

Study of the flavonoids of *Anemone obtusiloba* in the alpine meadow and the adaptation mechanism to the environment based on metabolic pathways

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Research Article

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Abstract

In this study, the main pigment composition of *Anemone obtusiloba* was analysed by UV-vis spectrophotometry, HPLC, and LC-MS. The results showed that there were two kinds of pigments in *A. obtusiloba*, carotenoids, and flavonoids. The absorbance spectra regularly changed with the depth of flower colour. Based on HPLC and LC-MS analyses, seven kinds of flavonoids were inferred, including luteolin-3-7-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-sophoroside, and myricetin-3-O-rhamnose, and the derivative of quercetin was identified as the main component in *A. obtusiloba*. Besides, according to the analysis of the flavonoid metabolism pathway, it could be determined that flavonoid 3'-hydroxylase (F3'H) was the key enzyme to increase the content of quercetin. This paper speculates that *A. obtusiloba* increases the content of quercetin by regulating flavonoid F3'H, so that it can deepen the flower colour to adapt to the environment.

1. Introduction

Anemone obtusiloba, commonly known as Padar, Rattanjog, or Kawashud, is a flowering plant of the family Ranunculaceae and a densely tufted perennial herb [1]. It is native to the Himalayan and mountainous regions of Myanmar occurring in the Alpine Himalaya from Kashmir to Sikkim at 2100-4200 m altitude and in the Nilgiri hills at an altitude above 1800 m [2]. It bears buttercup hermaphrodite flower and the stem is short and tufted with a single terminal flower, generally producing one to three individual stems with yellow, light-yellow, or white colour. At present, research mainly focuses on the medicinal value of *A. obtusiloba*, whose ethanolic extract contains saponins (obtusilobinin, obtusilobin [3], and obtusilobicinin [4]). It also contains protoanemonin, which is an irritating acid oil and is an enzymatic breakdown product of the glycoside ranunculin [5]. *A. obtusiloba* is used as a purgative and in the treatment of rheumatic joints, jaundice, spleen disorders, and anxiety neurosis. Besides, it is also used as an antidote to snakebite, while its seed oil is used to cure arthritis [6, 7]. However, there are few studies on its petal pigment.

In the Tibetan Plateau, *A. obtusiloba* has its own adaptive mechanism to the local environment. During the past years, there have been significant environmental changes in the plateau area, with an obvious decrease of white flowers and an increase of yellow and light-yellow flowers of *A. obtusiloba*. It is speculated that this phenomenon may be related to its adaptability to the environment and biological evolution. Flower colour is one of the most important traits of plants and is attributed to various pigments that are composed of three major classes of compounds including flavonoids, carotenoids, and anthocyanin [8]. Among these compounds, flavonoids are responsible for the yellow colour. At the same time, the number and position of phenolic groups in the chemical structure have a certain influence on the flower colour. The colour of the flower will change from yellow to orange and to red by the hydroxylation at C₃ [9]. However, increasing the hydroxylation of the B ring will change the colour to blue, and the methylation of the B ring will make it red [10]. Carotenoids are responsible for colours ranging from

yellow to red [11]. Cyanidin, which controls red, blue, purple, and other colours, can be divided into three types: pelargonidin, delphinidin, and anthocyanins [12].

Flavonoids represent a large subgroup of plant secondary metabolites including flavones, isoflavones, anthocyanins, flavanols, flavonols, and derivatives [13, 14] with various biological activities such as anti-inflammation, anti-cancer, etc. [15]. Over the past decade, studies have revealed the core biosynthesis pathways of flavonoids in various plants such as *Arabidopsis* [16], *Lactuca sativa* [17], *Camellia sinensis* [18], *Salvia miltiorrhiza* [19], *Oroxylum indicum* [20], *Chrysanthemum morifolium* [21], *Chrysanthemum indicum*, and *Scutellaria baicalensis* [22, 23]. Flavones are synthesized via the flavonoid pathway, which is part of phenylpropanoid metabolism [24]. Naringenin is a central intermediate in the biosynthesis of normal 4'-hydroxyflavones [25]. The enzymatic properties related to flavonoid accumulation in vitro have been confirmed [26]. Flavonoid biosynthetic genes can be classified into two categories: (i) early biosynthetic genes, such as genes encoding phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI); (ii) late biosynthetic genes, such as those encoding flavanone 3-hydroxylase (F3'H), dihydroflavonol reductase (DFR), and anthocyanidin synthase (ANS) [27]. CHS, the first key enzyme in the anthocyanin synthesis pathway, is affected by multiple factors, such as environmental stress, light, inducer, etc. [28]. There are six genes involved in anthocyanin synthesis in different tissues of grape, CHS, CHI, F3'H, DFR, leucoanthocyanidin reductase (LAR), and flavonoids glycosyltransferase (UGT), while the accumulation of anthocyanidin is absent in white grape. Therefore, the accumulation of anthocyanidin is not only related to tissue, but also related to the alternative gene expression. The study aimed to qualitatively and quantitatively identify the chemical composition using different analytical methods including ultraviolet-visible (UV-vis) spectroscopy, HPLC, and LC-MS, and to further analyse the key enzyme synthesizing this pigment.

2. Materials And Methods

2.1 Materials

The experimental materials light-yellow and yellow *A. obtusiloba* were collected in early July at the peak of flowering from Maqu County, Gansu Province of China (101.52E, 33.40N). The flowers were rinsed, and the stamens were removed. After fixating at 80°C for 30 min, they were dried at 60°C and crushed into powder with a pulveriser (Tianjin Taisite Instrument Co. Ltd., Tianjin, China). Then the powder sample was stored in a sealed bag and kept in a dark place before using.

2.2 Spectral analysis by UV-vis spectroscopy

2.2.1 Spectral analysis of flavonoids

The flavonoid extraction was identified according to the method of Murugesan et al. [29] with some modifications. To extract flavonoids, 0.1 g of flower powder was treated with methanol/formic acid (98:2, v/v) for 24 h in a Soxhlet apparatus under dark conditions. The filter residue was repeatedly extracted by the solvent. After the filtrate was combined and diluted to 10 mL, the spectral scanning was carried out by

a UV spectrophotometer (UV-3000, Shanghai JiaPeng technology Co., Ltd., Shanghai, China) with a range of 200–700 nm.

2.2.2 Spectral analysis of carotenoids

The carotenoids of *A. obtusiloba* were extracted based on the method reported by Cai Xuan et al. [30] with necessary modifications. Briefly, each sample of flower powder (0.1 g) was mixed with acetone/ethanol (1:1, v/v), and the mixture was repeatedly extracted 24 h in a Soxhlet apparatus under dark conditions. After the filtrate was combined and diluted to 10 mL, the spectral scanning was carried out with a range of 200–700 nm.

2.3 Qualitative and quantitative analysis of petal pigments

Flavonoids in petals of *A. obtusiloba* were extracted with methanol/formic acid (98:2, v/v) in darkness at 4°C for 24 h. Then the mixture was centrifuged at 12,000 rpm for 5 min at 4°C to remove the precipitants. The supernatant was collected and filtered through a 0.45 µm micropore membrane, and the as-prepared sample was stored for qualitative and quantitative analysis. The experiment was repeated three times for each sample[31].

The quantitative analysis of flavonoids was performed on HPLC-PAD (Waters 2685, USA) and HPLC-MS (Agilent 6460, USA). A C18 column of Inertsil Zorbax SB (4.6 mm × 250 mm, 5 µm, Shimadzu GL, Shanghai, China) was used to separate the individual flavonoids with mobile phase A (double distilled water containing 0.1% phosphoric acid) and mobile phase B (acetonitrile). The elution gradient was set as follows: 0 min, 1% B; 15 min, 8% B; 35 min, 22% B; 45 min, 42% B; 55 min, 75% B; 65 min, 100% B. Then, 2 µL of the sample was injected for HPLC analysis. The chromatogram showed horizontal coordinate and vertical coordinate, corresponding to the retention time (min) and response value (mAU), respectively. The flow rate was 0.2 mL/min, and the column temperature was 30°C. Since flavonoids are expected to be observed at a 300 nm wavelength by UV detector, the spectra were scanned in the range of 190–700 nm.

The MS conditions were set as follows: High purity nitrogen (99.999%) was used as a nebulizing (60 psi) and drying gas at a flow rate of 9.0 L/min. The vaporizer temperature was set at 350°C. Other parameters were rationally set including an ion spray voltage of 70–205 V and a scanning range of 100–1000 m/z.

2.4. Statistical analysis

The data were processed and presented as mean ± standard deviation (SD). Experiments were performed in triplicates and the statistical analysis was executed by one-way analysis of variance (ANOVA). Significant differences between groups were discerned at $p \leq 0.05$. Statistic software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA) was used for all the graphical and statistical evaluations.

3. Result

3.1 Spectral analysis using UV-vis spectrometer

3.1.1 Spectral analysis of flavonoids

As shown in the UV-vis spectrum of the flavonoid fraction of *A. obtusiloba* (Figure 1A), the maximum absorption peaks were observed at 268 nm (band II) and 330 nm (band I), respectively. There are two typical absorbance bands of flavonoids, band B (310–350 nm) for flavones and band A (250–290 nm) for flavonols [32,33,34]. Generally speaking, band II absorption could be caused by ring A-cyclobenzoic acid system, while band I was caused by ring B-cyclocinnamic acid system [35,36]. As shown in Figure 1A, the absorption peak at 268 nm indicated the presence of the A-cyclobenzoyl system, while the absorption peak at 300 nm suggested that there was a B-cyclocinnamoyl system in the flavonoid fraction of the flower extract. Based on the observation that band II was the main peak while band I was weak, we speculated that the cinnamoyl system might be destroyed [37]. This result showed that the extract from *A. obtusiloba* had the basic structure of flavonoids C6-C3-C6.

3.1.2 Spectral analysis of carotenoids

According to the UV-vis spectrum of the carotenoid sample from *A. obtusiloba* (Figure 1B), the maximum absorption peak was at 268 nm, which was consistent with the characteristic absorption peak of the carotenoid. With the deeper colour, the absorption peak of the carotenoid sample gradually increased. The total content changed regularly with the shading of the colour (Figure 1B). The maximum light absorption value of the pale-yellow sample was 18.36% less than that of the yellow sample (4.112 and 3.357 of the optical density (OD), respectively).

3.2 Qualitative and quantitative analysis of petal pigments

3.2.1 Qualitative analysis of petal pigments from Maqu

Based on the HPLC-PDA analysis of the flavonoid extract of yellow flowers of *A. obtusiloba* (Figure 2), 7 kinds of compounds were detected at 300 nm with a retention time ranging from 30 to 50 min. Meanwhile, there were 8 kinds of compounds detected in light-yellow of flavonoid fraction of *A. obtusiloba*. Zhao et al.[38] reported that the different colours of flowers were not determined by the structure of the compound, but by the change in the content of some pigment molecules. Studies have also found that the red colour of cotton is mainly caused by flavonoid accumulation. Except for the flavonoid-related enzyme genes in red cotton such as CHS and F3'H, other genes that regulate flavonoid biosynthesis have higher transcription levels than those in white cotton [39]. In the HPLC chromatogram (Figure 2A), peaks 3 and 8 were close to each other, suggesting that they might be isomers. Therefore, the flavonoid components of the two coloured flowers are supposed to be the same. Mostly, the peak intensity (response value) of yellow flowers (B) was higher than that of the light yellow (B) ones. Therefore, it could be suggested that the colour intensity of *A. obtusiloba* was caused by the content of pigment molecules. The components (Figure 2A) at peaks 3 (1.775 mg/g), 6 (1.56 mg/g), and 7 (2.687 mg/g)

were more dominant in light-yellow flowers, whereas peaks 3 (4.124 mg/g), 4 (2.754 mg/g), and 7 (2.651mg/g) were more dominant in yellow flowers (Figure 2B).

3.2.2 Quantitative analysis of petal pigments from Maqu

To further determine the specific type of pigment molecules, HPLC-MS analysis was carried out on yellow flowers with high total contents. The MS peaks of four standards (Quercetin, Kaempferol, Luteolin, and Rutin) were shown in Figure 3. According to the total ion chromatogram and positive ion mode of flavonoid from the yellow flower (Figure 4 and Figure 5), the analysis results in Table1 were obtained.

The molecular ion peak was observed at m/z 645.18 [M+H]⁺ of peak 7. The fragment at m/z 465 represented the loss of one glucose molecule. Then one glucose molecule was removed to obtain a fragment m/z 287, which was luteolin aglycone. It was speculated to be luteolin-3-7-O-glucoside since it was consistent with the result reported by Waage S.K et al. [40].

Table 1

HPLC-MS analysis results of flavonid from *Anemone obtusiloba* yellow flower

Peak No	Retention time(min)	Molecular ion(m/z)	Fragment ions (m/z)	Identification
7	31.88	645.18([M+H] ⁺)	465287248	luteolin-3-7-O-glucoside
6	33.24	625.15([M] ⁺)	465303274	quercetin-3-O-rutoside
5	36.19	611.2([M+H] ⁺)	445287263	kaempferol-3-O-sophoroside
4	42.58	483.1([M+3H] ⁺)	318146	myricetin-3-O-rhamnose
3	45.31	465.1([M+H] ⁺)	303287274	quercetin-3-O-galactoside
2	45.74	465.1([M+H] ⁺)	303287274	quercetin-3-O-glucoside
1	47.40	449.2([M+H] ⁺)	303274179	quercetin-3-O-rhamnoside

The fragment at m/z 303 corresponded to quercetin aglytin and its cleaved m/z 274 fragments were found in MS of peaks 6, 3, 2, and 1, which were presumed to be derivatives of quercetin. The [M+H]⁺ of peak 6 was m/z 625.15, which contained fragment ions m/z 465 and 303, indicating that two molecules of six-carbon sugar have been removed. As a result, it could be quercetin-3-O-rutoside [40,41]. Besides, a molecular ion peak was observed at m/z 465.1 [M+H]⁺ of peaks 3 and 2, and the removal of one six-carbon sugar molecule obtained m/z 303. This result could indicate that peak 3 was quercetin-3-O-galactoside and peak 2 was quercetin-3-O-glucoside [42-45]. Fragments at m/z 303 showed that the

removal of rhamnose and fragments at m/z 179 could be the loss of one water molecule representing the [M+H]⁺ peak 1. The result indicated that it might be quercetin-3-O-rhamnoside [46]. The fragment ion m/z 287 matched the molecular weight of kaempferol. Therefore, it could be speculated that kaempferol was contained in its structure. The fragment m/z 445 indicated the loss of two molecules of glucose, which was consistent with the peak of kaempferol-3-O-sophoroside reported previously [46]. The molecular ion peak 4 was observed at m/z 483.1 [M+H]⁺. Based on the characteristic ion of myricetin at m/z 318, and the glycosyl ion at m/z 146, the data were found to be consistent with the result of myricetin-3-O-rhamnose [47]. The content of the flavonoids was measured (Table 2). The content of quercetin-3-O-galactoside (4.124 mg/g) was found to be relatively higher than the other components. As a result, quercetin derivatives were dominant in flavonoids of *A. obtusiloba* flowers. The result is the same as the Hezuo sample plot [49].

Table 2

The content of main components of anthocyanin in Maqu *Anemone obtusiloba* (mg/g)

Peak No.	Component	Yellow flowers	Light yellow flower
7	luteolin-3-7-O-glucoside	2.754	2.687
6	quercetin-3-O-rutoside	0.982	1.56
5	kaempferol-3-O-sophoroside	1.2	0.394
4	myricetin-3-O-rhamnose	2.651	0.34
3	quercetin-3-O-galactoside	4.124	1.775
2	quercetin-3-O-glucoside	0.867	0.815
1	quercetin-3-O-rhamnoside	1.05	0.461

3.3 The key enzyme analysis in the regulation of anthocyanin in *A. obtusiloba*

Although the anthocyanins of *A. obtusiloba* are mainly composed of four sugar derivatives of luteolin, kaempferol, myricetin and quercetin, In these major flavonoids, quercetin with the highest relative abundance may be one of the main active ingredients. According to the metabolic pathway of flavonoids (Figure 6) [48], quercetin could only be synthesized from dihydroquercetin and catalysed by flavonol synthetase (FLS). There were two pathways of dihydroquercetin synthesis: one was eriodictyol catalysed by flavanone-3-hydroxylase (F3H), and the other was dihydrokaempferol catalysed by flavonoid-3'-hydroxylase (F3'H). However, eriodictyol could be also catalysed by flavone synthase II (FNSII) to form luteolin. In the study, the derivatives of luteolin (2.754 mg/g) were observed. Therefore, luteolin pathway

was not prohibited, and the activity of FNSII might be reduced or the gene expression of FNSII was down regulated to reduce the decomposition of luteolin. F3'H catalysed the synthesis of dihydrokaempferol and dihydroquercetin from senkyolin and naringenin, respectively. According to the reactions in Figure 6, F3'H showed a higher affinity with naringenin. So it was speculated that the affinity of FLS with dihydrophlorin was enhanced in the pathway of the synthesis of luteolin, which competed with dihydrokaempferol and dihydromyricetin to increase the content of quercetin. According to the content of flavonoids in *A. obtusiloba*, it was speculated that the dihydrokaempferol pathway improved the translation of F3'H to increase the content of dihydroquercetin. And the affinity between FLS and dihydroquercetin was enhanced, so quercetin content could be eventually increased.

Therefore, enhancing the translation of F3'H gene could not only increase the content of dihydroquercetin, but also increase the content of eriodictyol, which could indirectly increase the content of dihydroquercetin and finally achieve the purpose of increasing the content of quercetin.

4. Discussion

In recent years, with changes in the environment in the alpine region, the pigment of the unique plant *A. obtusiloba* has also been changed. In the Hezuo plot with lower altitudes, the pigment of *A. obtusiloba* has undergone significant changes to adapt to the environment, the pigment of the *A. obtusiloba* is obviously darkened. Compared with low altitude Hezuo plot, the higher the altitude, the deeper the color, In the Maqu plot with higher altitudes, and even no white flowers are observed (**Figure 7**). The main anthocyanins in the two places are the same, and the change trend of main pigments was consistent with the change of flower color (**Table3**) [49].

In this study, the main components and their key enzymes in different colourful flowers of *A. obtusiloba* were analysed by UV-vis spectroscopy, HPLC-PAD, and HPLC-MS. Seven kinds of components in flavonoids were speculated by HPLC-MS, including luteolin-3-7-O-glucoside, quercetin-3-O-rutside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-sophoroside, and myricetin-3-O-rhamnose. According to the results of qualitative and quantitative analysis, quercetin showed the largest proportion of sugar derivatives and the most obvious change of flower colour. It is the main chromogenic pigment that causes the difference in flower colour of *A. obtusiloba*. According to the flavonoid metabolic pathways, there are three main ways to increase the content of quercetin: (1) reducing the activity of FNS or attenuating the translation of FNSII gene to reduce the path of eriodictyol; (2) enhancing the affinity of FLS binding with dihydroquercetin to make dihydroquercetin superior in substrate competition; (3) enhancing the translation of F3'H genes, such as increasing the transcription factor in the promoter region to increase the content or activity of F3'H to increase the contents of dihydroquercetin and saugenin.

Table 3

The content of main components of anthocyanin in Hezuo *Anemone obtusiloba* (mg/g)[49]

Peak No.	Component	Yellow flowers	Light yellow flower	White flowers
7	luteolin-3-7-O-glucoside	2.057	1.533	0.666
6	quercetin-3-O-rutside	1.667	0.812	0.213
5	kaempferol-3-O-sophoroside	1.661	0.922	0.256
4	myricetin-3-O-rhamnose	1.627	1.024	0.211
3	quercetin-3-O-galactoside	2.325	1.431	0.769
2	quercetin-3-O-glucoside	1.255	0.613	0.075
1	quercetin-3-O-rhamnoside	2.459	0.92	0.233

Compared with the content of main pigment components in low altitude Hezuo plots (**Table3**), it can be analyzed that quercetin-3-o-galactoside of peak 3, myricetin-3-o-rhamnoside of peak 4 and luteolin-3-7-o-diglucoside of peak 7 are the main pigments with high content. However, the pigments that affect the color change are quercetin-3-o-galactoside, quercetin-3-o-rhamnoside, myricetin-3-o-rhamnoside and kaempferol-3-o-sophoroside, but the content of quercetin derivatives accounts for the highest proportion. Based on the results of this study, quercetin was identified as the main pigment of *A. obtusiloba*. The key enzyme to adapt to the environment was discovered to be F3'H. The main environmental factor causing pigment change may be light intensity. This paper speculates that *A. obtusiloba* increases the content of quercetin by regulating the expression or activity of F3'H to deepen the flower colour so that it could adapt to the environment. In this study, the evolution of *A. obtusiloba* response to the environment was analysed. This conjecture is also applicable to the adaptation to altitude changes. It provides a theoretical basis for the study of the evolution mechanism of plateau plants responding to the environment and provides a reference for the relationship between plateau environmental change and vegetation.

Declarations

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Conflicts of interest/Competing interests

No conflict of interest exists in the submission of this manuscript, and all the authors listed have approved the manuscript that is enclosed.

Statement

Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability/Availability of Data and Materials Statements

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Collect *Anemone obtusiloba* Premissions Statement

School of life sciences, Lanzhou University of Technology and College of grassland agricultural science and technology, Lanzhou University had given a permission to collect *Anemone obtusiloba* on the sample plot.

Collection of *Anemone obtusiloba* comply with relevant institutional, national, and international guidelines and legislation.

All these voucher specimens are deposited in the public collection providing access and Author Lv wanling and Professor Liu zuojun identified it.

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Figures

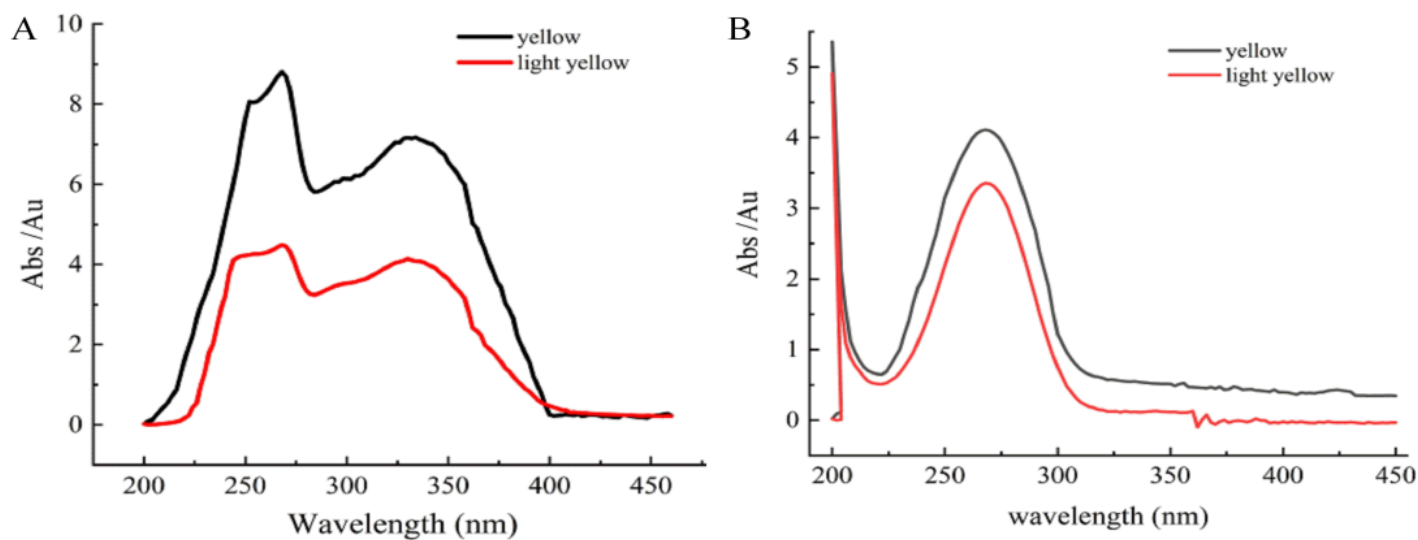


Figure 1

The UV-visible spectra of petal pigments in *Anemone obtusiloba* Flavonoid(A), Carotenoid(B).

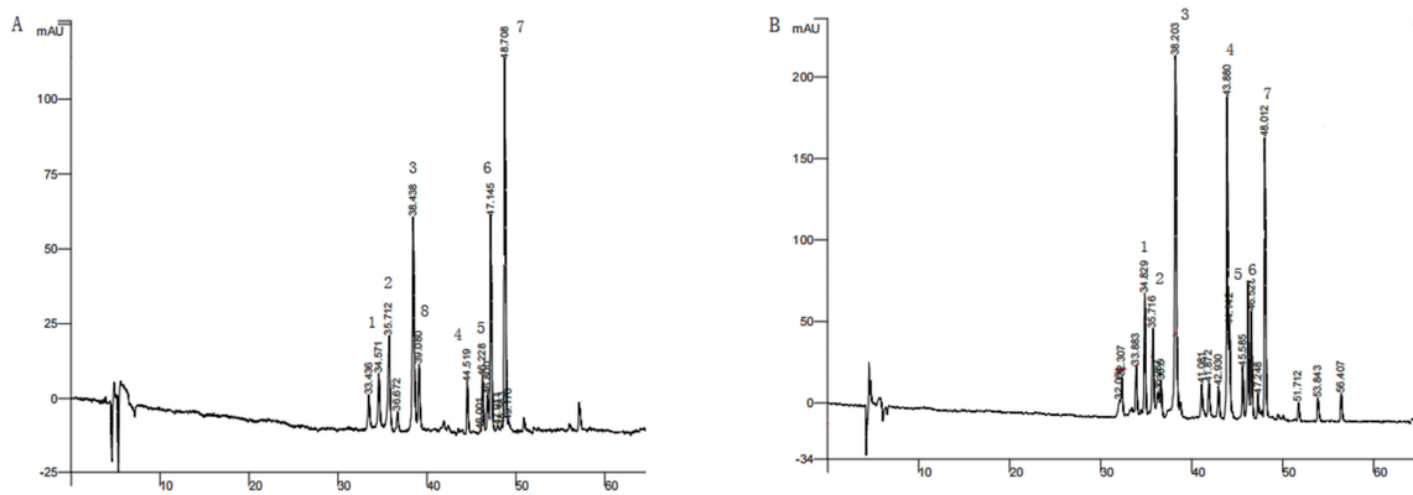


Figure 2

HPLC chromatogram of flavonoids from *Anemone obtusiloba* Light yellow(A),yellow(B)

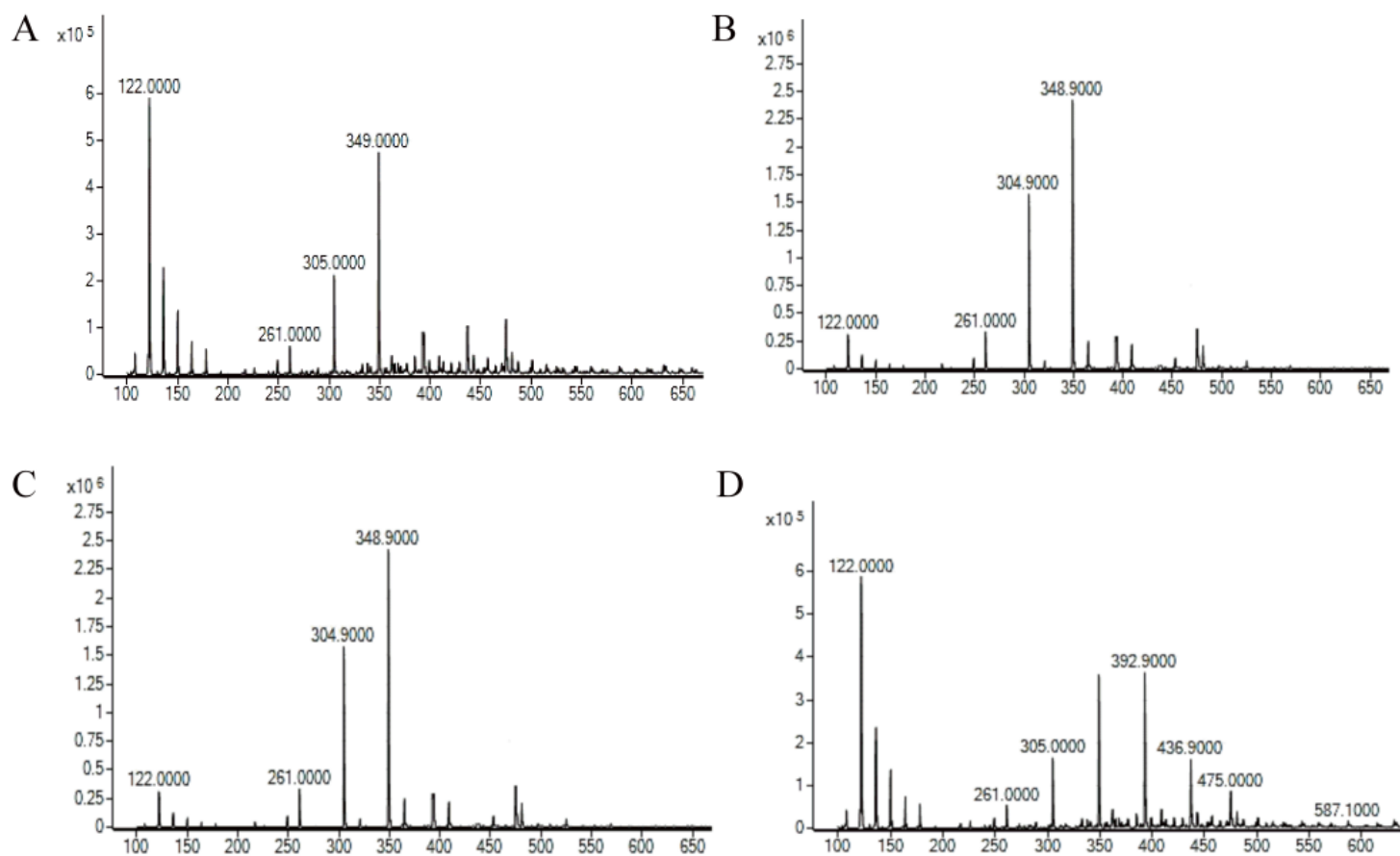


Figure 3

Total ion chromatogram of standards Lquercetin (A), Kaempherol (B), Luteolin (C), and quercetin-3-O-rutinoside (D)

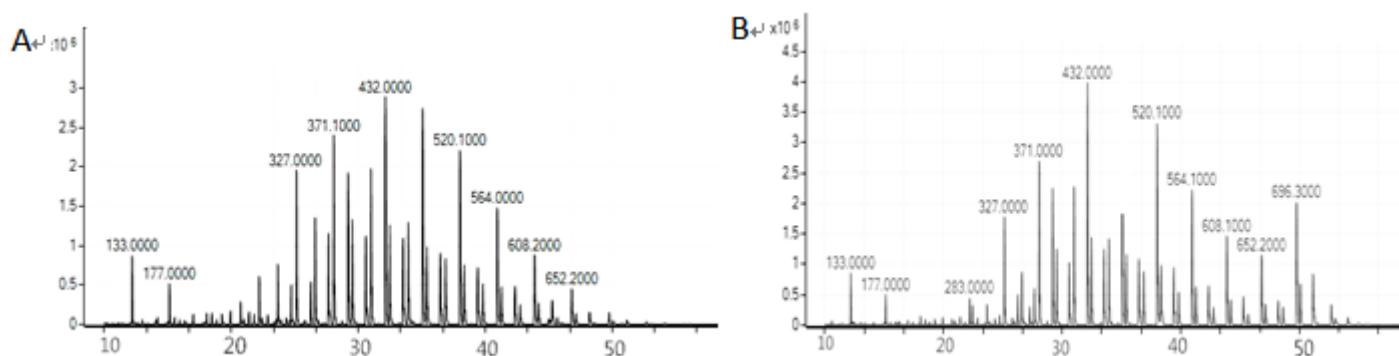


Figure 4

Total ion chromatogram of flavonoid from *Anemone obtusiloba* Light yellow(A),yellow(B)

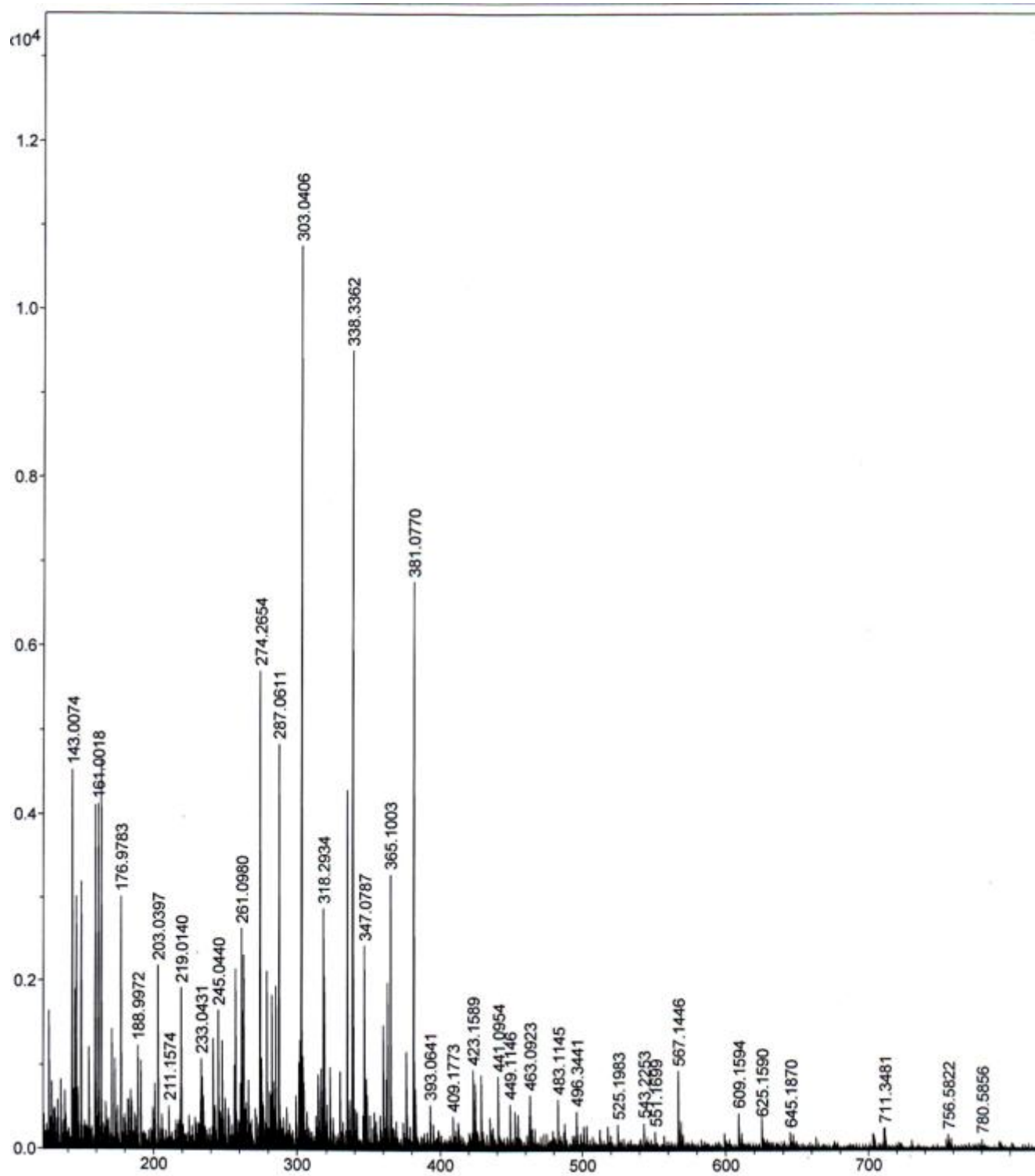


Figure 5

Cationic mass spectrograms of flavonoid from yellow *Anemone obtusiloba*

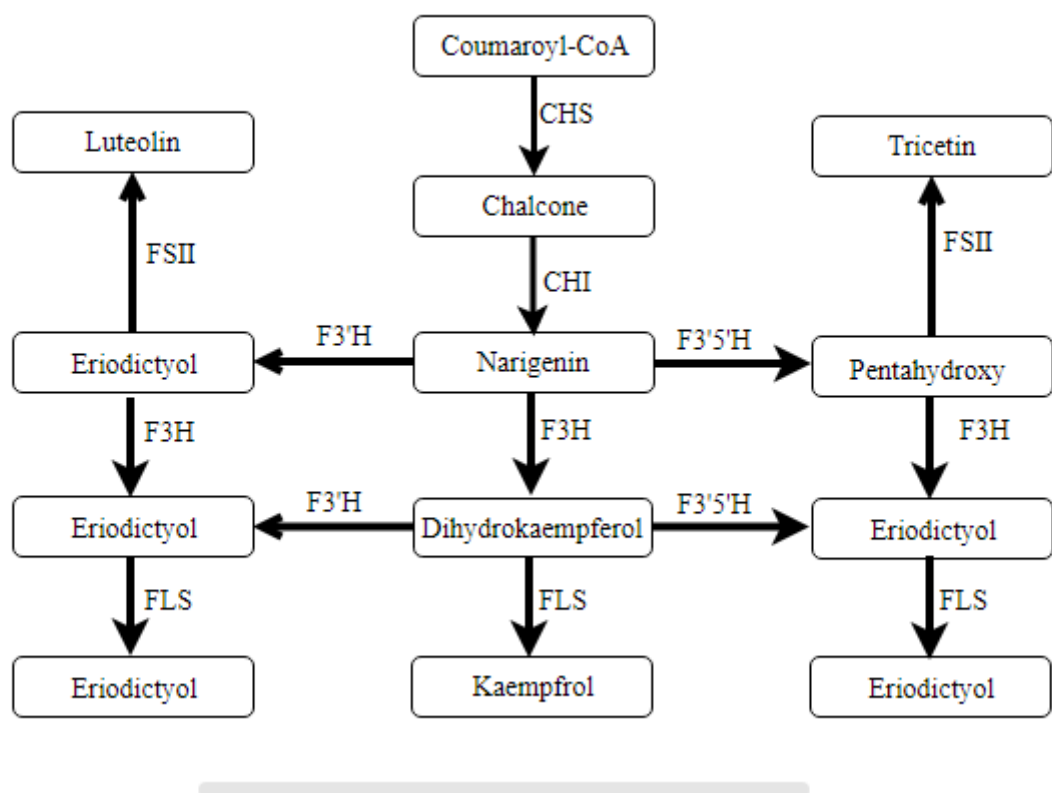


Figure 6

The metabolic pathway of flavonoid

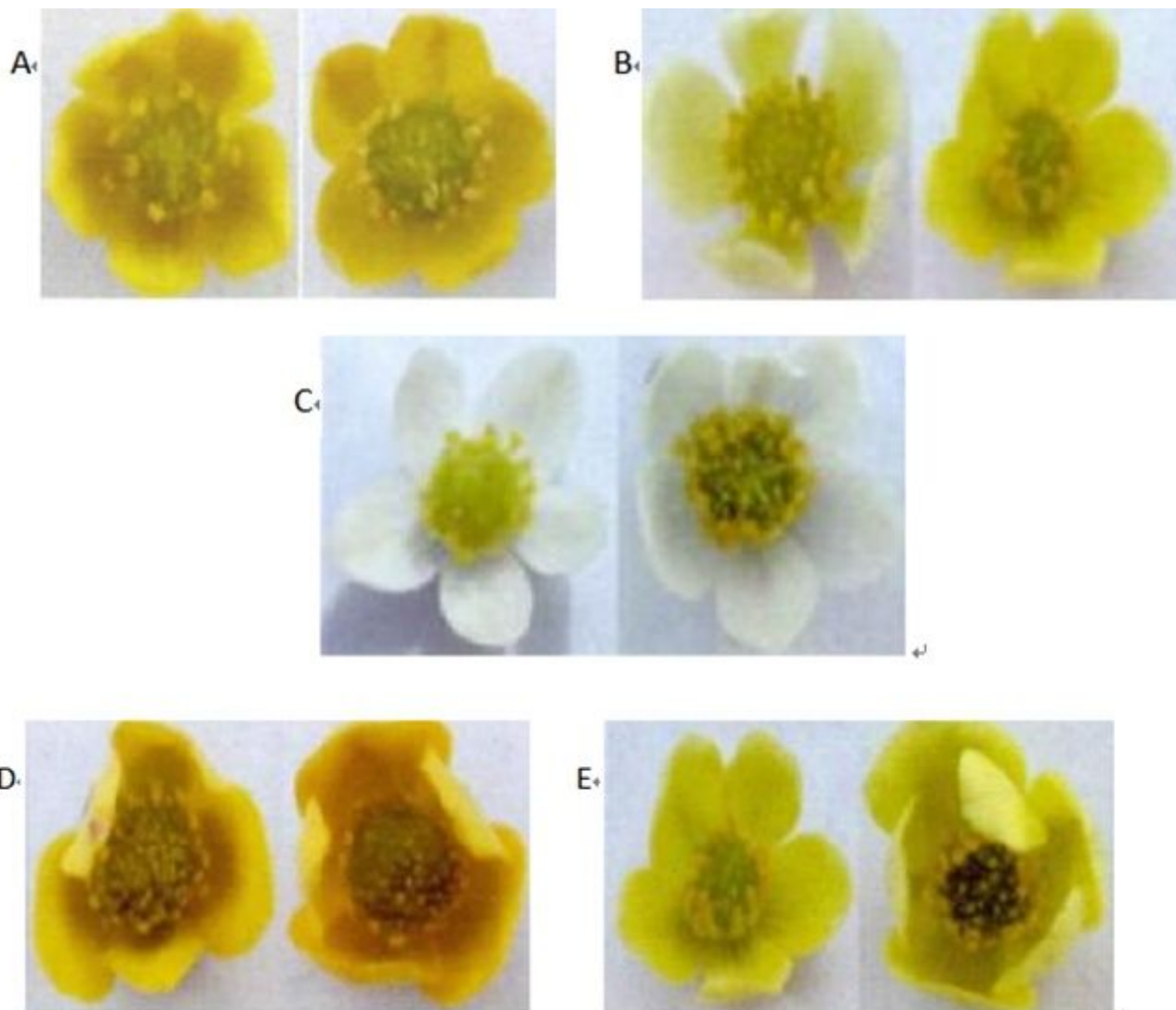


Figure 7

Three Pigment of *Anemone obtusiloba*. (A) Hezuo Yellow, (B) Hezuo Pale Yellow, (C) Hezuo White, (D) Maqu Yellow and (E) Maqu Pale Yellow.

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