HPLC CONDITION OPTIMIZATION FOR IDENTIFICATION OF FLAVONOIDS FROM *CARISSA OPACA*

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***ABSTRACT*:** *Phytochemicals are extracted from the plants and are used as nutraceutical agents due to their antioxidant, antimicrobial and anticancer properties. Extract of plant are rich in flavonoids and it is necessary to identify these phytochemicals which possess health promoting properties. For identification of phytochemicals High performance liquid chromatography (HPLC) is widely used, but there is need that identification condition of flavonoids for HPLC is to be optimized for rapid and efficient identification and quantification of flavonoids that can be utilized by industrialists. Seeking this present study was planned to optimize HPLC identification condition for quercetin and rutin trihydrate that possess several health promoting properties. In this study the standard compounds were scanned on spectrophotometer in the wavelength range of 200 nm to 800 nm to find the lambda max of these compounds. This lambda max was adjusted on HPLC and different flow rates were used to optimize identification and quantification condition at 28 ˚C. Lambda max was found to be 271 nm for quercetin and 274 nm for rutin trihydrate. Sample injection volume 80 µl was found best at the flow rate of 1.5 ml/min. best mobile phase was found to be solvent A (water to acetic acid solution (97:3 v/v)) and solvent B (methanol) in combination at the concentration of solvent A 20% and solvent B 80%. Using these condition the quercetin and rutin trihydrate content of Carissa opaca fruit was determined which was found to be 9.44 and 2.59 mg/gm dry weight respectively. The findings of this study can be utilized by pharmaceutical as well as nutraceutical industries for rapid identification and quantification of quercetin and rutin trihydrate in any sample using HPLC.*

KeyWords: HPLC, quercetin, rutin tri hydrate, extraction

INTRODUCTION

natural antioxidants from common wild plants and different fruits. Natural antioxidants can quench free radicals and breaks chain reactions [1]. Nutritionist and food scientist consent that plants have a great concern as a source of antioxidants and can contribute in the reduction of different diseases such as cardiovascular diseases and cancer. Synthetic drugs usage as antioxidant has several limitations because they are injurious to health and many of them possess carcinogenic effect. This is the reason that usage of synthetic drugs is strictly documented [2]. So it is the need of time to replace these synthetic substances with natural substances that have minimum side effects. In fact it is verified that intake of diets which are full of fruits and vegetables are associated with the decrease of chronic and degenerative diseases. Among antioxidant compounds considerable interest has been dedicated to phenolic compounds specifically flavonoids. For effective utilization of flavonoids it is required that method is to be optimized that can be efficiently used to check the presence of flavonoids as well as to quantify them. The present study is designed to optimize mobile phase as well as the detection wavelength for quercetin and rutin tri hydrate using spectrophotometer and HPLC.

Quercetin is the most commonly found flavonol in the diet. Quercetin is present in vegetables and fruits but the highest concentration is present in onion [3]. The importance of foods as a source of quercetin varies within different countries. According to Hertog tea is the major source of quercetin in Netherlands and Japan. While in Italy wine is the major source of flavonoids [4]. In the United States the daily intake of flavonols and flavones varies between 20 to 22 mg/d of which 73 to 76% was quercetin [5].

Several health benefits are associated with the ingestion of quercetin and rutin tri hydrate because they possess antibacterial, antiviral, antifungal, antioxidant and

In the present era, research has been focused towards anticarcinogenic properties [6, 7]. Beside from that flavonoids has also been shown to preventive against cardiovascular diseases, this is correlated with the French Paradox that is French people are less susceptible to cardiovascular diseases even though they consume high amount of fat this is due to the fact that they also consume vine made from grapes and grapes are rich source of phenolic and flavonoid compounds [8].

MATERIALS AND METHODS

Fruit of *Carissa opaca* was collected from forest area of Tehsil Kahuta District Rawalpindi. Identification of plant was confirmed by the Department of Forestry and Range Management Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. After collection the samples were brought to Department of Food Technology Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. The samples were cleaned and oven dried at 45 °C. After drying the samples were ground to fine powder and used for further analysis.

Extract preparation

Extraction carried out by mixing 50 mg of fruit of *C. opaca* with 6 ml of HCl (25%) and 20 ml methanol for one hour. (a) The extract was filtered in a volumetric flask of 100 ml. (b) the residue of filtration was extracted again with 20 ml methanol for 20 min. Both the extract (a) and (b) were mixed and made volume up to 100 ml. Extract (a) and (b) and volume was made 15 ml with the addition of methanol.

HPLC analysis of Flavonoids

HPLC system**,** system controller (SCL-10A), column oven (CTO-10AS), UV-Vis. detector (SPD-10A), C18 Column, auto injector (SIL-10AD) Shimadzu, Japan were used for the analysis of samples. The wavelength of the system was adjusted according to the readings of spectrophotometric data. Different samples injection volume and rates were used to optimize the best condition for rapid and efficient

determination of flavonoids in the samples. Once the condition optimized solutions of different concentration of standard compounds were used to develop standard curve for the determination of flavaonoids in the sample. Retention time and spectral characteristic of samples curves were compared against those of standard compound to find their flavonoid content. Limit of detection (LOD) and limit of quantification (LOQ) of the standard compound will be determined by the following formulas:

$$\text{LOD} = \frac{\text{Standard deviation of intercept b}}{\text{Slope of line}} \times 100$$

$$\text{LOQ} = \frac{\text{Standard deviation of intercept b}}{\text{Slope of line}} \times 100$$

RESULTS AND DISCUSSIONS

In this study reverse phase HPLC-UV was used for qualitative and quantitative analysis of quercetin and rutin tri hydrate in *Carissa opaca* fruit. Standard solutions of quercetin and rutin tri hydrate were prepared having concentration in the range of 6-125 ppm. Standard solution were scanned on UV-Visible spectrophotometer (UV-9200 Biotech Engineering Management Co. Ltd. UK) at wavelength ranging between 200-800 nm for determination of lambda maximum (Lambda max) of the standard compound. Lambda max is the wavelength for a certain compound at which the absorption of light is maximum for that that compound. The scanned curves of both the compounds are shown in Fig. 2. Which shows that average lambda max for quercetin is 271 nm while lambda max for rutin tri hydrate is 274 nm. These results in accordance with Stalikas [9], that phenols and flavonoids having benzene ring and conjugated double bonds absorb UV light. Flavonoid compounds having flavonoid skeleton mostly absorb UV light in the region of 200 to290 nm. The lambda max determined in this study for both the compounds fall in this region. This maximum absorbance is due to presence of A ring in the flavonoid skeleton. Beside from this wavelength these compounds absorb Visible light having wavelength of 465 nm that can be seen from the Fig. 2. but at this wavelength absorbance is less as compared to 271 or 274 nm. These results are also supported by the findings of Stalikas [9] that describes that the second maximum absorbance is found between 300 to 550 nm, this second maximum absorbance is due to C ring in the flavonoid skeleton and different substitutes present in the C ring.

After determination of lambda max the wavelength was set at system controller HPLC and different injection volumes and different combinations of mobile phase were used at different flow rates for obtaining optimum detection conditions. Fig. 3. shows the variation in the concentration of mobile phase, three different combination of mobile phase were checked first of all solvent A water to acetic acid (97:3 v/v) to solvent B methanol was used at 80%: 20% concentration, then at 50% : 50% and at 20% and 80%. From the Fig. 3. it is evident that best results are obtained when solvent A to solvent B ratio was 20%-80%. These results are supported by the findings of Henning who showed that aglycones of flavonoids are more soluble in methanol so by increasing the percentage of methanol in mobile phase up to 80% improved the separation and resolution of peaks [10]. So this was selected for the further analysis of samples. From the figure it is evident that as soon as the concentration of methanol in the mobile phase is increases the spectral characteristics of curves improves for better identification and quantification of flavonoid constituents. There is significant difference in the height of curves as shown in Fig. (a), (b) and (c). maximum height is achieved when methanol was 80 % in mobile phase that is height about 200 mAU as compared to 15 mAU when methanol was 20% in the mobile phase.

By running standard solution retention time of quercetin and rutin tri hydrate was determined which was found to be 0.94 sec for quercetin and 1.4 sec for rutin trihydrate. Different concentration of standard compounds were run on HPLC and standard curve was developed having linear equation of y = 50.841x - 229.45 for quercetin while for rutin trihydrate y = 14.712x - 70.575. The limit of detection and limit of quantification was found to 2.35 and 7.86 respectively for quercetin while for rutin trihydrate LOD and LOQ was found to be 3.43 and 11.46 respectively. Acid hydrolysis of sample flavonoid compounds hydrochloric acid was used because it has been found better for hydrolysis as compared to sulphric acid [11]. The HPLC chromatogram of *Carissa opaca* fruit is shown in Fig.4 for quercetin while (b) for rutin tri hydrate. The quercetin and rutin trihydrate content of *Carissa opaca* was found to be 9.44 mg/g and 2.59 mg/g respectively. Retention time for identification of quantification of flavonoids compounds is much lower described in this study as compared to others [11-14] Limited data is available about the flavonoid constituents of *Carissa opaca*. While comparing the querectin content of *Carissa opaca* is similar to other berries like whortle berry that has about 158 mg/kg quercetin content, but the quercetin content of *Carissa opaca* is higher than sweet rown berry, crowberry and buckthorn berry [15]. The rutin trihydrate content of *Carissa opaca* is compareable with that of tertary buck wheat which contains 0.8-1.6% on dry weight basis [16]. Among berries cranberries, aronia berries and mulberry contains rutin trihydrate [17]. The flavonoid constituent of a fruit depends upon species, and within species there may be variation due to region of cultivation of plant, climatic condition, soil fertility status etc. [18]

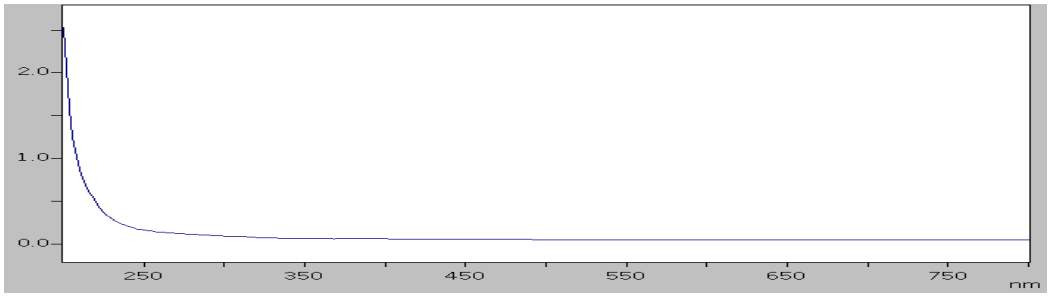


Fig. 1. Scanning of Methanol blank on UV-spectrophotometer

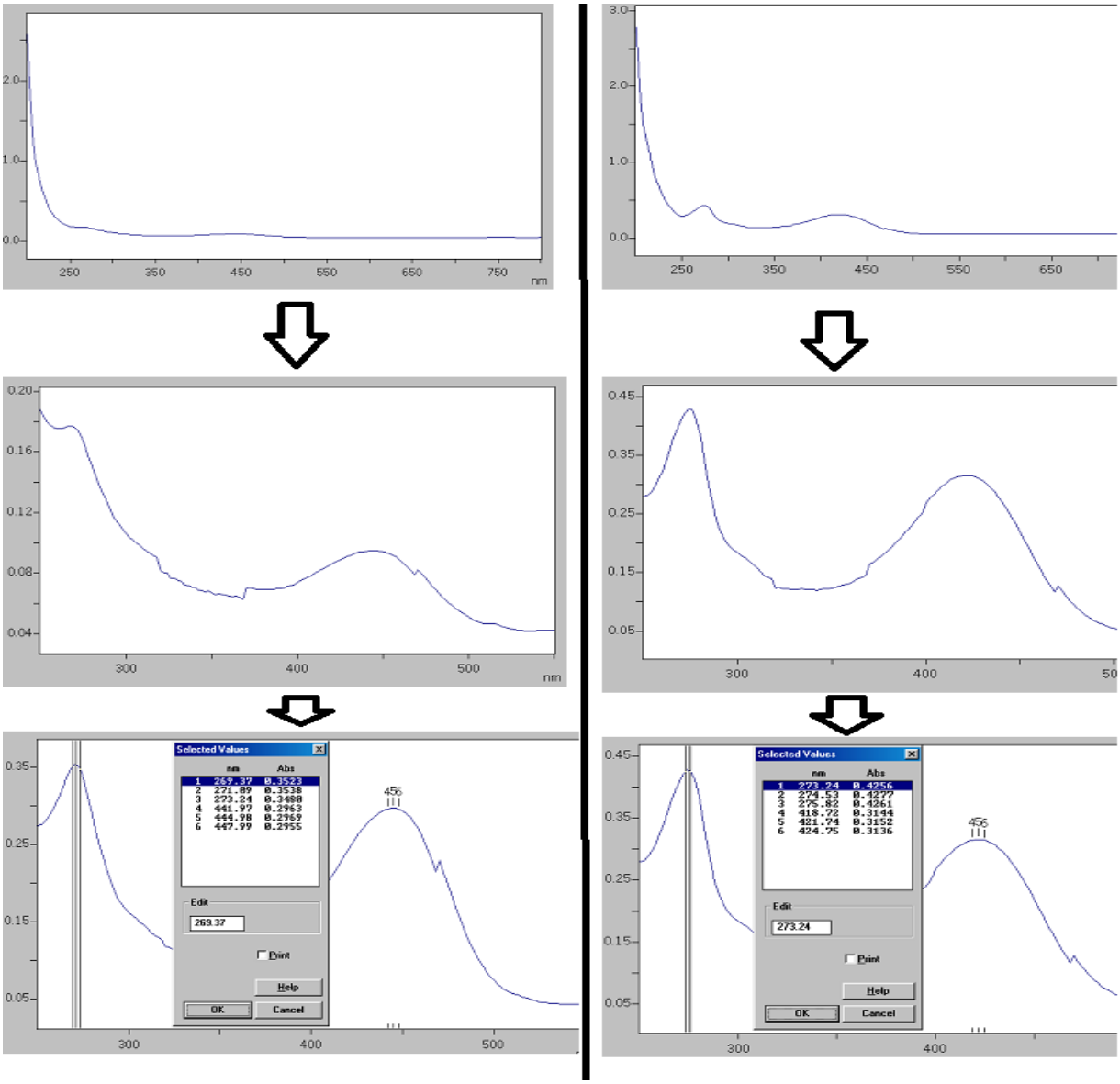


Fig.2. Lambda max determination of (a) Quercetin and (b) Rutin trihydrate Using spectrophotometer

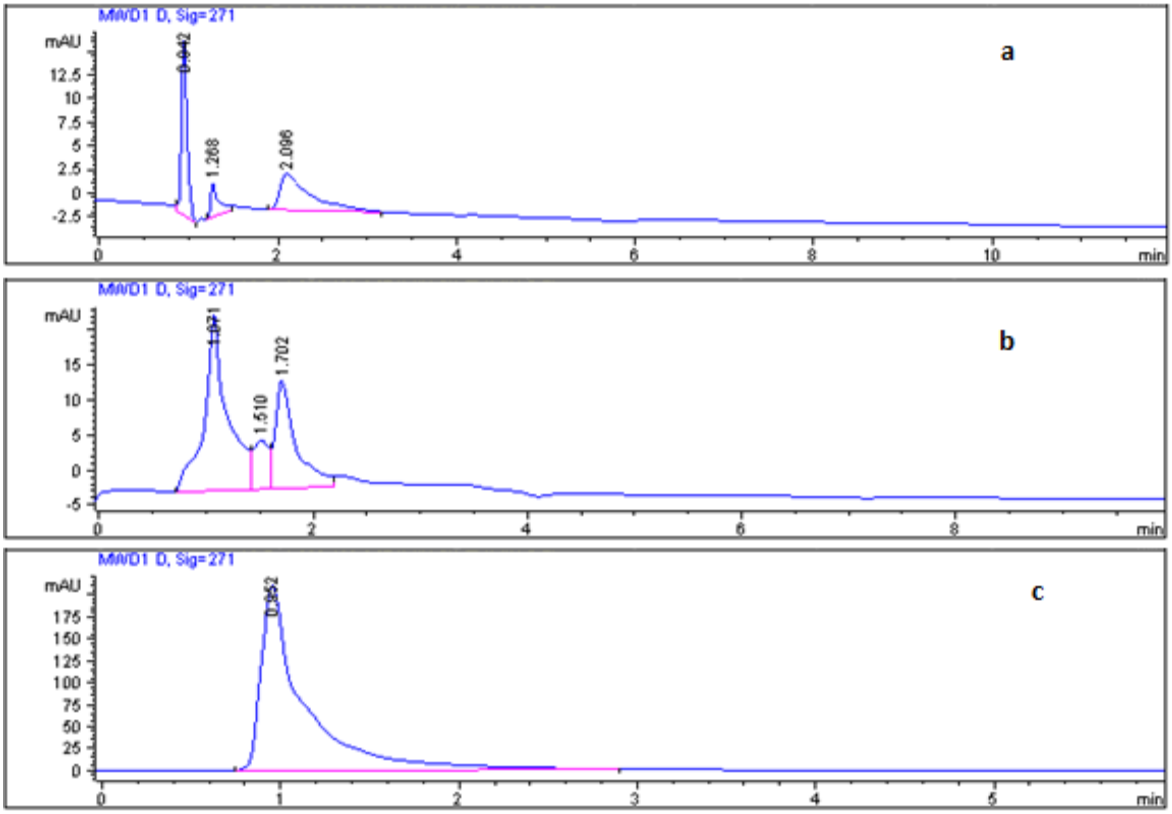
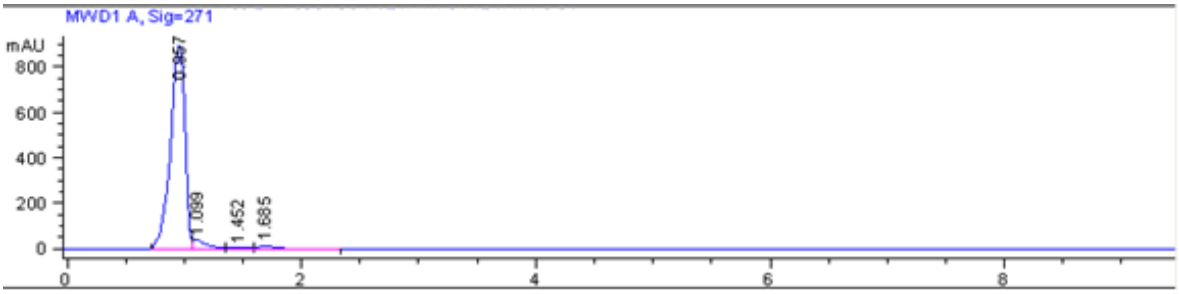


Fig. 3. Variation in the mobile phase used for standard solution of quercetin (a) solvent A to solvent B 80%:20%, (b)) solvent A to solvent B 50%:50%, (c)) solvent A to solvent B 20%:80%



. Fig. 4. HPLC chromatogram of *C. opaca* fruit at 271 nm, Peak 1 having retention time of 0.957 shows the presence of quercetin

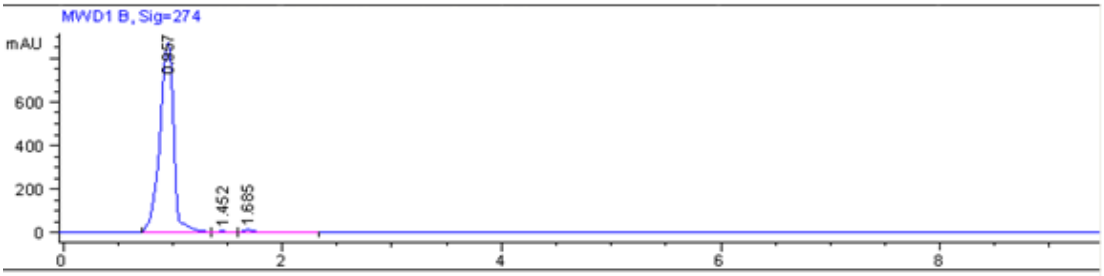


Fig. 5. HPLC chromatogram of *Carissa opaca* fruit at 274 nm, Peak 1 having retention time of 1.45 shows the presence of rutin tri hydrate in the sample

Peak No.	Retention time (min)	Type	Width (min)	Area (mAU*s)	Height (mAU)	Area
1	0.957	BV	0.1340	7692.94629	897.22272	93.6131
2	1.099	VV	0.1071	321.16217	41.13802	3.9081
3	1.452	VV	0.1663	74.67243	6.79209	0.9087
4	1.685	VB	0.1320	129.02779	14.47005	0.9087

Peak No.	Retention time (min)	Type	Width (min)	Area (mAU*s)	Height (mAU)	Area
1	0.957	BV	0.1373	7742.89502	874.23053	97.58
2	1.452	VV	0.1686	66.74122	6.05352	0.8412
3	1.685	VB	0.1319	124.55141	13.98138	1.5698

CONCLUSION

The presented method make us familiar with a method for rapid identification, quantification and reproducible method development of two important flavonoid compounds quercetin and rutin tri hydrate in acid hydrolyzed sample. Due to enormous number of flavonoids constituents in fruit and leaves samples, quantitative determination of flavonoids is complicated. Beside from method development this study shows that the fruit of *Carissa opaca* is rich source of two important flavonoid compounds quercetin and rutin tri hydrate, so the fruit of this plant can be further explored and can be used as an important constituents of nutraceutical foods. The findings of this study can be used by food and pharmaceutical companies for analysis of different plant based samples. Furthermore this study add value to the medicinal potential of plants due to presence of different bioactive components.

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