

# Content determination of the flavonoids in the different parts and different species of *Abelmoschus esculentus* L. by reversed phase-high performance liquid chromatograph and colorimetric method

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## ABSTRACT

**Background:** This research will establish the ultraviolet colorimetric method to determine the total flavonoid content in different species and different parts of *Abelmoschus esculentus* L. **Materials and Methods:** We establish the reversed-phase high-performance liquid chromatograph (RP-HPLC) method to determine the content of the three flavonoid glycosides in different species and different parts of the *A. esculentus*. Adopt the  $\text{NaNO}_2$ -Al  $(\text{NO}_3)_3$ -NaOH colorimetric method to determine the total flavonoid content; at the same time, adopt the RP-HPLC method to determine the contents of the three flavonoid glycosides. Using the methods of ultraviolet colorimetry and RP-HPLC, we determined and analyzed the total flavonoid content and the content of the three flavonoid glycosides in different species and different parts of *A. esculentus*. **Results:** There are great distribution differences of the total flavonoids and the three flavonoid glycosides in different species and parts of *A. esculentus*. Among them, the content of the effective constituents in the flower is relatively high, next is in the fruit. In the different species of *A. esculentus*, the content of the flavonoids of finger relatively high. The HPLC method established in this research is simple and convenient and its results are accurate and reliable. In addition, it has a very good repeatability. **Conclusion:** The results provided the reference data for the medicinal use of *A. esculentus* and it can be used in quality analyzing of its effective constituents.

**Key words:** *Abelmoschus esculentus*, flavonoid glycoside, reversed-phase high-performance liquid chromatograph, total flavonoids

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## INTRODUCTION

*Abelmoschus esculentus* L., scientific name “coffee abelmosk,” belongs to okra genus, *Malvaceae* family. It is annual herbal plants, which is widely cultivated in the northern part of China. Because its shape of the fruit is much like the goat horn, it is vividly called “goat horn beans,” or “lady finger okra.” *A. esculentus* loves the warm weather and has strong heat-resisting power,<sup>[1-4]</sup> so it can be used as a kind of rare vegetable in hot season. Now-a-days, *A. esculentus* has been designated as the first-choice vegetable as well as the health food for the elderly. In the United States, Japan and many other countries, it is called “botanical vigor” or “green

ginseng” and is a kind of excellent dietary health vegetable with very high nutritional value.

It is reported in the literature that there are many effective constituents such as flavone, polysaccharide, pectin, trace elements and amino acid in *A. esculentus*. It has the medical effects of anti-aging, anti-fatigue, strengthening people's immunity, protecting our livers and strengthening our kidney, reducing blood sugar, assisting our digestive power, getting fever away and improving our eyesight, invigorating the stomach and moistening our intestines,<sup>[5-14]</sup> etc.

There is much flavonoid in the different parts of *A. esculentus*. It has been known that flavonoid compounds are a very important bioactive substance existing in the plant world. According to the research reports, it can not only be used as a kind of medicine for preventing cardiovascular and cerebrovascular diseases, but can also have the following

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physiological activities like anti-lipid peroxidation, anti-aging, removing free-radicals, reducing blood fat, reducing cholesterol, reducing blood sugar level, anticancer and cancer prevention, immune regulation, etc. Therefore, this herbal plant has a wide application prospect in the areas of nutrition, health care and disease – prevention; it is also a extensively used natural oxidation inhibitor and preservative. It can be used as health food and cosmetics with very good development and practical value.

*A. esculentus* is appreciated more and more for its excellent medicinal and health care functions. Hence we can greatly enhance its added value and its utilization rate if we can make a deep-processing and development for this precious food-and-medicine plant. Through literature retrieval, there has been no report on establishing the reversed-phase high-performance liquid chromatograph (RP-HPLC) method to determine the flavonoid content in *A. esculentus*. This paper adopted the ultraviolet colorimetric method to determine the total flavonoid contents in *A. esculentus*. At the same time, we established the RP-HPLC method to determine the contents of the three flavonoid glycosides in different species and different parts of the *A. esculentus*. It provided the reference data for the quantitative analysis of its flavonoid constituents and for its further development and utilization.

## MATERIALS AND METHODS

### Instruments and reagents

Waters HPLC (Waters 2695) HPLC, binary solvent managing device, automatic sample-feeder, Waters 2996 photodiode array detector, binary high-pressure pump, chromatographic working station. UV-2102 PCS ultraviolet and visible spectrophotometer (Shanghai Unico Instrument Corp., Ltd.), Millipore Simplicity-type ultra-pure water maker (Millipore Corp.), Auto Science Solvent filtration device, KQ-250B-type supersonic cleaning device (Kunshan Supersonic Instrument Corp., Ltd.); R201B rotary evaporimeter (Shanghai Shensheng Biotechnological Corp., Ltd.). The methanol is chromatographical pure, the water is super-pure water, the other reagents are all analytical pure.

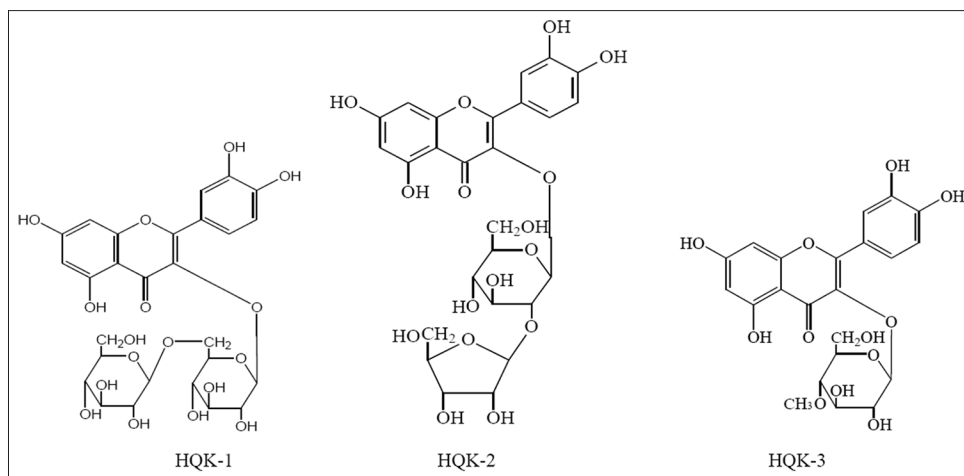
The *A. esculentus* sample used in the experiment was picked up in the vegetable test bases of Zhejiang Agriculture Academy and Zhejiang Agriculture and forestry University in August, 2010. It was also determined as the *Malvaceae* genus, *A. esculentus*. This plant includes six species: *A. esculentus* of finger, *A. esculentus* of red, *A. esculentus* of green, *A. esculentus* of pentagram, *A. esculentus* of five blessings, *A. esculentus* of pentagon. The samples are prepared for later use after being ground,

screened through 60-hole sift and dried at 40°C.

The three flavonoid reference samples of quercetin-3-*O*-gentiobiopyranoside (HQK-1), quercetin-3-*O*-[ $\beta$ -*D*-xyl-(1  $\rightarrow$  2)]- $\beta$ -*D*-glucopyranoside (HQK-2) and quercetin-4''-*O*-methy-3-*O*- $\beta$ -*D*-glucopyranoside (HQK-3) are all obtained by separation in our laboratory. With the determination by  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR,<sup>[15]</sup>  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR of HQK-1 as:  $^1\text{H}$ NMR (400MHz, MeOD):  $\delta$  7.690 (d, 1H,  $J$  = 2 Hz), 7.656 (dd, 1H,  $J$  = 8.4 Hz, 2.4 Hz), 6.858 (d, 1H,  $J$  = 8.4 Hz), 6.390 (s, 1H), 6.188 (s, 1H), 5.234 (d, 1H,  $J$  = 7.6 Hz), 4.143 (d, 1H,  $J$  = 7.6 Hz),  $^{13}\text{C}$  NMR (100MHz, MeOD):  $\delta$  179.5396 (C-4), 165.987 (C-7), 163.006 (C-5), 158.877 (C-2), 158.472 (C-9), 149.842 (C-3), 145.912 (C-4), 135.592 (C-3), 123.533 (C-6), 123.093 (C-1), 117.521 (C-2), 116.078 (C-5), 105.757 (C-10), 104.583 (C-1'), 103.984 (C-1''), 99.888 (C-6), 94.824 (C-8), 77.992 (C-5'), 77.872 (C-5''), 77.773 (C-3'), 77.601 (C-3''), 75.747 (C-2'), 75.082 (C-2''), 71.313 (C-4'), 71.284 (C-4''), 69.569 (C-6'), 62.507 (C-6'').  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR of HQK-2 as:  $^1\text{H}$ -NMR ( $\text{CD}_3\text{OD}$ , 400 Hz)  $\delta$ : 6.22 (1H, d,  $J$  = 2.4 Hz, H-6), 6.41 (1H, d,  $J$  = 2.0 Hz, H-8), 7.67 (1H, d,  $J$  = 2.4 Hz, H-2'), 6.91 (1H, d,  $J$  = 8.4 Hz, H-5'), 7.64 (1H, dd,  $J$  = 8.4 Hz, 2.4 Hz, H-6'), 5.50 (1H, d,  $J$  = 2.8 Hz, H-1''), 3.73 (1H, dd,  $J$  = 9.6 Hz, 2.0 Hz, H-6''), 3.57-3.62 (1H, m, H-6''), 4.80 (1H, d,  $J$  = 6.8 Hz, H-1'''), 3.96 (1H, dd,  $J$  = 11.6 Hz, 4.8 Hz, H-5''), 3.24-3.29 (1H, m, H-5''').  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{OD}$ , 100 Hz)  $\delta$ : 158.3 (C-2), 135.1 (C-3), 179.5 (C-4), 99.8 (C-5), 135.1 (C-6), 165.7 (C-7), 94.6 (C-8), 158.3 (C-9), 105.8 (C-10), 123.2 (C-1'), 117.4 (C-2'), 149.7 (C-3'), 146.0 (C-4'), 116.1 (C-5'), 123.4 (C-6'), 100.8 (C-1''), 82.2 (C-2''), 78.1 (C-3''), 70.9 (C-4''), 77.0 (C-5''), 62.3 (C-6''), 105.3 (C-1'''), 74.9 (C-2'''), 78.2 (C-3'''), 70.9 (C-4'''), 66.6 (C-5''').  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR of HQK-3 as:  $^1\text{H}$  NMR (400MHz, MeOD):  $\delta$  7.699 (d, 1H,  $J$  = 1.2 Hz), 7.577 (dd, 1H,  $J$  = 8.4 Hz, 1.2 Hz), 6.858 (d, 1H,  $J$  = 8.4 Hz), 6.381 (s, 1H), 6.189 (s, 1H), 5.250 (d, 1H,  $J$  = 7.6 Hz),  $^{13}\text{C}$  NMR (100MHz, MeOD):  $\delta$  179.504 (C-4), 166.023 (C-7), 163.060 (C-5), 159.029 (C-2), 158.474 (C-9), 149.862 (C-3), 145.917 (C-4), 135.625 (C-3), 123.200 (C-6), 123.075 (C-1), 117.562 (C-2), 116.005 (C-5), 105.700 (C-10), 104.306 (C-1'), 99.888 (C-6), 94.710 (C-8), 78.400 (C-5''), 78.122 (C-3'), 75.731 (C-2'), 71.122 (C-4'), 62.552 (C-6'), 55.114 (C-4'-OCH<sub>3</sub>). The structure of HQK-1, HQK-2, HQK-3 see Figure 1. Through the calculation by HPLC peak area normalization method, we get the purity all over 98%. The batch number of the reference sample Rutin, which was bought from China Pharmaceutical Biological Product Assaying Institute, is 10080-200306.

### The preparation for the standard solution

Weigh precisely the standard substance of Rutin 17.75 mg and use 70% ethanol solution to dissolve it and then put it to a 100 mL flask until it reaches the constant volume.



**Figure 1:** The structure of quercetin-3-O-gentiobiopyranoside, quercetin-3-O-[β-D-xyl-(1→2)]-β-D-glucopyranoside, quercetin-4"-O-methy-3-O-β-D-glucopyranoside

Weigh precisely a suitable amount of HQK-1, HQK-2 and HQK-3 and put them in the flask of 5 mL respectively. Then use methanol to dissolve them until they reach the desired degree scale. After shaking evenly, we will get the reference substance solutions of HQK-1, HQK-2 and HQK-3 with the concentrations 3.16 mg/mL, 0.325 mg/mL, 0.508 mg/mL respectively. At last, take 1 mL of the 3 reference substance solutions respectively and mix them in the 5 mL flasks. We will get the mixed reference substance solutions after using the methanol to make it to the constant volume.

#### The preparation for the test solution

Weigh precisely 1.0 g of the dried fruit, peel, leaf and flower of different species of *A. esculentus* which are screened through the 60-hole sift. Then put them in the triangle flask. Use 30 mL 70% methanol to extract 2 times supersonically each time for 30 min by using optimized extracting method and we will get filtered liquid after filtration. Combine the filtered liquid 2 times and put them onto the rotary evaporimeter to concentrate at 50°C by reducing the pressure. Then use methanol to dissolve and transfer it to 25 mL flask and then make it to a constant volume, we'll get the extract liquids of flower, fruit, leaf and peel of *A. esculentus*, which can be used in the HPLC determination.

#### RP-HPLC chromatographic condition and the system adaptability

Chromatographic Column: SunFire C<sub>18</sub> chromatographic column (4.6 mm × 250 mm, 5.4 μm); the mobile phase is methanol-the phosphoric acid water solution of 0.1% and. The ratio of the gradient elution is 47:53; the flow velocity is 0.8 mL/min; the detection wavelength of diode array detector is 255.6 nm; the column temperature is 28°C. The sample-feeding amount: 1-20 μL.

Under the above chromatographic condition, the separation degrees of each adjacent chromatographic peak are all >1.5, the tailing factor is between 0.95 and 1.05, the theoretical pedaling numbers are all over 8000, which satisfy the quantity requirement.

#### Investigation of the linear relationship

The content determination of the total flavonoids: weigh precisely the Rutin reference solutions 0, 0.5, 1.5, 2.5, 3.5, 4.5 mL in turn and put them in 10 mL flasks respectively and then add 70% ethanol to 5 mL respectively, shake them evenly after adding 1 mL of the 5% sodium nitrite solution. After 6 min, add 1 mL of the 10% aluminum nitrate solution and shake it evenly; 6 min later, add 3 mL of the 4% sodium hydroxide solution and add 70% ethanol until it reaches the constant volume to the desired degree and shake it evenly; After 15 min, use the corresponding reagent solutions as the blank reference to determine its absorbance at the wavelength 510 nm. Draw a standard curve by the absorbance A as the Y-axis and the concentration of the reference substance as X-axis (mg/mL). The results show that when the Rutin concentration is between 0.01775 and 0.159 mg/mL, it has a good linear relationship with its absorbance and its equation of linear regression is  $0.0057 \times -0.0256$ ,  $r = 0.9989$ .

The content determination of the flavonoid glycosides: weigh precisely in turn the mixed reference sample solutions 1.00, 2.00, 3.00, 7.00, 11.00, 15.00 μL and put them respectively into the liquid chromatograph. According to the chromatographic conditions, we test the peak area value of each constituent. Draw a standard curve by using the sample-feeding size (μg) as the X-axis and the integral value of the peak area as the Y-axis. The equation of linear regression and its related coefficient of HQK-1, HQK-2 and HQK-3 can be seen in Table 1, which shows that

there is a good linear relationship among the 3 flavonoid glycosides also shown in Table 1.

### The methodological investigation of RP-HPLC

#### Precision test

Take the mixed reference substance solution with a certain content, feed the sample 6 times in a row according to the chromatographic condition, write down the peak areas of the three constituents respectively and by calculating the corresponding peak areas of HQK-1, HQK-2 and HQK-3, we get the following relative standard deviation (RSD) values 0.30%, 0.21%, 0.56% respectively, which show that the precision of the instruments is very good.

#### Repeatability test

Weigh precisely six hares of the fruit powder of *A. esculentus* 1.0 g. Then analyze it according to the way of preparing the sample solution and the chromatographic condition and we get the RSD of the average content values of HQK-1, HQK-2 and HQK-3 are respectively 0.50%, 0.11%, 0.43%.

#### Stability test

Take the test solution of the fruit of *A. esculentus* and put it at the room temperature. Analyze it respectively at 0, 5, 10, 15, 20, 24 h according to the chromatographic condition, we get the RSD of the average content values of HQK-1, HQK-2 and HQK-3 are respectively 0.51%, 0.03%, 0.38%. The results show that the test solution is stable at 24 h.

The recovery rate test of the added samples: Take nine shares of the fruit powder of *A. esculentus* with a given content, each share 1.0 g. Weigh them precisely and then add the suitable amount of the mixed reference substance solution precisely. According to the way of preparing the sample solution, we make three shares of the test solutions with three different concentrations. Analyze them according to the chromatographic condition and calculate the added sample recovery rate. As a result, the average added sample recovery rate of HQK-1, HQK-2 and HQK-3 are 105.34%, 95.6%, 103.11% ( $n = 3$ ) respectively.

## RESULTS AND DISCUSSION

### The content determination results of the total flavonoid

Determine the total flavonoid contents in different parts and different species of *A. esculentus* by the  $\text{NaNO}_2$ -Al  $(\text{NO}_3)_3$ -NaOH colorimetric method. The results can be shown in Table 2. It can be seen from the experiment that in the different species of *A. esculentus*, the content of total flavonoid in *A. esculentus* of finger is relatively high; in different parts of *A. esculentus*, the total

content of flavonoid in the flower is relatively high, next comes the fruit, while the lowest content is in its peel.

### RP-HPLC content results of the flavonoid glycosides

Take the powder of *A. esculentus* 1.0 g in different species and different parts, weigh them precisely and analyze according to the way of preparing the test solutions and the chromatographic condition. Then calculate the contents of the three flavonoid glycosides, the results of which can be seen in Table 3. The HPLC chromatogram of the reference substance solution and the sample solution can be seen in Figures 2-5. From the experiment results, we can see that in different species of *A. esculentus*, the HQK-1 contents in flower and fruit are comparatively the highest, next comes HQK-3, while the content of HQK-2 is the lowest. The HQK-2 content in the leave is the highest comparatively and the contents of the three flavonoids in the peel are all low comparatively.

This research established the RP-HPLC determination method. During the process of the methodological research, we established the preparation of the test substance solutions and examined the different extraction solvents (methanol, ethanol and acetone) and the different extraction methods (supersonic wave, immersion extraction). We established the best extraction method for the three flavonoid glycosides in *A. esculentus* through the orthogonal experiment design, that is: using 70% of methanol solution

**Table 1: Regression equation of three kinds of glycosylflavones**

Glycosylflavones	Regression equation	<i>r</i>	Linear range/ $\mu\text{g}$
HQK-1	$Y=3.27 \times 10^6$ $X-7.64 \times 10^5$	0.999 2	0.632-9.480
HQK-2	$Y=3.08 \times 10^6$ $X-6.01 \times 10^4$	0.999 5	0.065-0.975
HQK-3	$Y=3.35 \times 10^6$ $X-1.43 \times 10^5$	0.999 0	0.102-1.530

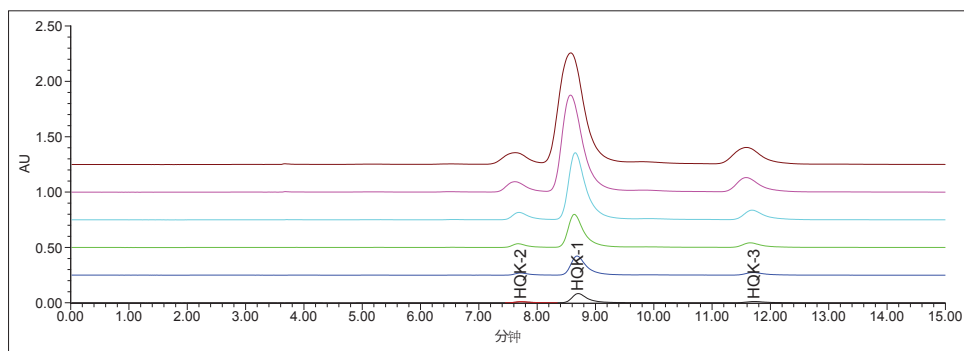
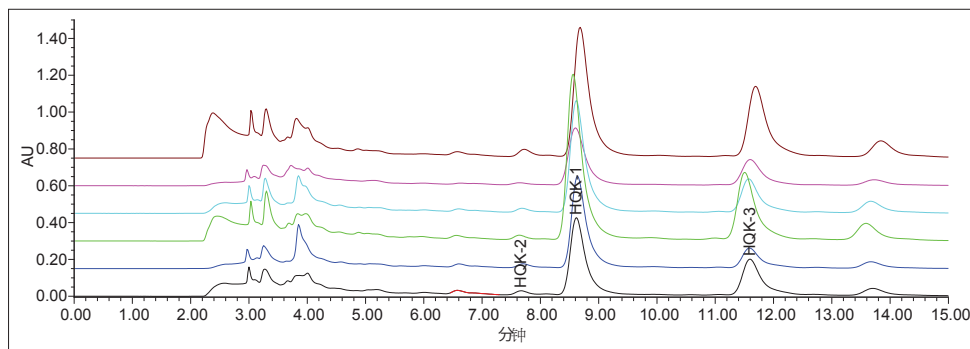
**Table 2: The content of total flavone in different species and part of *A. esculentus* (mg/g)**

Species	Fruit	Peel	Flower	Leave
<i>A. esculentus</i> of finger	28.34	6.03	37.60	10.55
<i>A. esculentus</i> of red	11.26	3.52	17.25	10.30
<i>A. esculentus</i> of green	12.51	4.11	20.38	11.01
<i>A. esculentus</i> of pentagram	22.77	4.58	27.85	6.32
<i>A. esculentus</i> of five blessings	17.21	4.79	25.50	9.17
<i>A. esculentus</i> of pentagon	8.49	3.73	12.50	7.44



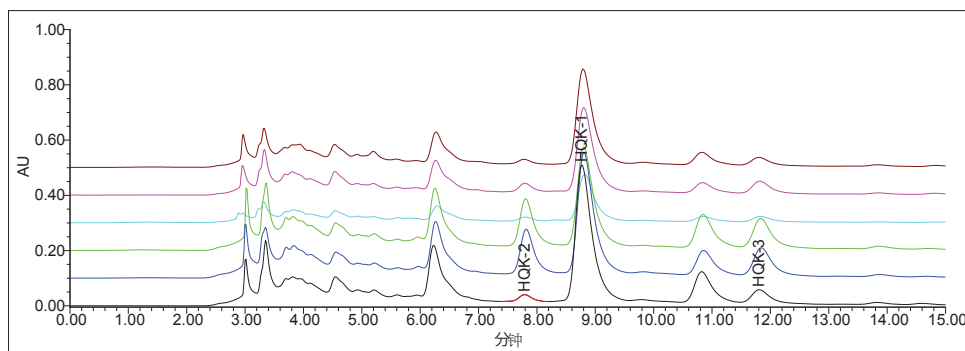
**Table 3: Content of three kinds of glycosylflavones in different parts and species of *A. esculentus* (mg/g)**

Different part	Glycosylflavone	Different species of <i>Abelmoschus esculentus</i>					
		<i>A. esculentus</i> of finger	<i>A. esculentus</i> of red	<i>A. esculentus</i> of green	<i>A. esculentus</i> of pentagram	<i>A. esculentus</i> of five blessings	<i>A. esculentus</i> of pentagon
Flower	HQK-1	6.676	5.897	4.803	5.646	4.071	4.584
	HQK-2	0.308	0.275	0.220	0.437	0.392	0.202
	HQK-3	0.790	1.549	1.635	0.030	0.686	0.475
Fruit	HQK-1	2.690	2.274	2.120	2.784	1.559	0.819
	HQK-2	0.128	0.136	0.132	0.133	0.078	0.240
	HQK-3	1.414	0.733	2.437	1.226	0.983	2.531
Leave	HQK-1	0.173	0.124	0.091	0.485	0.605	0.248
	HQK-2	0.817	1.641	0.999	1.222	1.238	1.062
	HQK-3	0.085	0.257	0.133	0.093	0.118	0.089
Peel	HQK-1	0.122	0.060	0.110	0.200	0.013	0.021
	HQK-2	0.013	0.479	0.708	0.072	0.070	0.064
	HQK-3	0.024	0.025	0.372	0.050	0.092	0.021

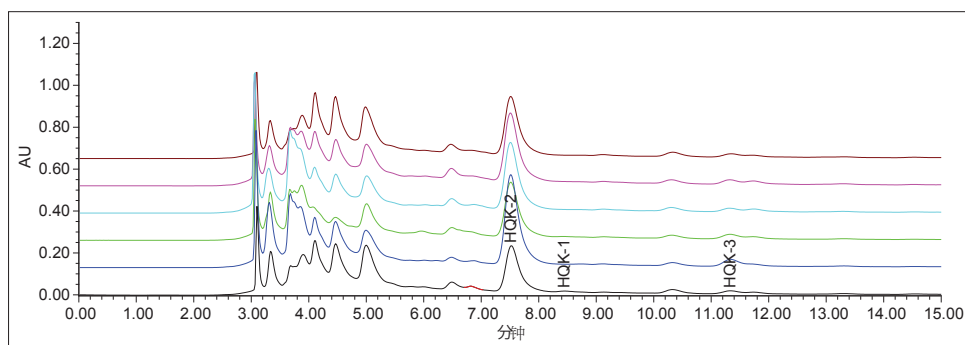
**Figure 2: HPLC chromatography of standard references in different volumes****Figure 3: HPLC chromatography of fruit from 6 species**

to extract 2 times, each time for 30 min, with 30 times amount of the solvents each time. As for choosing the detection wavelength, because in the experiments the components of the three flavonoid glycosides have the same mother nucleus skeleton and their ultraviolet absorption spectra are nearly the same. In addition, near 255 nm, 355 nm, there are two strong absorption bands and the absorption is the strongest at 255.6 nm. Therefore, we choose 255.6 nm as the detection wavelength. In this experiment, in order to reduce the interference and to

improve the sensibility of the method, when choosing the flowing phase, we compared the eluting systems of methanol-water, methanol-phosphoric acid liquid and found that we could improve the separation degree of the three constituents and meanwhile it could better the peak shape. Therefore, we adopted the methanol-0.1% phosphoric acid water as the flowing phase in this experiment. When choosing the flowing speed, we found that the flowing speed has a relatively big influence on the separation of the three flavonoid glycoside components. If we set the flowing speed



**Figure 4:** HPLC chromatography of flower from 6 species



**Figure 5:** HPLC chromatography of leave from 6 species

at 0.8 mL/min, it can make the three components separate quickly at the baseline. Hence we chose the flowing rate at 0.8 mL/min. When choosing the column temperature, we control the temperatures at 24°C, 28°C, 32°C and 36°C respectively. When the column temperature is over 30°C, the peak-shaping time of each component will advance and will pile up, so we set 28°C as the chromatographic column temperature.

## CONCLUSION

This research takes the total flavonoids and the three flavonoid glycosides as the objects of study. It makes an analysis for the content differences of the different species and different parts in *A. esculentus*. From the determining results, we can see that the content distributional differences of the total flavonoids and the three flavonoid glycosides in different species and different parts of *A. esculentus* are very great. Generally speaking, the content of flavonoids in the flower and fruit is very high, while very small in its leave and peel.

Of the different species, the content of the total flavonoids in *A. esculentus* of finger is the highest. In the different parts of the *A. esculentus*, the content of the total flavonoids in flower is the highest, next comes the fruit and the lowest content is in its peel. In the different species of *A. esculentus*, the content of HQK-1 is the highest in the flower and fruit, next comes the HQK-3 and the content of HQK-2 is the lowest. The

content of HQK-2 in the leave is the highest, while the content of the three flavonoid glycosides in the peel is relatively low.

The *A. esculentus* is a kind of health vegetable with very high nutritious value. It has been listed as the best green food in the new century. Recently in mainland China, its planting area and yearly yield are increasing very quickly after it was introduced in our country. The RP-HPLC method established by this research is both fast and accurate, with reliable results and very good repeatability. It provides the reference data for its medicinal use and its development. This method can also be used in making a quantitative analysis for the flavonoid components in *A. esculentus*.

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