	$(7.42\pm0.20)\times10^{-2e}$
Casein	0.10	$(2.37\pm0.06)\times10^{-2b}$	$(5.36\pm0.29)\times10^{-2c}$
Casein	0.20	$(1.76\pm0.02)\times10^{-2a}$	$(3.80\pm0.10)\times10^{-2a}$
DSPI	0.10	$(2.28\pm0.13)\times10^{-2b}$	$(4.37\pm0.16)\times10^{-2b}$
DDSPI	0.10	$(2.32\pm0.15)\times10^{-2b}$	$(4.49\pm0.11)\times10^{-2b}$
WPI	0.10	$(2.98\pm0.03)\times10^{-2d}$	$(5.97\pm0.03)\times10^{-2d}$
Casein + SDS	0.10 + 1.00	$(2.73\pm0.10)\times10^{-2c}$	$(7.21\pm0.33)\times10^{-2e}$

DSPI, DDSPI, WPI, and SDS denote dephenolized soybean protein isolate, thermal denatured DSPI, whey protein isolate, and sodium dodecyl sulfate, respectively. Different lowercase letters after the mean values as the superscripts in same column indicate that one-way ANOVA of the mean values is significantly different ($P < 0.05$). Coefficient (R^2) for regression analysis of the rate constants ranged from 0.979 to 0.999.