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Spectrophotometric and cloud point extraction methods to detect Quercetin Dihydrate in supplement formulations and urine samples

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Abstract. *The accurate detection and quantification of quercetin dihydrate (QRC) are vital for quality control, pharmacokinetic studies, and bioavailability assessments in pharmaceutical and biological samples. This study aimed to develop and validate a cloud point extraction (CPE) method combined with spectrophotometry for the sensitive and environmentally friendly detection and quantification of QRC in pharmaceutical formulations and spiked urine samples.*

Methods. The CPE method employed Triton X-114 as a non-ionic surfactant to extract QRC from samples. The extraction process was optimized by evaluating key parameters, including surfactant concentration, incubation temperature, extraction time, and centrifugation speed. Spectrophotometric analysis was conducted before and after extraction to assess the sensitivity and linearity of the method. The method was validated using spiked urine samples and pharmaceutical formulations of QRC, with recovery rates, limits of detection (LOD), and linearity evaluated to ensure accuracy and precision.

Results. The optimized CPE conditions included an incubation temperature of 65°C, a 5-minute extraction time, and centrifugation at 3500 rpm. The CPE method significantly improved the sensitivity of QRC detection, reducing the LOD from 0.0351 µg/mL (without CPE) to 0.0234 µg/mL (with CPE). The method exhibited excellent linearity ($r^2 > 0.998$) over a wide concentration range (1–12 µg/mL). High recovery rates (98.88% to 101.6%) and low relative standard deviations (RSD < 2%) were observed in pharmaceutical formulations and spiked urine samples, demonstrating the method's accuracy and precision. The enrichment factor was 1.75, and the preconcentration factor was 4.6.

Conclusions. The proposed CPE method combined with spectrophotometry provides a simple, sensitive, and environmentally friendly approach for QRC analysis. It offers significant advantages over conventional methods, including reduced organic solvent use and waste generation, making it suitable for routine analysis in pharmaceutical quality control and pharmacokinetic studies. The method's adaptability to complex matrices, such as urine, and its potential for broader applications, including the analysis of other polyphenolic compounds, were also demonstrated.

Keywords: *Quercetin dihydrate, cloud point extraction, spectrophotometry, pharmaceutical analysis, urine sample, green chemistry, Triton X-114.*

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Методи спектрофотометрії та екстракції за точкою хмарності для кількісного визначення кверцетину дигідрату у добавках та зразках сечі

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Резюме. *Точне виявлення та кількісне визначення кверцетину дигідрату (QRC) є важливими для контролю якості, фармакокінетичних досліджень та оцінки біодоступності в фармацевтичних та біологічних зразках. Метою цього дослідження було розробити та валідувати метод екстракції за точкою хмарності (CPE) у поєднанні зі спектрофотометрією для чутливого та екологічно безпечного виявлення та кількісного визначення QRC у фармацевтичних формулах та зразках сечі.*

Методи. Метод CPE використовував Тритон X-114 як неіонний поверхнево-активний агент для екстракції QRC із зразків. Процес екстракції був оптимізований шляхом оцінки ключових параметрів, таких як концентрація поверхнево-активної речовини, температура інкубації, час екстракції та швидкість центрифугування. Спектрофотометричний аналіз проводився до та після екстракції для оцінки чутливості та лінійності методу. Метод було валідовано з використанням зразків сечі з додаванням фармацевтичних формул QRC, із оцінкою відновлення, меж виявлення (LOD) та лінійності для забезпечення чутливості та точності.

Результати. Оптимізовані умови CPE включали температуру інкубації 65°C, час екстракції 5 хвилин та центрифугування на 3500 об/хв. Метод CPE значно покращив чутливість виявлення QRC, зменшивши LOD з 0,0351 мкг/мл (без CPE) до 0,0234 мкг/мл (з CPE). Метод показав відмінну лінійність ($r > 0,998$) в широкому діапазоні концентрацій (1–12 мкг/мл). Високі коефіцієнти відновлення (98,88% до 101,6%) і низькі відносні стандартні відхилення (RSD < 2%) були отримані в фармацевтичних формулах та зразках сечі, що свідчить про точність та прецизійність методу. Фактор збагачення становив 1,75, а фактор передконцентрації — 4,6.

Висновки. Запропонований метод CPE у поєднанні зі спектрофотометрією є простим, чутливим та екологічно безпечним підходом для аналізу QRC у добавках та зразках сечі. Він має значні переваги порівняно з традиційними методами, зокрема зменшену кількість використаних органічних розчинників та зменшення утворення відходів, що робить його придатним для рутинного аналізу в контролі якості фармацевтичних засобів та в фармакокінетичних дослідженнях. Запропонований метод можна адаптувати для складних матриць, таких як сеча, а також для більш широкого застосування, включаючи аналіз інших поліфенольних сполук.

Ключові слова: *кверцетин дигідрат, екстракція за точкою хмарності, спектрофотометрія, фармацевтичний аналіз, зразок сечі, зелена хімія, Тритон X-114.*

Introduction. The growing worldwide usage of medications and their environmental contamination has become a significant concern [1]. Human urine, with its varied composition, contains both nutrients and contaminants that can act as pollutants and resources [2]. Pharmaceuticals often enter the environment through human activities, as they are excreted in various forms after administration. Pharmaceutical contaminants, particularly hormones and antibiotics, pose risks to the safe use of source-separated urine. Wastewater treatment plants are recognized as major contributors to pharmaceutical impurities in the environment [3]. Therefore, developing a dependable, straightforward, and sensitive technique for analyzing quercetin dihydrate (QRC) in pharmaceuticals and real samples is of paramount importance.

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Flavonoids, the most prevalent class of bioactive polyphenolic phytochemicals, are abundant in the plant kingdom and various food sources [4, 5]. Comprising a 15-carbon skeleton (C6–C3–C6) [6], flavonoids are considered safe with a wide therapeutic range [7] and have attracted significant interest for their biological effects. Research shows that flavonoids have beneficial impacts on diabetes, obesity [8, 9], neurodegenerative diseases [10], cardiovascular diseases [11], autoimmune diseases [12], and cancer [13]. They also possess potent antioxidant, antimicrobial, and anti-inflammatory properties [14, 15].

Quercetin, a flavonol, is a plant pigment with a flavonol-type structure known as 3,3',4',5,7-pentahydroxyflavone [16]. It is found in green tea, onions, apples, and other fruits [17, 18]. Quercetin is a small molecule with strong antioxidant qualities and various biological activities, including anti-inflammatory, antiviral, and anti-carcinogenic effects [19-21]. In nature, quercetin is often present in glycosidic forms. It undergoes metabolism in the liver and gastrointestinal tract before being absorbed into the bloodstream. Its metabolites, detectable in blood and urine, are important for evaluating bioavailability [22, 23].

Cloud point extraction (CPE) combined with spectrophotometry is an environmentally friendly and cost-effective method for extracting and preconcentrating compounds [24, 25]. This technique is used across various domains, including pharmaceuticals, food samples, and natural materials [26, 27]. CPE combined with spectrophotometry is an environmentally friendly and cost-effective method for extracting and preconcentrating compounds [24, 25]. Despite the effectiveness of methods like high-performance liquid chromatography-ultraviolet detection (HPLC-UV), gas chromatography-mass spectrometry (GC-MS), and others for quercetin analysis [28-30], they are often time-consuming, reagent-intensive, and produce high waste, making them less compatible with green chemistry principles. Conventional liquid-liquid extraction methods also have limitations, such as health risks and environmental concerns.

Therefore, the present study aimed to address the lack of literature on the CPE approach for extracting QRC from pharmaceutical forms and urine samples, introducing a simple extraction procedure using Triton X-114 as a non-ionic surfactant.

Materials and Methods. *Ethical Considerations.* The collection and use of urine samples in this study were conducted in accordance with ethical guidelines. Prior to sample collection, informed consent was obtained from the volunteer participant, who was fully informed about the purpose of the study, the procedures involved, and any potential risks or benefits. The study protocol was reviewed and approved by Mustansiriyah University, protocol number 5349 dated 13/12/2023.

Instrumentation. A Shimadzu UV-VIS 1800 digital double-beam spectrophotometer (Kyoto, Japan) was used to measure the absorbance spectra of the drug. For the CPE tests, absorbance measurements were performed using 50 µL quartz cells (Cecil) with a 1 cm path length. The CPE experiments were conducted in a thermostatic water bath (Haake, Fe3) monitored with a mercury thermometer. Separation of the analyte was carried out in a Hettich EBA 21 centrifuge using 15 mL calibrated centrifuge tubes. For batch analysis, 1 cm silica cells were used with a PD-303 spectrophotometer (APEL, Japan). A Sartorius BL 210 S sensitive electronic balance was employed for all weighing procedures.

Reagents and standard solutions. All reagents and chemicals used were of analytical grade and did not require further purification. A stock solution of quercetin dihydrate (QRC) at a concentration of 100 µg/mL was prepared by dissolving 0.01 g of QRC in 5 mL of 0.1 M sodium hydroxide (NaOH) and diluting to 100 mL with distilled water in a volumetric flask (Carl ROTH, Germany).

The diazotized sulphadimidine (DSD) solution (0.005 M) was prepared by transferring 0.45 mL of sulphadimidine (33%) into a 100 mL volumetric flask, adding 4 mL of 1 M HCl, followed by 0.0345 g of sodium nitrate. The mixture was shaken and allowed to react for 5 minutes, and then distilled water was added to reach the 100 mL mark. A 0.1 M sodium hydroxide solution was prepared by diluting a concentrated 1 M NaOH solution in a 250 mL volumetric flask. Triton X-114 (10% v/v) was prepared by dissolving 3 mL of Triton X-114 in 50 mL of distilled water.

Pharmaceutical Samples. Fifteen Mega Quercetin capsules (1200 mg, Solaray, USA) were weighed, and an amount equivalent to 0.01 g of QRC was dissolved in 5 mL of 0.1 M NaOH. The solution was diluted to 100 mL with distilled water in a volumetric flask, stirred thoroughly, and filtered. The filtrate was further diluted with distilled water for analysis.

Spiked Urine Sample. A urine sample was collected from a healthy female volunteer and refrigerated without preservatives. Before the extraction process, the sample was brought to room temperature. To prepare the spiked sample, varying concentrations of pure QRC were added to 8 mL of urine. The mixture was centrifuged at 3500 rpm for 10 minutes. The supernatant was collected and diluted with distilled water to a final volume of 50 mL. This diluted sample was then processed using the standard extraction method for analysis.

General Batch Procedure. Serial dilutions of the QRC stock solution (100 µg/mL) were prepared in 10 mL calibrated flasks to achieve concentrations ranging from 2 to 18 µg/mL. To each flask, 2 mL of DSD solution (0.005 M) and 1 mL of NaOH (0.1 M) were added. The solutions were then diluted to the mark with distilled water and thoroughly mixed. At room temperature (approximately 25°C), the mixtures were allowed to stabilize for 5 minutes for the azo coupling reaction to reach maximum stability. The absorbance was measured at 440 nm using a spectrophotometer.

Cloud Point Extraction Procedure. A 1 mL aliquot of QRC solution (100 µg/mL) was mixed with 2 mL of DSD solution (0.005 M), 1 mL of NaOH (0.1 M), and 2 mL of Triton X-114 (10% v/v) in a 10 mL calibrated flask. The flask was then filled with distilled water. The mixture was heated to 65°C (above the cloud point temperature of the surfactant) in a thermostatic bath for 15 minutes to form a cloudy phase while avoiding the degradation of QRC. After heating, the mixture was centrifuged at 3500 rpm for 5 minutes to separate the surfactant-rich phase from the aqueous phase. The mixture was then cooled in an ice bath to below the cloud point temperature to facilitate phase separation. The aqueous phase was discarded, and the surfactant-rich phase, containing the extracted analyte, was collected for further analysis. The surfactant-rich phase was diluted with 1 mL of ethanol, and the absorbance was measured at 455 nm using a PD-303 spectrophotometer (APEL, Japan). A schematic of the cloud point extraction process is presented in Fig. 1.

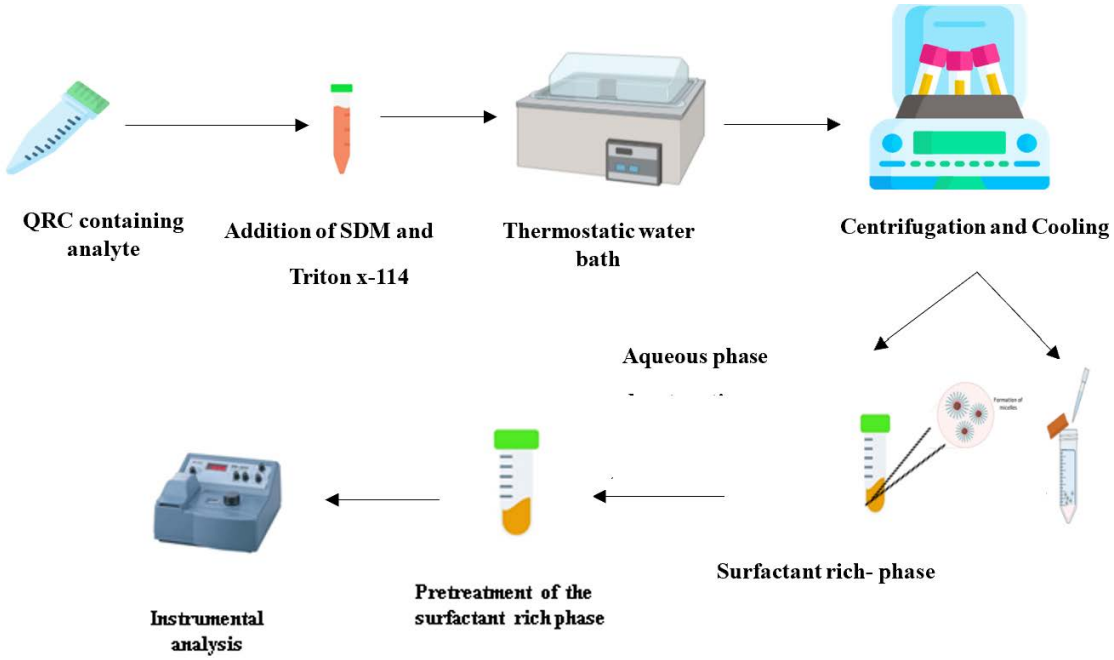

Fig. 1. Schematic representation of cloud point extraction process. Aqueous Solution: Contains QRC, DSD, and Triton X-114. Surfactant Micelles: Formed by the non-ionic surfactant Triton X-114. Heating: The solution is heated above the cloud point temperature to induce phase separation. Centrifugation: Separates the surfactant-rich phase (containing QRC) from the aqueous phase. Cooling: The mixture is cooled to room temperature to form a stable surfactant-rich phase containing the extracted analyte.

Results. The spectrophotometric approach demonstrated high sensitivity and efficiency in analyzing quercetin dihydrate (QRC) in pharmaceutical formulations and spiked urine samples. The coupling reaction between QRC and diazotized sulphadimidine (SDM) successfully produced an orange azo-dye product. Cloud point extraction (CPE) using Triton X-114 as a surfactant allowed for effective phase separation, enhancing the sensitivity of the analyte.

The absorbance spectra of the QRC-SDM coupling product showed peaks at 440 nm before extraction and 455 nm after extraction, confirming successful extraction (Fig. 2).

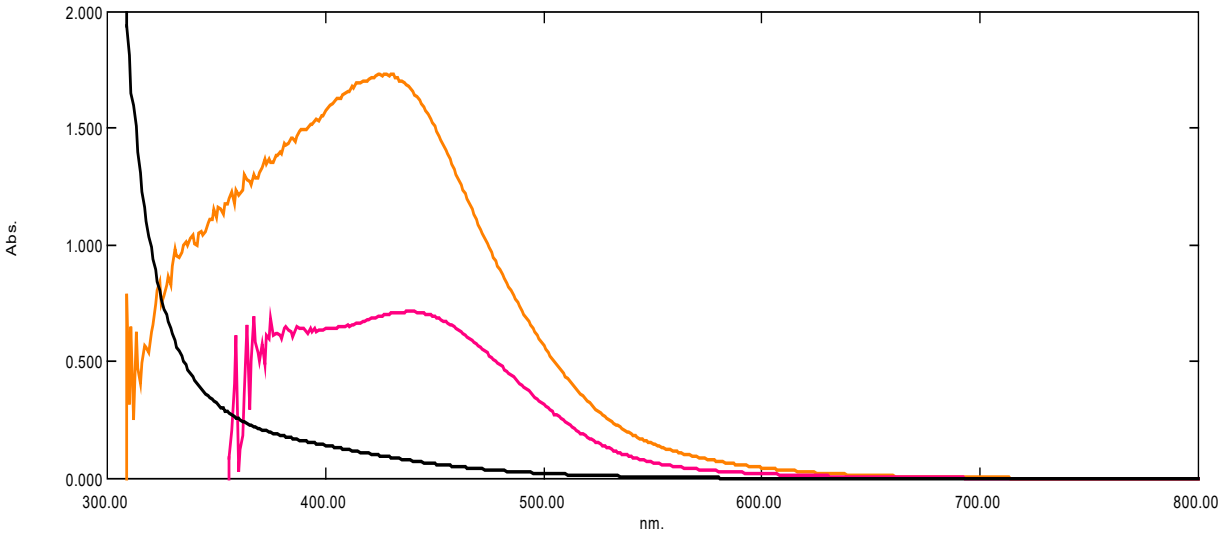

Fig. 2. Absorption spectra of the formed dye by coupling reaction of QRC treated with SDM before and after CPE, measured against a reagent blank.

Optimized conditions for the reaction (Table 1), including 1 mL of NaOH (0.1 M) and 2 mL of SDM (0.005 M), were critical for achieving maximum color intensity and efficient extraction (Fig. 3).

Table 1

Designated parameters of the CPE technique

Factor	Studied range	Chosen value	
		Spectrophotometry	CPE
Hydrochloric acid volume (mL)	1-4 (1 M)	4	...
SDM volume (mL)	0.5-3 (0.005 M)	2	...
Volume of base solution (mL)	0.5-3 (0.1 M)	1	...
Addition order and reaction time (min)	Different orders 1-45	QRC+R+B 5	...
Triton X-114 volume (mL)	0.5-3	...	1
Incubation time (min) and equilibration temperature (°C)	5-25 40-80	... ...	5 65
Separation time (min) and rate (rpm)	5-20 2000-3500	... ...	5 3500

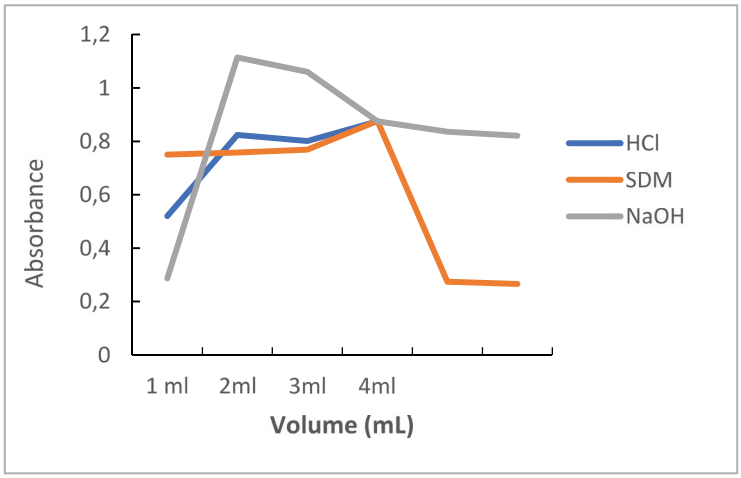

Fig. 3. Effect of volume of (a) HCl, (b) SDM, and (c) NaOH. The volume of HCl can influence the pH of the solution, which in turn affects the stability of the analyte and the surfactant micelles. Fine-tuning the reagent volume allows for precise control over complex formation and analyte recovery. The alkaline volume of the solution can significantly impact the extraction efficiency in cloud point extraction.

The molar ratio of QRC to SDM was determined to be 2:1, as supported by both Job's and mole ratio methods (Fig. 4).

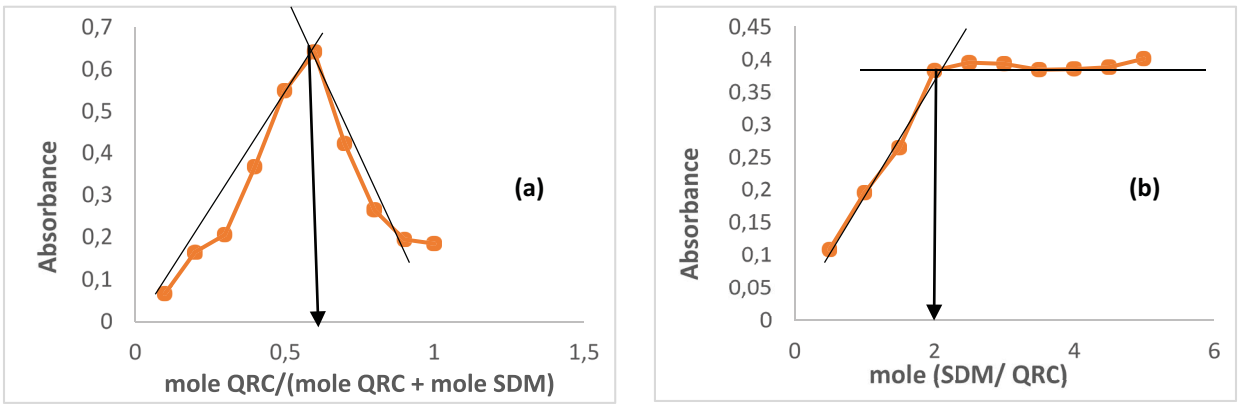

Fig. 4. (a) Job's method, (b) Mole ratio method.

The study also revealed that Triton X-114 outperformed other surfactants in promoting phase separation, with an optimal volume of 1 mL of Triton X-114 yielding the highest extraction efficiency (Fig. 5).

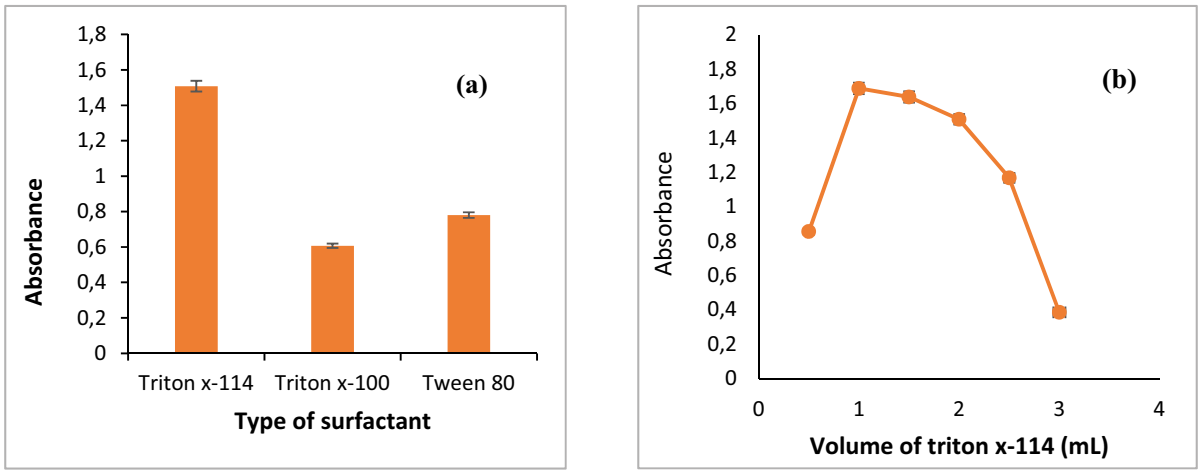

Fig. 5. Effect of (a) surfactant type and (b) Triton X-114 volume on extraction efficiency. The surfactant type and volume can influence micelle formation, phase separation, and analyte solubility in the extraction system. Higher surfactant concentrations may enhance the formation of micelles and improve extraction efficiency.

Optimal conditions for CPE were identified, with a temperature of 65°C, an incubation time of 5 minutes, and a centrifugation speed of 3500 rpm (Figs. 6-8). These conditions ensured efficient micelle formation and phase separation, which were crucial for analyte recovery.

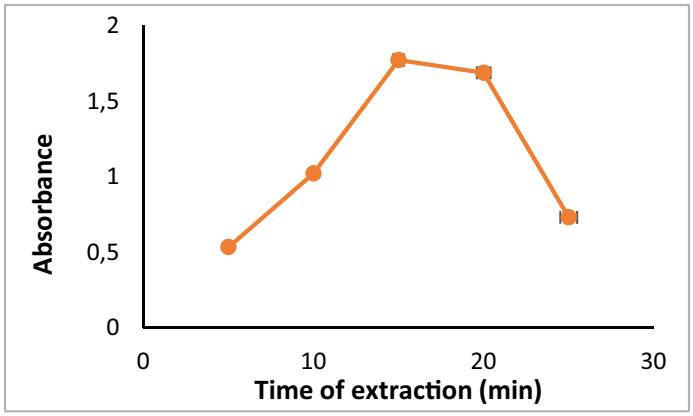

Fig. 6. Effect of extraction time on analyte concentration. Longer extraction times can lead to higher analyte concentrations in the extracted phase, but excessive extraction times may result in decreased efficiency or analyte degradation.

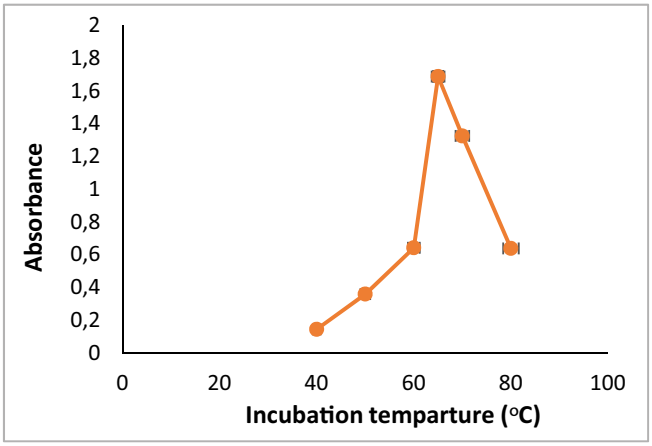

Fig. 7. Effect of incubation temperature on micelle formation and extraction efficiency. Precise control of the temperature during the cloud point extraction process is essential for promoting phase separation and maximizing analyte extraction. The cloud point temperature of the surfactant must be reached and maintained to ensure effective extraction.

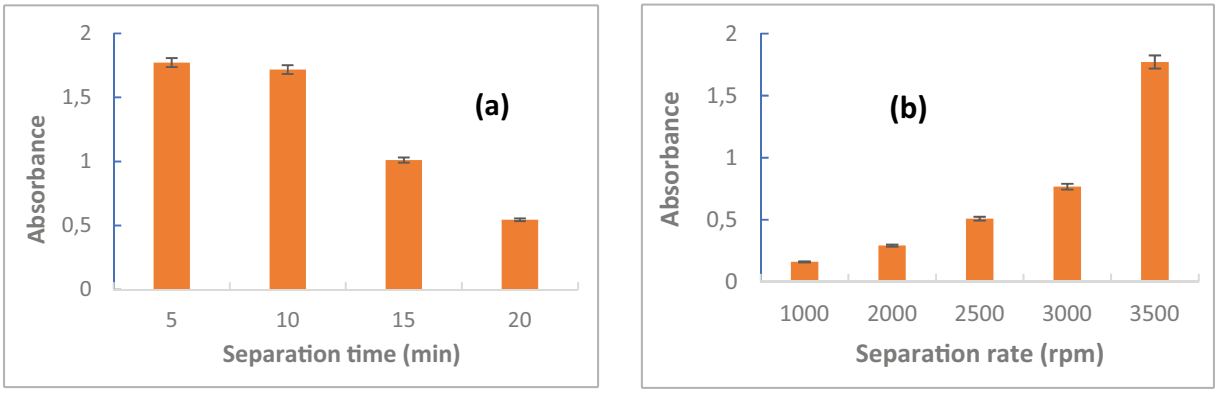

Fig. 8. Effect of (a) separation time on phase separation. Proper selection of separation time is essential to achieve successful separation of the cloudy phase containing the analyte. Longer separation times may lead to improved separation of phases and higher analyte concentrations in the extracted phase, and (b) Effect of separation rate on phase stability.

Calibration curves constructed for QRC, both with and without CPE, exhibited excellent linearity, with correlation coefficients (r^2) greater than 0.998, indicating strong reliability in the analytical method. The limit of detection (LOD) for QRC after CPE was determined to be 0.0234 μg/mL, a significant improvement compared to the non-extracted method, demonstrating the enhanced sensitivity provided by the extraction process (Table 2).

Table 2

Analytical features for the analysis of QRC using the proposed methods

Parameter	Value	
	Without CPE	With CPE
Calibration equation	Y=0.1003x+0.1304	Y=0.1754x-0.0172
Maximum wavelength, nm	430	455
Linearity range (μg/mL)	2-18	1-12
Linearity coefficient, r	0.9986	0.9983
Molar absorptivity, (L/mol.cm)	33928.481	59332.558
Slope, b (mL/μg)	0.1003	0.1754
Intercept, a	0.1304	0.0172
Sandell's sensitivity, (μg/cm^2)	0.0099	0.0057
Average of recovery%	99.568	99.797
LOD (μg/mL)	0.0351	0.0234
LOQ (μg/mL)	0.1170	0.0781
RSD (%)	< 0.18	< 0.19
$S_{y/x}$	0.02167	0.043370
S_a	0.01574	0.026692
S_b	0.00139	0.003626
Enrichment factor	...	1.748754
Preconcentration factor	...	4.6

The recovery rates of QRC from pharmaceutical formulations ranged from 98.88% to 101.6%, with relative standard deviations (RSD) below 2%, confirming the high precision and accuracy of the method (Table 3).

Table 3

Assay of QRC in pharmaceutical formulations and spiked urine samples

Sample	Spectrophotometric method	CPE method
	Conc. (µg/mL)	% Rec
Mega quercetin (1200 mg/capsule)	Spiked	Found ± SD*
6 µg/mL	5.998 ± 0.001	99.966
8 µg/mL	8.071 ± 0.001	100.897
10 µg/mL	9.862 ± 0.001	98.624
Urine sample	6 µg/mL	5.736 ± 0.002

* Average of five determinations; SD, standard deviation; Rec%, average of recovery; RSD%, average of relative standard deviation.

These results validate the effectiveness of the proposed CPE method for the quantitative analysis of QRC in complex matrices.

The calibration curves for QRC, both before and after cloud point extraction, exhibited excellent linearity, indicating strong method reliability. The post-extraction curve showed a notable improvement in sensitivity, as demonstrated by the higher slope and lower detection limit when compared to the pre-extraction curve. This highlights the effectiveness of the CPE process in concentrating the analyte and enhancing the overall sensitivity of the method. The comparison of these calibration graphs is shown in Fig. 9.

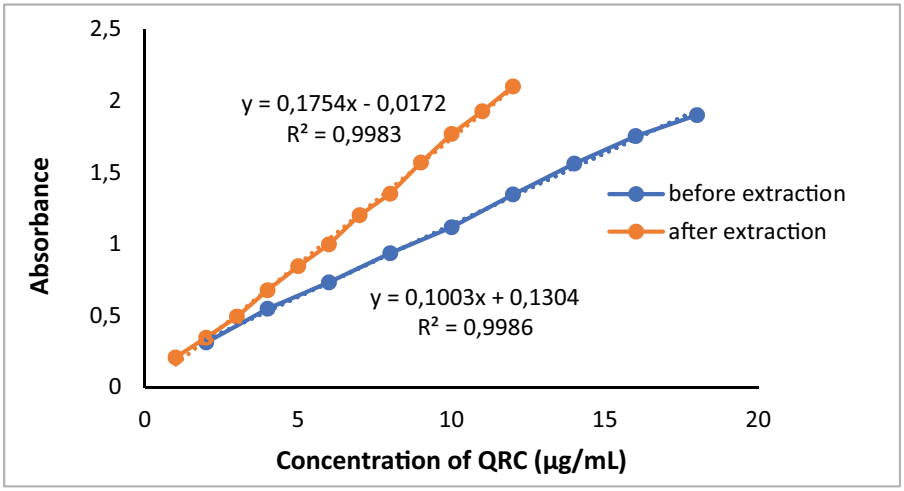

Fig. 9. Calibration graph of QRC (a) before extraction and (b) after CPE.

Discussion. The detection and quantification of quercetin dihydrate in supplement formulations and urine samples are crucial for quality control, pharmacokinetic studies, and bioavailability assessments [30, 31]. Various analytical methods have been developed and employed for this purpose, each with its own strengths and limitations [27-31]. The proposed CPE method combined with spectrophotometry has demonstrated significant advantages for the analysis of QRC in pharmaceutical formulations and urine samples. The CPE technique significantly improved the sensitivity of QRC detection compared to conventional spectrophotometric methods. The LOD decreased from 0.0351 µg/mL to 0.0234 µg/mL after extraction, indicating a substantial enhancement in the method's ability to detect trace amounts of QRC. This improvement can be attributed to the preconcentration effect of CPE, which achieved an enrichment factor of 1.75 and a preconcentration factor of 4.6.

Our study investigated various parameters affecting the CPE process, including surfactant type and concentration, incubation temperature, and separation conditions. Triton X-114 proved to be the most effective surfactant, likely due to its optimal cloud point temperature and micelle-forming properties. The optimized conditions (65°C incubation temperature, 5-minute extraction time, and 3500 rpm centrifugation speed) ensured efficient phase separation and analyte recovery.

The method demonstrated excellent linearity (r^2> 0.998) over a wide concentration range, both with and without CPE. The high recovery rates (98.88% to 101.6%) and low relative standard deviations (<2%) in pharmaceutical formulations and spiked urine samples validate the method's accuracy and precision. These

results suggest that the proposed method is suitable for routine analysis of QRC in complex matrices.

Compared to traditional extraction methods and chromatographic techniques, the CPE approach aligns well with green chemistry principles [24-26]. It reduces the use of organic solvents and minimizes waste generation, making it an environmentally friendly alternative for QRC analysis [24, 27].

In line with our study, several recent advancements in the field of quercetin analysis have highlighted the need for more efficient, cost-effective, and environmentally sustainable methods [17, 23, 28]. The CPE method offers a simpler and greener alternative, with comparable sensitivity and accuracy. The reduction in organic solvent consumption and the relatively rapid extraction process makes CPE a suitable method for laboratories aiming to reduce environmental impact without compromising analytical performance.

Moreover, the successful application of the CPE method to both pharmaceutical formulations and spiked urine samples demonstrates its versatility. Urine is a complex biological matrix, often posing challenges due to the presence of interfering substances [32]. The high recovery rates observed in the spiked urine samples suggest that the CPE method efficiently isolates QRC from such complex matrices, further validating its application in pharmacokinetic and bioavailability studies. The ability to detect low concentrations of QRC in urine is particularly beneficial for monitoring therapeutic levels, drug metabolism, and excretion in clinical settings.

Future considerations for this method could include further automation to enhance reproducibility and scalability, particularly in high-throughput environments. Incorporating automation would streamline the sample preparation process and allow for even more precise control over the extraction parameters. Additionally, further research into the applicability of this method for other structurally similar compounds could expand its utility in the analysis of other nutraceuticals or active pharmaceutical ingredients.

Despite the promising results, this study does have some limitations that should be addressed in future research. First, the CPE method demands precise control over experimental conditions such as temperature, surfactant concentration, and centrifugation speed. Any deviations from these optimized parameters could impact phase separation and analyte recovery, potentially affecting the method's reproducibility in less-controlled environments or large-scale applications. Second, while Triton X-114 is effective, its use involves manual intervention during phase separation, introducing variability and limiting scalability and automation potential, especially in high-throughput laboratories. Third, although the method has been validated for QRC in pharmaceutical formulations and spiked urine samples, its applicability to more complex biological matrices, like blood plasma or tissues, remains unexplored. These matrices often contain higher levels of interfering substances, which might reduce the method's efficiency and require further optimization. Additionally, while CPE offers environmental benefits, it still involves surfactants that may not be fully biodegradable. The long-term environmental impact of these surfactants should be considered, and future research could focus on identifying greener alternatives to Triton X-114. The suggested procedures, with a 95% confidence level, have been thoroughly validated, showing negligible variance in outcomes across classical and recently introduced means, indicating acceptable precision in determining quercetin. Finally, the study's focus on quercetin means that the method's performance with other polyphenols or structurally similar compounds has not been thoroughly tested, and its broader applicability should be investigated before generalizing its use.

Conclusions. In conclusion, the proposed CPE method combined with spectrophotometry provides a reliable, accurate, and environmentally friendly approach for the detection and quantification of quercetin dihydrate in both pharmaceutical and biological samples. Its sensitivity, precision, and alignment with green chemistry principles make it an excellent alternative to more resource-intensive analytical techniques, with the potential for broader applications in both clinical and industrial settings.

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Author's contributions.

Sadeem Subhi Abed: Conceptualization of the research, validation, resources, methodology, formal analysis, and manuscript proofreading.

Mayasa Mansour Mohammed: Writing – review & editing, statistical analysis, Writing – original draft, Validation, Resources, Methodology, Investigation, and Conceptualization.

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