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Determination of Procyanidins by High Performance Liquid Chromatography and Ultraviolet Spectrophotometer

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Proanthocyanidins are a mixture of poly polyphenols widespread in nature, in this experiment we establish a quantitative method for the determination of procyanidins, so as to provide references for its development and utilization. The contents of procyanidins is determined by HPLC and HPLC conditions are as follows: A diamonsil C18 column as separation column (250 × 4.6 mm i.d.,10 µm), mobile phase of methanol -0.4 % phosphoric acid (v:v = 1:1), flow rate of 1 mL/min, detection wavelength of 280 nm and column temperature of 30 °C, retention time is 2.55-2.78 min, HPLC standard curve: y = 142773x + 14416, R^2 = 0.9998, the linear relationship between the concentration of procyanidins and peak area are good in the range of 20-100 µg/mL, the recovery rates of the procyanidins is 104.83 % with RSD of 1.87 %, the detection limit is 1.25 µg/mL. The contents of procyanidins are determined by UV-spectrophotometer and UV conditions are as follows: ethanol as a constant volume solvent, the absorbance of procyanidins is measured at 471.5 nm. Determination of procyanidins standard curve is A = 0.7438 × C-0.0026, R^2 = 0.9997, linear range is 0.014-0.056 mg/mL.

Keywords: Procyanidins, HPLC, UV.

INTRODUCTION

Proantho cyanidins (PC) are a mixture of poly polyphenols, the chemical designation is 2-(3,4-dihydroxyphenyl)-2-[{2-(3,4-dihydroxyphenyl)-3,4-dihydro-5,7-dihydroxy-2*H*-1-benzopyran-3-yl}oxy]-3,4-dihydro-2*H*-1-benzopyran-3,4,5,7-tetrol, its structural formula is shown in Fig. 1. There are often a mixture of different degree of polymerization in plants, containing a variety of isomers, which is generally reddish brown powder, gas micro, astringent taste, soluble in water and most organic solvents[1-3], usually extract from grape seed or pine bark. These compounds have a variety of biological activity, which are known for its high efficiency, low toxicity, high bioavailability, which is a highly functional factors *in vivo* activity. Procyanidins exist widely in plants, which can be medicine and treated for cardiovascular disease, cancer and other features[4].

Procyanidins is now recognized as the most effective natural antioxidants, antifree radical oxidation, procyanidins is 50 times more vitamin E and 20 times the vitamin C and procyanidins can be rapidly absorbed completely, oral 20 min to reach the highest blood levels, the metabolic half-life[5-6] is up to 7 h. Because of its antioxidant activity, quantitative analysis of procyanidins become quite difficulties. In this study, we wish to find a convenient, accurate and high stability quantitative analysis method, lay the foundation for future research in all aspects of procyanidins.

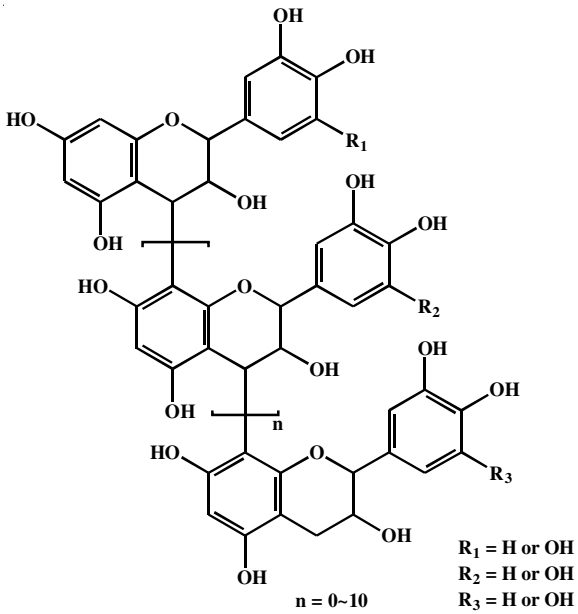

Fig. 1. Structure of procyanidins

EXPERIMENTAL

Vanillin (3-methoxy-4-hydroxybenzaldehyde) (Tianjin Guangfu Fine Chemical Research Institute); methanol (HPLC grade) (Honeywell Trading (Shanghai) Co., Ltd.; Procyanidins content = 95 % (Shanghai Golden Harvest Biotech Co., Ltd.); methanol (AR), hydrochloric acid, phosphoric acid (Beijing chemical Plant); distilled water; deionized water (homemade).

UVmini-1240, SHIMADZU ultraviolet spectrophotometer; HPLC analysis is performed using a SHIMADZU liquid chromatographic systems (Shimadzu company, Japan) HPLC system, A Venusil XBP C18 column (250 × 4.6 mm i.d., 10 μm) is employed for the separation of samples. All solutions are filtered through 0.22 μm membranes (Chromatography Science and Technology Co., Tianjin, China) before HPLC analysis; KQ-50DB ultrasonic cleaner; Microinjector; analytical balance; volumetric flask; Cuvette.

High performance liquid chromatography

Chromatography conditions: A Venusil XBP C18 column (250 × 4.6 mm i.d.,10 μm), mobile phase of methanol- 0.4 % phosphoric acid (v:v = 1:1), flow rate of 1 mL/min, detection wavelength of 280 nm and 30 °C as column temperature, sample amount is 10 mL, retention time is 2.55-2.78 min, theoretical plate number is 6000.

Preparation of procyanidins solution: 10.53 mg Procyanidins with constant weight is weighed precisely and put into 10 mL brown volumetric flask, then dissolved in methanol and diluted to 10 mL which get a concentration of 1 mg/mL standard solution.

Methodology investigation

Investigation of linear relationship: Standard solutions are taken 0.20, 0.40, 0.60, 0.80, 1.00 mL, respectively, put into 10 mL brown volumetric flask, then dissolved in methanol and diluted to 10 mL which get the concentration of 20, 40, 60, 80 and 100 μg/mL solution, the solutions of procyanidins are measured, respectively, filter through 0.22 μm microporous membrane and injected by the chromatographic conditions. Sample amount as abscissa (x), peak area as ordinate(y), the line relationship of the Procyanidins reference substance is investigated.

Limit of detection experiments: Using standard solution of procyanidins dilute to 10, 5, 2.50, 1.25, 0.63 μg/mL solution, filter through 0.22 μm microporous membrane, inject 10 μL into the HPLC system.

Precision test: The same control solution 80 μg/mL is injected for 5 times continuously, RSD of procyanidins peak areas is calculated.

Repeatability test: 0.01 g of 70 % procyanidins is weighed precisely, put into 10 mL brown volumetric flask, then dissolved in methanol and diluted to 10 mL which get a concentration of 0.7 mg/mL sample solution, taken 0.60 mL into 10 mL brown volumetric flask, then dissolved in methanol and diluted to 10 mL which get the concentration of 42 μg/mL, inject 10 μL into the HPLC system, the peak areas of procyanidins and RSD are figured out.

Stability test: The control solution 80 μg/mL is injected 10 μL into the HPLC system at 0, 2, 4, 8, 12 h, respectively, the peak areas of procyanidins and RSD are figured out.

Recovery test: 4 mg standard procyanidins is weighed accurately and transferred to a 10 mL brown volumetric flask and dissolved in methanol. This is taken as 100 % concentration, followed by preparing low concentration solution 80 % and high-concentration solution120 %. Injection volume used for assay studies is 10 μL, the average recovery rate and RSD are calculated.

Standard sample preparation: 7.37 mg of procyanidins reference substance with constant weight is weighed precisely and put into 10 mL brown volumetric flask, then dissolved in methanol and diluted to 10 mL. Shake well, put it aside, the concentration is 0.737 mg/mL procyanidins.

1 % vanillin preparation: 1 g of vanillin is weighed precisely, put into 100 mL volumetric flask, then dissolved in methanol and diluted to 100 mL.

Preparation of hydrochloric acid solution: Accurately pipette 8 mL from concentrated hydrochloric acid to100 mL volumetric flask diluted with water.

Investigation of linear relationship: Exact amount taken from the standard sample solution 0.2, 0.3, 0.4, 0.6, 0.8 mL solution placed in 10 mL brown volumetric flasks respectively and diluted to 10 mL with methanol solution, which get the concentration of 0.014, 0.021, 0.028, 0.042, 0.056 mg/mL standard solution, take 0.50 mL, respectively, put into 10 mL stoppered test tubes, add 5 mL mixed solution 1 % vanillin-hydrochloric acid solution(v:v = 1:1), placed in water bath at 30 °C, react 0.5 h in dark condition, then remove to room temperature, methanol is the reference solvent, by wavelength scanning of procyanidins solution, determine procyanidins positions of absorption peak and make a standard curve at the peak position.

Limit of detection experiments: Using standard solution of procyanidins dilute to 7, 3.50 μg/mL solution, take 0.50 mL, respectively, put into 10 mL stoppered test tubes, add 5 mL mixed solution 1 % vanillin-hydrochloric acid solution (v:v = 1:1), placed in water bath at 30 °C, react 0.5 h in dark condition, then remove to room temperature, the corresponding values are measured at the maximum absorption wavelength by UV, the detection limit obtained.

Precision test: Using standard solution of procyanidins dilute to 0.028 mg/mL solution, take 0.50 mL, put into 10 mL stoppered test tubes, add 5 mL mixed solution 1 % vanillin-hydrochloric acid solution (v:v = 1:1), placed in water bath at 30 °C, react 0.5 h in dark condition, then remove to room temperature. The corresponding values are measured at the maximum absorption wavelength by UV five times, RSD are figured out.

Stability test: Using standard solution of procyanidins dilute to 0.028 mg/mL solution, take 0.50 mL, put into 10 mL stoppered test tubes, add 5 mL mixed solution 1 % vanillin-hydrochloric acid solution (v:v = 1:1), placed in water bath at 30 °C, react 0.5 h in dark condition, then remove to room temperature. The corresponding values are measured at the maximum absorption wavelength by UV at 0, 2, 4, 8, 12 h, respectively.

RESULTS AND DISCUSSION

Methodology investigation in HPLC

Investigation of linear relationship: Chromatogram showing in Fig. 2, retention time is 2.55-2.78 min. As shown

in Fig. 3, the result indicate the content of procyanidins has good linear relationship with peak area in the range of 20-100 μg/mL, the regression equation is y = 142773x + 14416, R^2 = 0.9998.

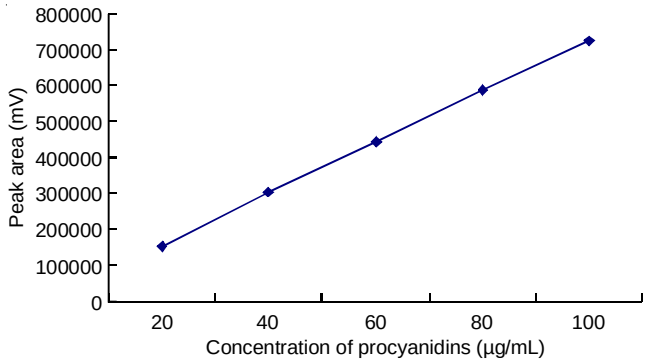

Fig. 3. Standard curve of procyanidins by HPLC

Limit of detection experiment: As shown in Table-1, the result indicate the detection limit is 1.25 μg/mL.

TABLE-1
EXPERIMENTAL DATA OF DETECTION LIMIT BY HPLC

Test no.	Content of procyanidins (μg/mL)	Peak area (mv)
1	10.00	85222
2	5.00	50214
3	2.50	31260
4	1.25	23798
5	0.63	220

Precision test: The RSD of reference procyanidins peak areas is 1.86 % (n = 5), which suggest the instrument is of good precision, the precision test data of procyanidins by HPLC is shown in Table-2.

TABLE-2
PRECISION TEST DATA OF PROCYANIDINS BY HPLC

Test no.	Peak area (mv)	RSD (%)
1	604507	
2	628435	
3	609492	1.86
4	601843	
5	600923	

Repeatability test: The precision test data of procyanidins by HPLC is shown in Tablel-3, the RSD of reference procyanidins peak areas is 1.34 % (n = 5), which suggest the instrument is of good repeatability.

TABLE-3
REPEATABILITY TEST DATA OF PROCYANIDINS BY HPLC

Test no.	Peak area (mv)	RSD (%)
1	310412	
2	312722	
3	317375	1.34
4	307460	
5	316664	

Stability test: As shown in Table-4, the result indicate RSD of procyanidins peak areas in control solution is 1.87 % (n = 5), which suggest the procyanidins samples are stable in 12 h.

TABLEL-4
STABILITY TEST DATA OF PROCYANIDINS BY HPLC

Test time (h)	Peak area (mv)	RSD (%)
0	619947	
2	632022	
4	638950	1.87
8	624235	
12	608373	

Recovery test: As shown in Table-5, the result indicate the average recovery rate of procyanidins in high, medium and low concentration groups are 103.36, 109.75 and 101.38 %. The average recovery rate of the procyanidins is 104.83 % with RSD of 1.87 %. These results show that the recovery is good, impurities do not interfere with the content of procyanidins samples measured.

Study on analysis procyanidins by ultraviolet spectrophotometer

Investigation of linear relationship: Methanol is the reference solvent, scan in the wavelength range 400-600 nm, the maximum absorption wavelength of procyanidins is 471.5 nm, make a standard curve at the peak position. As shown in Fig. 4, the result indicate the concentration of procyanidins has good linear relationship with absorbance in the range of 0.014-0.056 mg/mL, the regression equation is A = 0.7438 × C-0.0026, R^2 = 0.9997.

Limit of detection experiment: As shown in Table-6, the result indicated the detection limit is 6.5 μg/mL.

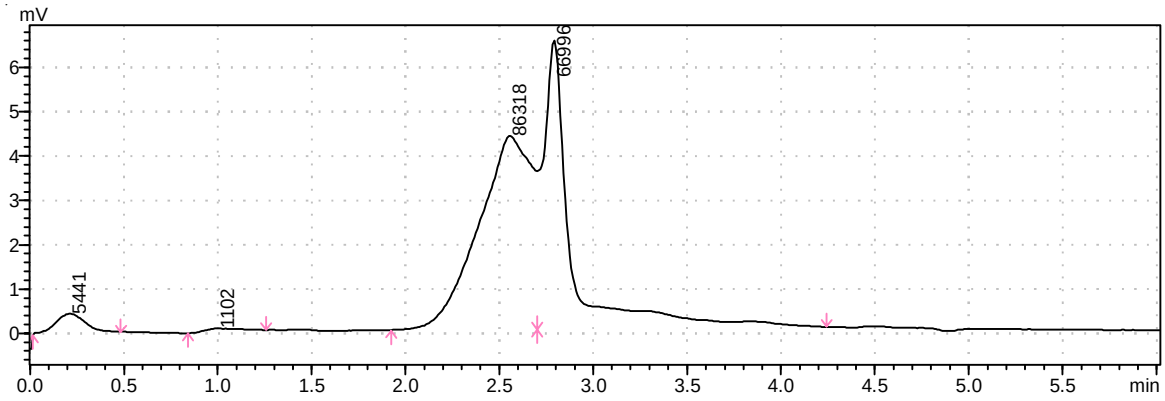

Fig. 2. Chromatogram of procyanidins sample

TABLE-5
RECOVERY TEST DATA OF PROCYANIDINS BY HPLC

Test no.	Addition amount of tested substance (mg)	Addition amount of reference substance (mg)	Actual measurement value (mg)	Recoveries (%)	Average recoveries (%)	RSD (%)
1	28.00	22.40	50.71	101.38		
2	28.00	28.00	58.73	109.75	104.83	1.87
3	28.00	33.60	62.73	103.36		

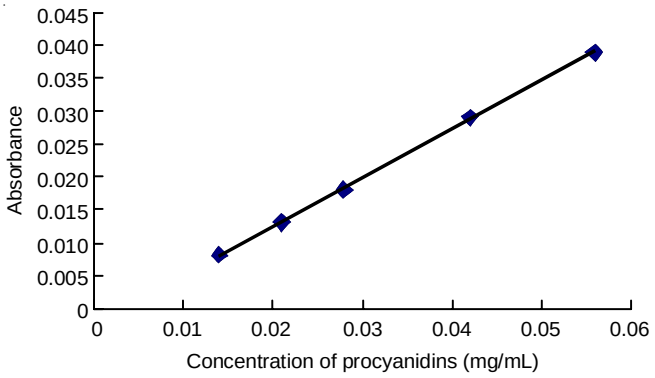

Fig. 4. Standard curve of procyanidins by UV

TABLE-6
EXPERIMENTAL DATA OF DETECTION LIMIT BY UV

Test no.	Content of procyanidins (mg/mL)	Absorbance
1	0.0065	0.003
2	0.0031	0.016

Precision test: The RSD of reference procyanidins is 1.08 % (n = 5), which suggest the instrument is of good precision, the precision test data of procyanidins by UV is shown in Table-7.

TABLE-7
PRECISION TEST DATA OF PROCYANIDINS BY UV

Test no.	Absorbance	RSD (%)
1	0.050	
2	0.051	
3	0.051	1.08
4	0.051	
5	0.050	

Stability test: As shown in Table-8, the result indicate RSD of procyanidins in control solution is 1.15 % (n = 5), which suggest the procyanidins samples are stable in 12 h.

TABLE-8
STABILITY TEST DATA OF PROCYANIDINS BY HPLC

Test time (h)	Absorbance	RSD (%)
0	0.047	
2	0.048	
4	0.048	1.15
8	0.047	
12	0.048	

Conclusion

We mainly study the qualitative and quantitative analysis method of procyanidins by HPLC and UV. Methodology investigation indicate HPLC standard curve: y = 142773x + 14416, R^2 = 0.9998, the linear relationship between procyanidins concentration and peak area are good in the range of 20-100 μg/mL, the procyanidins samples are stable in 12 h and the recovery rates of the procyanidins was 104.83 %. In UV analysis, ethanol is as a constant volume solvent, the absorbance of procyanidins is measured at 471.5 nm, determination of procyanidins standard curve is A = 0.7438 × C-0.0026, R^2 = 0.9997, linear range is 0.014-0.056 mg/mL.

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