Asian Journal of Chemistry; Vol. 26, No. 19 (2014), 6409-6413

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16396

Simultaneous Determination of Total Flavonoids and Organic Acids in Chrysanthemum by UV-Visible Spectrophotometry

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Received: 19 September 2013; *Accepted*: 8 January 2014; *Published online*: 16 September 2014; AJC-15928

A rapid method for simultaneous determination of total flavonoids and total organic acids in different kinds of chrysanthemum was developed. The content of total flavonoids was determined at 270 nm by UV method using luteolin as standard and that of total phenols was done at 510 nm using the color reaction with $Al(NO_3)_3$ using the colored luteolin as standard. The optimum extraction conditions of flavonoids from chrysanthemum were investigated by using orthogonal design. The content of total organic acids is the value for the differences between the content of total phenols and that of total flavonoids. Due to the reports of the methods of determining the content of total organic acids in chrysanthemum is few. This method can be relatively simple measure the content of total organic acids in chrysanthemum.

Keywords: Chrysanthemum, Flavonoid, Organic acid, UV-visible spectrophotometry, Orthogonal test.

INTRODUCTION

Chrysanthemum which was described functions were soothing asthma, Mingmu, dispelling wind and heat, curing high blood pressure and heart disease. It is widely used not only as a tea drink, but also as a traditional Chinese herbal medicine. Recent studies show that the biologically active constituents of Chrysanthemum contains organic acids and abundant flavonoids, which have been reported to have the functions of antioxidation, antibacteria, antivirus, antiulcerogenic, antiinflammatory, checking cough and dispelling phlegm[1,2]. So identification and determination of the content of total flavonoids and total organic acids in chrysanthemum play important role to control its quality and safety for clinical applications[3].

Extraction is the most important step in isolation and analysis of bioactive compounds from medicinal plants. Thus, it is essential to choose and optimize extraction conditions. Traditional extraction techniques, such as maceration, heating reflux and soxhlet extraction, are often effective but time consuming or labor intensive. In contrast, ultrasonic extraction can extract analytes from various matrices in a shorter time[4].

Besides, the measure of flavonoids was always achieved by UV-visible[5] and HPLC[6]. The quantification of total flavonoids was usually completed by UV-visible absorption at 500 nm and calculated against a standard reference in routine analyses[7-10]. Although a number of papers have reported on the quantification of total flavonoids in traditional herbal medicines, simultaneous determination of total flavonoids and total organic acids in chrysanthemum has not been reported.

In this work, the aim was to develop an ultrasound-assisted extraction (UAE) method and to measure of total flavonoids and total organic acids simultaneously in chrysanthemum with the use of UV-visible spectrophotometer, which has been proven to be simple and convenient, as well as sensitive and accurate.

EXPERIMENTAL

The determination was performed on a UV-3600 spectrophotometer (SHIMADZU Instrument Co., Ltd., Tokyo, Japan). JK-300B ultrasonic apparatus (Jin nike Instrument Co., Ltd., Anhui, People's Republic of China) was applied here and the outpower was 500 W, with a frequency of 40 kHz.

Chlorogenic acid and luteolin were abtained from Chinese Chemical and Biological Drugs Institute (Beijing, China), Chrysanthemum (chuju, gongju, hangbaiju) were purchased from Bozhou drugstore, Anhui province, China. Other chemicals were analytical grade and purchased from SCRC (Shanghai, China). All aqueous solutions were made up in doubly distilled water. Stock solutions of chlorogenic acid (380 mg L^{-1}) and luteolin (300 mg L^{-1}) were prepared in anhydrous ethanol, stored in the dark at 4 °C and were diluted to the desired concentrations with ethanol.

Sample preparation and determination: Air-dried chrysanthemum (chuju, gongju, hangbaiju) were grounded and shifted through a 0.75 mm sieve. The process of flavonoids extraction from chrysanthemum (chuju, gongju, hangbaiju) (1 g) was carried out in an ultrasonic cleaner JK-300B was mixed with 40 mL ethanol in a conical flask and sonicated for different time (from 10 to 50 min) at 25 °C, while the ratio of material to liquid ranged from 1:10 to 1:50. Flavonoids extraction was carried out according to orthogonal experimental design. The extracts were centrifuged (15 min, 3000 rpm), then filtered through filter paper. A total of each sample extraction was pipetted into a 250 mL volumetric flask. Sample extraction solution (1.0 mL) was accurately taken and put in 10 mL volumetric flasks. This solution was investigated at 270 nm. The content of total flavonoids was determined at 270 nm by UV method using luteolin as standard.

After that, a total of 2 mL of sample extraction solution from 250 mL volumetric flask was pipetted into a 10 mL volumetric flask. The solution was treated with 0.5 mL of the 5 % $NaNO_2$ solution for 6 min and evenly mixed, into which 0.5 mL of the 10 % $Al(NO_3)_3$ solution was added and shaked up; then after 6 min, 4 mL of the 4 % NaOH solution was added to it. The mixture was diluted to the volume with 60 % ethanol and allowed to stand for 15 min before analyzing against the blank solution. This solution was examined at 510 nm. The content of total phenols was done at 510 nm by UV method using the color reaction with $Al(NO_3)_3$ using the colored luteolin as standard. The content of total organic acids is the value for the differences between the content of total phenols and that of total flavonoids.

Optimization of flavonoids extraction: An orthogonal design [L_9 (3^3)] was applied to evaluating the effects of the following influencing factors: ratio of material to liquid (A), ethanol concentration (B), ultrasonic time (C). Nine extraction experiments were carried out in order to evaluate the best conditions for the extraction of flavonoids. Factors and levels test are displayed in Table-1.

TABLE-1
FACTORS AND OF THE ORTHOGONAL DESIGN

Factors	Levels		
	1	2	3
(A) Material to liquid	1:30	1:40	1:50
(B) Ethanol concentration (%)	20	40	60
(C) Ultrasonic time (min)	30	40	50

RESULTS AND DISCUSSION

Selection of detection wavelength: The absorption spectra (from 200 nm to 400 nm) of sample extraction, luteolin solution and chlorogenic acid solution were obtained (Fig. 1). The absorption peak of luteolin was at 348 nm (curve 3) and sample extraction at 333 nm (curve 2), while chlorogenic acid solution had strong absorptions at 333 nm (curve 1). So in the wavelength of the total organic acids have interference on the determination of flavonoid content. As shown in Fig. 1, the absorption peak of luteolin was very strong at 270 nm, while there was little absorption for chlorogenic acid at 270 nm. Comprehensively, 270 nm was chosen as the detection wavelength for the determination of total flavonoids.

The absorption spectra (from 400 nm to 600 nm) of sample extraction and luteolin solution using the color reaction with $Al(NO_3)_3$ were obtained (Fig. 2). The absorption peak of luteolin was at 510 nm (curve 5) and sample extraction at 520 nm (curve 4). According to spectra, 510 nm was chosen as the detection wavelength for the determination of total phenols.

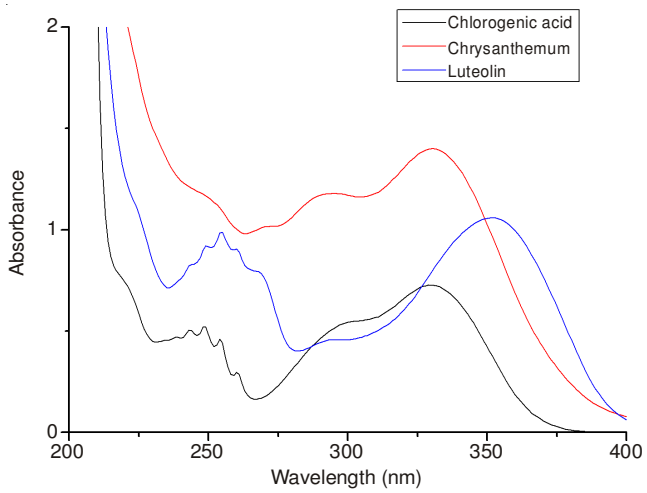

Fig. 1. UV spectra of sample extraction, luteolin and chlorogenic acid

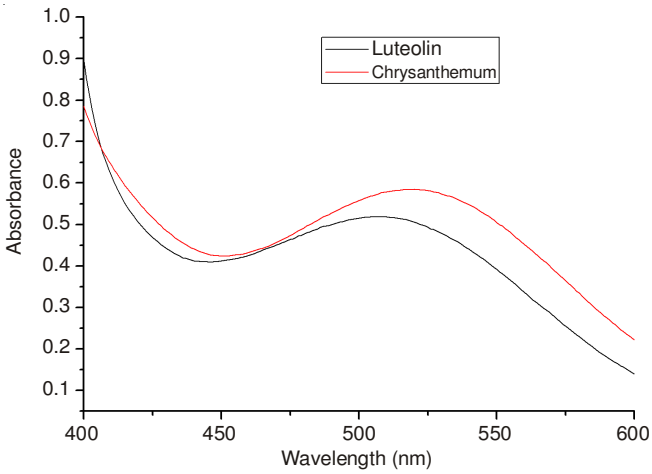

Fig. 2. UV spectra of sample extraction and luteolin using the color reaction with $Al(NO_3)_3$ from 400 nm to 600 nm

Effect of ethanol concentration on absorbance: Since flavonoids contain some hydroxyl groups, they often dissolve easily in ethanol or alcohol-water mixtures. Chrysanthemum sample was employed here, trying to find out the optimum solvent for sample extraction. One gram of fine powder was extracted with 40 mL of different concentration ethanol (20, 40, 60, 80 and 100 %) in ultrasonic bath at 25 °C, respectively. All the time of ultrasonic was 40 min. The relationship between ethanol concentration and the absorbance at 270 nm is shown in Fig. 3. It is observed that the absorbance kept increasing and reached the top when the ethanol concentration increases to 40 % and then began to fall sharply as the ethanol concentration continued growing. Hence, the optimum extraction solvent turned out to be 40 % ethanol.

Effect of ultrasonic time on absorbance: One gram of fine powder was extracted with 40 mL of 40 % ethanol concentration in ultrasonic bath at 25 °C. Different time of sonication

(10, 20, 30, 40, 50 min) was used in the experiment to get the maximum absorbance of flavonoids. The absorbance under different ultrasonic time is shown in Fig. 4. The absorbance of total flavonoids increased sharply within 40 min, but after 40 min, it had no difference. According to the Noyes-Whitney theory, the dissolution is fast at firstly and changed little when the active ingredient concentration between inner and outer diffusion layer reach equilibrium after a period of extraction. Therefore, 40 min was chosen as the extraction time.

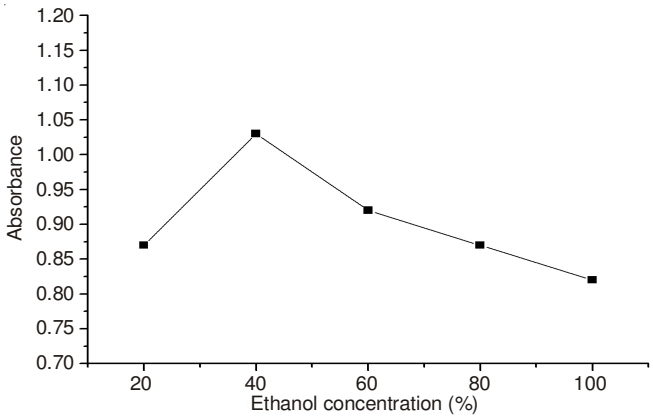

Fig. 3. Effect of the ethanol concentration on absorbance

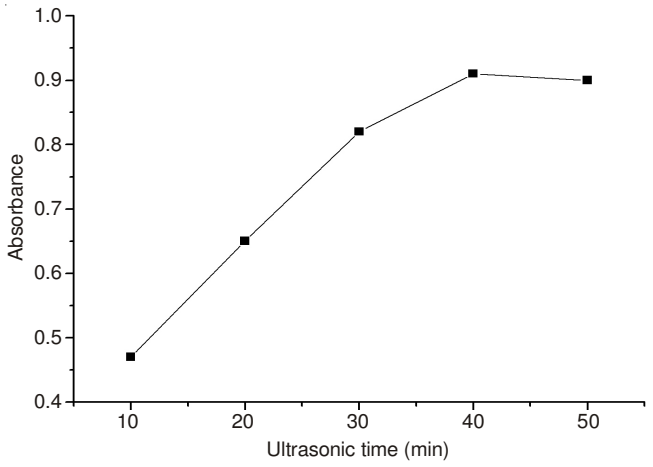

Fig. 4. Effect of ultrasonic time on absorbance

Effect of the ratio of material to liquid on absorbance: In this work, effect of the ratio of material to liquid on absorbance was investigated. Other extraction conditions such as ethanol concentration and ultrasonic time were fixed at 40 % and 40 min and the ratio of material to liquid was changed from 1:10 to 1:50. As shown in Fig. 5, it was found that the absorbance rose as the ratio of material to liquid is increased and reached maximum when the ratio was 1:40. However, there were few changes when the ratio improved. It was meant that more time and energy were required to condense the extraction solution in later separation process. So, the solid/ liquid ratio of 1:40 was suitable to reach the high absorbance for total flavonoids.

Optimization of the extraction condition of flavonoids from chrysanthemum: An orthogonal design [L_9 (3^3)] was applied to evaluating the effects of the extraction condition of total flavonoids from chrysanthemum. The experimental data

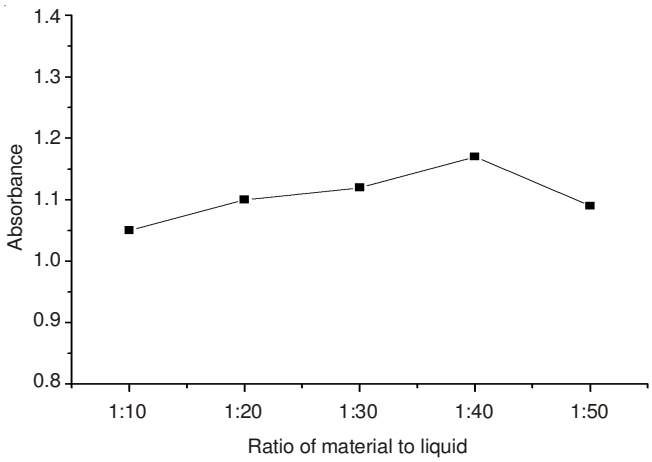

Fig. 5. Effect of the ratio of material to liquid on absorbance

obtained from orthogonal design and the analysis results were shown in Table-2. The results suggested that there were great difference in the absorbance between each experimental trail. From Table-2, it was seen that the influence factor to the absorbance declined in sequential order: B > C > A. The results demonstrated that the optimal conditions for the extraction of total flavonoids from chrysanthemum were $B_3C_1A_2$, that was to say 60 % of ethanol concentration, 0.5 h of ultrasonic time and 1:40 of material/solvent ratio.

TABLE-2
RESULT AND ANALYSIS SHEET OF ORTHOGONAL EXPERIMENT

No.	(A) Material to liquid	(B) Ethanol concentration (%)	(C) Ultrasonic time (min)	Absorption
1	1:30	20	30	0.896
2	1:30	40	40	1.018
3	1:30	60	50	0.979
4	1:40	20	40	0.936
5	1:40	40	50	0.944
6	1:40	60	30	1.192
7	1:50	20	50	0.956
8	1:50	40	30	1.006
9	1:50	60	40	0.960
K1	0.964	0.929	1.031	–
K2	1.024	0.989	0.971	–
K3	0.974	1.044	0.960	–
R	0.060	0.155	0.071	–

Method validation: A total of 300 mg L^{-1} of the luteolin standard solution was prepared by dissolving luteolin reference material in ethanol solution. Luteolin solution (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 mL) was accurately taken and put in 10-mL volumetric flasks, respectively. The following steps were similar to those sample treatment. The content of total flavonoids was determined at 270 nm by UV method. The regression equation was Y = 79.208X-0.0756 (r^2 = 0.9958), with a good linearity in the range of 6.0-60 µg/mL. The content of total phenols was done at 510 nm by UV method using the color reaction with $Al(NO_3)_3$ using the coloured luteolin as standard. The regression equation was Y = 21.385X-0.0299 (r^2 = 0.9949), with a good linearity in the range of 6 to 60 µg/mL.

1 mL of the chrysanthemum sample solution, put in 10 mL volumetric flasks and determined at 270 nm successively for five times. The average absorbance was 1.094 and the RSD attained was 0.57 %. 2 mL of the chrysanthemum sample solution, put in 10 mL volumetric flasks and determined at 510 nm successively for five times after coloration. The average absorbance was 0.348 and the RSD attained was 0.48 %, demonstrating that the instrument used had a high precision.

A series of chrysanthemum samples (chuju) were taken and treated according to sample preparation and colorimetric methods mentioned above. Average content of the total flavonoid calculated from regression equation (270 nm) were 29.59 mg g^{-1} with RSD < 1.7 % (n = 5). Average content of the total phenols calculated from regression equation (510 nm) were 33.14 mg g^{-1} with RSD < 2.4 % (n = 5), investigating a good repeatability.

After the extraction of Chrysanthemum, the sample mentioned above was dealt with according to the procedures for the determination of total flavonoids and total phenols; absorbances were measured every 10 min ranging from 0 min to 60 min. As shown in Figs. 6 and 7, it's clear that the absorbance of the solution was relatively stable (RSD = 0.76 %) for the determination of total flavonoids (270 nm) and (RSD = 1.09 %) for the determination of total phenols (510 nm) if the measurement was carried out within 1 h. Thus, all the analyses should be performed within 1 h.

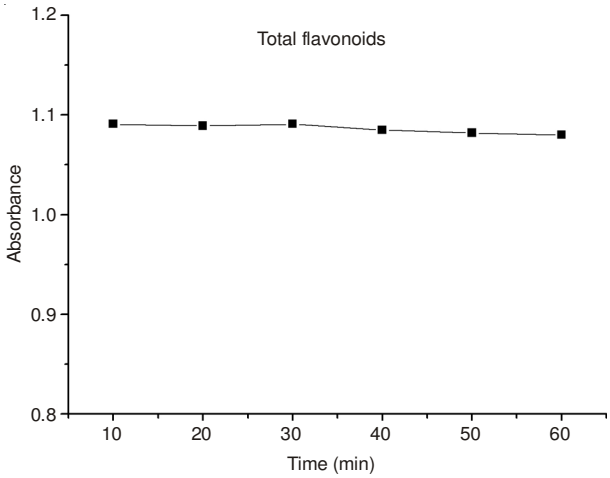

Fig. 6. Relationship between the analysis time and absorbance for total flavonoids

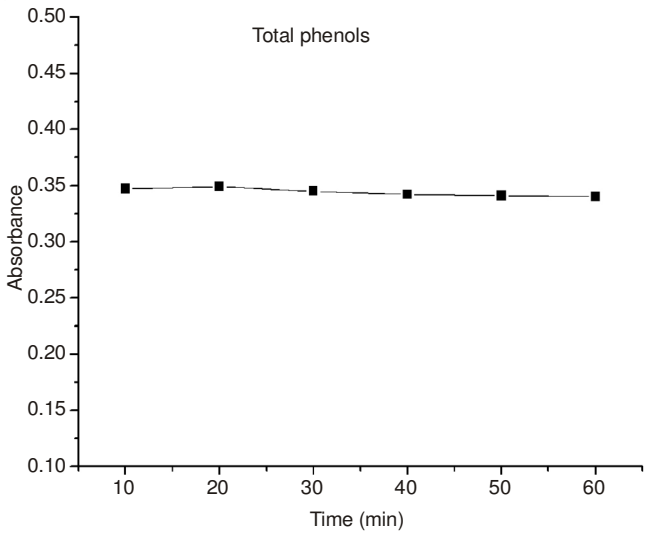

Fig. 7. Relationship between the analysis time and absorbance for total phenols

Recovery experiment was performed to evaluate the accuracy of the methods. 1 g of Chrysanthemum samples solutions (chuju) were spiked with 0.5 mL luteolin solution (containing 0.15 mg of luteolin) after the extraction. The spiked samples were analyzed in six copies. Recoveries of total flavonoids and total phenols obtained are shown in Table-3, which informed that the method possessed a nice accuracy.

Sample analysis: Under the optimum condition, the contents of total flavonoids and phenols in the different sample of chrysanthemum (chuju, gongju, hangbaiju) were determined according to the procedures described earlier. The comparisons in the different sample of chrysanthemum are shown in Table-4. As shown, the contents of total flavonoids and phenols in gongju were higher than others. The content of total organic acids is the value for the differences between the content of

TABLE-4
CONTENT OF FLAVONOIDS, PHENOLS AND ORGANIC ACIDS IN CHRYSANTHEMUM

Sample	Phenols (mg g^{-1})	Flavonoids (mg g^{-1})	Organic acids (mg g^{-1})
Gongju	52.60	40.32	12.28
Chuju	33.14	29.59	3.55
Hangbaiju	31.15	28.33	2.82

TABLE-3
RESULTS OF RECOVERY OF FLAVONOIDS AND PHENOLS

Compound	m_{sample} (mg L^{-1})	m_{add} (mg L^{-1})	m_{found} (mg L^{-1})	Recovery (%)	Average recovery (%)	RSD (%)
Flavonoids	0.0118	0.0150	0.0262	97.76	99.29	2.3
	0.0115	0.0150	0.0273	103.03		
	0.0123	0.0150	0.0279	102.22		
	0.0108	0.0150	0.0254	98.45		
	0.0119	0.0150	0.0263	97.77		
	0.0112	0.0150	0.0253	96.56		
Phenols	0.0177	0.0150	0.0314	96.02	100.21	2.1
	0.0172	0.0150	0.0316	98.14		
	0.0182	0.0150	0.0334	100.65		
	0.0168	0.0150	0.0332	104.43		
	0.0173	0.0150	0.0321	99.38		
	0.0179	0.0150	0.0338	102.70		

total phenols and that of total flavonoids. Therefore, the contents of total organic acids in gongju, chuju and hangbaiju were 12.28, 3.55 and 2.82 mg g^{-1}, respectively. The content of total organic acids in gongju was also highest.

Conclusion

At present, UV wavelength to detect the contents of total flavonoids in chrysanthemum is usually 348 nm, but chlorogenic acid has strong absorption at 333 nm. Therefore in the wavelength of the total organic acids have interference on the determination of content of total flavonoids. But the flavonoid has great absorption and organic acid has a minimum absorption at 270 nm, which selected as the detection wavelength. In this work, the content of total organic acids is the value for the differences between the content of total phenols and that of total flavonoids, simultaneous determination of total flavonoids and total organic acids in chrysanthemum has been completed.

This paper was attempted to evaluate the effects of three experimental factors, namely ratio of material to liquid, ultrasonic time and ethanol concentration on the simultaneous determination of total flavonoids and total organic acids in absorbance from chrysanthemum. Orthogonal design was used to optimize the extraction conditions. The results showed that ethanol concentration played a critical role for achieving higher extraction yields. But other factors (ultrasonic time and ratio of material to liquid) had secondary influence on the flavonoids yields.

Studies on total flavonoids and total organic acids determination of different kind of chrysanthemum samples clearly manifested that the contents of total organic acids in gongju, chuju and hangbaiju were different from each other. The content of total organic acids in gongju was also highest. The method established for the total flavonoids and total organic acids determination simultaneously in chrysanthemum was simple, direct and accurate, providing a valuable reference for quality control.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support provided by Department of Education Anhui Province (Grant No. 2012SQRL147 and Grant No. KJ2013Z047).

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