VALIDATION OF A METHOD FOR THE SIMULTANEOUS DETERMINATION OF CYNARIN AND CHLOROGENIC ACID IN CYNARA SCOLYMUS L. EXTRACT USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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TÓM TẮT

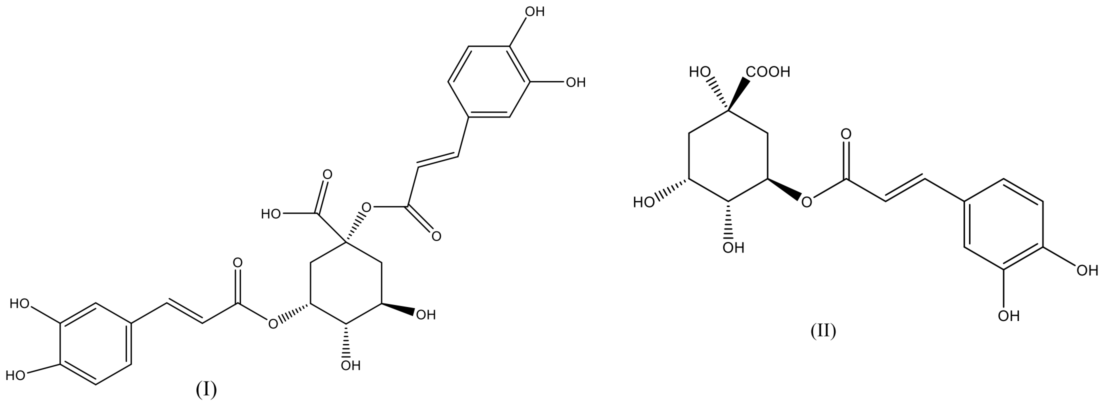
THẨM ĐỊNH PHƯƠNG PHÁP XÁC ĐỊNH ĐỒNG THỜI CYNARIN VÀ CHLOROGENIC ACID TRONG CAO CHIẾT CÂY ACTISO BẰNG SẮC KÝ LỎNG PHA ĐẢO HIỆU NĂNG CAO

Trong nghiên cứu này, phương pháp sắc ký lỏng pha đảo (RP-HPLC) được sử dụng để thẩm định xác định hàm lượng cynarin và chlorogenic acid trong cao trích cây actiso. Cynarin và chlorogenic acid được tách trên cột Shim-pack GISS C18 column (2,1 mm x 100 mm), kích cỡ hạt 1,9 µm, nhiệt độ 30 oC và đầu dò PAD đặt ở bước sóng 325 nm đối với cynarin và 321 nm đối với chlorogenic acid. Pha động là hỗn hợp MeOH và AcOH 5% ở tỉ lệ thể tích 10:90, tốc độ dòng 1,5 ml/phút. Tổng thời gian phân tích mỗi mẫu xấp xỉ 25 phút, trong đó thời gian lưu của cynarin và chlorogenic acid lần lượt là 10,939 phút và 21,969 phút. Khoảng tuyến tính của cynarin trong khoảng nồng độ từ 31,5 đến 73,5 µg/mL, phương trình hồi quy y = 16736x + 31934, hệ số tương quan r2 = 0,9998 và khoảng tuyến tính của chlorogenic acid ứng với nồng độ từ 39,0 đến 91,0 µg/mL, phương trình hồi quy y = 19094x + 43864, hệ số tương quan r2 = 0,9996. Giới hạn phát hiện và giới hạn định lượng của cynarin là 0,64 µg/mL và 1,93 µg/mL và của chlorogenic acid là 0,30 µg/mL và 0,90 µg/mL. Hiệu suất thu hồi từ 96,15% đến 100,73% đối với cynarin và từ 98,53% đến 103,14% đối với chlorogenic acid. Phương pháp được đánh giá qua độ đúng, độ chính xác, độ đặc hiệu, độ tuyến tính, độ ổn định và độ nhạy.

Từ khoá: *Cynarin, chlorogenic acid, actiso, HPLC.*

1. INTRODUCTION

Cynara scolymus L. was called artichoke which is originating from the Mediterranean region, which today is widely cultivated all over the world. In Vietnam, the plant is widely distributed in Da Lat, Sapa and Tam Dao [2, 8]. The leaves of artichoke has been long-used in traditional medicine and now included in British and European Pharmacopeia (BP/EP), the British Harbal pharmacopeia (BHP) and the Complete German Commission E Monographs [16]. In various studies, the pharmacological activities of artichoke, such as choleretic, lipid-lowering and anti-therogenic, hepatoprotective, antioxidative effects and inhibition of cholesterol biosynthesis were well documented [9, 12]. These therapeutic indications were due to the phytochemical compounds in artichoke. Artichoke content of phenolic acid constituent in particular cynarin and chlorogenic acid [3, 6]. C. scolymus leaves and extracts contain a complex of substances which were caffeoylquinic acids, flavonoids and sesquiterpenes. Among that natural compounds, chlorogenic acid, cynarin, luteolin and luteolin glycosides are considered as essential artichoke compounds [3].



(I)

(II)

Figure 1. Structure of cynarin (I) and chlorogenic acid (II)

Chlorogenic acid and cynarin have been examined by various methods: Including thin-layer chromatography (TLC), colorimetric methods, reverse-phase high performance liquid chromatography (RP-HPLC), and micellar electrokinetic capillary chromatography, … [1, 4, 7, 13, 14]. However, these methods were only determinaton each of chlorogenic acid and cynarin in extracts.

Base on the biological characterization of cynarin and chlorogenic acid in the artichoke extracts, a sensitive and selective analytical method was needed, which allowed for the simultaneously determination of cynarin and chlorogenic acid in artichoke extract samples.

In the present study, a method for simultaneously determination of cynarin and chlorogenic acid in leafves artichoke extract was undertaken to develop and validate. The procedure, based on the use of reversed-phase liquid chromatography with ultraviolet detection, is simple and rapid and provides accurate and precise results.

2. MATERIALS AND METHODS

2.1. Chemicals

- Cynarin, chlorogenic acid standard were purchased from TRC, Germany.

- All solvents of HPLC grade: Methanol (MeOH), ethanol (EtOH), water (H_2O), acetic acid (AcOH), ethyl acetat (EtOAc), and acetonitril (ACN) were purchased from Sigma Aldrich, Singapore.

2.2. Apparatus

The method was used the HPLC system with a Shimadzu LC-20ADXR model and Photodiode Array Detector (PAD). Shim-pack GISS C18 column (2.1 mm x 100 mm), 1.9 µm particle size, maintained at 30 °C with a CTO-10ASvp series oven was used for the chromatographic separation at a detection wavelength of 325 nm for cynarin and 321 nm for chlorogenic acid. The mobile phase of composition MeOH and 5% acetic acid solution in a ratio 10:90; v/v was selected for elution. Flow rate of mobile phase was 1.5 mL/min at a temperature of 30 °C and the injection volume was 10 µL. Data recording was integrated with a Labsolution LC software.

2.3. Preparation of stock and standard solutions

Standard stock solution of cynarin and chlorogenic acid (1000 mg/L) were prepared in mobile phase (MeOH and 5% acetic acid in a ratio 10:90; v/v) and then sonicated for 10 min. From this stock, standard solutions of cynarin (from 31.5 to 73.5 mg/L) and chlorogenic acid (from 39.0 to 91.0 mg/L) were prepared by diluting stock solution using mobile phase. Blank solution is mobile phase (MeOH and 5% acetic acid in a ratio 10:90; v/v) passing a 0.45-μm filter membrane. Spike samples were prepared using cynarin and chlorogenic acid standard solutions. Standard solutions, spike samples, sample solutions, and blank solution were daily prepared for one day.

2.4. Preparation of sample solution

The extract of artichoke samples was weighted about 0.5000 g in 25 mL of flask and added mobile phase to disolve. The volume was made up to the mark with mobile phase and then separated by 0.45-μm filter. This solution was measured using the RP-HPLC/PAD.

2.5. Validation of the RP-HPLC method

The validation parameters included in this study were specificity, trueness, precision, linearity, limit of detection (LOD), and limit of quantification (LOQ), system suitability. The validation method was followed the protocol guidelines on validation of analytical methods [5].

Specificity

The method was found to be specific when the test solution was injected. Test solutions were standard, blank, and sample solutions which were tested against potential interferences as specificity of a method. The cynarin and chlorogenic acid peak in the sample were identified by comparing with the peak of cynarin and chlorogenic acid in the standard. There was not any interference at the retention time of cynarin and chlorogenic acid in the chromatogram of blank solution and they have the same peak at the retention time of cynarin and chlorogenic acid in the chromatogram of standard solutions and sample solution.

Linearity

Five concentration levels ranging from 31.5 to 73.5 µg/mL for cynarin standard and 39.0 to 91.0 µg/mL for chlorogenic acid were injected into the chromatographic system (n=3) to determine the linearity of the RP-HPLC method. The peak area and retention time were recorded. The mean values of peak area ratio were plotted against concentrations to obtain the calibration curves. The relative standard deviation (%RSD) was determined for each concentration.

Limit of detection (LOD) & Limit of quantification (LOQ)

The LOD is defined as the smallest level of analyte that can be distinguished from noise, but not necessarily quantified. LOQ is defined as the lowest concentration at which can be determined with acceptable precision and accuracy. The precision expressed by relative standard deviation (RSD) is less than 10% and accuracy expressed by relative difference in the measured and true value is also less than 2%. LOD and LOQ were calculated:

$$\mathrm{LOD} = \frac{3 \times \mathrm{C_{min}}}{\mathrm{S/N}};\ \mathrm{LOQ} = \frac{10 \times \mathrm{C_{min}}}{\mathrm{S/N}}$$

Where

S/N: signal-to-noise ratio

C_{min}: The minimum concentration at which the analyte signal could be reliably detected.

Precision

The precision of the analytical method was studied by analysis of six sample solutions on the same day and for intermediate precision % RSD was calculated from repeated studies on different days to established precision.

Trueness

The recovery of the standards from sample was evaluated as the reliability and accuracy of the assay method. A known quantity of cynarin and chlorogenic acid standard solutions was added to pre-analyzed sample. The contents were reanalyzed by the proposed method and the percent recovery was reported. Calculate the percent recovery of each solution, the mean recovery at each concentration level and %RSD of the percent recoveries at each concentration level.

$$\%H = \frac{C_{standard+sample} - C_{sample}}{C_{standard}} \times 100$$

System suitability

Injected 10 µl of each cynarin and chlorogenic acid standard solutions, injected 6 times repeated at 100% concentration of standard solution. The result of system suitability was tested through theoritical plate of peak of cynarin and chlorogenic acid peak, tailing factor of peak, and RSD of retention time and area of peak. Theoritical plate of cynarin and chlorogenic acid peak is not less than 1900. Tailing factor of cynarin and chlorogenic acid peak is not more than 2. %RSD of retention time and area of cynarin and chlorogenic acid peak in 6 times repeated in standard solution is not more than 2%.

Stability in solution [15]:

Stability of solution will be determined within 72 hours with the same mobile phase. Store a portion of one sample solution and one standard solution from one of the verification parameters at room temperature and then inject into the chromatograph system at 0, 24, 48 and 72 hours.

Calculate the percentage content of cynarin and chlorogenic acid standard solution at time intervals compared to the initial time point:

$$\frac{A_{SS}}{A_S}\times 100$$

Where:

- A_S: Average conc. of cynarin or chlorogenic acid from the standard solution or sample solution at initial time point.
- A_{SS}: Average conc. of cynarin or chlorogenic acid from the standard solution or sample solution at time intervals.

If the solutions are not stable for 24 hours at ambient temperature, another portion of the same solutions will be continued to evaluate at 2 – 8 ºC up to 72 hours.

2.6. Statistical analysis

The statistical significance of the obtained results were analyzed using the Statistical Package for Social Sciences (SPSS) version 22.0. All data were expressed by mean ± SD. The significance of differences was established with the one-way analysis of variance (ANOVA), assuming that the significance level p is less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Quality control criteria for the RP-HPLC/UV method

Various proportions of mobile phase such as H_2O, MeOH, EtOH, EtOAc ACN, and AcOH solution were tested to establish the optimum mobile phase. Among these mobile phases, MeOH and 5% AcOH solution in a ratio 10:90; v/v was selected because of sharp and symmetric peaks. The RP-HPLC/PAD method showed quite quickly and effectively to determine the concentration of cynarin and chlorogenic acid. The optimal chromatography of cynarin and chlorogenic acid without any interference is shown in Figure 1. As seen in the chromatogram in Figure 2, the peak of cynarin and chlorogenic acid in the standard and sample solutions appeared at 10.939 and 21.969 minutes, respectively, while no peak of cynarin and chlorogenic acid appeared in blank solutions. The total time required for chromatographic analysis was 25.0 min. Besides, the UV spectra of standard and sample of cynarin and chlorogenic acid at various retention time throughout the peak were recorded by PAD detector. It was assured that the peak corresponds cynarin and chlorogenic acid only. The UV spectra of cynarin and chlorogenic acid were shown in Figure 3. The peak of cynarin and chlorogenic acid in the sample and different standard solutions absorbed at 325 nm and 321 nm, respectively, while no peak of cynarin and chlorogenic acid appeared in blank solutions.

The mobile phase: MeOH and 5% acetic acid in a ratio 10:90; v/v; flow rate: 1.5 mL/min at a temperature of 30 °C; wavelength: 325 and 321 nm for cynarin and chlorogenic acid.

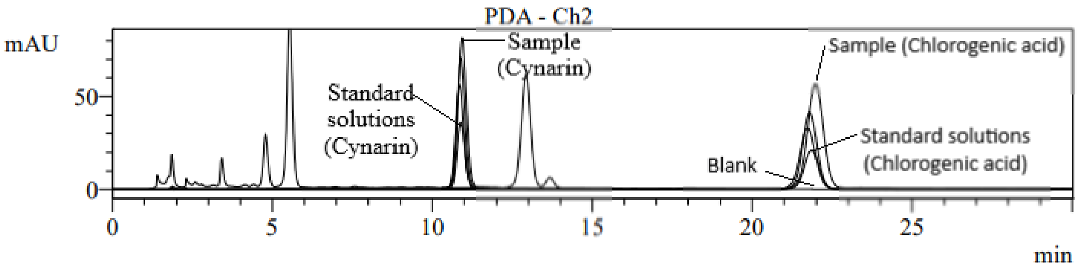


Figure 2. Representative chromatogram for cynarin and chlorogenic acid in different solutions.

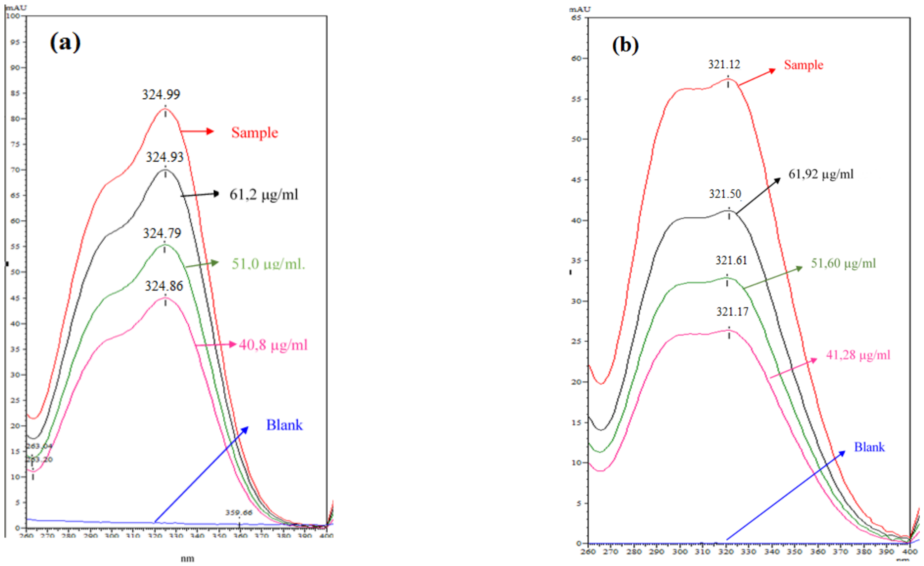


Figure 3. The UV spectra of cynarin (a) and chlorogenic acid (b) in different solutions.

Several authors have developed HPLC method for cynarin and chlorogenic acid analysis in many different matrices. María Rosario Alonso et al. developed and validated a method to determine cynarin in the biological samples. The retention time was found to be around 15.0±1.0 minutes with an analysis time of 22.0 min [14]. Serkan Özbilgin et al. developed a sensitive HPLC method with ultraviolet detection and the retention times of chlorogenic acid was 13.0±1.0 minutes with an analysis time of 20.0 min [13]. N. Mulinacci et al. analysed cynarin in commercial and laboratory extracts from artichoke leaves using HPLC/DAD, the retention times of cynarin was 30.18 min [11]. These methods was about 1.37 – 2.76 times slower than this method developed in analysis of cynarin and 0.59 times quicker than this method developed in analysis of chlorogenic acid.

Linearity was evaluated by repeated three times using different concentrations in the range from 31.5 to 73.5 µg/mL for cynarin standard and 39.0 to 91.0 µg/mL for chlorogenic acid. The linear regression was obtained by plotting the peak area values versus the cynarin and chlorogenic acid concentration. As seen in Figure 4, the standard curves were found to have a linear relationship ($r^2 = 0.9998$) over the analytical range of 31.5 to 73.5 µg/mL for cynarin and a linear relationship ($r^2 = 0.9996$) over the analytical range 39.0 to 91.0 µg/mL for chlorogenic acid. The relative standard deviation (%RSD) was determined for each concentration. The sensitivity of the analytical method was evaluated by determining the detection limit (LOD) and quantitation limit (LOQ) and the LOQ was defined as the lowest concentration with RSD $\leq$ 1.5%. For cynarin, the LOD and the LOQ were 0.64 µg/mL and 1.93 µg/mL, respectively. For chlorogenic acid, the LOD and the LOQ were 0.30 µg/mL and 0.90 µg/mL, respectively.

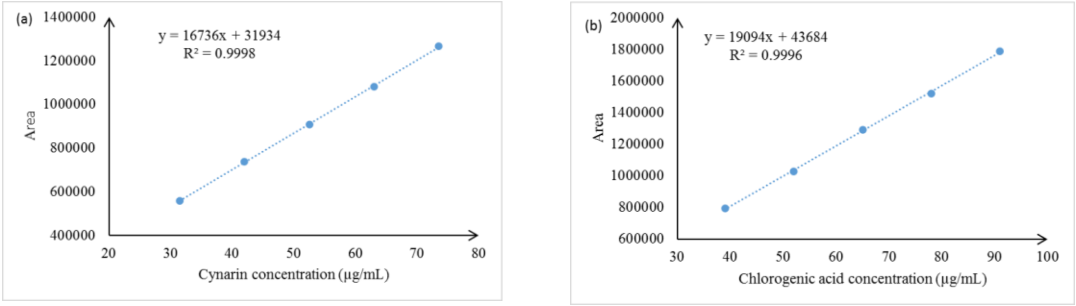


Figure 4. Cynarin (a) and chlorogenic acid (b) standard curve.

Validation results of assay precision and accuracy were shown in Tables 1. Intra-assay and inter-assay precision levels were assessed by analyzing the quality control samples (40, 50, and 60 mg/mL for cynarin and 40, 50, and 60 µg/mL for chlorogenic acid). After six replicate measurements at three concentration levels, the intra-day precision were shown lower than the accepted criteria (RSD ≤ 2%). The accuracy of the method within-day was between 98.8% and 101.8% for cynarin and 99.1% and 102.4% for chlorogenic acid. Inter-day precision was conducted during routine operation of the system and the result was tested through statistical evaluation revealed which relative standard deviation of cynarin and chlorogenic acid for six injections were less than 2%. The accuracy of the day-to-day data of this study was from 97.2% to 100.6% for cynarin and 96.2% and 100.7% for chlorogenic acid.

Table 1. Precision and accuracy data

Compound	Concentration (µg/mL)	Intra-assay (n=6, single day)			Inter-assay (n=6, six days)		
		Value found (mg/L) *	RSD (%) precision	Accuracy %	Value found (mg/L) *	RSD (%) Precision	Accuracy %
Cynarin	40	40.70 ± 0.09	0.22	101.8	40.23 ± 0.45	1.1	100.6
	50	50.82 ± 0.19	0.38	101.6	50.27 ± 0.85	1.7	100.5
	60	59.25 ± 0.15	0.26	98.8	58.32 ±0.67	1.2	97.2
Chlorogenic acid	40	39.98 ± 0.09	0.22	99.5	39.12 ± 0.32	0.8	97.8
	50	51.22 ± 0.19	0.38	102.4	50.36 ± 0.91	1.8	100.7
	60	59.47 ± 0.12	0.20	99.1	57.73 ± 1.12	1.9	96.2

* Mean ± SD (n=6)

Cynarin and chlorogenic acid recovery were measured based on recovery from artichoke powder matrix samples containing known concentrations of cynarin and chlorogenic acid. Recovery of cynarin from artichoke powder were found to be from 96.5% to 100.73%, and CV precision levels ranged from 0.18% to 0.70%. For chlorogenic acid, recovery was found to be from 98.53% to 103.14%, and CV precision levels ranged from 0.43% to 1.07% (Table 2).

Table 2. Recovery of cynarin and chlorogenic acid in artichoke powder

Compound	Artichoke extract (mg)	Sample Conc. * (µg)	Added Conc. (µg)	Observed Conc.* (µg)	Calculated conc.* (µg)	CV (%) precision	Recovery (%)
Cynarin	500.7	4206	3365	7439	3233	0.70	96.15
	500.6	4264	4260	8554	4290	0.18	100.73
	500.8	4207	5048	9147	4940	0.61	97.64
Chlorogenic acid	500.7	5558	4445	9969	4411	0.75	99.23
	500.6	5534	5555	11002	5468	1.07	103.14
	500.8	5559	6670	12217	6658	0.43	98.53

* Mean ± SD (n=5)

An integral part of chromatographic method are system suitability tests which are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared standard solutions. System suitability was studied by six replicate measurements at three concentration levels (40, 50 and 60 µg/mL for cynarin and 40, 50 and 60 µg/mL for chlorogenic acid). The parameters obtained were shown in Table 3. The RP-HPLC/PAD was suitability to determination of cynarin and chlorogenic acid.

Table 3. Results of suitability of cynarin and chlorogenic acid in solutions (n=6).

Parameters	Acceptance criteria	Results of cynarin	Results of chlorogenic acid
Theoretical plate	≥ 2000	8151	9679
Tailing factor	≤ 2.00	1.03	0.99
%RSD of retention time	≤ 2.0%	1.4	1.9
%RSD of area at 40, 50 and 60 µg/mL	≤ 2.0%	0.84%; 0.56%; 0.43%	0.81%; 0.52%; 1.58%

Table 4. Results of stability of cynarin and chlorogenic acid in solutions (n=6).

Compound	Solution	Conc. taken	24 h		48 h		72 h	
			Measured Conc. (µg/mL)	% degradation	Measured Conc. (µg/mL)	% degradation	Measured Conc. (µg/mL)	% degradation
Cynarin	Standard	40.92 µg/mL	40.83	0.25	40.63	0.72	40.37	1.37
	Sample	7.220 mg/g	7.181	0.54	7.173	0.64	6.973	3.41
Chlorogenic acid	Standard	40.00 µg/mL	39.60	3.24	39.33	3.90	39.20	4.23
	Sample	8.231 mg/g	8.118	1.37	7.969	3.18	7.821	4.98

Stability of cynarin and chlorogenic acid in standard and sample solutions after 24, 48 and 72 h were shown in Table 4. As seen in Table 4, there were marginal differences in degradation between standard and sample solution at ambient temperatures. After 72 h under ambient temperatures, degradation of cynarin in standard solution and sample solution were about 1.37% and 3.41%, respectively. Meanwhile, the degradation of chlorogenic acid in these solutions were about 4.23% and 4.98%, respectively after 72 h under ambient temperatures. The stability of the cynarin and chlorogenic acid in the solution was found to be until 72 h, sequence run times should be no longer than 72 h when analyzing cynarin and chlorogenic acid in the presence of its process impurities.

The method in this study has good accuracy, precision, linearity and efficientcy for quantification of cynarin and chlorogenic acid.

3.2. Estimation of cynarin and chlorogenic acid in extracts of artichoke

The proposed method was used to determine the concentration of cynarin and chlorogenic acid in artichoke extracts sample. Six replicate determinations were carried out and the results were summarized in Table 5.

Table 5. Results of RP-HPLC measurements of cynarin and chlorogenic acid in artichoke extracts sample (n=6).

Sample		Cynarin		Chlorogenic acid (%)	
		Level (%)	%RSD	Level (%)	%RSD
Leaf extract	No. 1	0.818 ± 0.015	1.1	1.110 ± 0.018	1.8
	No. 2	0.827 ± 0.011	1.4	1.208 ± 0.029	2.1
	No. 3	0.809 ± 0.011	1.5	1.171 ± 0.013	1.9
Flower extract	No. 1	0. 623 ± 0.014	1.2	0.918 ± 0.013	1.8
	No. 2	0.681 ± 0.014	1.4	0.981 ± 0.021	1.7
	No. 3	0.662 ± 0.017	1.3	0.951 ± 0.018	2.0

4. CONCLUSIONS

The RP-HPLC method with PAD detector described in this paper has good accuracy, precision, linearity and suitable for the quantitative determination of cynarin and chlorogenic acid in artichoke extracts sample. The most outstanding advantages of this method were:

(i) Quickly analysis of cynarin and chlorogenic acid (retention times were 10.939 for cynarin and 21.969 min for chlorogenic acid, the total time for chromatographic analysis was 25 min);

(ii) The optimized mobile phase composition for simultaneously determination of cynarin and chlorogenic acid were MeOH and 5% AcOH with ratio 10:90; v/v; mobile phase was quite simple and low cost for RP-HPLC analysis;

(iii) High recovery (96.15% to 100.73% for cynarin and 98.53% to 103.14% for chlorogenic acid).

Therefore, the rapid and sensitive developed method can be used for the routine analysis of cynarin and chlorogenic acid in artichoke extracts sample.

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