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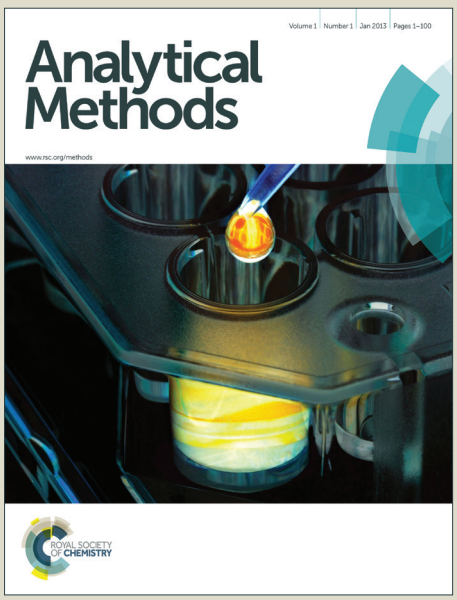

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MEKC method for naringenin from natural and biological samples

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Abstract

The proposed work describes the development of a micellar electrokinetic chromatographic (MEKC) method for the determination of naringenin in real samples including grapefruit juice and human blood serum using PDA detector. The effects of different CE parameters such as concentration and pH of the running buffer, voltage, injection time and concentration of sodium dodecyl sulfate (SDS) were optimized. Under the optimized conditions, naringenin could be well determined within 6 min using 40 mM borate buffer, 40 mM SDS at pH 9.0, at applied voltage of 25 kV. For the quantitative determination of naringenin (flavonoid aglycone) in grapefruit juice, the naringin (flavonoid glycoside) was hydrolysed and the resulting aglycone was identified and quantified. The calibration curve was linear in the studied concentration range from 0.1 to 50 µg/mL (R^2=0.995). The detection limit and limit of quantification was found to be 0.05 and 0.19 µg/mL respectively.

Key words: CE, flavonoids, naringenin in grapefruit juice and human blood serum

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1. Introduction

It has been recognized for many years that a diet rich in vegetables and fruits protects against chronic diseases. [1] Citrus fruits and juices are among the most important nutrient-dense foods. They contain a range of important nutrients essential for human health such as vitamin C, vitamin A and minerals. [2] The well-established benefits of citrus products are mainly due to the presence of most important class of secondary plant metabolites, namely flavonoids.[3] In recent years, the grapefruit (*Citrus paradise*Mact.(Rutaceae)) has received much attentions because of its nutritional and antioxidant properties. Besides ascorbic acid, it is also abundant in flavonoids, which are reported to be the important part of active ingredients.[4]

Grapefruit is cultivated principally to obtain the juice.[5] Naringin (4' ,5, 7-trihydroxyflavanone-7-rhamnoglucoside) is a major flavonoid present in grapefruit.[6] It is present in grapefruit juice at concentrations of up to 800 mg/L[7]. It possess anti-inflammatory, antiulcer, superoxide scavenging and antioxidation activities.[6] Naringenin belongs to the class of flavonoids called the flavanones and is derived from the hydrolysis of glycone forms of this flavanone, such as naringin or narirutin. The chemical name of naringenin is 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, and it has a molecular weight of 272.26 ($C_{15}H_{12}O_5$).

Naringenin is almost insoluble in water and is soluble in organic solvents such as alcohol.[8] It may be potentially useful as pharmacological agent, as anticancer, in the treatment or prevention of atherosclerosis, with a number of antiatherogenic activities, such as antioxidant, anti-inflamatory, antithrombotic and vasodilator.[9]

Due to various health benefits of naringenin and the steady progress of medicinal sciences, analytical techniques for their characterization and quantification are required.[10] Several methods for the determination of naringenin have been described. They include HPLC–UV,[11-13] HPLC with photodiode-array,[14] gas chromatography,[15] gas chromatography with mass spectrometry [16] and liquid chromatography with mass spectrometry.[14] However, these methods tend to be rather time-consuming, use toxic solvents, and sometimes require development of extremely complex gradients for the separation.[17]

During the past decade, capillary electrophoresis (CE) has become one of the versatile approaches for highly-efficient separation. CE has many advantages, such as minimal requirements for sample and running buffer, shorter analytical period and high separation efficiency.[18] CE has been applied to the separation of flavonoids since 1991. [19] Most studies that use CE for the analysis of flavonoids are in the field of natural product research, including the analysis of plants,[20-22] vegetables,[23] herbs[24] and other plant or fruit-derived products.[25-27]

Ganzera et al. [28] developed and validated a micellar electrokinetic capillary chromatography (MEKC) method for the simultaneous determination of flavonoids in Arnica Montana. Zhang, et al. [29] used capillary zone electrophoresis (CZE) for simultaneous determination of flavonoids in chrysanthemum. Huck et al. [10] analyzed flavonoids by CE-UV and CE-ESI-MS. A CE method was established for the analysis of flavonoid mixture consisting of 5-methoxyflavone, biochanin A, hesperetin, and naringenin obtained from plant extracts. MEKC separation and identification of flavonoids obtained from cruciferous plants, [30] from honeys, [31] from Scute and Coptis herbs,[32] and from green tea samples [33] have been reported.

Wang et al. [34] determined nine flavonoids most often seen in grape wine, namely apigenin, baicalein, naringenin, luteolin, hesperetin, galangin, kaempferol, quercetin and myricetineby means of CZE. Separation of flavonoid -3-O-glycosides differing in their sugar moiety and flavonoid-7-O-glycosides differing in their aglycones were separated as borate complexes by CE [35] and MEKC [36], applying UV detection. A group of flavonoids of special interest to wine quality were separated by MEKC with diode array detection [18]. Rutin and quercetin have been separated and determined in the flowers of Sophora Japonica L. by CE with electrochemical detection .[37] Liang et al. [20] characterized flavonoids in extracts from four species of Epimedium by

MEKC with diode array detection. Another MEKC method is developed for the determination of potentially anticarcinogenic flavonoids in various types of wines .[38] Five flavonoids (hesperidin, naringin, hesperedin, naringenin and rutin) have been separated and determined in grapefruit peel and juice by CE with electrochemical detection .[4] Lu et al. [39] analyzed flavonoids by non-aqueous CE with 1-ethyl-3-methylimidazolium ionic-liquids as background electrolytes.

The main objective of this work is the determination of biologically important naringenin from real sample matrices such as blood serum and grapefruit juice. For which a simple, rapid and sensitive method using micellarelectrokinetic chromatography (MEKC) is developed.

2. Results and discussion

2.1. Method optimization

The flavonoids were analyzed by CE using an optimized method. Different parameters including the pH of the buffer, borate and SDS concentration, applied voltage and injection time were optimized to determine naringenin. Using a photodiode array detector UV spectra of naringenin was obtained at 319 nm (Fig.1).

Buffer selection

In order to obtain the optimum CE conditions, three buffers namely sodium hydrogen phosphate (pH, 7.5-9.0), sodium acetate (pH, 6-7) and sodium tetraborate (pH, 8-10) were investigated. Among these buffers, the best results were obtained with sodium tetraborate in terms of peak shape, peak height and shorter migration time. Therefore it was selected and further optimized.

Effect of SDS+ buffer concentration

To improve the intensity of the signal, SDS was added (20 – 100 mM) in borate buffer and the effect of SDS+ buffer concentration was studied from 20-100 mM with intervals of 20 mM of each. Fig. 2(a) describes the effect of SDS+ buffer concentration on the absorbance and migration time of analyte. The absorbance was found to be highest at buffer concentration of 100 mM but the analysis time increased to 6 min and the baseline noise was also found to be greater. The best resolution with the least migration time was obtained at a buffer concentration of 40 mM, therefore 40 mM was selected for further studies.

Effect of buffer pH

The pH of the running buffer affects the electro-osmotic flow as well as the overall charge of the analytes, which determine the migration behavior and affect the determination of the analytes [21]. Keeping buffer concentration at 40 mM, a variation in the pH value from 8 to 10 was tested in increments of 0.5. Fig. 2(b) indicates an increase in migration time with increasing pH of buffer solution. Buffer of pH 9 was selected because of reasonable migration time and absorbance.

Effect of applied voltage and injection time

The effect of applied voltage was examined in the range of 10-30 kV under the selected conditions. The influence of applied voltage on absorbance and migration time is exhibited in Fig. 2(c). The migration time showed a decreasing trend with increasing applied voltage but the capillary current also keep on increasing which resulted in heating and baseline noise. On the basis of these experiments, 25 kV provided the best response in terms of analysis time, current intensity and efficiency.

Finally, in order to improve the detection and quantification limits, different injection times were tested between 4-12 s, at intervals of 2 s, at a constant pressure of 0.5 psi and voltage of 25 kV. Fig. 2(d) indicates that absorbance increases with

increasing injection time upto 10 s. Therefore 10 s was selected as the optimum injection time to get greater absorbance as there were very small changes on migration time.

Electropherogram of naringenin in concentration range of 0.1 to 50 µg/mL obtained at optimum conditions is shown in Fig. 3. It can be seen that naringenin is well determined within 5.5 min.

2.2 Validation of the method

Linearity

Fig. 4 shows the calibration curve for naringenin in the concentration range from 0.1 to 50 µg/mL. The response was linear with coefficient of determination (R^2) = 0.995. The limit of detection (LOD) and limit of quantification (LOQ) measured as signal to noise ratio of 3:1 and 10:1 were obtained as 0.05 µg/mL and 0.19 µg/mL respectively.

Precision and accuracy

Precision of the method was calculated both inter and intraday. The quantitation was repeatable in terms of migration time and peak height, inter (n=5) and intraday (n=5) with relative standard deviation (RSD) within 1.0-2.5 and 2.0-3.0% respectively. Accuracy of method was confirmed by spiking a known amount of analyte in sample solution and calculating the recovery, which was in the range of 81-98%.

2.3 Sample analysis of commercial grapefruit juice

The optimized MEKC method was applied to determine the amount of naringenin in grapefruit juice. Hydrolysed grapefruit juice was run for recording electropherogram. Fig. 5 shows that the naringenin was present in the juice sample and was successfully identified and quantified. Identification of peak was based on comparison of migration time and UV spectra to those of standard by using photodiode array detector. Quantitation was done by external calibration plot and the amount of naringenin was found to be 115.32 µg/mL. These results are in good agreement with literature values. The naringenin concentration was measured in different commercial grapefruit juices ranging from 4 to 126 µg/mL using HPLC and their results showed that all tested commercial grapefruit juice products contained naringenin, with variation in content form brand to brand.[40-41]

2.4 Sample analysis of human blood serum

A blank blood sample was first collected before the administration of grapefruit juice and it was analysed by developed method. No peak was observed in blank sample, as shown in Fig. 6. The blood samples were then collected after 2 hrs administration of the grapefruit juice and were treated using the procedure given. The electropherogram was obtained by analyzing blood sample and it was observed that a peak appeared at 5.5 min. Table 1 shows the % recoveries from serum which were calculated by spiking the known amount of naringenin in serum samples. These findings are in general agreement with the literature. Yil et al[42] developed a HPLC method for measuring naringenin in biological fluids and its disposition from grapefruit juice, and the recovery of naringenin was found to be 96%.

3. Experimental

3.1. Standards and reagents

Naringenin was purchased from Sigma Aldrich (United Kingdom). Boric acid and sodium hydroxide were purchased from E. Merck (Germany). Sodium dodecyl sulphate (SDS) and ascorbic acid were purchased from Fluka (United States & Switzerland). Sodium borate decahydrate was obtained from Daejung Chemicals (Korea). Methanol and Hydrochloric acid were purchased

from Fischer chemicals (United Kingdom) and Sigma Aldrich, respectively. All reagents were HPLC grade and used without further purification. Double deionized water was used throughout the experiments.

The background electrolyte (BGE) was prepared by dissolving the appropriate amounts of SDS and sodium borate decahydrate in doubly deionized water to obtain the final concentration. The stock standard solutions of 200 mg/L of naringenin was prepared by dissolving the appropriate mass of the flavonoid in 20 mL of methanol and then diluted with doubly deionized water to 100 mL. The pH of the running buffer was adjusted with either 0.1M NaOH or 0.1M HCl.

3.2 Instrumentation and Electrophoretic conditions

All experiments were conducted with a Beckman P/ACE MDQ capillary electrophoresis instrument (Beckman coulter Inc., USA) equipped with a photodiode array detector with 32 Karat software. Uncoated fused-silica capillaries (PolymicroTechnologies, Phoenix) with dimensions of 57 cm total length, 50 cm effective length, 75 µm inner diameter and 375µm outer diameter were used.

New capillaries are conditioned by flushing with methanol for 10 min, 1M NaOH for 30 min and double deionized water for 10 min, at 20 psi, while the capillary temperature was maintained at 25^{o}C as recommended by the company (Beckman coulter Inc., USA). Whereas at the beginning of the each day, the capillary was regenerated and conditioned by washing in sequence, with methanol for 1 min, water for 0.5 min, hydrochloric acid (0.1 M) for 2 min, water for 0.5 min, sodium hydroxide (0.1 M) for 2 min, water for 0.5 min, and, finally, running buffer for 2 min. Between runs, the capillary was washed with methanol for 2 min, water for 0.5 min and BGE for 2 min.

Samples were injected by means of the autosampler using hydrodynamic method (10 s at 0.5 psi). The running buffer was 40 mM sodium borate decahydrate (pH 9) containing 40 mM SDS and the applied potential was 25 kV.

The pH of solutions was measured using a inoLab pH meter.

3.3 Sample preparation

3.3.1 Commercial grapefruit juice

Commercial grapefruit juice was purchased from a super store of Hyderabad, Pakistan. The reported method [43] was employed for hydrolysis for grapefruit juice with some modifications. A 5 mL of the juice was hydrolysed in 5 mL of 1.2 M HCl in 50% aqueous methanol. To the hydrolysis mixture, 3 mg of ascorbic acid was added. After refluxing at 80^{o}C for 90 min, the extract was allowed to cool and sonicated. The extract was then filtered through 0.45 µm cellulose acetate filter paper and the clear solution was run for recording electropherogram.

3.3.2 Preparation of blood samples

The experiments conducted were performed with compliance with relevant laws and institutional guidelines of University of Sindh, Jamshoro, Pakistan and also the institutional committees; Scrutiny Committee and Board of Advance Studies and Research has approved all the experiments. A total of seven healthy volunteers (5 female + 2 male) aged between 25-40 yrs have taken part in this study. After their written consent, blood samples were collected before and after 2 hrs administration of the grapefruit juice. Each blood sample was collected by vein puncture in a clean screw capped vial and centrifuged at 5000 rpm for 10 min. Serum was separated and 5 mL of methanol was added and the contents were mixed for 2 min. The samples were again centrifuged at 5000 rpm for 10 min and filtered through 0.45 µm cellulose acetate filter paper and the clear solution was then run for recording electropherogram.

4. Comparison of proposed method with reported method

The proposed method has been compared with a reported method for the determination of naringenin in citrus fruits by HPLC. [44] As shown in Table 2, the reported method was linear in working range of 10-60 µg/mL whereas the proposed method is linear in working range of 0.1-50 µg/mL. Limit of detection (LOD) and limit of quantification (LOQ) for naringenin in reported method is 1.11 µg/mL and 3.37 µg/mL, whereas in proposed method it is 0.05 µg/mL and 0.19 µg/mL, respectively. Naringenin has been determined within 15.15 min in reported method on HPLC whereas by using CE it has been determined within a very short time of 6 min and the solvent system used in proposed method is borate and SDS whereas in reported method acetonitrile is used which is very toxic and not environmental friendly.

5. Conclusion

This paper describes the development of a rapid, easy and sensitive MEKC method for the determination of naringenin in grapefruit juice and human blood serum, using PDA detector. Under optimized conditions, naringenin could be well determined within 6 min in a 40 mM borate buffer containing 40 mM SDS at pH 9 and an applied voltage of 25 kV. A good linear range (0.1-50 µg/mL), LOQ (0.19 µg/mL), and LOD 0.05 µg/mL, values shows that method can be used over wide range of sample concentrations. The method was successfully applied for the determination of naringenin in grapefruit juice and human blood serum.

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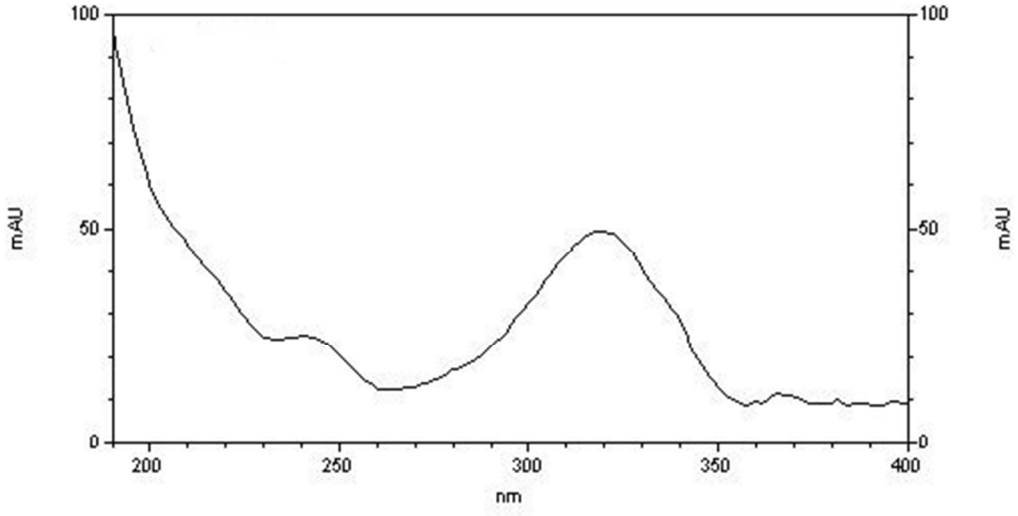

Fig 1. UV spectra of naringenin

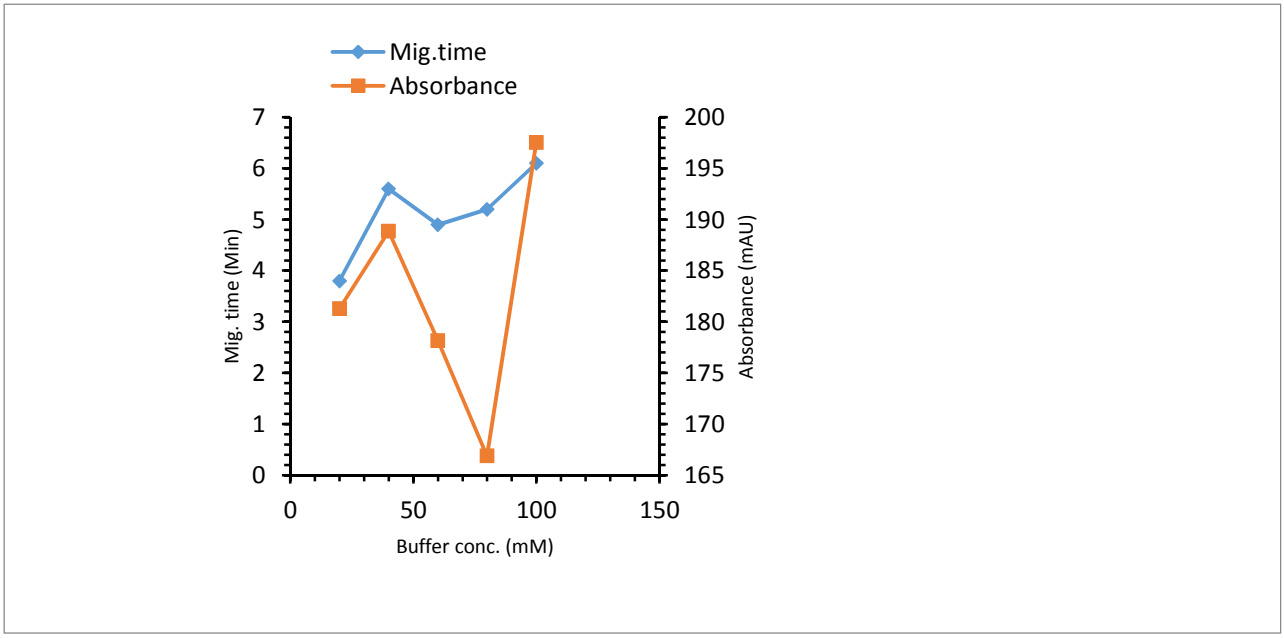

Fig 2(a). Effect of buffer concentration on migration time and absorbance

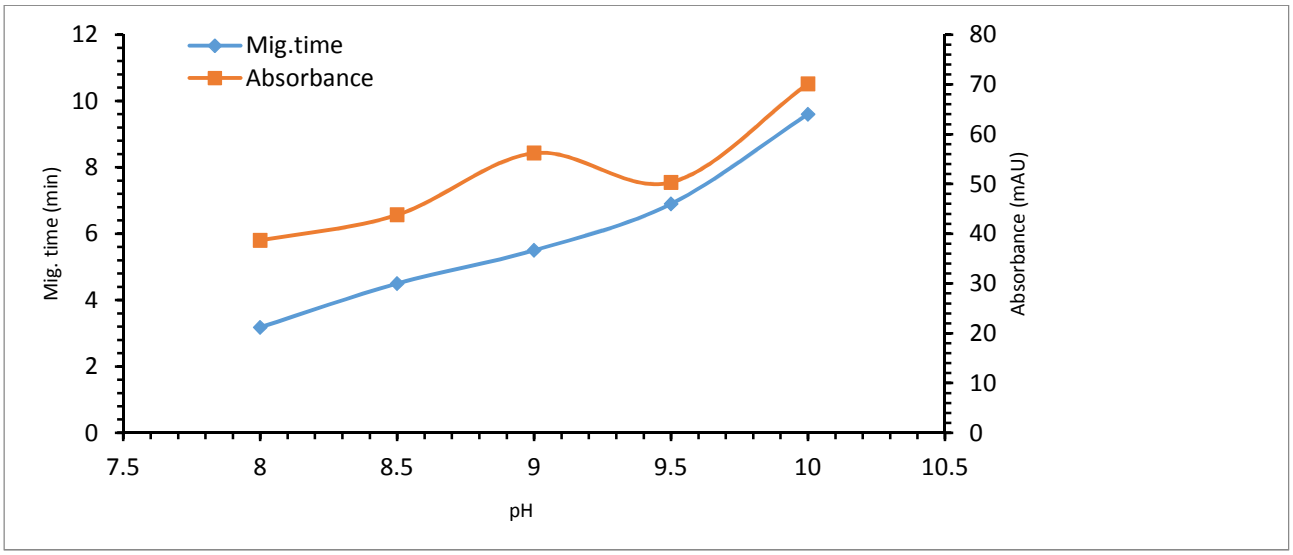

Fig 2(b). Effect of buffer pH on migration time and absorbance

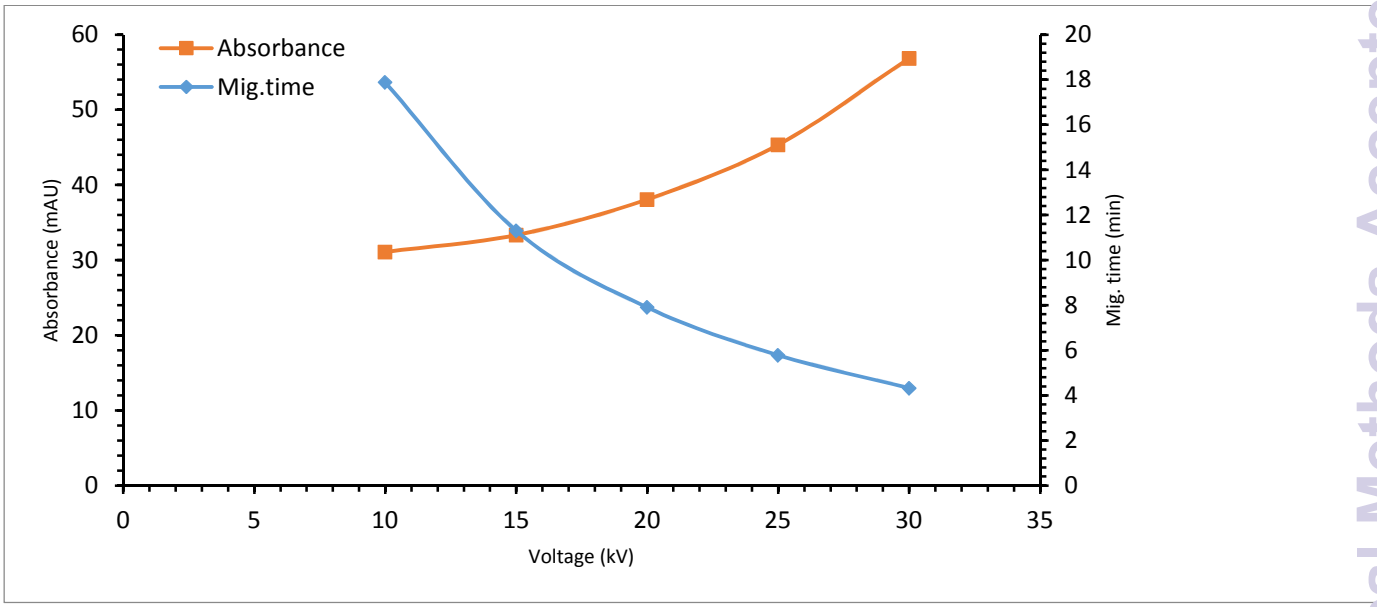

Fig 2(c). Effect of applied voltage on migration time and absorbance

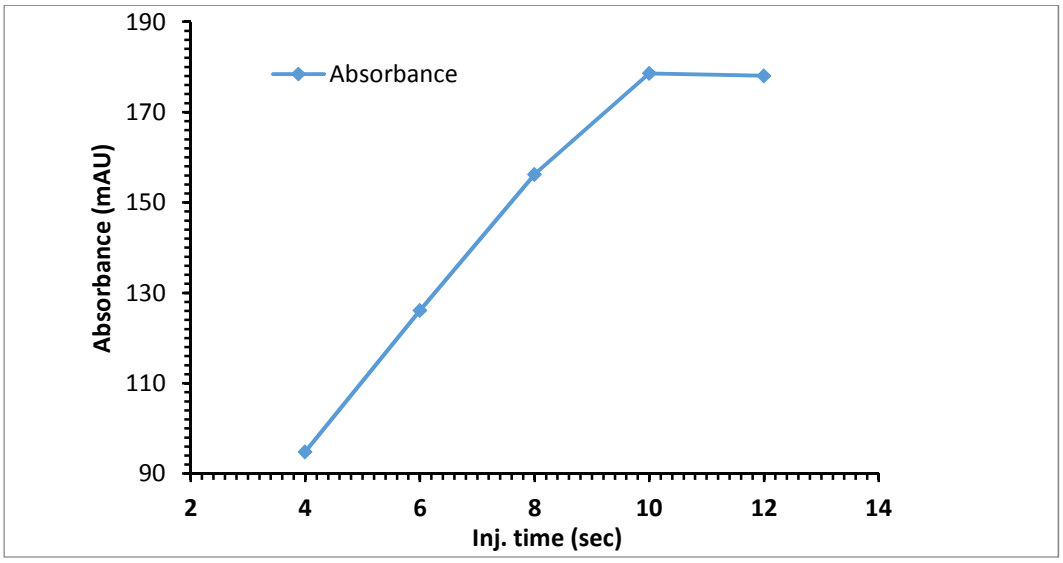

Fig 2(d). Effect of injection time on absorbance

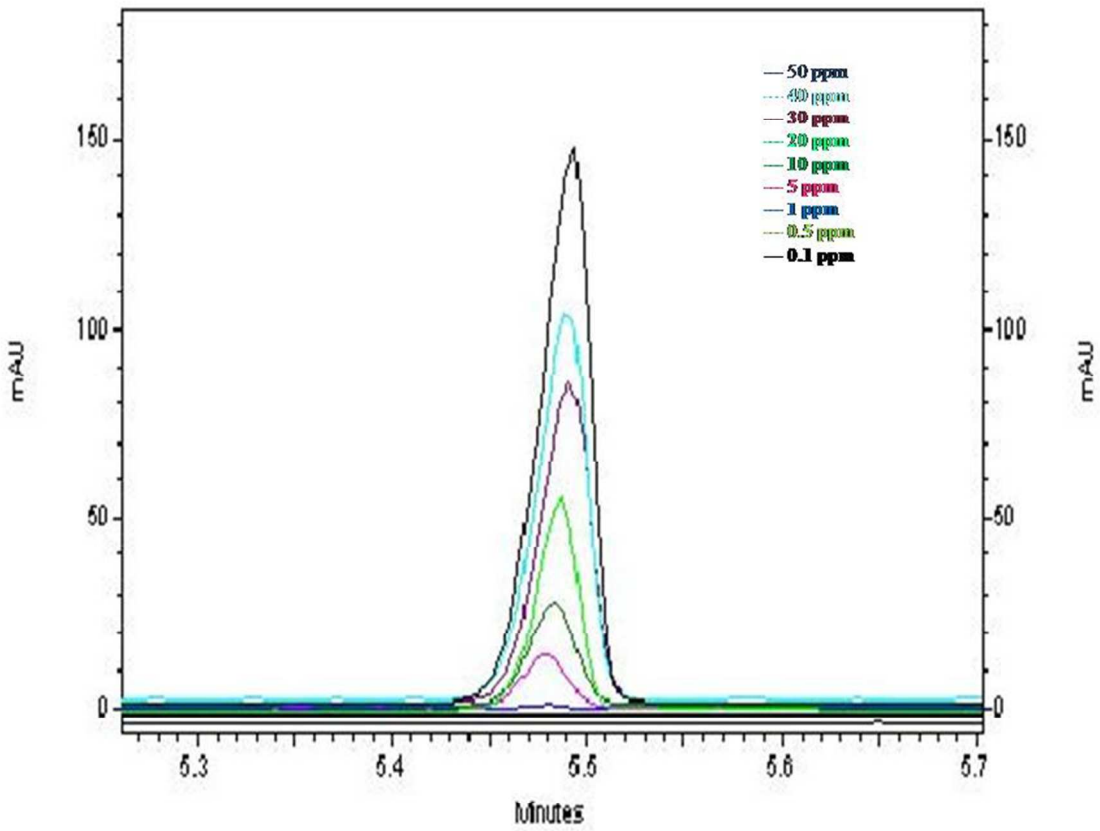

Fig 3. Electropherogram of naringenin in concentration range from 0.1-50 µg/mL. Hydrodynamic injection at 0.5 psi for 10 sec, 25 kV, uncoated fused-silica capillaries with dimensions of 57 cm total length, 50 cm effective length, 75 µm i.d. and 375µm o.d., 25 ^{0}C. and 40mM borate buffer solution containing 40mM SDS at pH 9 were used.

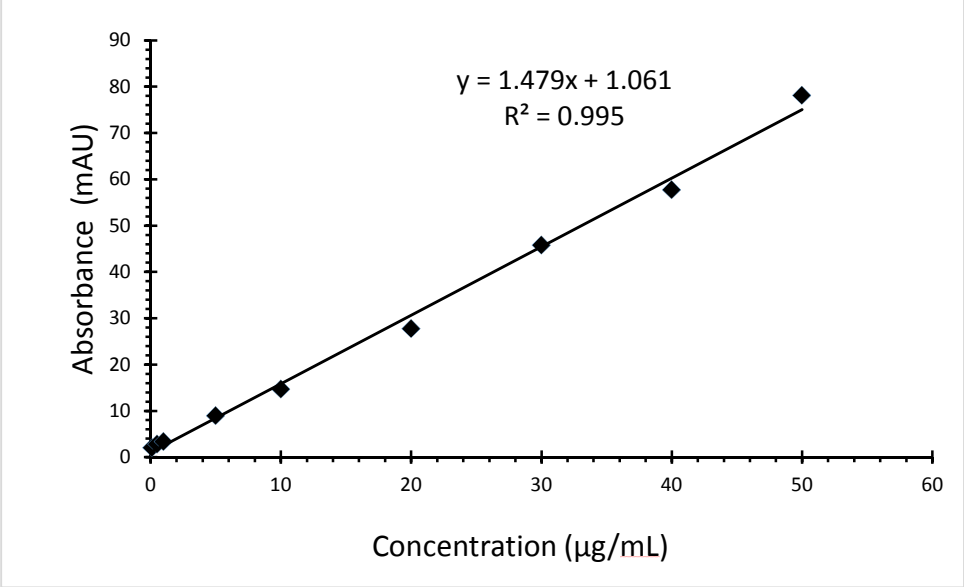

Fig. 4. Calibration plot of naringenin in concentration range from 0.1-50 µg/mL

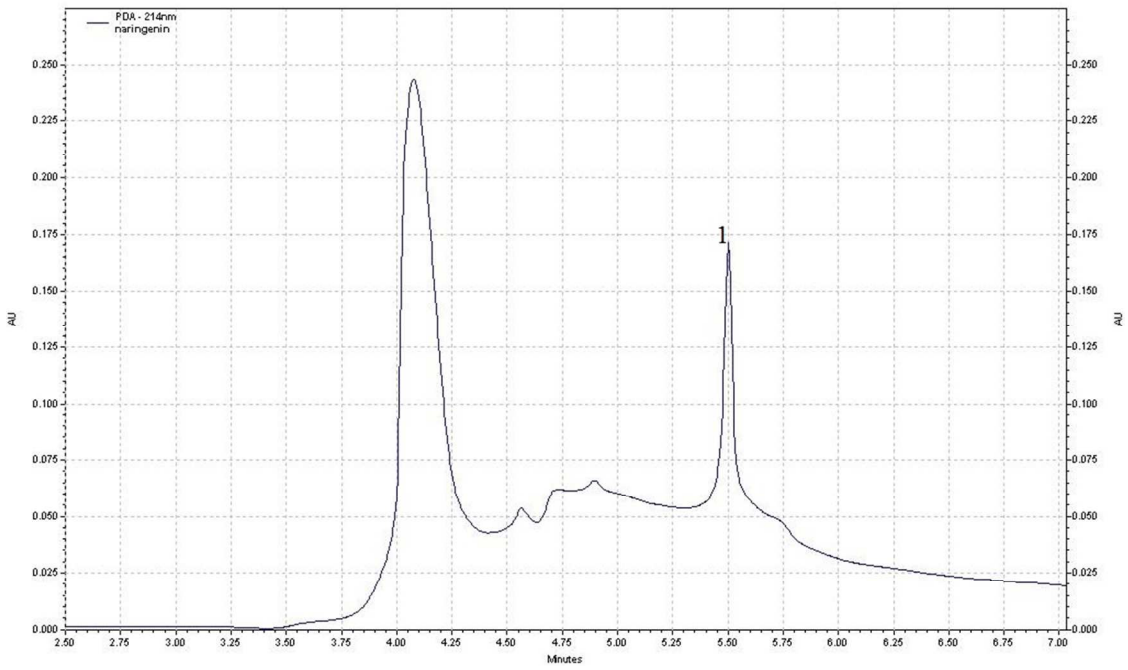

Fig 5. Electropherogram obtained from hydrolysis of commercial grapefruit juice under optimum conditions, 1.naringenin at 5.5 min

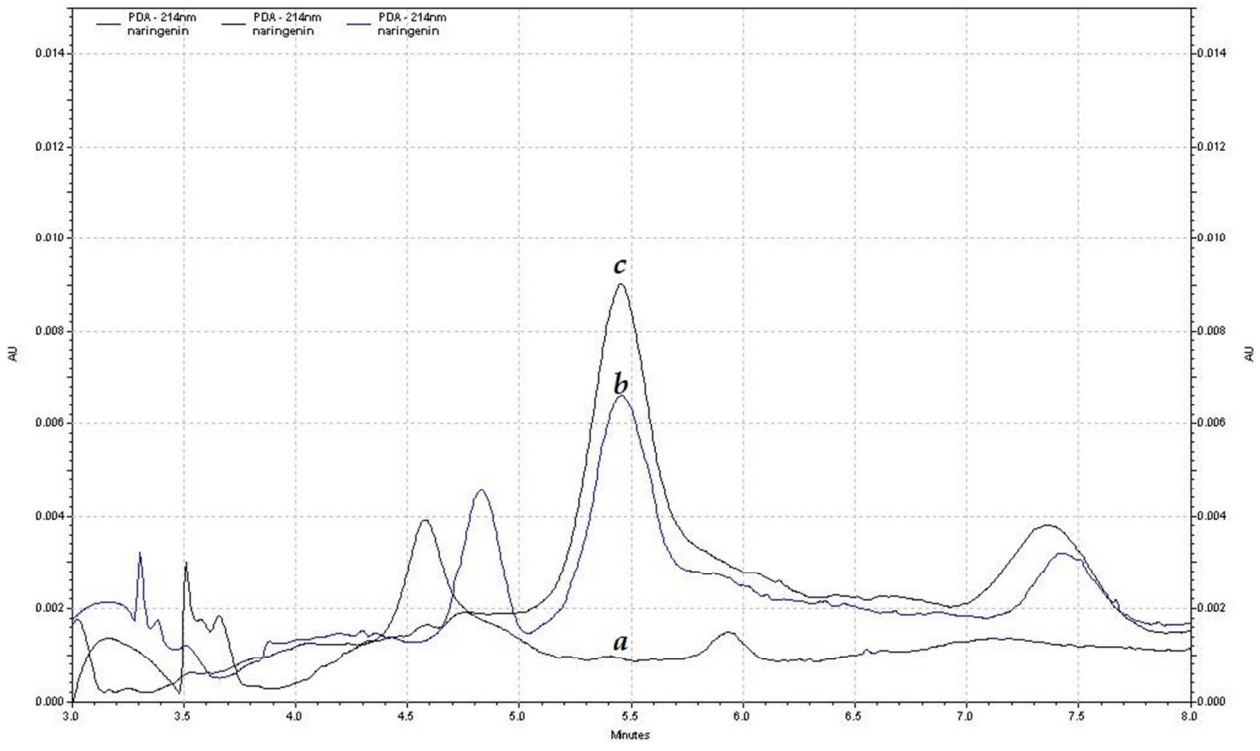

Fig. 6.Electropherogram of (a) blank , (b) blood sample and (c) spiked blood sample.

Table 1. **Percent recoveries calculated from blood samples**

Sample	Found amount (µg/mL)	Added amount (µg/mL)	Found amount after spiking (µg/mL)	% Recovery
Blood sample #1	11.27	10	9.81	98%
Blood sample #2	6.69	10	8.18	81%
Blood sample #3	10.06	10	8.92	89%
Blood sample #4	9.67	10	9.75	97%
Blood sample #5	9.10	10	9.68	96%
Blood sample #6	8.89	10	9.51	95%
Blood sample #7	10.17	10	9.44	94%

Table 2. **Comparison of proposed method with reported method**

Parameter	**Proposed method**	**Reported method[41]**
Range	0.1-50 µg/mL	10-60 µg/mL
LOD	0.05 µg/mL	1.11 µg/mL
LOQ	0.19 µg/mL	3.37 µg/mL
Run time	6 min	15.15 min
Solvent system	Borate + SDS	Acetonitrile + water