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Original Article

QUALITY CONTROL OF CINNAMIC ACID IN TRADITIONAL MEDICINAL PLANTS IN JORDAN USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: The objective of the study: is the analysis of cinnamic acid (CA) from commercial herbal drugs, and from dried plant material used in traditional medicine in Jordan market qualitatively and quantitatively by using (HPLC) high performance liquid chromatography.

Methods: The chromatographic conditions comprised of a reversed-phase RP-8 column with a mobile phase consisting of a mixture of Acetonitrile: Phosphate buffer solution (35:65 v/v) with flow rate 1 ml/min. The detection was carried out at 269 nm, with retention time of 17.3 min. The standard curve was linear over the range of (20-400) µg/ml with correlation coefficient of the linear calibration graphs for the analytics exceeded (0.9987).

Results: A Simple, sensitive and specific HPLC method was developed and validated for a qualitative and quantitative analysis of cinnamic acid from commercial herbal drugs, and from dried plant material used in traditional medicine in Jordan market.

Conclusion: The highest concentration of cinnamic acid was found in *Cinnamon verum*(Lauraceous) (4330 µg/g) while the other plants contain insignificant amount of cinnamic acid as it is a key intermediate precursor for many plant metabolites. The cinnamic acid and derivate were successfully separated using pH 2.5.The developed method was successfully applied to estimate the amount of cinnamic acid in the main traditional medicinal plants in Jordan market.

Keywords: Cinnamic Acid,, Cinnamon, Sumac, Cranberry, Artichoke, Bay Laurel, Mango leaves, HPLC, Validation.

INTRODUCTION

Cinnamon and its components is one of the medicinal plants [1-3] that have been used to improve various diseases caused by insufficient blood circulation so the use of cinnamon as a dietetic food supplements [4, 5]. The conditions of HPLC methods varied; for Main oral liquid, the mobile phase consisted of methanol – 0.1% water – H_3PO_4 (60:40) with flow rate 1.0 ml/min and UV detective wavelength was set at 278 nm [6]. The HPLC analysis of cinnamic acid in maifukang particles utilized a diamonsil C18 column (5µm, 250mm×4.6mm) with mobile phase of acetonitrile–0.4% phosphoric acid (30:70), flow rate 1.0 ml/min detection wavelength was at 277 nm [7]. In suhexiang pills, separation was performed on Hypersil C18 column with mobile phase of methanol and 0.1% acetic acid (61:39) at 270 nm with 1.0 ml/min flow rate [8]. The RP-HPLC method of Guanxinsuhe pills was performed with Hypersil C18 column (250mm×4.6mm, 5µm) and the mobile phase was a mixture of methanol and 1% acetic acid (61:39) with a flow rate of 1.0 ml/min and detection wavelength was 315 nm [9]. The RP-HPLC method for determination of CA in liyanlingtablets was used with irregular C18 column, mobile phase of methanol – 1% glacial acetic acid (52:48) at the detection wavelength of 274 nm [10]. Determination of cinnamic acid in storax by HPLC was carried out using ODS C18 column withmobile phase of methanol: water: acetic acid (58:42:0.3) and detection wavelength was 271 nm [11]. Cinnamic acid in Guilongke chinning capsules was determined using C18 column with MeCN-0.1% H_3PO_4 solution (30:70) as mobile phase and detection at 285 nm [12]. Furthermore, several HPLC methods have been developed for determination of cinnamic acid in plasma. Examples include determination of cinnamic acid in rat plasma using a kromasil C18 column (250mm×4.6mm×5µm) eluted with mobile phase methanol– acetonitrile– water–glacial acetic acid (25:20:55:0.3) at flow rate 1.0 ml/min with UV detection at 278 nm [13]. Determination of cinnamic acid in rabbit plasma was performed by employing a column of kromasil C18 (250mm×4.6mm, 5µm) and a mobile phase of methanol– acetonitrile– water–glacial acetic acid (10:22:55:0.5) at flow rate 0.8 ml/minwith UV detection at 270 nm [14]. The HPLC method used for determination of cinnamic acid in human plasma used C18 column and the mobile phase was acetonitrile- 0.1 mol/L KH_2PO_4 solution (30:70) at flow rate 1.2 ml/min the UV detection wavelength was 272 nm were used [15-17]. The developed HPLC methods are summarized in table (1)

Cinnamic acid and Cinnamic acid derivatives

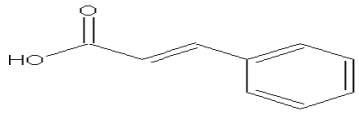
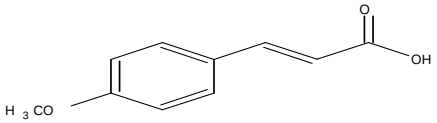
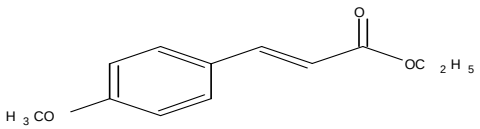


Fig. (1): Cinnamic acid, (CA)



4-methoxy-trans-CA



4-methoxy-trans-CA ethyl ester

Fig. 2: Cinnamic Acid Derivatives (CADs)

This study is an attempt to found a suitable, sensitive, selective, accurate, precise and specific HPLC method to quantification of Cinnamic acid (CA) and derivatives (CADs) in some traditional medicinal plants in Jordan to use CA as a marker in cinnamon capsules by same HPLC method.

MATERIAL AND EQUIPMENTS

Chemicals

The chemicals used in this study were highest analytical grade available. CA standard was obtained from (Finzelberg HG, Germany); magnesium stearate was from (Mallinckrodt chesterfield, st.lauis USA); fumed silicone dioxide (Aerosol 200) from (Degussa AG, Frankfurt, Germany); Talc from (Merck); methylcellulose from (colorcon), acetonitrile (HPLC grade) from (VWR,PROLABO); monobasic potassium phosphate (Scharlau, Spain); orthophosphoric acid from (Acros, Germany); Ethanol 96% from (Merck, Germany); graduated pipettes, volumetric flask and other glassware from precicolor (HBG) class A Germany, Cinnamon tablet (Cirkulin).

Plants

I-Dried plant materials of traditional herbs in Jordan used in the isolation process of Cinnamic Acid, where purchased from (Finzelberg HG, Germany), certified GMP and ISO 9002, each of these plants is in powder form for CA detection, i.e.

1- *Cynarascolymus* (Asteraceae), Artichoke [leaves]

2-*Cinnamoumverum*(Lauraceae), Cinnamon [barks]

3- *Rhuscoriaria* (Anacardiaceae), Sumach [seeds]

4- *Vacciniumoxycoccos* (Ericaceae), Cranberry [juice]

5- *Laurusnobilis* (Lauraceous), Bay laurel [leaves]

6- *Mangiferaindica* (Anacardiaceous), Mango [leaves]

II- Other samples were obtained:

1- From local Jordanian market such as Cinnamon tablet (Cirkulin)®.

2- Laboratory prepared capsule of; Cinnamon, Placebo, and CA capsules

Equipment

High performance liquid chromatography system [HPLC] (Thermo, USA) with Quaternary pump (Spectra System pump 1000), auto sampler with variable loop Injector (AS1000) and a UV detector dual λ absorbance (Spectra UV 1000), C8-3 column: 250*4.6 mm, particle size (5µm), Metler Toledo analytical balance (AB204, Switzerland), Sonicator (Elmasonic 545H, Germany), pH 720 meter (Metler Toledo MP225), spectrophotometer (Thermo, USA). Water bath (Julabo SW-21C), Water distiller (Millipore, France), 0.45 µm nylon membrane filters (Vacuubrand membrane–vacuum pump M22C, Germany). The integrator system was (Pentium 4 Intel Inside) program with windows XP and Chromo Quest Version 3.0 to control HPLC parts and record chromatograms.

Experimental data

HPLC experiment was achieved at ambient temperature, on a reversed phase column (RP-8) using a mobile-phase consisting of 65 volume of 0.43 w/v of KH_2PO_4 in distilled water adjusted to pH value 2.5 using 85% orthophosphric acid and 35 volume of acetonitrile. Three different formulations were prepared (table 2) the cinnamon capsule were used in study.

Table 1: HPLC conditions

Analyzed sample	Column	Mobile phase	Flow rate ml/min	Detection wave length (nm)
Maian oral liquid	Kromasil C18(250mm×4.6mm×5µm)	methanol─ 0.1% water─ H_3PO_4 (60:40)	1.0	278
Maifukang particles	Diamonsil C18 (5µm, 250mm×4.6mm)	acetonitrile─0.4% phosphoric acid (30:70)	1.0	277
Suhexiang pills	Hypersil C18	methanol and 0.1% acetic acid (61:39)	1.0	270
Guanxinsuhe pills	Hypersil C18 (250mm×4.6mm,5µm)	methanol and 1% acetic acid (61:39)	1.0	315
Liyanling Tablets	irregular C18 column	methanol─ 1% glacial acetic acid (52:48)	--	274
Storax	ODS C18	methanol: water: acetic acid (58:42:0.3)	--	271
Guilongkechuanning capsules	C18	MeCN-0.1% H_3Po_4 solution (30:70)	--	285
Rat plasma	Kromasil C18(250mm×4.6mm×5µm)	methanol─ acetonitrile─ water─glacial acetic acid (25:20:55:0.3)	1.0	278
Rabbit plasma	Kromasil C18 (250mm×4.6mm, 5µm)	methanol─ acetonitrile─ water─glacial acetic acid (10:22:55:0.5)	0.8	270
Human plasma	C18	acetonitrile- 0.1 mol/L KH_2PO_4 solution (30:70)	1.2	272

Table 2: Cinnamon capsule preparation

Type Of Capsule	CA Extract(mg)	Cinnamon Extract(mg)	Methylcellulose (mg)	Talc(mg)	Aerosol (mg)	Magnesium stearate(mg)	Total Weight(mg)
Cinnamon cap	-------	112	151.56	6.07	0.143	13.9	283.673
Cinammic acid cap	0.485	------	151.56	6.07	0.143	13.9	172.158
Placebo cap	-----	------	151.56	6.07	0.143	13.9	171.673

RESULT AND DISCUSSION

The study was carried on three stages:

Stage 1: Method development

Two different wave lengths max were obtained by scanning of C.A working standard with concentration of (2µg/ml) using UV-Vis Spectrophotometer (210nm, 269nm). Different pH range was tried on from (2.5-4.5) It was found that the best results were at pH 2.5. Ethanol was replaced by acetonitrile as diluents Standard curve was obtained by plotting AUC at 269 nm against CA concentration. A calibration curve should consist of minimum six to eight non-zero samples. A sufficient number of standards should be adequately for establishing the relationship between concentration and response. Linearity, LOD and LOQ were detrminated.

Stage 2: Cinnamic Acid Method Validation

For validation of cinnamic acid some tests were applicable like, linearity specificity and selectivity, accuracy and precision. Detection Limit (DL) and Quantification Limit (QL) Ratios of 3:1 and 10:1 signal-to-noise were considered acceptable for estimation of the DL, QL respectively.The robustness of the method was established as the relative deviation from mean assay value of (normal condition) obtains less than ± 2.0% for standards [18]. The short-term stability (sample solution stability test SSST) was examined by analyzing duplicate or triplicate replicates of three samples at room temperature for 24 h. The concentration of CA after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately. The determination of CA and theredegradesin pharmaceutical dosage was determined by the forced degradation study data. Forced degradation sample was tested for each condition: Acid and based-induced degradation product, photo degradation (where the samples keptin UV light for 24 h), and thermal degradation (the sample was heated at 50º C on a boiling water bath for 30 min), oxidant (H_2O_2) degradation. The non stress sample was used as control; the stressed samples were evaluated relative to the control sample with respect to the % assay.

Stage 3: Identification and Quantification of CA

I. Preparation of Capsule

Three different formulations were prepared using different amount of methylcellulose as dry binder, talc as anti-adherent, and calculated amount of cinnamon powder and CA standard which equivalents to 40 capsule of each type according to polyphenolic compound formulations [19, 20] Table (2) shows the cinnamon capsule formulations that were used in the study.

II. Capsule testing

1- Mass Variation: USP 28 procedure was used in the determination of capsule weight uniformity. Using the balance, each capsule was weighed,the contents of each capsule were emptied into a flask and the empty capsule shell was weighed and the weight of the contents of each capsule was determined.

2. Uniformity of Drug Content: Random samples of 20 capsules from the prepared cinnamon and CA capsule were tested for the uniformity of drug content by calculating the weight of active ingredient in each capsule.

III. Identification of Cinnamic Acid

The identification of CA was performed as a part of assay. The capacity factor, theoretical plate, retention time of CA peak in the chromatogram of the test solution in three sequential replicate of injections was compared to that of standard solution.

IV. Assay of Cinnamic Acid

The presence of CA in all plant material and samples (tablet and capsule) were detected. This method was applied for all 5-plants except for cinnamon plant and samples. The method was modified as follows: extracted in 100 ml acetonitrile; because it contains much more CA than others, Cinnamon Capsule, CA standard. Good separation conditions should be satisfactory when analyzed peaks have baseline separation with adjacent peaks within acceptable resolution and constant retention time. Thus, HPLC separations of CA were evaluated on C8-3 column 250X4.6 mm, 5µmby using a mixture of (0.43%) monobasic potassium phosphate buffer (KH_2PO_4)(the pH of the solution was adjusted to 2.5 with orthophosphric acid) and acetonitrile (65:35v/v) at a flow-rate of 1.0 ml/min at 269 nm.

Cinnamic acid Quantitation

I. Calibration Curve of CA assay by HPLC method.

The external standard method used to obtain the regression equation is shown in Figure 3, where the results in Table 12 indicate that the response is linear over therange of (20-400) µg/ml of CA.

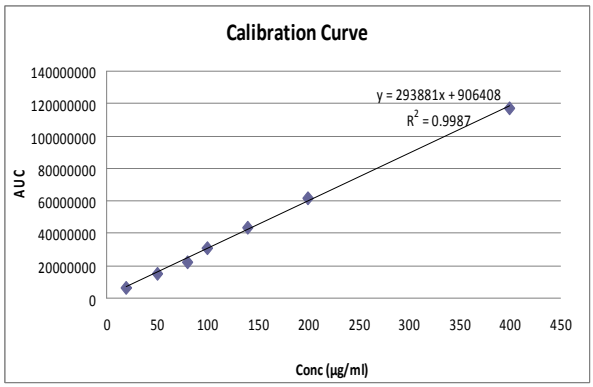


Fig. 3: Standard Calibration Curve of Cinnamic Acid.

Shows a typical chromatogram of CA standard. The average retention time of the standard was (17.5min).

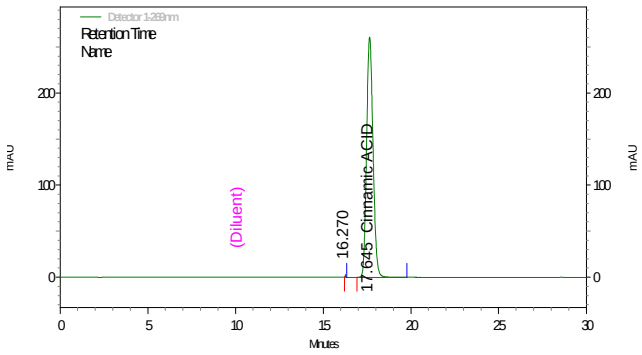


Fig. 4: Typical Chromatogram of CA Control (standard) Sample.

In order to obtain quantitative extraction of dried plant material, variables involved in the procedure were optimized, and the acceptance criteria for assay was from (95-105%), with respect to personal and instrumental errors and RSD ≤2 [21-23].

Method validation

A validation study involves testing multiple attributes of a method to determine that it can provide useful and valid data when used routinely.

I- Linearity Test and Range

The summary of linearity parameters is shown in the table (3) This method was specific for CA assay, no peak was observed in the chromatogram of placebo solution as seen in Figure (5), there was no peak at the retention time of CA at (17.3 min) as in chromatogram of analytical validation standard solution, and there was no interfering peak around the retention time of C.A.

Table 3: Show the regression Line Parameters of CA Analytical Procedure

Linearity parameters	Results
Correlation coefficient	0.9987
Slope of the regression line	293881
Y-intercept	906408

The limit of detection (LOD) (3:1) and limit of quantization (LOQ) (10:1) were expressed as µg/ml from 0.02 and 0.06 µg/ml, respectively

Table 4: Recovery of CA

Sample Preparation	% Simulated Dosage Nominal	% Amount Recovered	% Recovery
1	80	77.88	97.35
2	100	100.92	100.92
3	120	119.82	99.85
Mean ($\tilde{x}$)			99.37
+ Standard Deviation (SD)			1.83
% Relative Standard Deviation (RSD)			1.84

Table 5: the Precision of the Method in the Studied Range

Precision	Acceptance Criteria	Results	
Repeatability	Injection Repeatability The RSD≤1	Concentration (µg/ml)	The RSD of CA concentration
		1× 10 40(µg/ml) 3 × 3×2	0.05
	Analysis Repeatability The RSD of CA concentration in the AVSS should not be more than 2.5%.	% of Label Claim	The RSD of CA concentration
		80%→ 77.88%	2.34
		100%→ 100.92%	0.20
		120%→ 119.82%	0.30
Intermediate precision (inter-day precision)	The RSD of CA concentration in the AVSS should not be more than 2.5%.	3 × 2 Concentration (µg/ml)	The RSD of CA concentration
		81.75%	1.84
Reproducibility (inter-laboratory precision) (intra-day precision) (24hr stability)	The RSD of Cinnamon in the AVSS should not be more than 2.5%.	2 × 3 %assay	The RSD of CA concentration
		81.75% 83.33%	1.84 2.19

RSD: relative standard deviation; AVSS: Analytical Validation Standard Solution

The RSD of peak areas of CA should not be more than 2%. The accuracy of the method was established by recovery studies, so accuracy was evaluated at three concentrations of 80%, 100%, and 120 % assay. The recovery % was 97.35, 100.9 and 99.85%, respectively as shown in Table (14).

Table 6: Robustness of CA

Parameter	Normal (Original)	Changed conditions
Mobile Phase and pH	(pH 2.5) phosphate buffer: Acetonitrile	(pH 2.7-2.4) phosphate: Acetonitrile
Aqueous Mobile Phase ratio	(KH_2PO_4) 650:350	(KH_2PO_4) water 630:350
Injection volume	10	20
% Assay cinammic acid	99.02%	99.05%
% RSD of assay value for CA 0.03%		

Results indicate that the individual recovery of CA ranges from 100.92% to 97.35% with mean recovery of 99.37% and % relative standard deviation of 1.84%. The recovery of CA by the proposed method is satisfactory as % relative standard deviation is not more than ± 2.0% and mean recovery between 97.35 - 100.92%. Accuracy will pick up recovery problems that could be encountered during the

sample preparation and the chromatographic procedures. However, it does not count the effect of the manufacturing process.

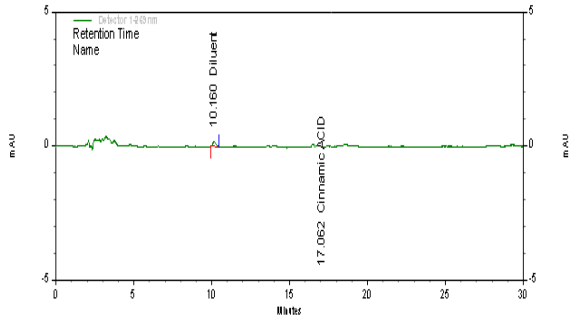


Fig. 5: Typical Chromatogram of Placeb I- Accuracy and Precision Test

Precision on the other hand, was the results of three levels: repeatability, intermediate precision and reproducibility. RSD for all three levels of precision were all within the accepted limits. The %RSD <2 for standard in repeatability, intermediate precision, and the %RSD <3 for samples in reproducibility step as shown in Table (5) [24, 25].

III- Robustness Test

Robustness was determined by analyzing the same sample at normal operating conditions and also by changing some operating analytical conditions such as change in pH and aqueous ratio composition which were chosen among others to show robustness.

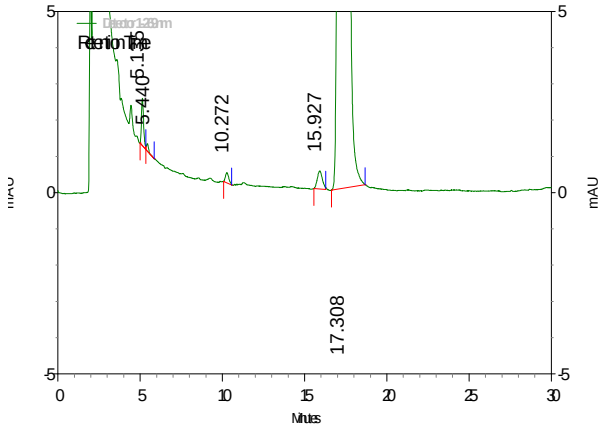
The results of robustness which are summarized in Table (6) showed that % assay was 99.02 and 99.05 for normal and changed condition respectively. The robustness of the method is established if the % RSD <2 and % recovery is 95-105 [18].The deliberate aforementioned changes in parameters of CA method gave RSD=0.03% which is not a significant change, and recovery of 99%. So our method is valid for this test.

IV. Stability- indicating property

Table 7: Stressed study data of CA

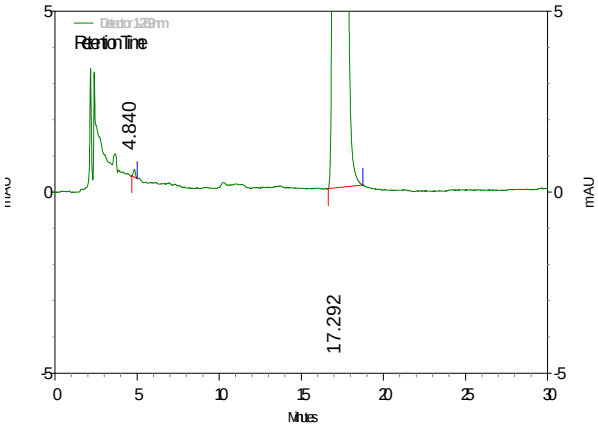
S. No.	Condition	% Assay % CA	%Total degradation
a.	Non stressed sample	98.02	Nil
b.	Acid(0.1N)	70.7	29.3%
c.	Base (0.1N)	98.77	Nil
d.	Oxidant H_2O_2 (3%)	100.32	Nil
e.	UV (254)	96.22	Nil
f.	Thermal(50°C)	99.07	Nil

As shown in table (7) it is sensitive to acidic conditions only. As the chromatogram of no stress treatment sample (as control) showed no additional peak, retention time (RT) of CA at 17.3min, and 98.02% recovery Figure(6), the chromatogram of acid degraded sample showed CA at 17.2min with 70.7% recovery. The chromatogram of alkali showed no additional peaks, RT of CA at 17.3 min 98.77% recovery. The chromatogram of hydrogen peroxide sample showed no additional peaks, RT of CA at 17.4 min 100.32% recovery. The chromatogram of UV sample showed no additional peaks, RT of CA at 17.4 min 96.22% recovery. The chromatogram of thermally treated sample showed no additional peak, RT of CA at 17.4 min 99.07% recovery.



(a) Acid degraded sample of CA

Accordingly, we found out that it is sensitive to acidic conditions only. The absorption spectrum of this compound didn't change significantly under the influence of pH. This observation suggests that conjugated non phenolic aromatic acids such as CA are stable at high pH [26]. As a result, the presented chromatographic method is simple and easy to use in routine tests. The reversed-phase HPLC method allows successful separation of CA from other component and could be used for rapid quality-control analysis of various plants and commercially available herbal drugs. By testing this method, using validation parameters, we were able to show that the presented chromatographic conditions were suitable for estimating CA, and reproducible [27].

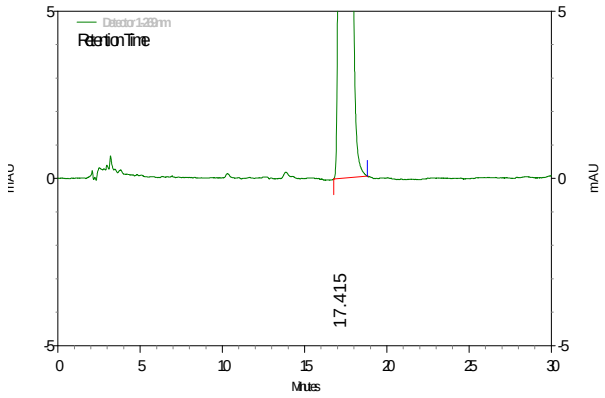


(b): Alkali degraded sample of CA

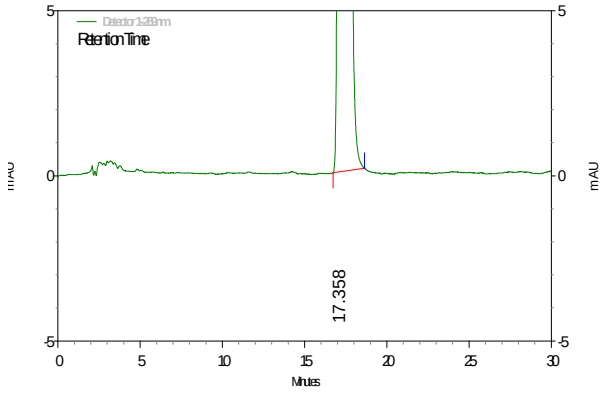
V. System Suitability Test

On the basis of results of this analytical validation, the values for N, T, and K´ were calculated. The capacity factor, k´, which should be more than 2, was found to be 169.616 for CA. The column efficiency, which usually should be more than 2000 theoretical plates, was determined to be 10782 for CA. The tailing factor was determined as 1 which is less than usual pharmacopoeia standard (less than 2.0). The relative standard

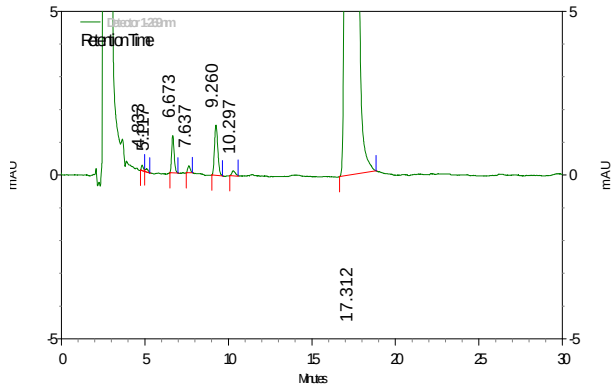
deviation for replicate injections was also less than 2.0%. The stability of the test solution was checked up to 24 hours and it was found that it was stable.



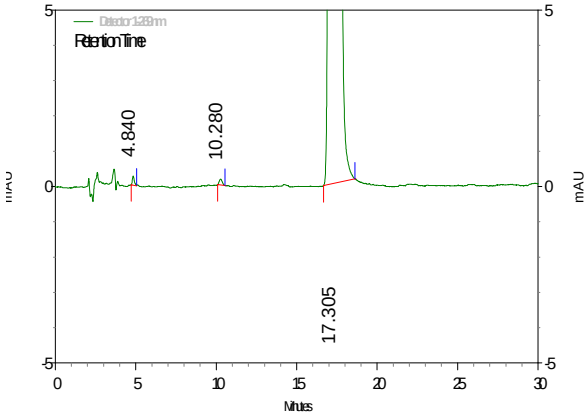
(b) UV degraded sample of CA



(c) Thermal degradation at 50°C



(d) Hydrogen peroxide degraded sample of CA



(e) H_2O degraded sample of CA

Fig. 6: Typical Chromatogram of Stressed Sample ;(a) Acid degraded sample of CA, (b) Alkali degraded sample of CA,(c) UV degraded sample of CA,(d) Thermal degradation at 50ºC sample of CA, (e) Hydrogen peroxide degraded sample of CA, (f) H2O degraded sample of CA.

3.3 Sample Analysis

The developed method enabled us to test six traditional medicinal plants commonly used in the Hashemite Kingdom of Jordan for CA. Many plants were analyzed for quantization of their CA contents (figure 8) it has been found that the amount of CA extract differs significantly between plant families and the largest amount of CA is extracted from crude herbs of cinnamon which contain 4330µg /g as shown in table (8).

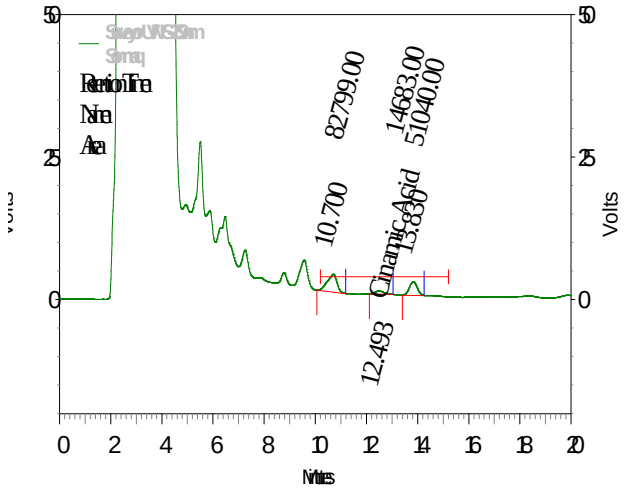
The maximum variations can be made without the need for validation rather than verification of method performance under the new conditions. Includes among others are changes in columns length (±70%), change in column diameter (±25%), particle size (can reduced by as much as 50%), and flow rate (±50%).

Table 8: CA Concentration in Plant Samples

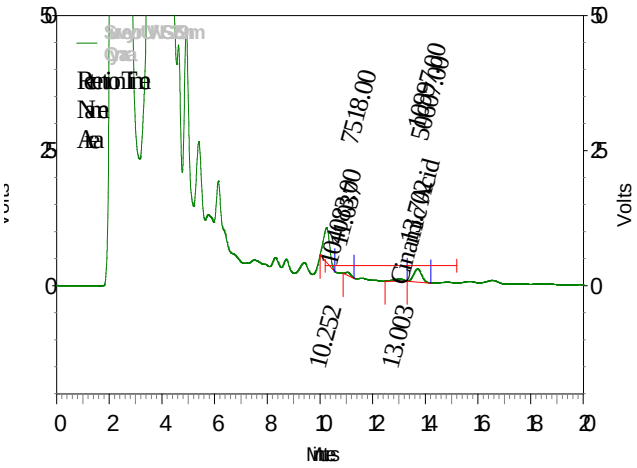
Plant	Mean CA Concentration µg/g
Cinnamon	4330.0
Cranberry	16
Ghar	3.8
Sumach	3
Cynara	3
Mango	2.2

3.4 Analysis of Marketed Formulation

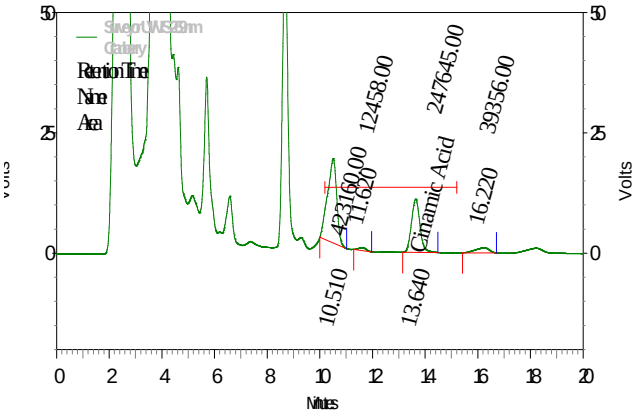
The drug content of cinnamon tablet was found to be 0.4890mg/tab with a % RSD of 0.90%. It was noted that no degradation of CA had occurred in the marketed formulation that were analyzed using this method as shown in table (9) The low RSD value indicates the suitability of this method for routine analysis of CA in pharmaceutical dosage form. The recovery of CA capsule 0.485mg/Caps and negligible Mass Variation with Uniformity of Content as shown in Table (10).



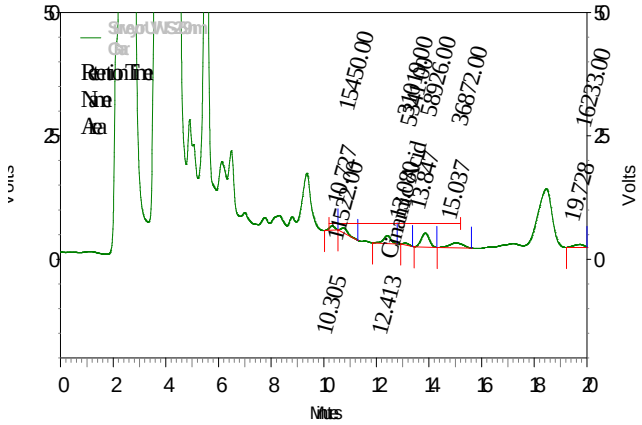
(a)Sumach



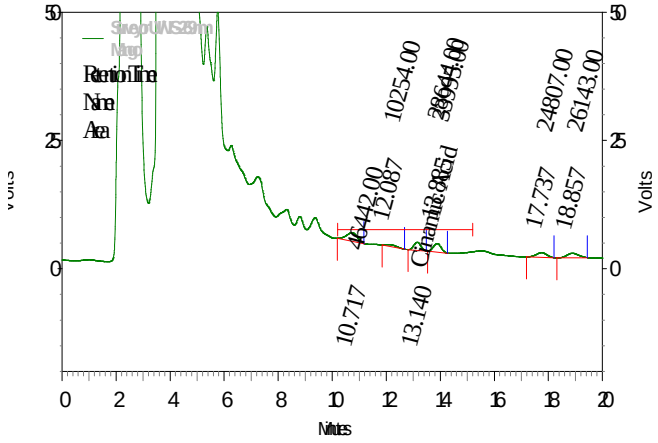
(b) Artichoke



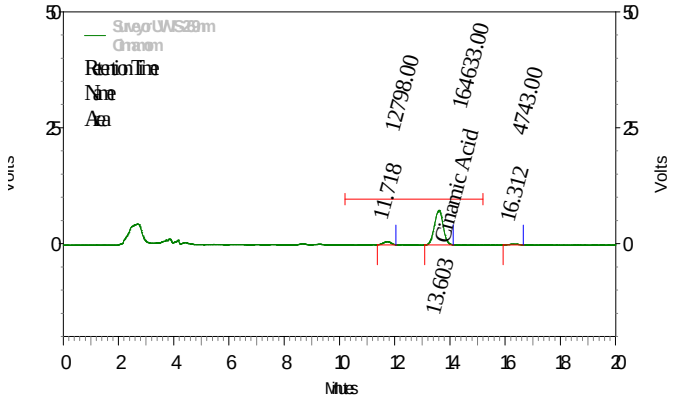
(c) Cranberry



(f) Bay Laurel



(e)Mango



(g) Cinnamon

Fig. 8: Typical chromatogram of CA extract from plants. (a) Sumach plant, (b) Artichoke plant, (c) Cranberry juice, (d) by Laurel leaves, (e) Mango leaves, (f) Cinnamon plant

Table 9: Recoveryof CA in (capsule and tablet)

Samples	Excipients weight in mg	weight cinnamon mg	Total Weight Mg	Theoretical WT of CA mg/tab or cap	%Recovery
Market tablet	489.8	130.2	620	0.4890	99.84
Prepared capsule	171.673	112.0	283.70	0.485	100.0

Table 10: Weight and Content Uniformity of cap of cinammic acid

No. of tested cap	Ave content uniformity	Ave wt of Finished cap
20 cap	100.1%	283.7 g

CONCLUSION AND RECOMMENDATIONS

The developed HPLC method for analysis covers all the requirements of validation. It is precise, specific, accurate and stability indicating.

Statistical analysis proves that the method is reproducible and selective for the analysis of CA in pharmaceutical dosage form and covers all the requirements of validation (specificity, accuracy, intra-day and inter-days precision, reproducibility, DL, QL, linearity). The method can be used to determine the purity of the drug available from various sources. As the method separates the drug from its recipients, it can be employed as stability indicating method of assay.

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