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Opinion

Detection and Development on Total Flavonoids by the Way of $Al(NO_3)_3$ Ultraviolet Colorimetry

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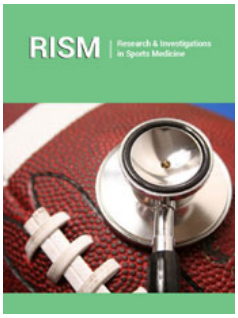

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Abstract

Flavonoids are mainly distributed in higher plants and almost exist in all green plants of Traditional Chinese Medicine (TCM). In this paper we detect the total flavonoids from the standards and samples arising from special flavonoids frameworks by the way of $Al(NO_3)_3$ ultraviolet colorimetry so as to analyze the features of wavelength degree and peak strength in the graph of ultraviolet spectrum. The diverse positions and strengths of absorption band 200-600nm in the ultraviolet spectrum are distinct taking the rutin reference and total flavonoids from *Corydalis conspersafor* instance. We can find there are 4 pairs peaks & peak valleies (325 and 335; 345 and 355; 360 and 365; 375 and 380) between 300nm and 400nm through scanning the rutin standard solution. There is one strong peak at the 242nm and one weak peak at about 510nm. The important result is that the absorbance always is highest at the 345nm and the absorbance is from 0.133 to 1.487.After that, we put forward some thoughts for the further researches for the verification of chemical and biochemical reaction mechanism by the Al(NO3)3 ultraviolet colorimetry, which can be used for the assay of flavonoids with special structures from the diverse families.

Keywords: Flavonoids; Structure and origin; Characteristics of UV; Rutin; $Al(NO_3)_3$ Ultraviolet colorimetry

Introduction

The discovery of flavonoids has the long-standing and abundant history. In the 1930s, the Hungarian scientists Rusznyak and Szent Györgyi identified a substances which is named 'Citrin' from the fruit of lemon' peels [1]. However, at the beginning the substance was designated as vitamin P (P for permeability), one kinds of the member from the vitamin families. Later Bruckner and Szent Györgyi found it was a mixture of the flavonoid's hesperidin and eriodictyol glucoside including rutin. So far flavonoids compound has been identified for much more than 6000 varieties from all kinds of plants [2].

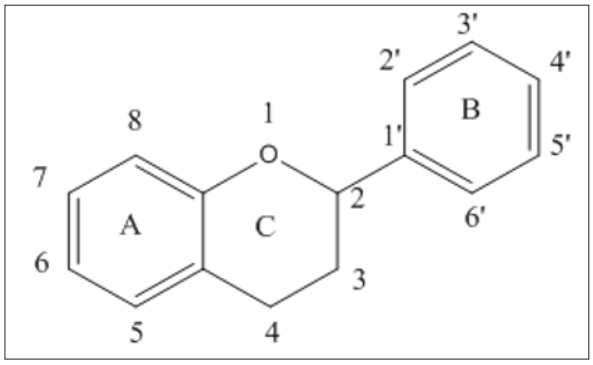

Figure 1: 2-phenylchromogenic ketones+$NaNO_2$+$Al(NO_3)_3$+NaOH→complex structure?

Flavonoids are a class of compounds widely existing and distributed in nature, with 2-phenylchromone (flavone) and C_6-C_3-C_6 framework compounds (Figure 1). There is a keto carbonyl group in their molecules. The oxygen atom on the first position is alkaline and can

form salt with strong acid. Flavonoids have diverse pharmacological activities such as antibacterial [3], antiallergic [4], anticancer [5] and antiviral [6] activities etc. reported in the TCM.

In general, the UV absorption spectrum of most flavonoids in methanol consists of two main absorption bands. The absorption band I (Cinnamoyl ring) is between 300~500nm and the absorption band II (Benzoyl ring) is between 200~280nm [7]. If aluminum salt was added, Al^{3+} formed a stable complex with flavonoids, and band I moved significantly to the long wavelength direction (red shift); in the strong alkaline solution of $NaNO_2$, its absorbance value was the highest at 510nm [8]. Therefore we can infer the structures of various flavonoids for these changes in detailed and detect the contents of the flavonoids if we can carry out the researches laws regarding the special structures flavonoids by $Al(NO_3)_3$ Ultraviolet colorimetry.

Materials and Methods

Collection and treatment of *Corydalis conspersa*

The plants were collected in Jigang mountain, Huangnan Prefecture, Qinghai Province, in August 2017 and identified by Professor Lin Pengcheng (Qinghai Nationalities University) identified as a Tibetan medicine Corydalis conspersa [9]. After cleaning, the samples were divided into roots, stems, leaves and flowers, then dried in the shade, crushed through 80 mesh sieves for further study.

Materials

Rutin standard substance (HPLC, purity≥98%, Shanghai Yuanye Biotechnology Co., Ltd); Ethanol (analytical pure, Tianjin Fuyu Fine Chemical Co., Ltd); Purified water (Hangzhou Wahaha Group Co., Ltd.); Nitrite Sodium (AR, Yantai Shuangshuang Chemical Co., Ltd); Aluminum nitrate (Shanghai Guangnuo Chemical Technology Co., Ltd.); Sodium hydroxide(Chinese Medicine Group Chemical Reagent Co., Ltd.).

Instruments

UV -Vis2450 spectrophotometer (Japan island); Cuvette (Shimadzu, Japan); HH-6 digital display constant temperature water bath pan (Changzhou Aohua Instrument Co., Ltd.); AL204 electronic balance (METTLER TOLEDO Instrument Co., Ltd., Switzerland); DFT-100 high speed powder (Shanghai Dingguang Machinery Equipment Co., Ltd.)

Preparation of standard substance solutions

Weigh 10.9mg rutin standard, add appropriate amount of 60% ethanol, heat to dissolve, and fix the volume in a 100ml volumetric flask after room temperature to prepare rutin standard with the concentration of 0.1mg•ml^{-1}. Shake well and set aside. Take an appropriate amount of rutin standard solution and scan it in the range of 200-700nm to observe the absorption wavelength of rutin [9].

Preparation of sample solutions

The root powder of *C. conspersa* was taken, and 0.1g powder was precisely weighed (3 times were determined in parallel under each experimental condition), and then put into a 250ml flask with a condensation tube, put it in a constant temperature water bath, add a 30ml, 40ml, 50ml, 60ml amount of 60% ethanol to extract total flavone, and keep the volume to 100ml volumetric flask using the 60% ethanol for standby.

Detection of absorption wavelength conditions

Take 2.0ml, 4.0ml, 6.0ml, 8.0ml, 10.0ml rutin standard and 0.5ml sample solution respectively, add 2.5ml of 5% sodium nitrite solution, 2.5ml of 10% aluminum nitrate solution, 20.0ml of 4% sodium hydroxide solution, and finally add 60% ethanol solution to fix the volume to 50.0ml. After adding reagent in each step above, ultrasonic wave for 5min, shake well, and stand for 15min. The absorbance was measured at different wavelength with a 1.0cm quartz cuvette, recording the spectra and peaks [9]. The reaction equation is as follows (1).

Results

Rutin standard substance solutions

From Figure 2, we can find there are 8 peaks and peak valleys between 300nm and 400nm, at the same time there is one strong peak at the 242nm and one weak peak at about 510nm. We summary the wavelength position and absorption of the peaks in Table 1. It shows that the absorbance is changing up and down because the 4 pairs peaks & peak valleies (325 and 335; 345 and 355; 360 and 365; 375 and 380) with the different wavelengths at the same concentration. The important result is that the absorbance always is highest at the 345nm of the same concentration and the absorbance is between 0.133 and 1.487.

Table 1: The average absorbance of standard solution from the corresponding concentration and diverse wavelength (three parallel repetitions).

Concentration(mg/ml)	Different wavelengths and absorbance							
	325	335	345	355	360	365	375	380
0.00436	0.231	0.277	0.287	0.271	0.247	0.239	0.191	0.133
0.00872	0.449	0.484	0.658	0.648	0.586	0.52	0.428	0.302
0.01308	0.569	0.623	0.85	0.873	0.786	0.703	0.566	0.404
0.01744	0.742	0.79	1.223	1.136	1.037	0.844	0.677	0.5
0.0218	0.907	0.904	1.487	1.376	1.199	1.018	0.81	0.567

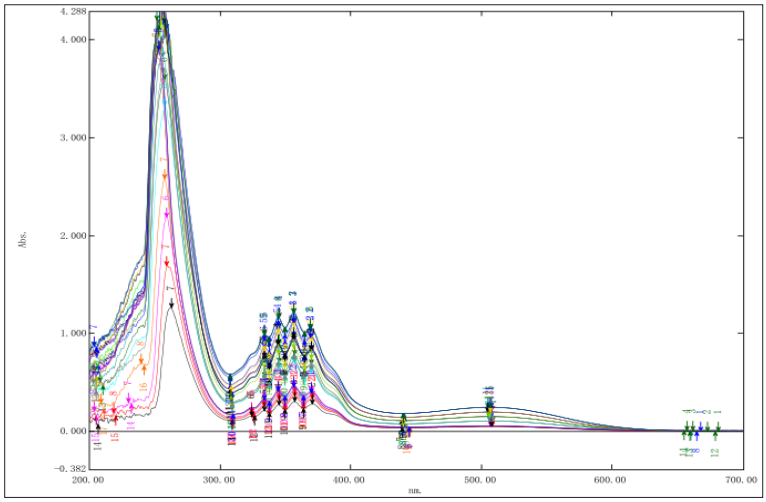

Figure 2: The UV scanning graph of rutin standard substance solutions.

Sample solutions

From Table 2 and Figure 3 compared to the Figure 2, we found that there are the series of peaks & peak valleies between 310 and 400nm the same as the standard solutions. There shows the highest absorbance while using the material liquid ratio of 50 ml: 0.1g and the most contents of flavonoids.

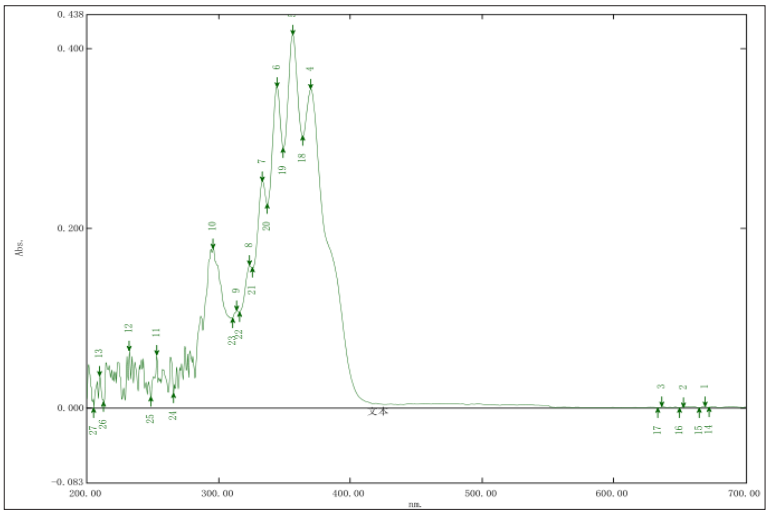

Figure 3: The UV scanning graph of sample solution.

Table 2: The average absorbance of sample solution at 345nm wavelength (three parallel repetitions) from the diverse material liquid ratio (30ml:0.1g; 40ml:0.1g; 50ml:0.1g; 60ml:0.1g).

Times	30	40	50	60
1	0.262	0.292	0.469	0.398
2	0.264	0.278	0.419	0.372
3	0.246	0.283	0.421	0.347
Average	0.257	0.284	0.43678	0.372

Discussion

The commonly methods used to determine the contents of flavonoids is ultraviolet spectrophotometry, which is mainly used for the determination of total flavonoids, which can be divided into direct determination (determination at the maximum absorption wavelength of UV spectrum compared to the standard reference substance) and metal complex salt colorimetry (refers to the determination of total flavonoids). Flavonoids with specific structure react with aluminum salt, magnesium salt, lead salt, zirconium salt and other reagents to form colored complex, which can be used for the identification and content determination of some types of flavonoids. In this paper, we determinate the total flavonoids through colorimetry of aluminum nitrate or aluminum chloride in alkaline conditions at the maximum absorption wavelength of the colored complex.

The total flavonoids were extracted by ethanol under different conditions, the flavonoids with 5-hydroxy, 3-hydroxy or o-dihydroxy were complexed with Al $(NO_3)_3$ and $NaNO_2$. After the preliminary

study, it was found that there were 4 pairs absorption peaks in the range of 300-400nm after adding the complexing reagent. However, some flavonoids have no maximum absorption or weak absorption near 500 nm. Some non-flavonoid substances have maximum absorption or strong absorption near 500nm [10]. Therefore, with rutin as the reference substance and Al $(NO_3)_3$-$NaNO_2$-NaOH as the chromogenic reagent, the method for the determination of total flavonoids at 510nm is not specific and firstly we should scan the treated sample solutions through the UV spectrophotometer to define the detected wavelength having the maximum absorbance.

Conclusion

It is important to clarify the relationship between the absorption peaks and flavonoids with special structures. We can adopt the below methods to verify to clearly know the reactions courses. Firstly add one kind of reagent as the reaction equation (1) into the standard solution of different structures changing the concentrations of the reagent and scan the solutions using the UV spectrophotometer to summary the laws of graph alterations step by step. It is huge works and how do they complex with each other among groups? Secondly observe and analyze the intensity of absorption peaks because of the altered concentration. Thirdly detect the reaction speeds or changes of graphs through adding or removing the reagents one by one so as to verify the key steps during the reactions process. Finally, we develop the certain combined instruments to auto-operate the reagents to the solution not only improving the accuracy and efficient, but also saving the cost of manual labor and material resources.

The solutions and approaches to all aspects of the problems are the key technical difficulties and challenges in the future researches. We must deeply discover and verify the reaction courses accompany the advanced technology to well explain the relative structures and biochemical reaction mechanisms.

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