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# Stopped-Flow Injection and Spectrophotometric Methods of Quercetin Dihydrate Determination by Using Two Reagents (2,4-Dichloroaniline and *p*-Aminoacetophenone)

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A stopped flow injection and spectrophotometric method were used to estimate of quercetin dihydrate in pure and supplement preparations were suggested. This method was depend on coupling reaction between quercetin dihydrate and diazotized reagents 2,4-dichloroaniline (method A) and *p*-aminoacetophenone (method B) in alkaline medium to form water soluble with orange colour dyes. These dyes are steady and have a maximum absorbance at 420 nm in method A and 413 nm in method B. Calibration graphs showed that a Beer's law is obeyed over the range of concentration 0.4-50 and 0.4-45 μg $mL^{-1}$ for quercetein dihydrate with a detection limit 0.3715 and 0.385 for method A and B, respectively. The sample through put is 27 and 24 for method A and B, respectively. The suggested methods were successfully applied for the evaluation of quercetin dihydrate in dietary supplements.

**Keywords: Flavonoids, Quercetin dihydrate, Stopped flow injection, Spectrophotometric methods.**

## INTRODUCTION

Phytochemicals (flavonoides) are biologically active compounds and present in edible foods that when ingested, have the ability to delay the onset or prevent of disease [1]. Flavonoids consist of a very large collection of polyphenolic compounds having a benzo-γ-pyrone structure and far and wide existing in plants. They are synthesized by phenylpropanoid pathway. Available reports include to explain that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of drug activities [2,3]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [4]. The chemical environment of flavonoids depends on their degree of hydroxylation, substitutions, essential class and degree of polymerization [5]. Quercetin dihydrate is a type of flavonoids which is found in green tea, onions, berries, citrus fruit, apples, garlic, *etc*. [6].

High concentrations of quercetin occur in onion and three predominant forms of quercetin in onion are quercetin aglycone, quercetin-3,4′-O-diglucoside and quercetin- 4′-O-glucoside [7-9]. Flavonoids have capability to induce human defending enzyme systems. Several studies [10,11] have suggested protective effects of flavonoids against many infective (viral diseases and bacterial) and degenerative diseases such as cancers, age-related diseases and cardiovascular diseases.

Several methods [12-14] were proposed in the literature to determine quercetin in the samples. These methods have largely been replaced to high-performance liquid chromatography (HPLC), which has become one of the most important tools in the identification and separation of flavonoids from raw plant extracts. This paper is described colorimetric methods for estimation of quercetin dihydrate by using the coupling reactions with diazotized 2,4-dichloroaniline and *p*-amino-acetophenone in alkaline medium. 2,4-Dichloroaniline and *p*-aminoacetophenone was found to be a valuable coupling reagents after diazotization reaction. The proposed methods are considered as a new method for determination of flavonoids in stopped flow injection method. In addition, these methods have been suitably applied for the estimation of quercetin dihydrate in pure and supplement dietary preparations.

## EXPERIMENTAL

All spectral and absorbance measurements were approved by means of a Shimadzu UV/visible- 260-digital double beam spectrophotometer using 1 cm quartz cell and flow cell with 50 microliter internal volume. A two-channel manifold peristaltic pump (SHENCHEN, LabM1 model, China) was utilized for SFI-spectrophotometric determination of quercetin dihydrate. Injection valve (KNAUER (6-Port/3-channel), Wissenschaftliche Geräte GmbH) was applied to fitting injection volumes of standard solutions and samples. Stopped-flow system is a local made and consists of two parts: (a) gives a timer for the time needed to access the inject sample to the measuring cell and (b) gives

the stop time of the sample segment in the measurement cell. Flexible vinyl tubing (0.5 mm, i.d.) was used for the peristaltic pump. Reaction coil (RC) was of Teflon (0.5 mm, id.).

Stopped flow injection procedure can increase the sensitivity by increasing the reaction time in a stopping period, thereby raising more product. There is a stopping of the flow to hold an injected zone of sample in a flow cell for promoting the reaction to take place without dispersion [15].

**Standard solution of quercetin dihydrate (m.w. = 338.27 g/mol):** Stock solution of 100 μg $mL^{-1}$ is prepared by dissolving 0.01 g of pure compound in 50 mL absolute ethanol and complete the volumetric flask by absolute ethanol.

**Diazotized *p*-aminoacetophenone solution (0.005 M) (m.w. = 135 g/mol):** Prepared daily by dissolving 0.0337 g of *p*-aminoacetophenone in 3 mL distilled water and 2.5 mL HCl (1 M) in 50 mL volumetric flask. Cooled the mixture at 0-5 ºC for 5 min using an ice bath. After 5 min, added with mixing (0.01725 g) of sodium nitrite (0.005 M), then the volume is made up to the mark with distilled water.

**Diazotized 2,4-dichloroaniline solution (0.0005 M) (m.w. = 162 g/mol):** Prepared daily by dissolving 0.0040 g of 2,4-dichloroaniline in 3 mL absolute ethanol and 2 mL HCl (1 M) in 50 mL volumetric flask. Cooled the mixture at 0-5 ºC for 5 min using an ice bath. After 5 min, added (0.00172 g) of sodium nitrite (0.0005 M) with constant stirring to the mixture and the volume is made up to the mark with absolute ethanol.

**Sodium hydroxide (0.1 M):** Prepared by dissolving 1 g of NaOH pallets in 250 mL of distilled water.

### Supplements diatery preparation

**Mega quercetin (Solary dietary supplements (USA) (1200 mg/capsules):** Stock solution of 100 μg $mL^{-1}$ was prepared by dissolving of 0.01 g of the supplement in 50 mL absolute ethanol (1.2 g in each capsule) and the volume is made up to the mark with absolute ethanol.

**Quercetin dihydrate (bulk dietary supplements (USA) (100 g for one package):** Stock solution of 100 μg $mL^{-1}$ was prepared by dissolving 0.01 g of compound in 50 mL absolute ethanol and the volume is made up to the mark with absolute ethanol.

**General procedure of stopped flow injection method:** The manifold used for the determination of quercetin dihydrate was designed to provide different reaction conditions for increasing the absorbance signal caused by the reaction of diazotized 2,4-dichloroaniline and *p*-aminoacetophenone with quercetin dihydrate in sodium hydroxide medium. Maximum absorbance was achieved when the sample (quercetin dihydrate 30 μg $mL^{-1}$) was inserted into a stream of diazotized 2,4-dichloroaniline (0.0005 M) and *p*-aminoacetophenone (0.005 M) and then mixed with NaOH (0.1 M) and the maximum spectrum value was measured at 420 and 413 nm in method A and B, respectively (Figs. 1 and 2).

By stopping the flow, the residence (reaction) time (the elapsed time after sample and reagent are mixed together prior to detection of the reaction product), can be prolonged without increasing the length of reaction coil, thus avoiding an increase of dispersion. The stopped flow injection method can increase sensitivity of the measurement by increasing the residence time [16,17].

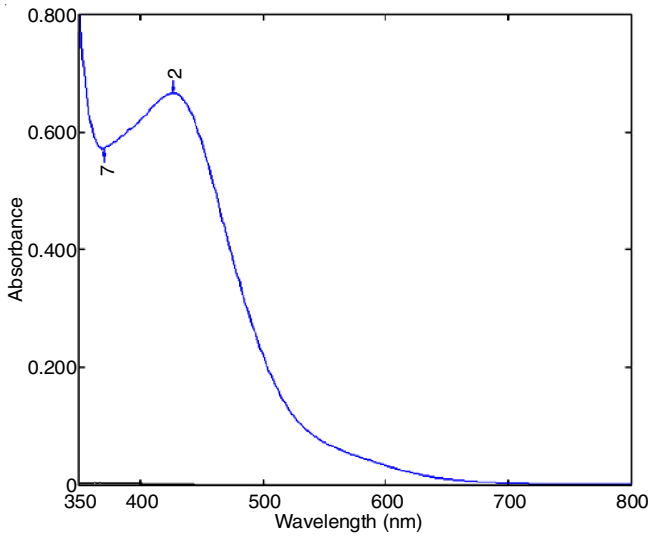


Fig. 1. Absorption spectrum of 100 μg $mL^{-1}$ quercetin dihydrate treated are showed under procedure and measured against blank reagent of diazotized 2,4-dichloro aniline and sodium hydroxide and the blank reagent measured against distilled water

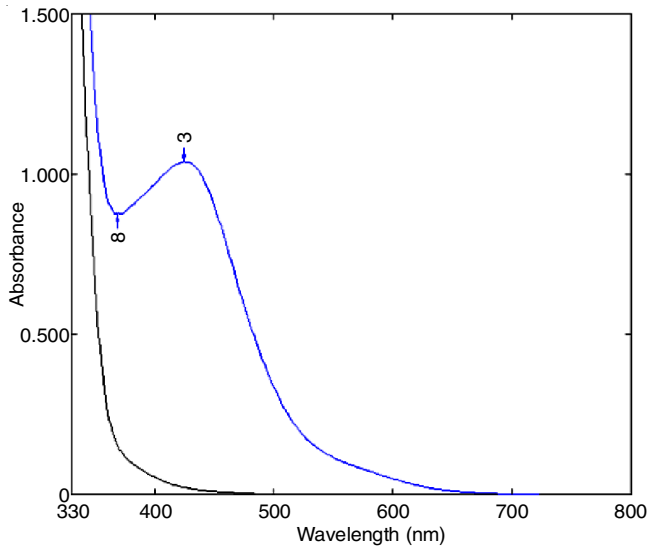


Fig. 2. Absorption spectrum of 100 μg $mL^{-1}$ quercetin dihydrate treated are showed under procedure and measured against blank reagent of diazotized *p*-amino acetophenone and sodium hydroxide and the blank reagent measured against distilled water

Travelling time is the time from injection to the stopping time depend on the physical parameters in which increase with increase reaction coil and sample manifolds parameters, the travelling time after each injection was studied and established that sample zone achieved the flow cell 30 and 25 s of the two methods after each injection and the time intervals between stopping and resuming the pump flow, in the range (10-260 s) were also studied, it was injected 30 μg $mL^{-1}$ for method A, 35 μg $mL^{-1}$ for method B. It found after 100 and 120 s, the stopping time was selected as the optimum interval time in two methods, respectively (Figs. 3 and 4). After the optimization of the physical parameters, the next step is washing which took 30 and 20 s for method A and B, respectively.

## RESULTS AND DISCUSSION

The affecting factors on the stability and sensitivity of coloured product of reaction between diazotized 2,4-dichloroaniline,

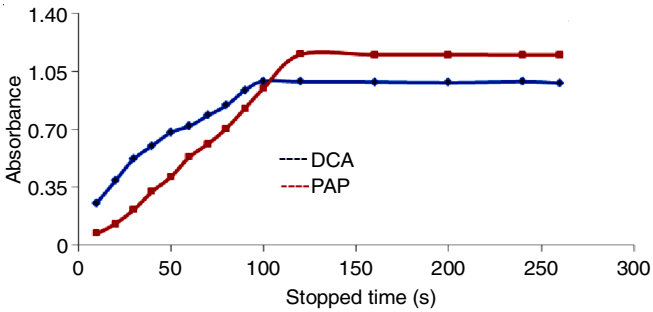


Fig. 3. Effect of stopped time (using 30 μg $mL^{-1}$) of quercetin dihydrate (method A), (using 35 μg $mL^{-1}$) of quercetin dihydrate (method B)

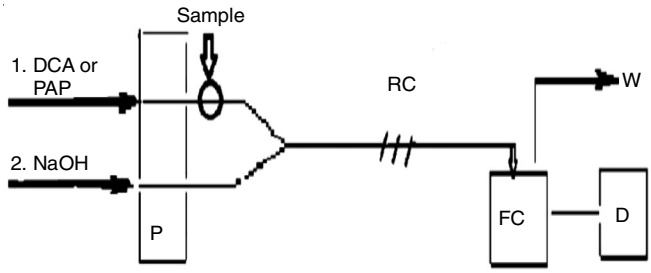


Fig. 4. A schematic diagram of sFIA manifold where: (1) 2,4-dichloro aniline and *p*-amino aceto phenone (2) sodium hydroxide; RC = reaction coil, P = peristaltic pump; Sample = injection sample quercetin dihydrate; D = detector ; Fc = flow cell; W = waste

*p*-aminoacetophenone and quercetin dihydrate in an alkaline medium were precisely studied. The impact of different physico-chemical stopped flow injection analysis (sFIA) parameters on the absorbance of the coloured product were enhanced as followed:

**Effect of order of mixing:** Three different reaction manifolds were used to perform three different paths of the reaction for kinds of sFIA methods for method A and B, using a preliminary conditions that effect of variations in sFIA manifolds on the absorbance of colourd product. The results exhibited that the manifolds (Fig. 4) gave the best absorbance for sFIA, for method A and B, respectively and were chosen for further use.

**Effect of reagent concentration:** The effect of differnt concentrations in method A ($5 \times 10^{-5}$ to $1 \times 10^{-3}$ M of 2,4-dichloroaniline) and B ($1 \times 10^{-4}$ to $7 \times 10^{-3}$ M of *p*-aminoacetophenone) were examined. It was established that the absorbance increased as the concentration of method A increased up to $5 \times 10^{-4}$ M and for method B increased up to $5 \times 10^{-3}$ M. After this concentration, the absorbance was slightly changed (Fig. 5) therefore, concentration (0.0005 M, 0.005 M) for both methods were found to be the most suitable concentration for a maximum absorbance and optimized for further procedure.

**Effect of sodium hydroxide concentration:** Several concentrations of sodium hydroxide vary between 0.01-0.3 M were examined and the highest absorbance intensity was present with 0.1 M for method A and for method B (Fig. 6).

**Effect of total flow rate:** The impact of flow rate on the sensitivity was considered in the range of (1-6 mL $min^{-1}$). In sFIA. the absorbance was slightly changed with increase in the flow rate, this may be resulted from the stopping time in sFIA that would permit to increase sensitivity due to the complete reaction. The results showed that a total flow rate of 2 and 2.5 mL $min^{-1}$ for both methods A and B, respectively, provided

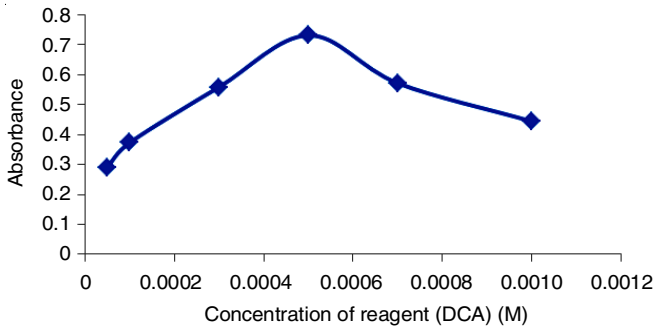


Fig. 5a. Effect of concentration of diazotized 2,4-dichloro aniline (M)

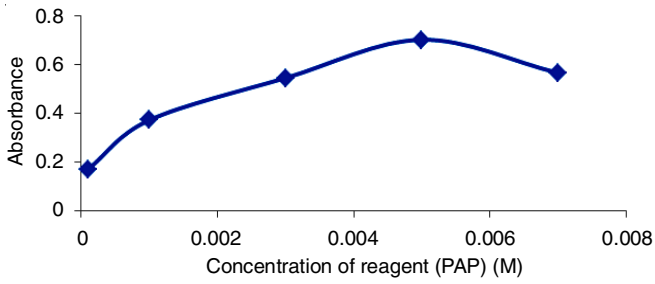


Fig. 5b. Effect of concentration of diazotized reagent (*p*-amino aceto phenone) (M)

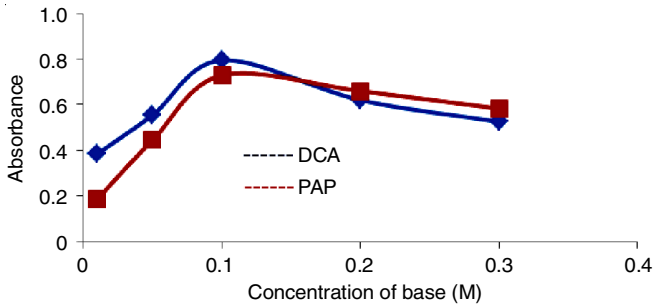


Fig. 6. Effect of sodium hydroxide concentration

the greatest absorbance (Fig. 7) and was optimized in all the experiments.

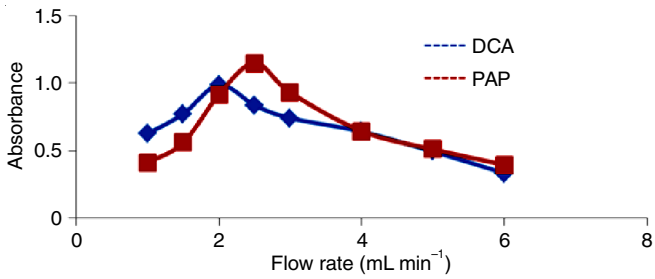


Fig. 7. Effect of total flow rate in method A and B

**Effect of reaction coil length:** Coil reaction length was searched in the range of 25-250 cm. The results achieved (Fig. 8) showed that a coil length of 150 and 100 cm was adequate to create an efficient mixing of both streams and gave the largest absorbance for both methods A and B, respectively.

**Effect of injected sample volume:** The volume of injected sample was adapted between 74 and 302 μL using altered lengths of sample loop. The results showed that the injected sample of 153 and 102 μL for methods A and B, respectively, gave the highest absorbance (Fig. 9) and was optimized in all the experiments.

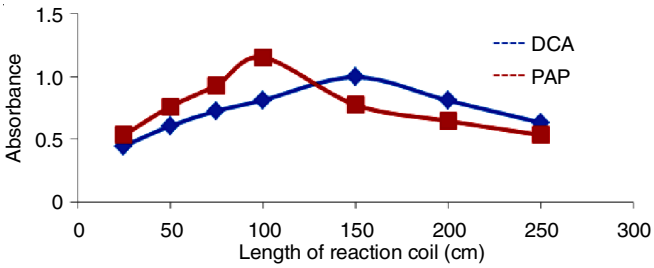


Fig. 8. Effect of reaction coil in method A and B

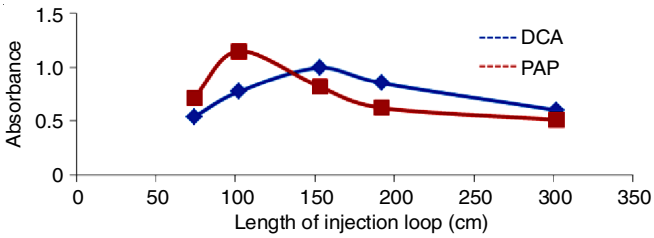


Fig. 9. Effect of sample injection volume

Selected optimum conditions: The optimization variables of sFI-spectrophotometric that gave the optimal conditions in both methods A and B are shown in Table-1.

TABLE-1
OPTIMIZATION OF sFIA VARIABLES

| Parameters | Method A | Method B |
|---|---|---|
| Sodium hydroxide (M) | 0.1 | 0.1 |
| Type of reagent (M) | 0.0005 | 0.005 |
| Total flow rate (mL $min^{-1}$) | 2 | 2.5 |
| Reaction coil (cm) | 150 | 100 |
| Sample volume (µL) | 153 | 102 |
| Stopped time (s) | 100 | 120 |
| Flow time after injection at which pump is stopped (s) | 30 | 25 |
| Washing time (s) | 30 | 20 |

**Calibration graphs:** A two series of solution containing (0.4 to 50 and 0.4 to 45 µg $mL^{-1}$) of quercetin dihydrate were set by suitable dilution of the stock solution. Calibration graphs for the estimation of quercetin dihydrate were achieved using the optimal conditions (Table-1) for both methods A and B, respectively. The statistical handlings for calibration graphs are shown in Table-2.

**Accuracy and precision:** The accuracy and precision of the proposed methods for the determination of quercetin dihydrate using both stopped FIA methods (A and B) were studied applying four different concentrations of standard quercetin dihydrate.

TABLE-2
ANALYTICAL VALUES OF STATISTICAL TREATMENTS FOR THE CALIBRATION GRAPHS

| Parameters | Method A | Method B |
|---|---|---|
| Regression equation | Y = 0.0218x + 0.2981 | Y = 0.0285x + 0.1696 |
| Correlation coefficient ($r^2$) | 0.9976 | 0.9983 |
| Linearity percentage ($r^2$ %) | 99.76 | 99.83 |
| Dynamic range (µg $mL^{-1}$) | 0.4-50 | 0.4-45 |
| Molar absorptivity (L $mol^{-1}cm^{-1}$) | 7374.286 | 9640.695 |
| Slope, b (mL $\mu g^{-1}$) | 0.0218 | 0.0285 |
| Intercept, a | 0.2981 | 0.1696 |
| Standard deviation of the residuals, Sy/x | 0.000129 | 0.000211 |
| Standard deviation of the slope, Sb | 0.0000154 | 0.0000211 |
| Standard deviation of the intercept, Sa | 0.00065 | 0.000432 |
| Sandell's sensitivity, S (µg $cm^{-2}$) | 0.04587 | 0.03508 |
| Sample through-put ($h^{-1}$) | 27 | 24 |
| LOD (µg $mL^{-1}$) | 0.1114 | 0.1157 |
| LOQ (µg $mL^{-1}$) | 0.3715 | 0.385 |

The results (Table-3) indicate a agreeable values of accuracy and precision.

**Pharmaceutical applications:** Solutions of diatery supplements were prepared as similar to quercetin dihydrate solution. The suggested methods were successfully applied for the determination of quercetin dihydrate in the dietary supplements, by injecting the three different concentrations of the sample using the optimal conditions and the sFIA manifold (Fig. 4) for both sFIA methods (A and B, respectively). The results are summarized in Table-4.

**Analytical application:** To appraise the suitability of the present methods, the results achieved were evaluated with those obtained from the standard method (it found that quercetin dehydrate was dissolved in 20 mL methanol and diluted up to 100 mL by distilled water to get 100 µg/mL concentration of quercetin dihydrate. This solution was subjected to scanning between 200-400 nm and absorption maxima at 372 nm for quercetin dihydrate were determined) [18].

The results obtained by two different methods (Table-5) were statistically compared, using the Student t-test and F-test at 95 % confidence level. In all cases, the calculated t- and F-values did not go beyond the theoretical values, which indicate that there is no considerable difference between each of both methods in terms of accuracy and precision.

## Conclusion

The appliance of diazotization-coupling reaction between quercetin dihydrate and either of diazotized 2,4-dichloroaniline

TABLE-3
ACCURACY AND PRECISION OF THE PROPOSED METHODS

| Proposed method | Concn. of quercetin dihydrate (µg $mL^{-1}$) | | E (%) | Rec (%) | RSD (%) |
|---|---|---|---|---|---|
| | Present | Found | | | |
| Method A | 16 | 15.860 | -0.875 | 99.125 | 0.800 |
| | 20 | 20.500 | +2.500 | 102.500 | 0.657 |
| | 28 | 27.830 | -0.607 | 99.393 | 0.4842 |
| | 30 | 31.090 | +3.630 | 103.630 | 0.4333 |
| Method B | 10 | 9.870 | -1.300 | 98.700 | 0.332 |
| | 20 | 20.500 | +2.500 | 102.500 | 0.290 |
| | 30 | 29.620 | -1.260 | 98.740 | 0.411 |
| | 38 | 38.080 | +0.210 | 100.210 | 0.036 |

TABLE-4
APPLICATION OF THE PROPOSED sFIA METHODS FOR ESTIMATION OF QUERCETIN DIHYDRATE IN CAPSULES FORMS

| Proposed method | Flav. Form | Conc. of QRC | | E (%) | Rec (%) | RSD (%) |
|---|---|---|---|---|---|---|
| | | Present | Found | | | |
| Method A | Quercetin dihydrate (100 g package) | 16 | 16.050 | +0.315 | 100.315 | 2.587 |
| | | 20 | 20.454 | +2.270 | 102.270 | 1.564 |
| | | 28 | 28.022 | +0.081 | 100.081 | 1.303 |
| | | 30 | 31.000 | +3.330 | 103.330 | 0.5162 |
| | Mega quercetin (1200 mg/capsule) | 16 | 15.770 | -1.430 | 98.570 | 0.887 |
| | | 20 | 20.310 | +1.580 | 101.580 | 1.821 |
| | | 28 | 27.790 | -0.750 | 99.250 | 1.156 |
| | | 30 | 30.910 | +3.030 | 103.030 | 1.1812 |
| Method B | Quercetin dihydrate (100 g package) | 10 | 9.830 | -1.700 | 98.300 | 3.916 |
| | | 20 | 20.680 | +3.400 | 103.400 | 2.240 |
| | | 30 | 29.620 | -1.260 | 98.740 | 1.299 |
| | | 38 | 38.110 | +0.280 | 100.280 | 0.931 |
| | Mega Quercetin (1200 mg/capsule) | 10 | 9.900 | -1.000 | 99.000 | 2.828 |
| | | 20 | 20.570 | +2.800 | 102.800 | 1.361 |
| | | 30 | 30.505 | +1.683 | 101.683 | 1.150 |
| | | 38 | 38.110 | +0.280 | 100.280 | 1.016 |

TABLE-5
COMPARISON OF THE PROPOSED sFIA METHODS WITH STANDARD METHOD USING t- AND F-STATISTICAL TESTS

| Drug forms | Rec (%) | | |
|---|---|---|---|
| | Proposed method | | Standard method |
| | Method A | Method B | |
| Quercetin dihydrate pure | 99.259 | 100.0375 | 99.699 |
| Mega quercetin | 98.910 | 100.180 | 100.000 |
| Quercetin dihydrate | 100.198 | 100.940 | 99.853 |
| S | $S^2 1 = 0.3098$ | $S^2 1 = 0.23535$ | $S^2 2 = 0.02265$ |
| T (2.776)* | 1.179 | 1.8259 | $(n_1 + n_2 - 2 = 4)$ |
| F (19)* | 13.679 | 10.3900 | $(n_1 - 1) = 2, (n_2 - 1) = 2$ |

and *p*-aminoacetophenone in alkaline medium to the sFI-spectrophotometric estimations of the quercetin dihydrate in dietary supplements is reported. The sFIA system has the advantages of low reagent consumption, need of small sample volume, time saving reproducibility, simplicity, high sample throughput (27 and 24 injection $h^{-1}$ for both methods A and B, repectively) and large dynamic range of concentration. The sFIA method can increase sensitivity measurement by decreasing the detection limit and increasing the reaction time. The proposed methods can be applied to the analysis of a wide concentration range of quercetin dihydrate in real samples with agreeable results. The proposed methods are inexpensive, simple and the procedure does not comprise any critical reaction conditions or tedious sample preparation steps. F- and t- test at 95 % confidence level showed that there was no significant difference between the proposed method and the standard method during its appliance to the analysis of supplements samples.

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