RP-HPLC Method for the Analysis of Quercetin in Kurka Sativa with Green Solvent

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Abstract: Background: Quercetin is a major representative of flavonol sub-class of flavonoids which has a nutritional value and active moieties of many medicinal plants with potent antioxidant, anti-diabetic, anti-tumor, antiviral and anti-inflammatory properties. Kurka Sativa one of the most consumable herbs in Saudi Arabia, has not been analyzed yet by using green solvent.

Objectives: A simple, sensitive, accurate, and precise reversed phase liquid chromatography method was developed and validated for the quantitative estimation of flavonoid quercetin from the extract of Kurka Sativa.

Method: The flavonoid was analyzed on RP-HPLC using Lichrosphere-180, C\textsubscript{18}, column (25\text{cm} \times 4.6\text{mm ID}, 5\text{μm}) with UV detector system. The mobile phase consisting Methanol and water having β-cyclodextrin (5mM) with 0.1% ortho-phosphoric acid (70:30 v/v) solution was used with flow rate of 1.0 mL min\textsuperscript{-1} and detection was performed at 370 nm wavelength.

Results: The data showed that the linear ranges of Quercetin were 0.2-14 μg mL\textsuperscript{-1} (r=0.997), intraday and intermediate precision at three concentration level as % RSD (0.4550, 0.7251, 0.5075; 0.4859, 0.7999, 0.5618), accuracy at three concentration level as % recovery (102.3 ± 0.8074, 101.17 ± 0.9074, 101.46 ± 0.1856 ), limit of detection; LOD (3.64042E-05 g mL\textsuperscript{-1}) and limit of quantitation, LOQ (0.00011 μg mL\textsuperscript{-1}) and robustness ( in significant variations expressed as % RSD 0.13).

Conclusion: The method was applied to quantitative analysis of Quercetin in plant extract of Kurka Sativa and was found to be simple, rapid and efficient.

Keywords: RP-HPLC, quercetin, Kurka Sativa, validation, green solvents.

INTRODUCTION

Quercetin, chemically is 2-(3, 4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, major representative of flavonol sub-class of flavonoids widely distributed in fruits and vegetables. It is a major component of nutritional supplements, available in so many medicinal plants an active moiety. They have potent antioxidant, anti-diabetic, anti-tumor, antiviral and anti-inflammatory properties [1]. Most consumable foods in Saudi Arabia known as Jarjeer leaves in Arabic and commonly known as Salad rocket (Kurka Sativa, ES), is a major source of quercetin as well as common fruits such as green apple, onion, green tea, lemon as well as many seeds, flowers, barks, and leaves are a major source of quercetin.

It is sparingly soluble and chemically unstable in aqueous intestinal fluids, and poorly orally absorbed [2]. Due to its poor aqueous solubility, it has posed great challenge to be analyzed in natural sources.

Literature revealed the presence of some HPLC method with DAD and UV detector for the analysis of flavonoid quercetin from different medicinal plant with other component, alone and inhuman plasma [3-8].

Chen et al., [3] developed the method for the separation of quercetin, luteolin and apigenin and to quantify them in extractive solutions from Marchantia convoluta with an HPLC-DAD system using a mobile phase, consisted of methanol-acetonitrile-acetic acid-phosphoric acid-H\textsubscript{2}O (200:100:10:100, V/V). Sri, et al. [4], developed the method for the quantitative estimation of quercetin in human plasma by using mobile phase consisted of methanol and water with ortho-phosphoric acid (0.1 %) in the ratio of 77:23 v/v. Phani et al., [5] developed the method for the quantitative analysis of flavonoid quercetin in the extract of Solanum trilocatum by using a mobile phase consisted of methanol-acetonitrile-water (60:20:20 v/v/v) with HPLC-UV detector. Liu et al., [6], developed the method for the quantification of quercetin from Chinese plant by using mobile
phase consisted of methanol-0.2% phosphoric acid (65:35 v/v) solution by HPLC with UV detector. Gulati, et al. [7], developed the HPLC method for the quantitative estimation of Quercetin from alcoholic extract of fruit of Emblica officinalis, by using mobile phase consisted of Acetonitrile: Water: Acetic acid (10:90: 0.2 V/V), with PDA detector. Verma et al. [8], developed the HPLC method for fingerprinting of quercetin in different natural sources.

The above reported method used the methanol and acetonitrile in large proportion, but they suffer from a number of drawbacks from the environmental point of view. Alternative, greener mobile phases are required.

Literature revealed the application of cyclodextrin (CD) in liquid chromatography as mobile phase. Buha et al. [9], developed the method for analysis of atenolol. Yane zet al. [10], developed the method for the analysis of estron and estradiol. Oros et al. [11], reported the chiral separation capacity of cyclodextrins (CD) for the separation of enantiomers with markedly different biological activity. A number of studies dealing with the application of cyclodextrins (CDs) and its derivatives for the increase of the separation capacity of chromatographic systems have been increased considerably. Because of their versatility, cyclodextrin (CDs) have found application in many special branches of chromatography such as liquid chromatography (LC), gas chromatography (GC), size exclusion chromatography (SEC), gel permeation chromatography (GPC), and electrically driven separation methods (CE, CZE), and ultra-performance liquid chromatography (UPLC).

The use of β-cyclodextrin (β-CD) and (2-hydroxypropyl)-β-cyclodextrin (Hβ-CD) as mobile phase additives is proposed to increase the proportion of water in the mobile phases without loss in the resolution or efficiency of the separations.

As there is no reported method on HPLC with β-cyclodextrin (β-CD) inclusion complex liquid chromatography of quercetin, hence it is proposed to develop RP-HPLC methods with UV detection for the estimation of quercetin in most consumable plants in Saudi Arab. For the surety of method performance of the developed methods, these were validated according to the ICH guidelines 1996.

EXPERIMENTAL

Reagents and Chemicals

Standard Quercetin was purchased from Sigma Aldrich, St.Louis. Methanol (HPLC grade) was purchased from Fisher scientific U.K Ltd, UK. Orthophosphoric acid and hydrogen peroxide were purchased from E.Merck (Mumbai, Maharashtra, India). High pure water was prepared using Millipore purification system. The whole plant of Eruca Sativa was collected from local market alkharg, Kingdom of Saudi Arab.

Instrumentation

A Waters model HPLC equipped with quaternary 1525 controller pumps, Waters 2489 tunable absorbance detectors, Waters 2707 plus auto sampler with Rheodyne injector fitted with a 20 μL loop and Breeze 2 software was used.

Chromatographic Condition

The binary mobile phase consisted of 70% of Methanol and 30% of water having β-cyclodextrin (5mM) with 0.1 % orthophosphoric acid was circulated through a stainless steel analytical column (Lichrosphere-100, RP 18, 25cm × 4.6mm ID, 5μm) at flow rate of 1.0 mL min⁻¹. The variables UV-VIS detector was set at 370 nm. All the analysis was performed at ambient temperature and the volume of solution injected into the column was 10 μL.

Preparation of standard solution and calibration solution: About 5 mg of standard quercetin was accurately weighed and dissolved in 50 mL ethanol as diluent to get the concentration of 100 μg mL⁻¹. For the preparation of calibration plot, suitable aliquots were transferred into 10 mL volumetric flask and diluted with mobile phase to get concentrations 2, 4, 6, 8, 10, 12 and 14 μg mL⁻¹ of quercetin. 10 μL of each of the solution was injected into the column using optimized chromatographic conditions.

Preparation of sample solution: The Eruca Sativa (ES) fresh leaves were purchased in October, 2013, from Al-Kharj local vegetable market, Saudi Arabia, and identified by expert taxonomist Mr. Osman Ali Elmakki. ES leaves were shade dried, coarsely pulverized, and placed in glass percolator with methanol at room temperature for 72 h (percolation method). The collected percolate was dried under reduced pressure in vacuum. The obtained methanolic extract of Eruca Sativa (MEES) was later used for the HPLC analysis.

About 1gm of methanolic extract of Eruca Sativa was weighed. A quantity of extract equivalent to 5 mg of quercetin was placed in a 50 mL volumetric flask, dissolving it with ethanol as a diluent. This solution was sonicated for 20 min to dissolve. Once the time had elapsed and the volumetric flask reached the environmental temperature (25°C), this solution was filtered through whatman filter paper no-44 to get the clear solution. An 800 μL of aliquot was transferred to 10 mL of volumetric flask and the final volume was made with same mobile phase. Out of this solution obtained, a proportion was taken &filtered through a Flupore Hyphobic PTFE membrane filter (F.H. membrane, 0.45 μm) using micro syringe in an HPLC vial. Each of the solution prepared was injected in triplicate into the Chromatograph and observations.

METHOD VALIDATION

Specificity: specificity is the ability of the method to measure the analyte response in the presence of all potential impurities. The specificity was checked by stressing the pure samples of the drug, under extreme conditions, such as 0.1 M HCl and 0.1 M NaOH, at 70°C for 6 h. The stability of the drug to oxidation was studied by stirring a solution containing 1% (w/v) of the drug and 3% H₂O₂ for 2 h. The resulting solutions were appropriately diluted with mobile phase and injected for HPLC analysis [12].

Linearity: To carry out this study five concentration levels, within the range of 80 to 120% of the target analyte concentration i.e. concentration ranging from 6.4-9.6 μg mL⁻¹ were prepared, and analyzed immediately after their preparation. These analyses were performed in triplicate.
**Precision:** Precision was carried out at two levels of ICH suggestions i.e. repeatability and intermediate precision. Repeatability or intra-day precision was carried out by nine determinations at three concentration levels, 80%, 100% and 120% in one laboratory on one day. Intermediate precision was carried out by analyzing the same sample in second day as same way. Results of repeatability and intermediate precision were expressed in terms of, SD, % RSD and SEM.

**Accuracy:** The Accuracy of an analytical method is the closeness of results obtained by that method to the true value of the sample. It was expressed as recovery (%) and was determined by the standard addition method. The pre-analyzed samples were spiked with an extra 80, 100 and 120% of the standard and then re-analyzed. The experiment was performed in triplicate. Recovery (%) and RSD (%) were calculated for each concentration.

**Detection Limit & Quantitation Limit**

Limits of detection (DL) and quantitation (QL) were determined by the standard deviation method. For determination of DL and QL, a blank sample was injected in triplicate and the standard deviation (Sy/x) of the blank response was calculated. DL and QL were then determined from the slope, S, of the calibration plot and Sy/x by use of the formulae:

\[ DL = 3.3 \times \frac{Sy}{x} / S \]

\[ QL = 10 \times \frac{Sy}{x} / S \]

**Robustness:** The study of robustness was carried out to evaluate the influence of small but deliberate variation in the chromatographic condition on results from analysis of the drug. The Mobile Phase flow rate was changed to 0.1 ± 0.25 mL min⁻¹ and the concentration of methanol in mobile phase was changed 70 ± 1% (v/v).

**Estimation of quercetin from the Methanolic extract of Eruca Sativa**

The quantification of analytes in plant extract was determined by recording area of all peaks. Corresponding concentration of quercetin against respective area value was determined by using the calibration curve. The statistical analysis of checking uniformity in batches was also performed.

**RESULTS**

**RP-HPLC Optimization Condition:**

The best mobile phase consisted of Methanol and water having β-cyclodextrin (5mM) with 0.1 % orthophosphoric acid (70: 30 v/v) solution was used with the flow rate of 1.0 mL min⁻¹ and detection was performed at 370 nm wavelength. The standard (reference) and sample peaks of Quercetin are shown in Figs. (1 and 2) respectively.

**System-Suitability Tests:** System-suitability tests were performed to measure the number of theoretical plates, tailing factor (asymmetry), capacity factor (k), and repeatability of retention time (RSD, %) of the analytes calculated by using system software (Breeze 2). The results are listed in Table I.

![Fig. (1). HPLC chromatogram obtained from standard of quercetin (tR 2.66 min) under normal conditions.](image)

![Fig. (2). HPLC chromatogram obtained from quercetin in a test sample.](image)
Method Validation

It was decided to use an isocratic mobile phase to achieve optimum resolution of the analytes. In this method there was no interference at the elution time of the analytes from peaks of degradation products produced under acidic, alkaline, or oxidizing conditions. The retention times, t<sub>R</sub>, of quercetin was 2.66 ± 0.02 min. After treatment with only alkali (0.1 M NaOH), degradation products of quercetin were observed at t<sub>R</sub> 3.3 and 5.3 min. Typical chromatograms are illustrated in Fig. (3).

Response to the compounds was linear function of concentration in the ranges 6.4-9.6 μg mL<sup>-1</sup> (correlation coefficient 0.997). Linear regression data are given in Table II.

Test solutions containing low, medium, and high concentrations (80, 100, and 120%) of quercetin were used to determine recovery (accuracy), intraday and intermediate precision. Recovery of quercetin was (102.3 ± 0.8074, 101.17 ± 0.9074, 101.46 ± 0.1856) in low, medium and high concentration as respectively. Results from measurement of accuracy and intraday and intermediate precision are given in Tables III and IV. The limits of detection and quantitation for quercetin were (3.64042E-05 g mL<sup>-1</sup>) and (0.00011 μg mL<sup>-1</sup>) respectively. The chromatogram of placebo is given in Fig. (4). The reliability of the method during normal use was checked by the assessment of robustness. RSD of retention time (t<sub>R</sub>, min) and tailing factor ranged from 0.396 to 1.487% when mobile phase composition and flow rate were
varied slightly. Results from the assessment of robustness are given in Tables V. The Applicability of method was presented by assay results of quercetin in plant extract of *Eruca Sativa* expressed as % recovery (102.0 ± 0.6074). The results are given in Table VI.

**DISCUSSION**

A robust, specific, accurate, precise and sensitive HPLC method was developed for the estimation of quercetin in most consumable food available in Saudi Arab i.e. *Eruca Sativa* commonly known as Jarjeer in Arabic and Salad Rocket in English.

During optimization of RP-HPLC, we initially used mobile phase comprising organic phase with green solvent. So we tried the β-Cyclodextrin in different molar concentration as green solvent. Since quercetin peak obtained was asymmetrical, we re-adjusted the mobile phase and found that methanol with water having 5mM cyclodextrin and 0.1% ortho-phosphoric acid at ration of (70: 30v/v) yielded a highly symmetric quercetin peak at flow rate of 1.0 mL min⁻¹ at room temperature.

Since quercetin was reported to have the maximum absorption at 260-273 (5,7) and 360-370 nm (6, 13) we tested and found that the shape of the peak was more symmetric at the detection UV wavelength of 370 nm using Water 1525.
system with column (Lichrosphere-100, RP 18, 25cm × 4.6mm ID, 5μm).

The developed method was validated as per ICH guideline 1996 by using all validation parameters. The results of all the validation parameters of quercetin were found to follow specificity (absence of any interference of analyte peak with degradation product under acidic, alkaline and oxidative condition), showed the main peak of analyte at 2.66± 0.02 min, degradant peak at 3.3 and 5.3 min, linearity (0.997), intraday and intermediate precision at three concentration level as % RSD (0.4550,0.7251, 0.5075; 0.4859,0.7999, 0.5618), accuracy at three concentration level as % recovery (102.3 ± 0.21%, 101.17± 0.907, 101.46 ± 0.1856 ), limit of detection; LOD (0.0011μg mL-1) and limit of quantitation, LOQ (0.00011μg mL-1) and robustness ( in significant variations expressed as % RSD 0.13).

The Applicability of method was presented by assay results of quercetin in plant extract of *Eruca Sativa* expressed as % recovery (102.0 ± 0.6074).

HPLC method with diode array detector (DAD) and UV detector was used for the analysis of flavonoid quercetin from different medicinal plants with other component, alone and in human plasma [3-7]. The study [3] with diode array detector (DAD) the optimal chromatographic conditions were obtained on Kromasil RP-C18 column (250×4.6mm, i.d, 5μm), at ambient temperature (28±1°C). The mobile phase comprises methanol-acetonitrile-acetic acid-phosphoric acid-H₂O (200:100:10:10:200, V/V) at the flow rate of 0.6 mL min⁻¹ at 352 nm. The method showed the very long retention time (18.3 min) with multicomponent mobile phase i.e. time consuming. In another method with same detector [7] using the mobile phase consisted of Acetonitrile: Water: Acetic acid (10:90: 0.2 V/V), at 273 nm with retention time 6.7 min. showed the long retention time by using acetic acid in mobile phase which is very corrosive nature with pungent smell.

The method with UV detector was established for the analysis of quercetin in human plasma [4], chromatographic conditions were obtained on RPC-18 column (250 mm× 4.6 mm I.D.; particle size 5 μm; 30 °C. having mobile phase methanol and water with ortho-phosphoric acid (0.1 %) in the ratio of 77:23 v/v at 370 nm showed the low sensitivity (0.2μg mL-1) with retention time 2.7 min. In case of biological sample it was the very interfering area for the detection in the presence of blank matrix. Another method [5, 6] with UV detector for the analysis of quercetin from plant extract, using mobile phase methanol-acetonitrile-water (60:20:20 v/v/v), methanol-0.2% phosphoric acid (65:35 v/v), at 262 nm, 360 nm with retention time 2.03 min, 9 min respectively. Both methods have very short and very long retention time with poor sensitivity.

We used HPLC for the determination of quercetin content because it is a relatively simple method with green solvent having very simple composition of mobile phase as compared with HPLC combined with PDA and UV having multicomponent mobile phase using large proportion of or-

### Table V. Robustness study

<table>
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<tr>
<th>S. No</th>
<th>Chromatographic changes (factor)</th>
<th>Level</th>
<th>Mean Retention time (t₉)±SD</th>
<th>SEM</th>
<th>% RSD</th>
<th>Mean Tailing factor (t₉)±SD</th>
<th>SEM</th>
<th>% RSD</th>
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<tr>
<td>A</td>
<td>Flow rate (ml/min)</td>
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<tr>
<td></td>
<td>0.9 -1</td>
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<td>2.52±0.01</td>
<td>0.0057</td>
<td>0.396</td>
<td>1.2±0.01</td>
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<td></td>
<td>1.0 0</td>
<td></td>
<td>2.66±0.015</td>
<td>0.0081</td>
<td>0.566</td>
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<td>1.145</td>
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<td>2.69±0.021</td>
<td>0.0120</td>
<td>0.776</td>
<td>1.4±0.028</td>
<td>0.0120</td>
<td>1.480</td>
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<tr>
<td>B</td>
<td>% Of organic phase</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>69 -1</td>
<td></td>
<td>2.92±0.020</td>
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<td>0.684</td>
<td>1.21±0.015</td>
<td>0.0886</td>
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<td>2.66±0.015</td>
<td>0.0088</td>
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<td>1.31±0.01</td>
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<td>71 +1</td>
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<td>2.51±0.025</td>
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<td>1.35±0.02</td>
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</table>

Average from three determinations (i.e. n = 3)

### Table VI. Assay results of quercetin from *Eruca Sativa*

<table>
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<tr>
<th>S.No</th>
<th>Contents</th>
<th>Conc. Taken (mg/g)</th>
<th>Conc. found (mg/g)</th>
<th>% Mean Recovery</th>
<th>% RSD</th>
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<td>5</td>
<td>5.2</td>
<td>102.0</td>
<td>0.21</td>
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</table>

* N=3: Average of three injections, RSD: Relative Standard Deviation.
Organic phase, suffered from a number of drawbacks from the environmental point of view.

CONCLUSION

The RP-HPLC method was successfully developed in this study for the determination of Quercetin contents in *Eruca Sativa* as most consumable foods in Saudi Arabia. The data showed that RP-HPLC is a powerful technique for this purpose which has the potential benefits of high sensitivity, accuracy, reproducibility and time saving.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES


