European Journal of Chemistry

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# Development and validation of a simple and rapid UPLC method for the *in-vitro* estimation of (-)-epigallocatechin-3-gallate in lipid-based formulations

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RESEARCH ARTICLE

10.5155/eurjchem.9.1.7-12.1661

Received: 17 October 2017
Received in revised form: 14 November 2017
Accepted: 29 November 2017
Published online: 31 March 2018
Printed: 31 March 2018

KEYWORDS

Caffeine
Validation
Method development
Lipid-based formulation
Epigallocatechin gallate
Ultra-performance liquid chromatography

ABSTRACT

(-)-Epigallocatechin gallate (EGCG) is a catechin found in green tea that has potential health benefits, such as anti-oxidant, anti-carcinogenic and anti-inflammatory effects. A rapid and sensitive Ultra-Performance Liquid Chromatographic (UPLC) method was developed and validated for the estimation of (-)-epigallocatechin-3-gallate in lipid-based formulation. The UPLC method was conducted on C18 analytical column (50 mm × 2.1 mm, 1.8 µm particle size). The mobile phase consisted of a mixture of acetic acid (1%, *v*:*v*; pH = 3), acetonitrile and water at volume ratio of 13:15:72 delivered at a flow rate of 0.5 mL/min. The diode array detector (DAD) acquisition wavelength was set at wavelengths 210 and 280 nm. Caffeine was used as internal standard. The tested validation parameters, i.e., selectivity, linearity, accuracy, precision, and sensitivity (Limit of detection and limit of quantification) were determined at both wavelengths. Results revealed that caffeine and EGCG peaks were eluted at retention times of 0.55 and 0.85 minutes, respectively. The calibration curve was linear over the concentration range of 10-60 µg/mL, with coefficients of determination ($r^2$) of 0.9993 and 0.9998 nm at 210 and 280 nm, respectively. All the validation parameters were found within the acceptable range. The proposed method was successfully applied for the quantitation of EGCG in lipid-based formulation and statistical analysis with a reported method showed no significant difference at $p < 0.05$. Therefore, the proposed analytical method for EGCG can be considered as a rapid, selective and accurate analytical method that can be used for the quantitative analysis of EGCG.

**Cite this**: *Eur. J. Chem.* **2018**, *9(1)*, 7-12 **Journal website**: www.eurjchem.com

## 1. Introduction

Green tea is one of the most popular beverages that have numerous health-promoting benefits upon regular consumption [1]. In recent years, scientists throughout the world have been investigating the beneficial effects of green tea and its major abundant catechin; EGCG [2]. EGCG active pharmaceutical ingredient (API) is official in United States Pharmacopoeia (USP) [3]. The data obtained from *in vitro*, *in vivo*, and human studies that were conducted on EGCG have proven its pronounced cardiovascular and metabolic health benefits [4]. In addition, several studies have demonstrated its strong antioxidant property and its multiple anti-cancer effects [5]. EGCG formulations have been available in the market as nutraceutical tablets, however they are of low effectiveness since EGCG possesses poor systemic absorption, low bioavailability and high systemic clearance that was reported by previous studies [6,7]. Formulating EGCG in lipid-based formulations can provide a solution to the problems associated with the use of EGCG.

Due to the role of EGCG as a therapeutic agent, the qualitative and quantitative evaluation of EGCG is of crucial importance. Quantitative determination of total catechins in tea leaves was carried out using UV-visible spectrophotometry [8], however, High-Performance Liquid Chromatography (HPLC) presents the most frequently cited technique used to separate, identify and quantify catechins [9]. Several HPLC analytical methods have been reported for EGCG quantitation such as HPLC with UV detection with gradient elution [10-13], and HPLC with isocratic elution [14,15]. Other studies analyzed EGCG by using HPLC with MS-electrospray detection [16-18].

Ultra-Performance Liquid Chromatography analysis offers numerous advantages over HPLC. Of the most important features offered are shortened analysis time, and reduction in the volumes of organic solvents used while maintaining

separation efficiency [19-21]. A UPLC method combined with DAD and mass spectroscopic (MS) detection was used to analyze three different types of teas where 68 compounds were identified and quantified, indicating that the UPLC method is a promising alternative to conventional HPLC technique [22,23]. In the current study, a simple, precise and sensitive UPLC method was developed and validated for the fast determination of EGCG in lipid-based formulation.

## 2. Experimental

### *2.1. Instrumentation*

UPLC system (Agilent 1290 Infinity LC system, Germany) equipped with G4204A Quat pump and G4212A photo Diode Array Detector (DAD) was utilized. PURELAB flex water purification system was used. Bath sonicator (BRANSONIC 3510E-DTTH, USA) was used for sonication. Rotary evaporator RII, HB equipped with air vacuum pump, model V-700 (Buchi, Zurich, Switzerland) was used for the preparation of lipid-based formulation. Sartorius Model CPA2245 balance was used for weighing purposes.

### *2.2. Chemicals and reagents*

(-)-Epigallocatechin-3-gallate was purchased from Bulkactives Company, USA (Purity ≥95%). Caffeine was purchased from Himedia, Mumbai, India (Purity >99%). Tween 80 was purchased from Oxford Laboratory, Mumbay, India. Soybean lecithin was kindly supplied as a gift from Cargill Inc., Germany. Acetonitrile and acetic acid of HPLC grade were purchased from Sigma-Aldrich, Germany. Water was obtained from PURELAB flex water purification system.

### *2.3. Chromatographic conditions*

The analytical column ZORBAX RRHD Eclipse Plus C18 (50 mm × 2.1 mm id, 1.8 µm particle size) was used. The mobile phase consisted of a mixture of acetic acid (1%, *v*:*v*; pH = 3), acetonitrile and water at volume ratio of 13:15:72. It was pre-filtered through a Millipore 0.22 µm filter followed by sonication prior to use for analysis and pumped at a flow rate of 0.5 mL/min. The DAD was set in the range of 200-400 nm with channel A set at wavelength 210 and channel B at wavelength 280 nm. The sample injection volume was 10 µL and a total run time of 5 minutes was applied. The column temperature was set at 20±3 °C. Caffeine was used as internal standard (IS) and EGCG peaks were identified by comparing their retention times and chromatograms with that of caffeine.

### *2.4. Preparation of solutions*

*Standard stock solutions*: 100 µg/mL EGCG and 50 µg/mL caffeine were prepared by dissolving an accurately weighed amount of 10 mg of EGCG and 5 mg of caffeine, respectively in 100 mL HPLC water obtained from PURELAB flex water purification system having pH = 5.5. The solution were filtered through a Millipore 0.22 µm filter and sonicated prior to injection.

*Working standard solutions*: Working standard solutions (10, 20, 30, 40, 50 and 60 µg/mL) of EGCG were prepared by serial dilution of the stock solution with water, prior to analysis, and each dilution was spiked with 1 mL of the IS (caffeine) of concentration 50 µg/mL, filtered through a Millipore 0.22 µm filter and sonicated prior to injection.

*Pharmaceutical formulation solutions*: The formulation is a pharmaceutical vesicular formulation that was prepared in the Pharmaceutical Technology Labs at Future University in Egypt according to the proportions listed under "Preparation of solutions" section using thin film hydration technique. The lipid-based formulation containing 2.5 mg/mL EGCG, 200 mg soya phosphatidylcholine and 10 mg tween 80 was prepared by thin film hydration technique. A volume of 0.25 mL of the formulation was transferred to 10 mL volumetric flask, spiked with 1 mL standard caffeine solution (Concentration 50 µg/mL) and diluted to 10 mL using HPLC water. The samples were then filtered through a Millipore 0.22 µm filter and sonicated prior to injection.

### *2.5. System suitability*

The system suitability test (SST) is an essential part of the analytical method that is used to verify effectiveness of the final operating system [24]. The test was performed by injecting the standard sample in triplicate and the SST parameters were calculated as reported by USP [25], which include capacity factor (k'), selectivity factor (α), resolution factor ($R_s$), column efficiency (number of theoretical plates, N) [26].

### *2.6. Analytical method validation parameters*

After method development and optimization, the assay procedures were validated in terms of selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) according to the ICH guidelines [27].

#### *2.6.1. Selectivity*

The selectivity of the chromatographic method is the ability of the method to accurately measure the analyte response in the presence of all interferences [28]. It was demonstrated by comparing the chromatograms of EGCG standard solution, EGCG-loaded lipid-based formulation and the lipid-based formulation without the drug to detect any possible interference between EGCG and the excipients used in the pharmaceutical formulation. The peaks were evaluated for lack of interference at the retention time of EGCG and IS [29,30].

#### *2.6.2. Linearity*

Previously prepared working standard EGCG solutions spiked with IS were used to construct the calibration curve. The peak area ratio (PAR) of EGCG/caffeine was plotted versus EGCG concentration. Linearity was assessed by computing the best fitting line equation and the coefficient of determination ($r^2$) between the nominal concentrations added and the measured PARs by linear regression data analysis [31-33].

#### *2.6.3. Precision*

Precision of the method was determined by repeatability (Intra-day precision) and intermediate precision (Inter-day precision). Intra-day repeatability was obtained by analyzing three freshly prepared samples having concentrations of 15, 35 and 55 µg/mL at three different times in one day. Inter-day repeatability was determined by analyzing freshly prepared solutions having the same concentrations on three consecutive days. All injections were carried out in triplicate. The precision of the assay was calculated in terms of percentage relative standard deviation (% RSD) of the replicates. %RSD values less than 2% were considered acceptable (≤ 2%) [34,35].

#### *2.6.4. Accuracy*

Accuracy of the analytical method is the measure of how close the experimental value is to the true value [35]. It was determined by analyzing five freshly prepared standard EGCG

**Table 1.** System suitability testing parameters of the developed method at 210 and 280 nm.

| Parameter | Value | |
|---|---|---|
| | 210 nm | 280 nm |
| Capacity factor (k') | 3.06 | 2.42 |
| Resolution factor ($R_s$) | 5.12 | 5.06 |
| Selectivity factor (α) | 2.35 | 2.41 |
| Number of theoretical plates (N) | 2561 | 2546 |

solutions of concentrations 15, 35, 45, 50 and 55 µg/mL of three replicates each.

Accuracy was estimated for each concentration by comparing the nominal concentration to the estimated concentration, as calculated from the straight line equation of the calibration curve. The recovery percentages (mean±%RSD of three replicates) of EGCG were calculated [36].

#### *2.6.5. Sensitivity*

Sensitivity of the method was determined by estimating the limit of detection as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions and the limit of quantification as the lowest concentration of analyte in a sample that can be quantified with acceptable precision and accuracy under the stated experimental conditions [36,37].

$$LOD = 3\ \sigma/S \text{ and } LOQ = 10\ \sigma/S \quad (1)$$

These two parameters were calculated based on the signal-to-noise ratio (S/N) of 3 and 10, respectively [38]. The test concentrations at LOD and LOQ were injected six times and the standard deviation of the regression line obtained from the calibration curve (σ) and the slope (S) was determined.

#### *2.6.6. Statistical analysis*

Statistical comparison between the developed method and the reported HPLC one was performed using Student's *t*-test and *F*-test value [39].

## 3. Results and discussion

### *3.1. Optimization of the chromatographic method*

In the analysis of EGCG by the proposed method, different parameters affecting the chromatographic performance of EGCG were carefully studied in order to achieve the most suitable chromatographic system, including detection wavelength, mobile phase composition, pH of the mobile phase and injection volume and flow rate of the mobile phase.

*Selection of the appropriate detection wavelength:* Several detection wavelengths were reported for the detection of EGCG. Some authors reported an optimal detection sensitivity at a wavelength of 280 nm [40], while others demonstrated higher UV sensitivity for EGCG at 205, 210 and 231 nm [41-43]. Under the current described conditions, the best results were achieved at wavelengths 210 and 280 nm, with 210 nm exhibiting better sensitivity.

*Mobile phase composition:* The composition of the mobile phase was optimized in order to provide sufficient selectivity and sensitivity in a short separation time. The mobile phase that resulted in optimum results was composed of a mixture of acetic acid, acetonitrile and water at volume ratio of 13:15:72, (*v*:*v*:*v*).

*pH of the mobile phase:* The effect of pH of the mobile phase was also studied over the range of 2.5-4.5 and it was observed that increasing the pH of the mobile phase above 3 caused peak tailing. Therefore, pH = 3 was selected as the optimum pH value for the mobile phase yielding the best peak shape with the optimum resolution.

*Injection volume of the mobile phase:* Different injection volumes (from 5-10 µL) were studied to detect the optimum injection volume producing good peak shape, and the injection volume of 10 µL was selected.

*Flow rate of the mobile phase:* The effect of flow rate of the mobile phase on the retention of EGCG was investigated over a range of 0.4-1.0 mL/min. Flow rate of 0.5 mL/min was selected as the optimum flow rate since it provided the optimum peak shape within a reasonable retention time. After optimization of these variables, good separation of the drug and IS from their mixture was achieved under the specified conditions. Caffeine and EGCG peaks were eluted at retention times of 0.55 and 0.85 min, respectively, with good baseline at 210 and 280 nm, with good peak shapes and resolution.

### *3.2. System suitability*

System suitability testing was performed during the development and optimization of the proposed method in order to ascertain the effectiveness of the overall operating system. The results are shown in Table 1. It was found that the values of the calculated parameters were within the acceptable limits at 210 and 280 nm, where the capacity factor was between 2-10, resolution between the two eluted peaks was >2, selectivity factor >1 and number of theoretical plates > 2000.

### *3.3. Method validation*

#### *3.3.1. Selectivity*

The selectivity of the proposed method was determined by comparing the chromatograms of EGCG standard solution, EGCG-loaded lipid-based formulation and lipid-based formulation without the drug (each spiked with 1 mL caffeine standard solution of concentration 50 µg/mL), at 210 and 280 nm. The chromatogram of EGCG standard solution Figure 1 (i) presented 2 peaks with retention times of 0.55 and 0.85 min, respectively, with good baseline at the 2 wavelengths. The chromatogram of EGCG-loaded lipid-based formulation Figure 1 (iii) also showed 2 peaks with retention times similar to EGCG standard solution at the 2 wavelengths, while the chromatogram of lipid-based formulation without the drug Figure 1 (ii) showed only 1 peak at the retention time of caffeine at both wavelengths, indicating that the components of the lipid-based formulation did not interfere with the analysis. As observed, the chromatogram peaks exhibited good resolution, indicating the high selectivity of the method.

#### *3.3.2. Linearity*

Two calibration curves were obtained by plotting the mean PAR of EGCG/caffeine against their corresponding concentrations, at 210 and 280 nm. The results are listed in Table 2. The PAR was linear over the concentration range 10-60 µg/mL, at both wavelengths (210 and 280 nm), as illustrated in Figure 2. The linear regression equations of the calibration curves were $y = 0.1887x - 0.0571$ and $y = 0.0572x + 0.0222$, for 210 and 280 nm, respectively; where $y$ is PAR of EGCG/caffeine and $x$ is EGCG concentration in µg/mL.

**Table 2.** UPLC calibration data of EGCG in water at 210 and 280 nm (n = 3).

| EGCG concentration (µg/mL) | PAR±SD (mAU) | |
|---|---|---|
| | 210 nm | 280 nm |
| 10.0 | 1.859±0.099 | 0.605±0.021 |
| 20.0 | 3.738±0.212 | 1.160±0.044 |
| 30.0 | 5.488±0.056 | 1.743±0.004 |
| 40.0 | 7.462±0.065 | 2.283±0.015 |
| 50.0 | 9.529±0.038 | 2.901±0.032 |
| 60.0 | 11.197±0.084 | 3.459±0.025 |

**Table 3.** Accuracy study of EGCG at wavelength 210 and 280 nm *.

| $C_{nominal}$ | PAR (Mean±SD) (mAU) | | $C_{Estimated}$ (Mean±SD) (µg/mL) | | % Recovery (Mean±%RSD) | |
|---|---|---|---|---|---|---|
| (µg/mL) | 210 nm | 280 nm | 210 nm | 280 nm | 210 nm | 280 nm |
| 15 | 2.795±0.008 | 0.888±0.004 | 15.116±0.044 | 15.135±0.065 | 100.771±0.295 | 100.90±0.429 |
| 35 | 6.528±0.036 | 2.042±0.006 | 34.898±0.189 | 35.304±0.112 | 99.707±0.539 | 100.87±0.318 |
| 45 | 8.440±0.051 | 2.620±0.009 | 45.032±0.273 | 45.421±0.172 | 100.071±0.606 | 100.94±0.379 |
| 50 | 9.477±0.039 | 2.889±0.029 | 50.527±0.209 | 50.128±0.523 | 101.054±0.418 | 100.26±1.043 |
| 55 | 10.348±0.029 | 3.189±0.036 | 55.139±0.152 | 55.370±0.637 | 100.253±0.277 | 100.67±1.150 |

* $C_{nominal}$: Normal (added) concentration; $C_{estimated}$: Estimated (found) concentration; SD: Standard deviation; %RSD: Relative standard deviation.

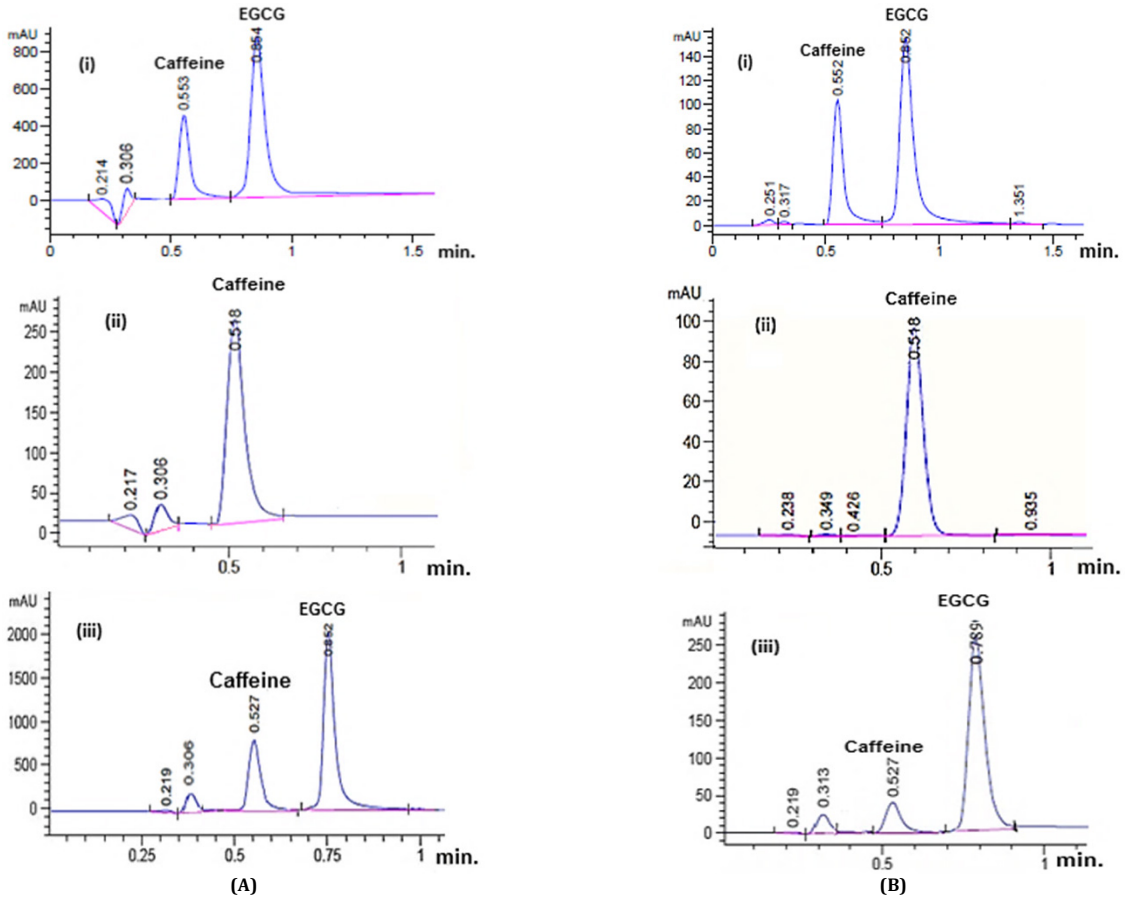


**Figure 1.** HPLC chromatograms of (i) EGCG standard solution, (ii) lipid-based formulation without the drug and (iii) EGCG-loaded lipid-based formulation (each spiked with 1 mL caffeine), at (A) 210 nm and (B) 280 nm.

The determined coefficient of determination ($r^2$) was found to be 0.9993 and 0.9998 over the concentration range used for 210 and 280 nm, respectively. A coefficient of determination near unity suggests the linearity of the described method.

### *3.3.3. Precision*

The assay method showed acceptable precision with %RSD values ranging from 0.017 to 0.725% for the intra-day assay and from 0.172 to 0.482% for the inter-day assay, at 210 nm. At 280 nm, the assay method showed precision with %RSD values ranging from 0.441 to 1.076% for the intra-day assay and from 0.214 to 0.985% for the inter-day assay. These low values of %RSD during the intra-day and inter-day analysis indicate the precision of the current method [44].

### *3.3.4. Accuracy*

The overall accuracy results were expressed as percent recovery (mean±%RSD) of EGCG. As clearly demonstrated in Table 3, the recovery of EGCG in the different solutions, assayed at 210 nm, ranged from 99.707 to 101.054%, with %RSD values below 1%, indicating good accuracy of the method. The recovery of the EGCG assay method at 280 nm showed recovery percentages ranging from 100.26 to 100.94%,

**Table 4.** Statistical analysis of the proposed method and the reported method for the assay of EGCG.

| Parameter | Proposed method | | Reported method |
|---|---|---|---|
| | 210 nm | 280 nm | |
| Mean | 100.136 | 100.23 | 100.63 |
| SD | 1.414 | 1.064 | 1.187 |
| *n* | 6 | 6 | 5 |
| Variance | 1.998 | 1.132 | 1.409 |
| Student's *t*-test | 0.630 (1.83 *) | 0.584 (1.83 *) | |
| F-test | 1.418 (2.77 *) | 1.245 (2.77 *) | |

* Theoretical *t*- and F-values at $p = 0.05$.

**Table 5.** Summary of the validation study of EGCG at 210 and 280 nm.

| Parameter | Value | |
|---|---|---|
| | 210 nm | 280 nm |
| Linearity | | |
| Linear regression equation | $y = 0.1887x-0.0571$ | $y = 0.0572x+0.0222$ |
| $r^2$ | 0.9993 | 0.9998 |
| LOD (µg/mL) | 0.528 | 1.225 |
| LOQ (µg/mL) | 1.6003 | 3.712 |
| Precision | | |
| % RSD Intra-day | 0.424 | 0.712 |
| % RSD Inter-day | 0.277 | 0.713 |
| Accuracy | | |
| Average recovery (%) | 100.369 | 100.727 |

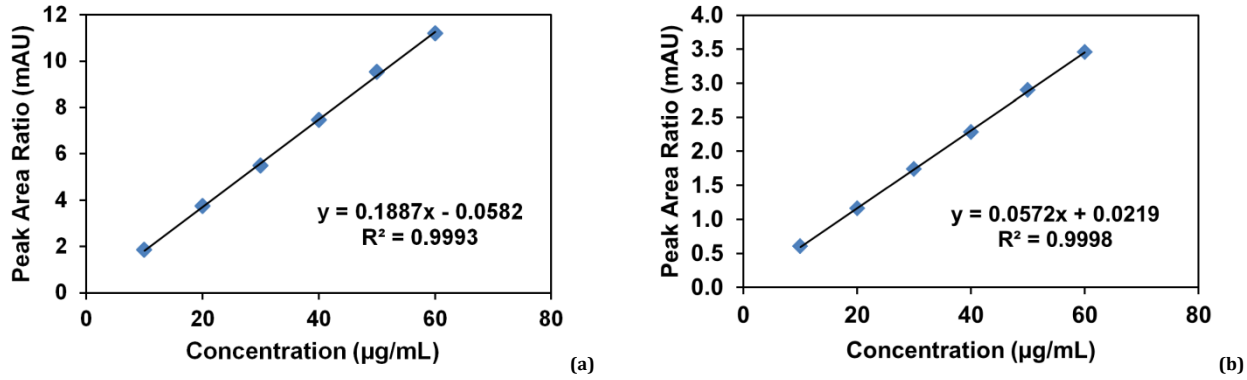


**Figure 2.** Standard Calibration curve for EGCG in water over the concentration range 10-60 µg/mL at $\lambda_{max}$; (a) 210 and (b) 280 nm.

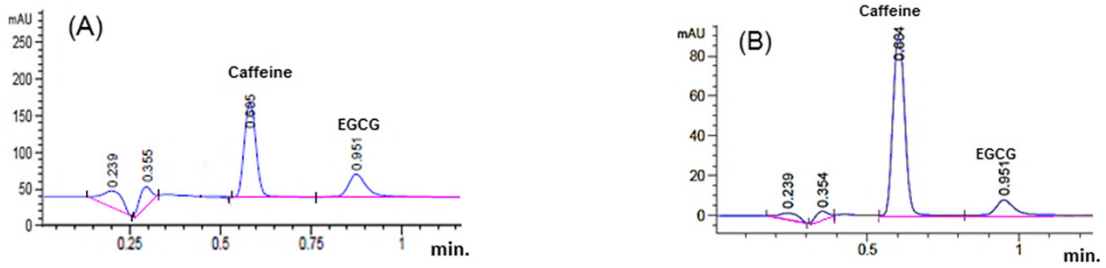


**Figure 3.** Typical chromatograms of LOQ of EGCG at (A) 210 and (B) 280 nm.

with %RSD values from 0.318 to 1.15%, indicating good accuracy as well.

### *3.3.5. Sensitivity*

The sensitivity of the UPLC method was determined by calculating LOD and LOQ values. The LOD and LOQ of EGCG of the proposed method at 210 nm were found to be 0.5280 and 1.6003 µg/mL, respectively. When measurement was carried out at 280 nm, the LOD and LOQ were 1.225 and 3.712 µg/mL, respectively. This supports the suitability of the proposed UPLC method, at both wavelengths for quantitation of EGCG. However, LOD and LOQ values suggest that quantitation at 210 nm was more sensitive. Figure 3 shows the chromatograms of 1 µg/mL of EGCG standard solution at 210 and 280 nm.

### *3.3.6. Statistical analysis*

Statistical analysis of the results obtained by the proposed method and the reported method [39] was performed and the results are listed in Table 4. It was found that the calculated *t*- and F-values were less than the theoretical ones [45,46], indicating no significant differences between the proposed method and the reported one.

Based on the results illustrated in Table 5, the developed method was suitable for the quantification of EGCG. However, the analysis at 210 nm showed better sensitivity, precision and accuracy than 280 nm, delineating the aforementioned wavelength for futuristic analysis of EGCG.

## 4. Conclusion

This study was conducted to develop a method for the rapid estimation of EGCG in water using UPLC. The results of validation study carried out on this UPLC method deduced that this method was rapid, selective, precise, accurate, and reproducible. Results also revealed that 210 nm was a more sensitive detection wavelength than 280 nm.

## Acknowledgements

This research was carried out in Pharmaceutical Technology Labs in Future University in Egypt.

## Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

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