FLUORESCENCE STUDIES ON FLAVONOID DIGLYCOSIDES AND CORRESPONDING DEGLYCOSYLATED MONOGLYCOSIDES[**]

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A fluorescence quantitative detection method suitable for high-throughput screening of rhamnosidase catalytic activity on naringin was established. Detection of prunin at $\lambda_{ex}/\lambda_{em}$ = 360/450 nm was based on the fluorescence difference after heating under alkaline conditions. Detection of naringin at $\lambda_{ex}/\lambda_{em}$ = 325/400 nm was based on the difference in fluorescence in phosphate buffer. The method is suitable for prescreening many samples. A similar method can detect the fluorescence of hesperidin and hesperitin-7-o-glucoside, which provides a reference for the rapid detection of diglycosides and corresponding deglycosylated monoglycosides.

Keywords: *fluorescence, naringin, prunin, diglycoside, monoglycoside.*

ФЛУОРЕСЦЕНТНЫЕ ИССЛЕДОВАНИЯ ФЛАВОНОИДНЫХ ДИГЛИКОЗИДОВ И ДЕГЛИКОЗИЛИРОВАННЫХ МОНОГЛИКОЗИДОВ

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УДК 535.37:612.396.175

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(Поступила 29 сентября 2021)

Разработан метод количественного определения флуоресценции, подходящий для высокопроизводительного скрининга каталитической активности рамнозидазы в отношении нарингина. Обнаружение прунина при $\lambda_{ex}/\lambda_{em}$ = 360/450 нм основано на различии флуоресценции после нагревания в щелочных условиях, обнаружение нарингина при $\lambda_{ex}/\lambda_{em}$ = 325/400 нм — на различии флуоресценции в фосфатном буфере. Предлагаемый метод подходит для предварительного отбора образцов и позволяет обнаруживать флуоресценцию гесперидина и гесперитин-7-о-глюкозида, что обеспечивает эталон для быстрого определения дигликозидов и соответствующих дегликозилированных моногликозидов.

Ключевые слова: *флуоресценция, нарингин, прунин, дигликозид, моногликозид.*

Introduction. Flavonoids are considered indispensable components in a variety of nutraceutical, pharmaceutical, medicinal, and cosmetic applications [1]. This is attributed to their anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme function [2–7]. Therefore, their occurrence in the human diet participates in preventing degenerative diseases [7]. In nature, flavonoids exist mostly in the form of glycosides [8]. Flavonoid glycosides are deglycosylated prior to intestinal uptake, whereas aglycones can freely penetrate through cell membranes [9, 10]. Some deglycosylated flavonoids have increased biological activity, the natural hydrolytic activity of glycosidases is widely employed in biotechnological deglycosylation processes of flavonoids, which produce respectively aglycones or partially deglycosylated flavonoids [11]. Therefore, the detection of flavonoids and deglycosylated flavonoids has widespread applications.

[**]Full text is published in JAS V. 90, No. 1 (http://springer.com/journal/10812) and in electronic version of ZhPS V. 90, No. 1 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

Gas chromatography, capillary electrophoresis, and thin-layer chromatography have been employed for the detection of flavonoids. The most important analytical methods are based on liquid chromatography (LC) [12–15]. When high-throughput screening is required, these methods are time consuming. For example, site-saturation mutagenesis is widely used to substitute targeted residues to any other naturally occurring amino acid, which modifies different enzyme properties [16]. This technology often needs to be combined with high-throughput screening. The method within this paper distinguishes flavonoids and their corresponding deglycosylated flavonoids.

Fluorescence detection is advantageous for high-throughput screening due to high sensitivity, simple operation, and fast response. Generally, molecules with fluorescence have the following molecular characteristics: conjugated π bond system, which contains aromatic rings; rigid-plane structure; substituent electron donating groups, such as −OH, −NH2, −NR2, and −OR; and singlet excited state S_1 of π,π^* type. Flavonoids contain conjugated rigid structures that may produce endogenous fluorescence or sensitized fluorescence through C-ring cleavage reactions [11]. There are few studies of the fluorescence properties of flavonoids, with most discussing the binding of flavonoids to proteins or as protein quenchers. Few papers used the principle of fluorescence analysis to study flavonoids [17]. Heating flavonoid aglycone under alkaline conditions opens the pyran ring. Li et al. and Zhang proved that different aglycones showed distinguished fluorescences [11, 18]. However, few studies distinguish flavonoid diglycosides from their corresponding deglycosylated monoglycosides [11, 17, 19].

Distinguishing flavonoid diglycosides and corresponding deglycosylated monoglycosides is a focus of this study. Naringin is a natural flavonoid diglycoside found in Chinese herbal medicines and citrus fruits [20]. Naringin and its corresponding deglycosylated monoglycoside, prunin, possess numerous biological properties [21, 22]. Naringin is a promising candidate owing to its multifaceted effect on bone tissues [23]. Prunin is a candidate for further development as a human enterovirus A71 therapeutic agent [24]. Both are important in debittering fruit juice [25, 26]. We optimized the heating and alkaline conditions and established a fluorescence detection method to distinguish them. To test the generality of the method, other flavonoid diglycosides and their corresponding deglycosylated monoglycosides were also investigated, including rutin and isoquercetin; hesperidin, neohesperidin and hesperitin-7-o-glucoside; and narirutin and prunin.

Experimental. All fluorescence spectra were recorded using a Cary Eclipse molecular fluorescence spectrometer (Agilent, Australia). All pH measurements were carried out with a FE28 pH meter (Mettler Toledo, China). Temperature was controlled using a DK-8D digital constant temperature water bath (Guohua, China). NaOH (AR, TCM), disodium hydrogen phosphate (AR, TCM), citric acid (AR, TCM), rutin, naringin, hesperidin, prunin (purchased from Xi'an Xiaocao Plant Technology Co., Ltd.), neohesperidin, narirutin, isoquercitrin, and hesperitin-7-o-glucoside (purchased from Shanghai Yuanye Biotechnology Co., Ltd.). Phosphate buffer solutions were prepared by mixing 0.2 M disodium hydrogen phosphate and 0.1 M citric acid solution; all aqueous solutions were prepared with distilled water and stored at 25°C.

Prepare pH 6.0, 0.06 mM phosphate buffer solutions of naringin and prunin. To detect prunin, each mixed with an equal volume of 6M NaOH, the mixed solutions were heated in a water bath at 90°C for 2.5 h and then placed at room temperature for 3.5 h. The scanning three-dimensional fluorescence spectrum was obtained under the conditions of excitation light 320–400 nm, emission light 400–500 nm, slit 20, and voltage 600 V. To detect naringin, each was heated in boiling water for 10 min and then placed at room temperature for 3.5 h. The scanning three-dimensional fluorescence spectrum was obtained under the conditions of excitation light 280–400 nm, emission light 350–500 nm, slit 20, and voltage 600 V.

Considering the very high concentration of NaOH, the effect of phosphate buffer on the final pH can be ignored. All experiments were performed by mixing the solution with a strong base in equal volumes. To investigate the effect of water bath time, the final prunin or naringin solution was compared with a blank containing the phosphate buffer mixed with a strong base in equal volumes. The water bath temperature was 90°C, bath time was set at 2.0–3.5 h, placement at room temperature for 3.5 h, and the fluorescence intensity at $\lambda_{ex}/\lambda_{em}$ = 360/450 nm was scanned.

The longer the placement time at room temperature, the weaker the fluorescence, with naringin itself being very low and not an object of investigation. In order to cool to room temperature and investigate the effect of placement time on the main detection substance prunin, the placement time was set at 2.0–3.5 h and the bath time was set at 2.5 h.

In order to investigate the effect of placement time at room temperature on naringin, 0.06 mM naringin in pH 6.0 phosphate buffer and blank-pH 6.0 phosphate buffer were in a boiling water bath for 10 min, then placed for 2.0–3.5 h, the fluorescence intensity was scanned at $\lambda_{ex}/\lambda_{em}$ = 325/400 nm.

The water temperature was set at 70–100°C, the bath time was set at 2.5 h, and the placement time was set at 2 h. The concentration of NaOH was set at 2–6 M, the bath time was set at 2.5 h, the placement time was set at 2 h, and the bath temperature was set at 90°C. According to the results of single-factor experiments, prunin was dissolved in phosphate buffer solution (pH 6.0) containing 0.06 mM naringin to make standard solutions of different concentrations (0–0.06 mM). The sample was mixed with the volume of 6 M NaOH, in a water bath at 90°C for 2.5 h, placed at room temperature for 2 h, and scanned for fluorescence intensity at $\lambda_{ex}/\lambda_{em}$ = 360/450 nm without subtracting the blank. Naringin was dissolved in phosphate buffer solution (pH 6.0) containing 0.06 mM prunin to make standard solutions of different concentrations (0–0.08 mM). The boiling water bath was for 10 min and the placement time at room temperature was 2 h. The fluorescence spectrum at $\lambda_{ex}/\lambda_{em}$ = 325/400 nm was scanned and the standard curve was fitted without subtracting the blank.

A series of mixed solutions of naringin and prunin with different concentrations were prepared. One part was added with equal volume of 6M NaOH, and then heated in water at 90°C for 2.5 h and placed at room temperature for 2 h. The other part was heated in boiling water for 10 min and placed at room temperature for 2 h. Without subtracting the blank, the fluorescence at $\lambda_{ex}/\lambda_{em}$ = 360/450 and 325/400 nm was scanned and compared with the values obtained from the standard curve.

The detection limit was determined by measuring the blank 20 times according to the method of the International Union of Pure and Applied Chemistry (IUPAC). In order to extend the application of this method, we studied some other flavonoids and their hydrolysates, including rutin and isoquercetin; hesperidin, neohesperidin, and hesperitin-7-o-glucoside; and narirutin and prunin. Excel 2016 was used for the statistical analysis. Data were graphed using OriginPro 2018 (OriginLab, USA) and GraphPad Prism 8 (GraphPad Software, USA). Three replicates were set for each group of samples, performing three parallel determinations. The data are expressed as the mean ± SD.

Results and discussion. *Fluorescence spectra of naringin and prunin.* Naringin and its hydrolysate prunin are 7-hydroxy-substituted flavanones [18]. In general, the fluorescence of naringin overlaps with that of prunin, and the fluorescence of naringin is stronger than that of prunin, which is not conducive to the quantitative analysis of prunin. However, we found that the ring opening product of prunin has one more fluorescence peak than that of naringin after a water bath under alkaline conditions.

According to the difference in fluorescence from Fig. 1, we chose $\lambda_{ex}/\lambda_{em}$ = 360/450 nm as the conditions for detecting prunin. Although the fluorescence intensity of prunin is not the strongest under this condition, the fluorescence intensity of naringin is weak. Under alkaline conditions, naringin and prunin will become chalcone type, and further heating will lead to the pyrolysis and ionization of hydrogen atoms [19]. The structure of prunin is one less rhamnosyl than naringin, which may lead to inconsistency in the degree of cleavage of glycosidic bonds (Fig. 2). We also find that after heating under strong alkaline conditions, the end product of naringin has a glucose group, and the end product of prunin has a group O^- at the same positi on. O^- has strong electronegativity and electron-withdrawing effect, but at the same time, conjugates with benzene ring and has an electron-donating effect stronger than its electron-withdrawing effect, resulting in an electron-donating group, thereby accounting for the difference in their fluorescence in Fig. 1. We speculate that the electron-donating group at this position may enhance the fluorescence of flavonoids in the form of chalcone.

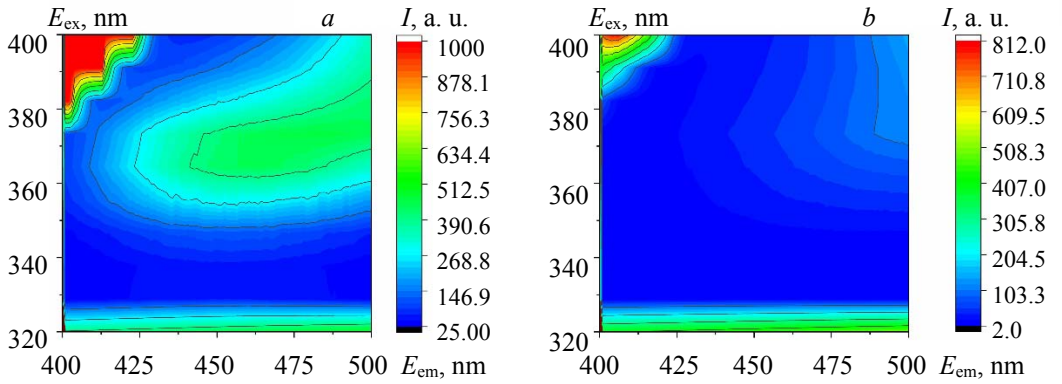

Fig. 1. The fluorescence spectra of prunin (a) and naringin (b) after a water bath under alkaline conditions.

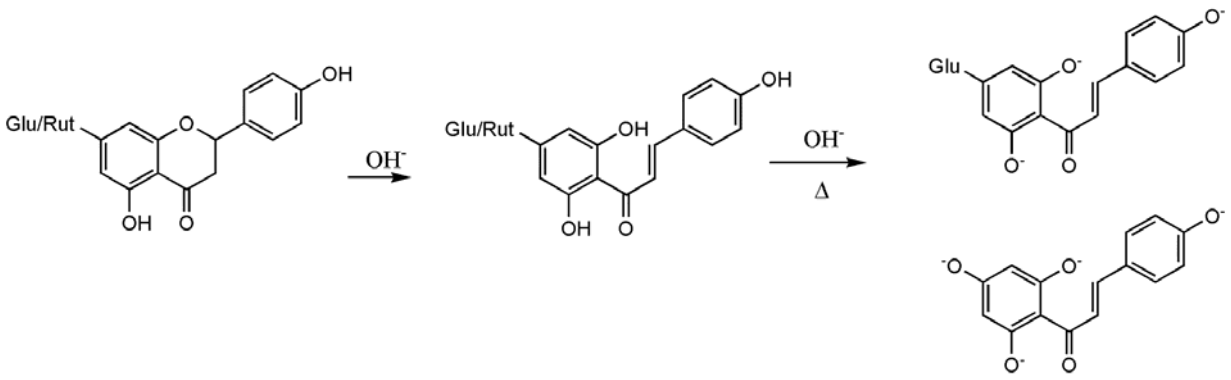

Fig. 2. Pyrolysis and ionization process of naringin or prunin [17].

Phosphate buffer is a common enzyme reaction media. Reaction is terminated in boiling water bath for about 10 min. Therefore, the fluorescence of naringin and its hydrolysate prunin in phosphate buffer were investigated after 10 min in a boiling water bath and placement for 3.5 h at room temperature to inspect differences between them.

Figure 3 shows that the fluorescence of naringin is strong at $\lambda_{ex}/\lambda_{em}$ = 325/400 nm. Prunin has almost no fluorescence here, which can be used as the basis for the quantitative analysis of naringin. In this case, both of them exist as molecular forms [17] and different than the ionization state after heating under strong alkaline conditions (Fig. 2), when the two exist in molecular form; that is, in the form of flavanone, compared with the glucose group of prunin, the rutinose group of naringin has an enhanced effect on the fluorescence of flavanone. Li et al. found that the electron-donating group has the effect of weakening fluorescence for flavanone, which is contrary to the usual [18]. Therefore, compared with the glucose group, the rutinose group may have a stronger electron-withdrawing effect, the more rhamnosyl of naringin is the reason for its high fluorescence. This also explains the significant difference in fluorescence between the two in Fig. 3.

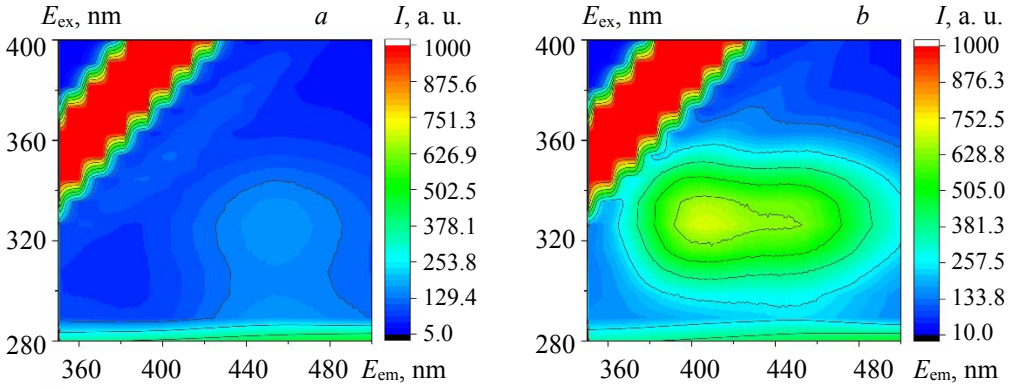

Fig. 3. The fluorescence spectrum of prunin (a) and naringin (b) in phosphate buffer solution.

Condition optimization. In order to obtain more convenient fluorescence detection conditions of prunin and naringin, the water bath time, temperature, placement time, and NaOH concentration, which affect fluorescence intensity, were optimized.

Figure 4a shows that with the extension of water bath time, the fluorescence of prunin gradually increases, whereas the fluorescence of naringin increases first and then decreases. In Figure 4b, in order to cool to room temperature, the minimum cooling time is set at 2 h. With the increase in placement time, the fluorescence of prunin decreases gradually, but placement time has no effect on the detection of naringin. Considering the consistency with the detection of prunin, the placement time is selected as 2 h. Figure 4c shows that the increase in NaOH concentration will lead to the increase in fluorescence intensity of prunin, whereas the fluorescence intensity of naringin first increases and then decreases. Figure 4d shows that the increase in temperature will lead to an increase in the fluorescence intensity of prunin and naringin. However, the flu-

orescence of naringin is weak, and the boiling water bath is inconvenient. To sum up, considering the time cost and convenience, the detection conditions of prunin are as follows: mix with 6 M NaOH, water bath for 2.5 h at 90°C, placing for 2 h, the corresponding detection of naringin is a boiling water bath for 10 min and placing for 2 h.

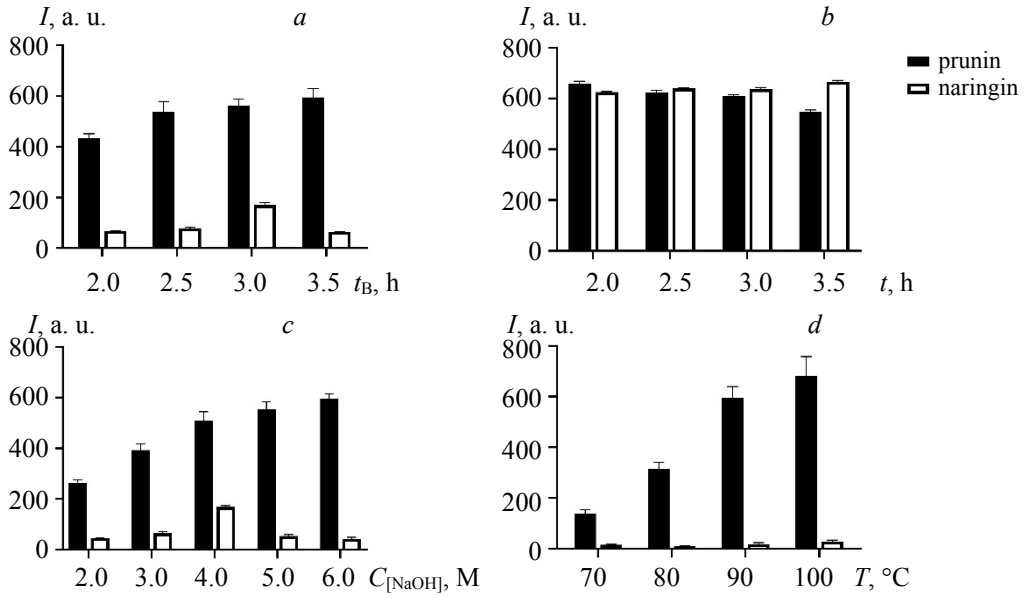

Fig. 4. Effect of different factors on the fluorescence intensity of prunin and naringin: (a) bath time, (b) placement time, (c) NaOH concentration, (d) temperature.

Concentration standard curve. In general, the detection samples contain both prunin and naringin. Therefore, the solution of the standard curve was selected as phosphate buffer with a certain concentration of nondetection substance to reduce the error.

Figure 5a shows that the fitting linear equation of prunin is $y = 9607.8x + 177.53$, $R^2 = 0.9914$. Figure 5b shows that the fitting linear equation of naringin is $y = 8400.9x + 159.55$, $R^2 = 0.9917$, and the results show that the fitting is good within the linear range and meets the requirements of prescreening.

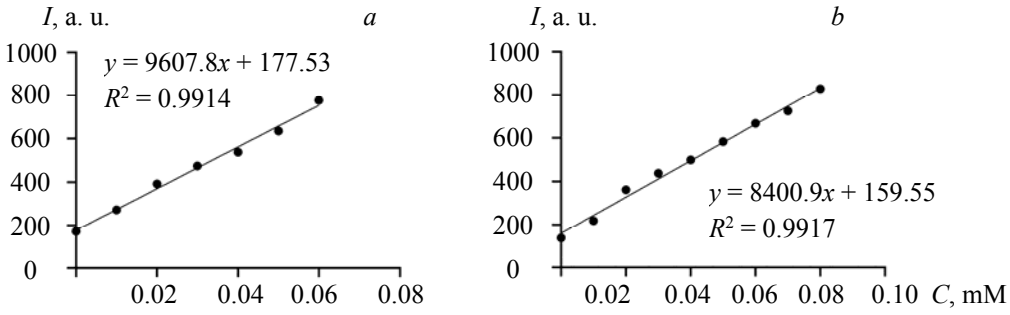

Fig. 5. Standard curve of prunin (a) and naringin (b).

Recovery rate of standard addition. Table 1 shows that, except for sample No. 9, the recoveries of prunin and naringin are between 94.5–126.3 and 81.6–120.6%, which meet the requirements for high-throughput prescreening. The recovery of sample No. 9 deviates from the normal size; the reason may be that the concentration of both is too low. In practical applications, such as high-throughput screening of rhamnosidase activity, the total concentrations of naringin and prunin are generally fixed, and they are not very low concentrations, so our method still has practical application value.

TABLE 1. Prediction of Concentrations and Recoveries of Prunin and Naringin in Samples Based on Standard Curve

Sample No.	Additive concentration, mM		Fluorescence intensity, a.u.		Predicted concentration, mM		Recovery, %	
	Prunin	Naringin	Prunin	Naringin	Prunin	Naringin	Prunin	Naringin
1	0.06	0.08	766.58	737.13	0.0613	0.0688	102.2	85.9
2	0.06	0.04	775.08	453.42	0.0622	0.0350	103.7	87.5
3	0.06	0.02	905.35	362.17	0.0758	0.0241	126.3	120.6
4	0.03	0.08	470.50	741.67	0.0305	0.0693	101.6	86.6
5	0.03	0.04	524.91	452.24	0.0362	0.0348	120.5	87.1
6	0.03	0.02	531.27	296.62	0.0368	0.0163	122.7	81.6
7	0.01	0.08	268.33	858.29	0.0095	0.0832	94.5	104.0
8	0.01	0.04	271.84	524.54	0.0098	0.0434	98.2	108.6
9	0.01	0.02	328.31	268.15	0.0157	0.0129	156.9	64.6

Detection limit. According to the formula of the IUPAC the detection limit of naringin is $X_L = X_0 + KS_0 = 158.94+3\times9.91 = 188.67$, and the detection limit of prunin is $X_L = X_0+KS_0 = 179.34+3\times9.53 = 207.93$. The fluorescence detection limits of naringin and prunin reach 188.67 and 207.93 a.u., respectively; the corresponding concentrations of naringin and prunin are 0.0035 and 0.0032 mM, which shows good sensitivity.

Fluorescence differences of other valuable flavonoids. We found that the fluorescence of prunin and naringin could be distinguished after heating under strong alkaline conditions, and established the quantitative detection method. In order to expand its application, some common flavonoids in practical application were tested in Table 2.

TABLE 2. Wavelength Range in Fluorescence Detection of Some Flavonoids Under Two Method Conditions

Flavonoid		Method A		Method B	
Diglycoside	Monoglycoside	λ_{ex}, nm	λ_{em}, nm	λ_{ex}, nm	λ_{em}, nm
Rutin	Isoquercitrin	300–550	350–700	300–550	350–700
Hesperidin	Hesperitin-7-o-glucoside				
Neohesperidin	Hesperitin-7-o-glucoside			280–500	320–600
Narirutin	Prunin				

N o t e. Slit 20, voltage 600 V. In method A, mix 0.12 mM flavonoids with 6 M NaOH in equal volume, water bath at 90°C for 2.5 h, and place for 2 h. In method B, heat 0.06 mM flavonoids in boiling water bath for 10 min, and place for 2h.

For these common diglycosides and corresponding monoglycosides, we detected their fluorescence. Table 3 shows that the fluorescence of diglycosides is different from that of monoglycosides in phosphate buffer solution. Rutin has no fluorescence, and isoquercitrin has fluorescence. The fluorescence peak positions of hesperitin-7-o-glucoside are different than those for hesperidin and neohesperidin. The fluorescence of narirutin is stronger than that of prunin. However, after heating under strong alkaline conditions, only hesperidin and corresponding hesperitin-7-o-glucoside show a difference in fluorescence than that in phosphate buffer. The applicability of this method is closely related to the structure of flavonoids, and the specific relationship needs further experiments.

TABLE 3. Fluorescence Difference of Some Flavonoids Under Two Method Conditions

Flavonoid		Method A	Method B
Rutin	Isoquercitrin	−	+
Hesperidin	Hesperitin-7-o-glucoside	+	+
Neohesperidin	Hesperitin-7-o-glucoside	−	+
Narirutin	Prunin	−	+

N o t e. The sign + indicates that the fluorescence differences of diglycoside and corresponding monoglycoside are obvious and can be distinguished; the sign − indicates that the fluorescence differences of diglycoside and corresponding monoglycoside are not obvious or cannot be distinguished.

Conclusions. A fluorescence detection method for the rapid detection of naringin and prunin was established. The common diglycosides and corresponding deglycosylated monoglycosides in practical application were tested by similar methods. Compared with fluorescence detection of flavonoid aglycones studied by former researchers, the applicability of this method is broadened to flavonoid glycoside. Compared with the LC-based method, our method is more convenient for high-throughput screening. The recovery rate of standard addition in this study is not ideal when the concentrations of naringin and prunin are both low. Considering that the situation appears rarely in practical application such as high-throughput screening of rhamnosidase activity, the applicability of our method is still valid. In addition, in order to further develop this method, several common flavonoid glycosides in application were detected. Only hesperidin and corresponding hesperitin-7-o-glucoside are applicable. Therefore, the relationship between the structure of flavone glycosides and the applicability of this method need further study.

Acknowledgments. We greatly appreciate useful discussion from Qi Chen, Sun Jiang, and Zhang Yichun, appreciate the funding support from the National Natural Science Foundation of China (No. U1805235), the Science and technology project of Xiamen City, Fujian Province, China (No. 3502Z20203058), and the Central financial award project of the modern agricultural industrial park in Pinghe County, Fujian Province, China (No. PH2020) for this project.

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