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Cloud Point Extraction-Spectrophotometric Method for the Determination of Amoxicillin Trihydrate in Different Matrices Using Derivatization Reactions.

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ABSTRACT

Two simple eco-friendly visible spectrophotometric methods (A and B) have been designed for the determination of Amoxicillin Trihydrate (AMXT) in different samples after cloud-point extraction (CPE). The methods are based on the oxidative coupling reaction of the drug AMX with oxidized form of 2, 4-dinitrophenylhydrazine (2, 4-DNPH) by sodium periodate in alkaline medium to give a dark purple-colored product (method A) and reaction with 4-aminoantipyrine (4-AAP) in the presence of sodium periodate in alkaline medium to give an orange-colored product (method B). The colored products can be easily extracted by a nonionic surfactant Triton X-114 and AMXT determined spectrophotometrically at λ_{max} of 554 nm and 479 nm respectively. All experimental parameters that impact on CPE efficiency of the colored products were thoroughly studied by the using classical optimization. Under the established optimum conditions, the enrichment factors were found to be of 147.55 and 54.20 fold, achieving the detection limit of 0.077 and 0.17 µg mL^{-1}, with linear range of 0.1-2.0 µg mL^{-1} (r = 0.9992) and 0.3-7.0 µg mL^{-1} (r=0.9997) for the drug AMXT in method A and B respectively. The reaction stoichiometry in both methods was evaluated by limiting logarithmic method and was found to be 1:2 (AMX: 2, 4-DNPH) and 1:1 (AMX:4-AAP) for method A and B respectively. The mean percent recovery was 97.77 ± 2.159 % in urine sample; 99.25± 0.85 % in drug injection, and the precision (RSD %) ranged between 0.06-1.02% and 0.80-4.50 % for AMX in method A and B, respectively. The proposed methods were compared with reported methods published in chemical literature and used for the determination of AMXT in various samples.

Keywords: Amoxicillin Trihydrate, 2, 4-Dinitrophenylhydrazine, 4-Aminoantipyrine, Oxidative- coupling reaction, Cloud point extraction, Spectrophotometry

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INTRODUCTION

The introducing of chemical derivatization reactions in the development of cloud point extraction method for extraction and pre-concentration of medicaments in different matrices is really became attractive avenue after the success achieved in our work which recently published[1]. The importance of this work indeed was reflected in improving the spectral properties of the drugs, selectivity and sensitivity of the procedure as well as the use of simple instrument available in drug detection, and avoid the use the most costly instrumentations (HPLC, GC, MS etc.) which were recently used in international pharmacopoeias [2-3]. This, in fact, encouraged us for continuing to present other works in this trend for the purpose of devising other new methods in determining the drug Amoxicillin trihydrate (AMXT) not only in their pure forms or pharmaceutical formulations, but also when they are present at trace concentration in biological and environmental matrices such as the wastes of the pharmaceutical industries.

Amoxicillin (Figure 1) chemically known as (2*S*, 5R, 6R)-6-[[(2*R*)-Amino (4 hydroxy-phenyl) acetyl] amino]-3, 3–dimethyl–7–oxo–4–thia–1–azabicyclo [3.2.0] heptane–2–carboxylic acid is widely used as an antibacterial drug in the treating of infections caused by gram-positive gram-negative bacteria [4]. It is specifically used in treating of the urinary tract infections, some respiratory infection and bacterial meningitis and many strains of Hemophilus influenza, ear infections especially for otitis media-infection, inflammation of middle ear and acute bacterial sinusitis in children, bladder infections, pneumonia and gonorrhea [5].

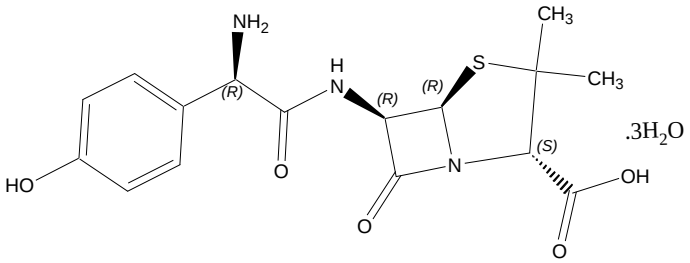

Figure 1: Chemical structure of Amoxicillin Trihydrate ($C_{16}H_{25}N_3O_8S$; M.wt. 419.45 g mol^{-1})

Since the therapeutic uses of amoxicillin are quite wide, establishment of newer methods is a must for the determination of AMXT not only in pharmaceuticals but in other matrices such as biological and environmental. Although the drug amoxicillin (AMXT) is official in British, United State, Indian pharmacopoeias [6-8], it found that there are enormous research publications been dedicated in chemical literature to assay this drug in the pharmaceuticals and few in biological samples by using different instrumental techniques including spectrometric (spectrophotometry, colorimetry, fluorimetry, NMR and NIRs) chromatographic (HPLC, GC, HPTLC and electrophoresis), and electrochemical (potentiometry and voltammetry,) methods. However, the most commonly techniques used in determining of AMX were the spectrophotometric and HPLC methods. Due to the simplicity, rapidity, cost-effective and time-saving, enough sensitivity and available in many laboratories, many efforts exert by authors to improve the selectivity and sensitivity of the spectrophotomtric or colorimetric methods using derivatization reactions with various chromogenic reagents in order to expand the applications of this technique for assay of AMX drug in the biological and environmental samples. Of these chromogenic reagents used in derivatization reactions for AMX assay include, p-amino benzoic acid or procain [9], p-nitoaniline [10], 2,4-dinitrophenylhydrazine [11-12], 4-aminoantipyrine [13], o-nitroaniline [14], N,N-dimethyl-p-phenylenediamine [15], chloramines-T [16], benzocaine [17], metol [18], bromocresol green [19] and luminal [20]. Even though these methods have adequate sensitivity but not free from the matrix interferences, which could have resulted from some additives present in pharmaceutical formulations, perhaps through their participation in diazotization reaction. Therefore elimination of interfering compounds from drug marix before its measurement is a must..

In the present work, a new approach was adopted for the designing two novel analytical methods for extraction and determination of the drug AMXT in different matrices by using cloud point extraction combined with spectrophotometry. The methods were based on the oxidative-coupling reaction of AMXT with two chromogenic reagents such as 2, 4-Dinitrophenylhydrazine (2, 4 DNPH) and 4-Aminoantipyrene (4-AAP) in the presence of alkaline oxidizing agent to form the colored products which can easily extract into a non-ionic surfactant Triton X-114 and AMXT determined spectrophotometrically at respective wavelength maxima. The

main reason for chosen this type of chemical derivatization reaction because it had widely used by many authors in routine spectrophotometric analysis of the medicaments in pharmaceutical formulations and there is no published report about the use of this type of reaction with CPE-spectrophotometry in the determining of the drugs so far.

MATERIALS AND METHODS

Reagents and chemicals

The chemicals used in this work are of high purity and used as received. Doubly distilled water was used for the preparation of all solutions and for the final rinsing of glass wares. A pure grade (95.5%) of amoxicillin trihydrate (AMX) was obtained from Sigma Aldrich (USA). A stock solution of 1000 µg mL^{-1} (or 0.0020 M) for the drug AMX was prepared by dissolving 0.0955 g in minimum amount of water and diluted to mark with water in a 100 mL volumetric flask. This solution was transferred to a brown bottle and stored in the refrigerator. The working solutions were daily prepared by appropriate dilutions in water. An amount of 5 mM 2,4-Dinitrophenyl hydrazine (DNPH; 99%, BDH, UK) is freshly prepared by dissolving 0.09907 g of DNPH in 2 mL concentrated sulphuric acid, transferred in to a 100 mL volumetric flask and diluted to the mark with distilled water. An amount of 5 mM 4-aminoantipyrene (4-AAP; 99.9%, SCHUCHARDT, Germany) is freshly prepared by dissolving 0.10162 g of 4AAP in a 100 mL volumetric flask and diluted to the mark with distilled water. 5 mM Sodium periodate solution(BDH, UK) is prepared by dissolving 0.10695 g of $NaIO_4$ and diluting to 100 mL with distilled water in a volumetric flask. Triton X-114 (purity >99.9%), was purchased from AMRESCO LLC (Solon, USA). A 10% (v/v) of Triton X-114 was prepared by diluting 10 mL with water in a 100 mL volumetric flask. An amount of 0.5 M of sodium hydroxide (BDH), sodium carbonate (BDH) and potassium hydroxide (Riedel De-Haenag, Germany) was prepared by dissolving an appropriate amount of base in water. An amount of 0.5 M ammonia (BDH, England) solution was prepared from concentrated solution (13.4 M) by transferring 3.73 mL into a 100 mL volumetric flask and diluted to mark with water. 1.0 mM of potassium ferricyanide (99%; Sigma-Aldrich, USA) is prepared by dissolving 0.0329 g of $K_3Fe(CN)_6$ in 5 mL water and diluted to mark in a 100 mL volumetric flask. 1.0mM of ferric chloride (99%; BDH,UK) is prepared by dissolving 0.0270 g of $FeCl_3.6H_2O$ in little amount of water and diluted to mark in a 100 mL volumetric flask.

Instrumentation

The main instrument employed in this work is a Shimadzu double-beam UV-Vis Spectrophotometer model UV-1800 (Kyoto, Japan) equipped with 5-mm optical path cell for scanning the absorption spectrum of the resulting colored products beside the absorbance measurements of the two target drugs under study. Thermostatic water bath model WNB7-45 Experts (England) is used throughout the CPE experiments. For solution pH measurements, a portable pH/mV/C meter HI 83141 (HANNA, Romania) is used.

Recommended CPE Procedure

Method A

In a series of 10 mL volumetric flasks, an amount of AMX standard or sample solutions in the concentration range of 0.1-2.0 µg mL^{-1}, 0.06 mL of 5 mM $NaIO_4$, 0.08 mL of 5 mM 2, 4-DNPH and 0.2 mL of 0.5 M NaOH were added. The content of each flask was kept aside for 10 min at room temperature to complete the reaction. Then, 0.8 mL of Triton X-114 (10%) was added to each flask, mixed well and diluted to mark with water. The content of each flask was transferred into a 10 mL centrifuging tubes and kept in the thermostatic bath at 60°C for 25 min. Separation of the phases was done by centrifugation at 3500 rpm for 20 min. The aqueous phase was easily removed by pipette. The surfactant-rich phase that contains the colored product was dissolved in 1.0 mL ethanol and the absorbance of the product measured at 554 nm against a reagent blank prepared under similar conditions. The remaining AMX in aqueous solution was determined by traditional spectrophotometry at λ_{max} of 274 nm in order to determine the distribution ratio (D) and extraction efficiency (%E).

Method B

In 10 mL volumetric flasks, an amount of AMX standard or sample solutions in the concentration

range of 0.3-7.0 µg mL^{-1}, 0.8 mL of 5 mM 4-AAP, , 1.6 mL of 5 mM $NaIO_4$ and 0.1 mL of 0.1 M NH_4OH were added and the content of each flask was kept aside for 15 min at room temperature to complete the reaction. Then 1.6 mL of Triton X-114 (10%) was added in each flask, mixed well and dilutes to mark with water. The content of each flask was transferred into a 10 mL centrifuging tubes and kept in the thermostatic bath at 60°C for 5 min. Separation of the phases was done by centrifugation at 3500 rpm for 20 min. The aqueous phase was easily removed by pipette.

The surfactant-rich phase that contains the colored product was dissolved in 1.0 mL ethanol and the absorbance of the product measured at 479 nm against a reagent blank prepared under similar conditions. The remaining AMX in aqueous solution was determined by traditional spectrophotometry at λ_{max} of 274 nm in order to determine the distribution ratio (D) and extraction efficiency (%E).

Preparation of Samples

Pharmaceutical Formulations: three selected medicaments from different international manufacturers in the form of vials containing 500 mg of active AMXT were purchased from the drugstores in Baghdad/Iraq. The powder of ten vials were mixed, homogenized, and the content of one vial (0.500 g) which equivalent to 500 mg of active drug was completely dissolved in sufficient amount of water, then transferred into 100 mL volumetric flasks and diluted to mark with water. This solution contains 5000 µg mL^{-1} of AMXT from which 100 µg mL^{-1} was prepared by dilution. An amount containing different concentrations of the prepared drug sample solution were transferred to a 10 mL volumetric flask and each solution followed the recommended CPE procedure for AMXT (method A and B) and the content of the drug was measured spectrophotometrically at the respective λ_{max} for three repeated measurements.

Urine: four urine samples collected from volunteers (two males aged from 45-50 years and two females aged 37-39 years) who were randomly designated to inject a single vial of Amoxicillin Pharma Roth (Germany) containing 500 mg AMXT. None of the volunteers was taking any medication at the time of this administration. The samples were collected from the volunteers at three different times (2, 4, 6 and 8 hrs) after injection and then kept in the refrigerator until analysis. The urine samples were thawed at ambient temperature, then 1.0 mL of each sample was pipetted into 10 mL centrifugal tubes and subjected to the recommended CPE procedure (method A) and the drug content was determined by spectrophotometry at λ_{max} of 554 nm.

Tap Water: About one liter of tap water was randomly collected from the chemistry department of College of Science / University of Baghdad/ Iraq. A 0.5 mL of water sample was transferred into three 10 mL volumetric flasks and spiked with 0.1, 0.3, and 0.7 mL of 100 µg mL^{-1} AMXT standard solution. To each flask, all reagents were added and left for 15 min at room temperature for complete reaction and then followed the recommended CPE procedure (Method B). Content of the drug AMXT was determined spectrophotometrically at λ_{max} of 479 nm for three repeated measurements.

Blood serum: blood sample was chosen from one volunteer via withdrawing 5 mL from the vein using a medical syringe. The sample was transferred immediately into a sterile plastic centrifugal tube, kept aside for 15 min and centrifuged at 6000 rpm for 10 min to separate the serum from the whole blood. To ensure a complete separation of blood matrix, 50 µg of trichloroacetic acid was added and the content re-centrifuged for 10 min. Three portions of 0.5 mL serum sample were transferred into three 10 mL volumetric flasks and spiked with 0.7, 1.0 and 1.5 µg mL^{-1} of AMXT standard solutions and then subjected to the general CPE procedure(method A) . The content of AMXT drug in the colored product was determined spectrophotometrically at 554 nm.

Statistical Analysis

Minitab version 17 (Minitab Inc., State College, PA, USA) (29) and Excel 2010 (Microsoft Office®) were was to carry out all statistical calculations such as regression and correlation analysis, ANOVA and significance tests.

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RESULTS AND DISCUSSION

Absorption spectra

In a preliminary study, the absorption spectra of the colored products produced by the coupling reactions between AMXT drug and 2, 4 DNPH or 4-AAP in the alkaline media were recorded without CPE (Figures 2a and 3a). The products were the dark purple and orange-colored exhibiting a maximum absorption peak (λmax) at 554 nm, and 479 nm of the two products respectively while the blank reagent appeared at 360 nm. These findings were adopted in the optimization conditions of CPE for both reactions. The absorption spectra of the two colored-products formed in method A and B were also recorded against the corresponding reagent blank between (190 to 1100 nm) or (200-800 nm) after obtaining optimum conditions according to the recommended CPE procedures using a Shimadzu model UV-1800 equipped with 1.0- cm quartz cell. Figure 2b shows the absorption spectrum of the extracted AMXT-DNPH product against reagent blank, exhibiting again λ_{max} of 554 nm and giving a molar absorptivity of $3.57x10^5$ $L.mol^{-1}.cm^{-1}$.

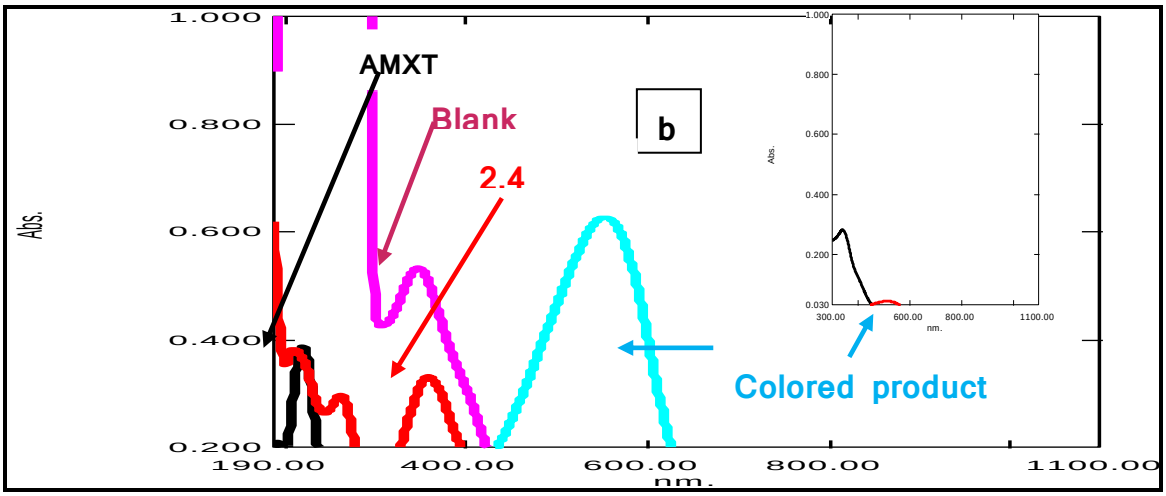

Figure 2: (a) Absorption spectra of the colored product (A) against reagent blank before CPE and (b) after CPE at optimum conditions; 1.0 µg mL^{-1} AMXT, $3x10^{-5}$ $NaIO_4$, $4x10^{-5}$ M DNPH , 0.01 M NaOH, 0.8% TX-114.

Figure 3b shows the absorption spectrum of the extracted AMXT-AAP product against reagent blank, exhibiting again λ_{max} of 479 nm and giving a molar absorptivity of 1.00 $x10^5$ $L.mol^{-1}.cm^{-1}$. Whilst the individual pure AMX and DNPH or AAP solutions have been displayed absorption maxima at 274, 360 and 330 nm, respectively. Consequently, the wavelength maxima at 554 and 479 nm for the two colored-products were used throughout this study for micro amounts of AMXT drug.

Preliminary studies

Type of oxidants and alkaline media as well as the reaction time was preliminary investigated for the reaction of AMXT drug with 2.4-DNPH and 4-AAP. It found that sodium periodate was the best oxidant amongst other like potassium persulphate, potassium ferricyanide and ferric chloride because it gave a highest absorbance value in both methods. Different inorganic bases and the reaction time were verified such as sodium hydroxide, potassium hydroxide, ammonium hydroxide and sodium bicarbonate added at concentrations of $1.0x10^{-5}$ M and $1.0x10^{-3}$M in final volume of 10 mL solution. Best results in terms of spectroscopic sensitivity were obtained with NaOH and NH_4OH, while the reaction times of 10 and 15 min were the best for completeness the reactions between the drug and the selected reagents described in the method A and B respectively.

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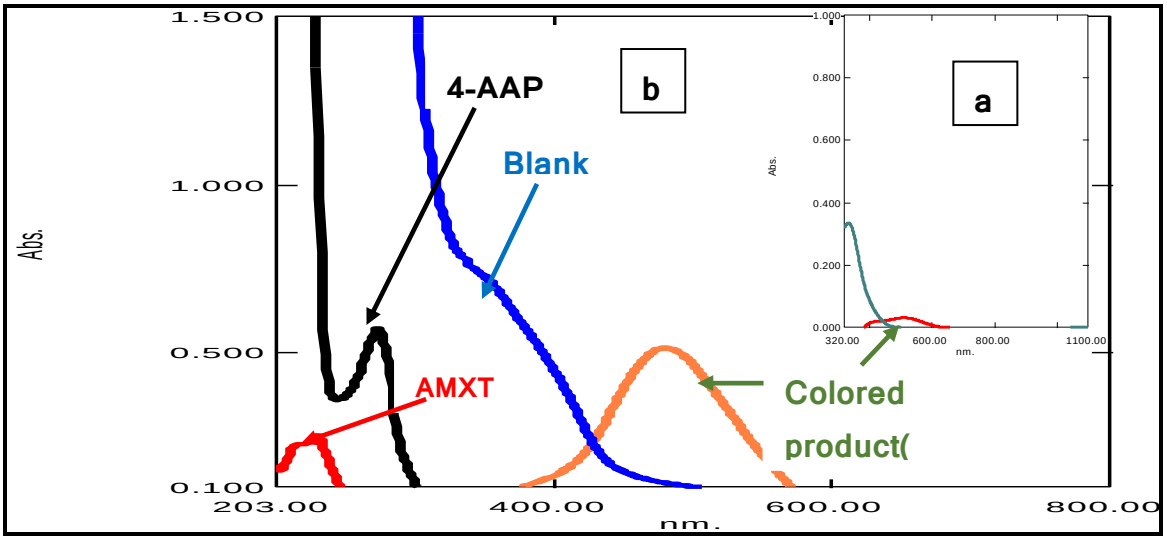

Figure :3 (a) Absorption spectra of the colored product (B) against reagent blank before CPE and (b) after CPE at optimum conditions; 3.0 µg mL^{-1} AMXT, 4.0x10^{-4} AAP, 8.0x10^{-4} $NaOI_4$, 0.001 M NH4OH, 1.6% TX-114.

Optimization of CPE methodology

A series of experiments has been conducted to study the effect of both reactions and CPE variables in order to maximize the sensitivity of the detection system and to enhance the extraction efficiency of the CPE for AMXT drug. In this regard, the variables such as, the concentration of oxidants, reagents, alkaline medium, Triton X-114 amount, equilibration temperature and incubation time, were investigated.

In the following experiments, a specified concentration of AMXT standard solution was taken to 10 mL volumetric flasks into which the reagents added and diluted to mark with water followed by CPE procedure using a classical optimization in which one variable is altered while the others fixed. The absorbance of a series of solutions was measured at 554 and 479 nm versus reagent blank for method A and B respectively.

Effect of oxidant concentration

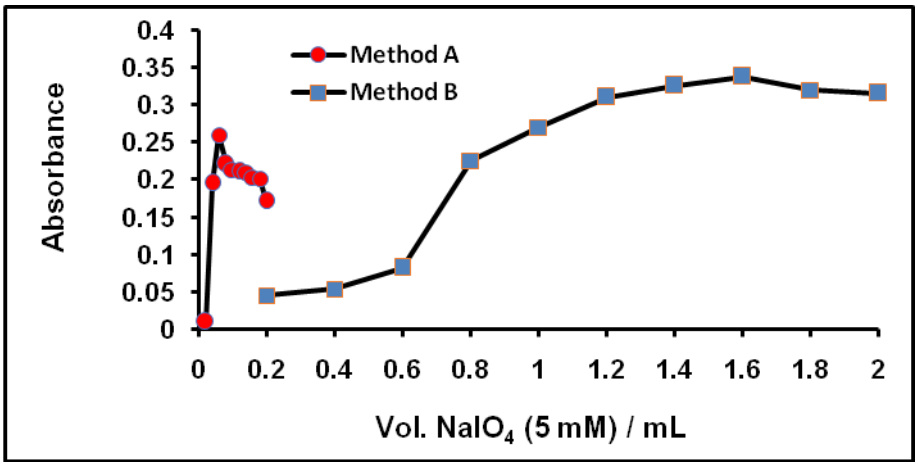

Figure 4: Effect of sodium periodate concentration on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); 2,4-DNPH (0.05 mM); NaOH(10 mM) reaction time (10 min);TX-114 (1.0%); CP temperature (55 ^{o}C); incubation time (15 min). Method B: AMXT (3.0 µg mL^{-1}) ; 4-AAP(0.4 mM); NH_4OH (1.0 mM) ; reaction time (15 min); TX-114 (1.0%); CP temperature (55 ^{o}C); incubation time (15 min)]

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In both methods, the influence of the concentration of sodium periodate was studied using different volumes of 5 mM $NaIO_4$ solution. The results (Figure 4) revealed that the reaction of 2.4-DNPH and 4-AAP with AMXT is proceeding with increases the volumes of each reagent, giving a proportional increase in the absorbance of the reaction products up to 0.06 and 1.6 mL after which a sudden or a gradual decreases in the absorbance values occur. Thus, 0.06 and 1.6 mL were selected as optimal volume of 5 mM $NaIO_4$ for the reaction of 2.4-DNPH and 4-AAP with AMXT respectively.

Effect of reagent concentration

The impact of concentration of 2,4-DNPH and 4-AAP was investigated using different volumes of 5 mM of each reagent solutions. Figure 5 shows that the optimum volume of 5 mM 2,4-DNPH was of 0.08 mL (0.04 mM in 10 mL final solution) and 0.8 mL of 5 mM 4-AAP (0.4 mM in 10 mL final solution) were enough to give maximum absorbance, high stability of the colored products and subsequently the best extraction efficiency for the determination of AMX drug in the two reaction systems. At lower or higher concentrations of each reagent than optimal, less intensely colored products were observed so any excessive amount of reagent was not needed. Therefore, 0.08 mL of 5 mM 2,4-DNPH and 0.8 mL of 5 mM 4-AAP in 10 mL solution were used in further experiments.

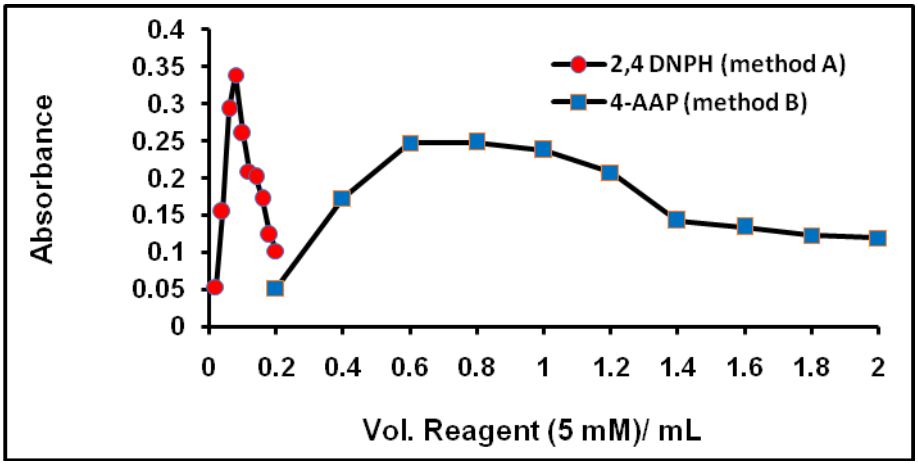

Figure 5: Effect of reagents concentration on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); $NaIO_4$ (0.03 mM); NaOH(10 mM) reaction time (10 min);TX-114 (1.0%); CP temperature (55 ^oC); incubation time (15 min). Method B: AMXT (3.0 µg mL^{-1}) ; NaIO4(0.8 mM); NH_4OH (1.0 mM) ; reaction time (15 min); TX-114 (1.0%); CP temperature (55 ^oC); incubation time (15 min)]

Effect of alkaline medium

It was found that an alkaline medium is an important variable and most favorable compared with acidic or neutral medium to facilitate the oxidative coupling reaction between the reagents and phenolic compounds **[12, 13].** It was shown in the preliminary study that the sodium hydroxide and ammonium hydroxide were the best in the reaction of AMX with 2,4-DNPH and 4-AAP reagents respectively, via an electrophilic substitution at the phenolic ring and nucleophilic substitution with phenolic moiety of AMX to form the colored products respectively **[21]**. Studies for the optimization of NaOH and NH_4OH concentration revealed that the optimum volume was 0.2 mL of 0.5 M NaOH (10 mM in 10 mLsolution) and 0.1 mL of 0.1 M NH_4OH(1.0 mM in 10 solution) gives maximum absorption signal of the coloured products between AMX and both chromogenic reagents as displayed in Figure 6.

Effect of Triton X-114 amount

Figure 7 depicts the impact of variation of Triton X-114 amount on the absorbance signal of the colored products. Different volumes ranges of Triton X-114 (10% v/v) were used in this study at previously optimum conditions. As shown in Figure 7, the absorbance for both products increases by increasing the Triton X-114 amount up to 0.1 and 1.6 mL of 10% (v/v) which equivalent to 0.8% and 1.6% Triton X-114 in a

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final 10 mL solution for method A and B respectively and then suddenly decreased at higher amounts. Therefore, 0.1 and 1.6 mL of 10% (v/v) Triton X-114 were used as the optimum amount in this study.

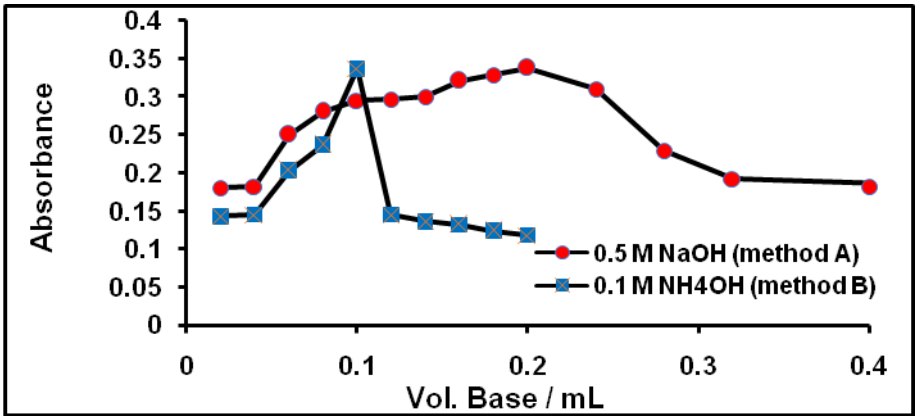

Figure 6: Effect of alkaline medium concentration on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); 2,4 DNPH (0.04 mM); $NaIO_4$ (0.03 mM); reaction time (10 min);TX-114 (1.0%); CP temperature (55 ^oC); incubation time (15 min). Method B: AMXT (3.0 µg mL^{-1}) ;4-AAP (0.4 mM); $NaIO_4$(0.8 mM); reaction time (15 min); TX-114 (1.0%); CP temperature (55 ^oC); incubation time (15 min)].

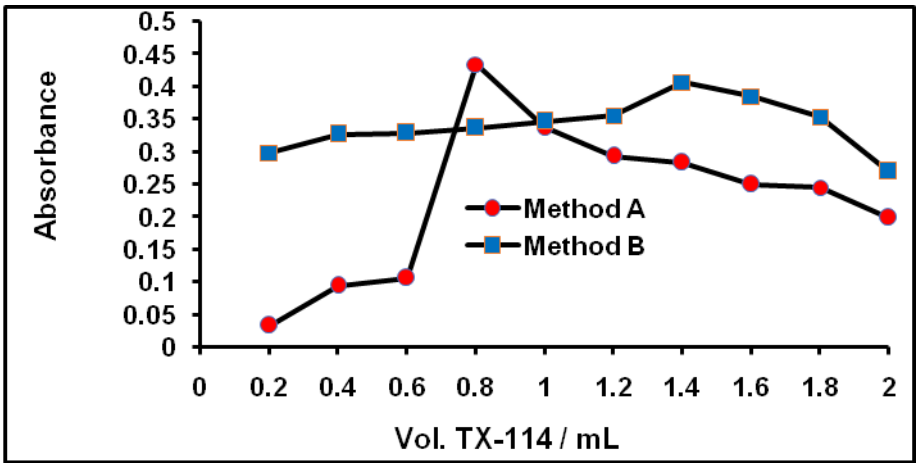

Figure 7: Effect of surfactant amount on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); 2,4 DNPH (0.04 mM); $NaIO_4$ (0.03 mM); NaOH (10 mM) reaction time (10 min); CP temperature (55 ^oC); incubation time (15 min). Method B: AMXT (3.0 µg mL^{-1}) ;4-AAP (0.4 mM); $NaIO_4$(0.8 mM); NH_4OH(1.0 mM) ; reaction time (15 min); CP temperature (55 ^oC); incubation time (15 min)]

Effect of equilibration temperature

The influence of the equilibrium temperature on extraction of the two colored products was examined due to its importance for the efficient separation of the phases, which reflect certainly the magnitude of pre-concentration factor of an analyte**[22].** Therefore, a study was carried out by varying the temperature from 25^oC to 70 ^oC in search of optimum values. The results in Figure 8 disclosed that the absorbance signal started to increase from 25^oC up to 60 ^oC. At higher temperature than 60 °C, a sudden decrease in absorption signal has occurred which most probably due to thermal decomposition and instability of the colored products as well as increase diffusion of micelles in aqueous solution, which lead to increase in surfactant-rich phase volume resulting in decreasing the extraction efficiency **[23-24]**. Thus, the temperature of 60 ^oC was selected to fulfill efficient separation conditions of the two colored products.

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Effect of incubation time

The effect of incubation time on the absorption signals of the two colored products was studied using different heating times starting from 5 min until 50 min at cloud point temperature of 60 ^{o}C. The maximum absorption signal was achieved at 25 min for AMX-DNPH product and gradually decreased thereafter, while 5 min was a sufficient for the maximum extraction of AMX-AAP product as showed in Figure 9. It was also noted that the centrifugation speed and time of 20 min at 6000 rpm were suitable to separate two phases.

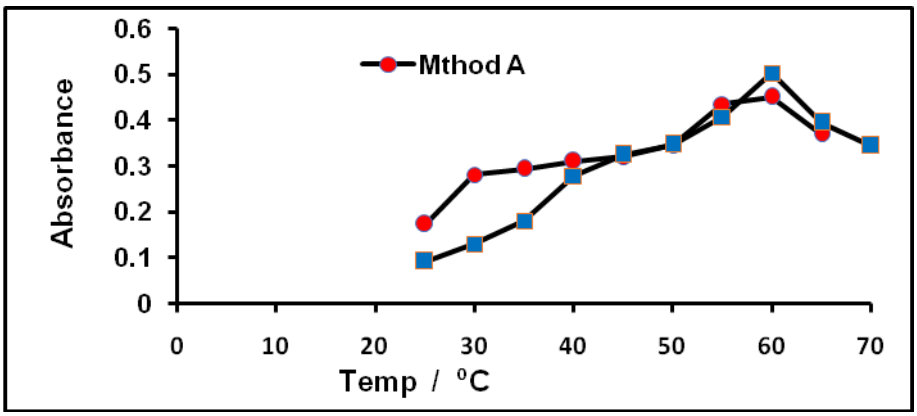

Figure 8: Effect of equilibration temperature on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); 2,4 DNPH (0.04 mM); $NaIO_4$ (0.03 mM); NaOH (10 mM) reaction time (10 min); TX-114 (0.8%); incubation time (15 min). Method B: AMXT (3.0 µg mL^{-1}) ;4-AAP (0.4 mM); $NaIO_4$(0.8 mM); (NH_4OH(1.0 mM) ; reaction time (15 min); TX-114 (1.6%); incubation time (15 min)]

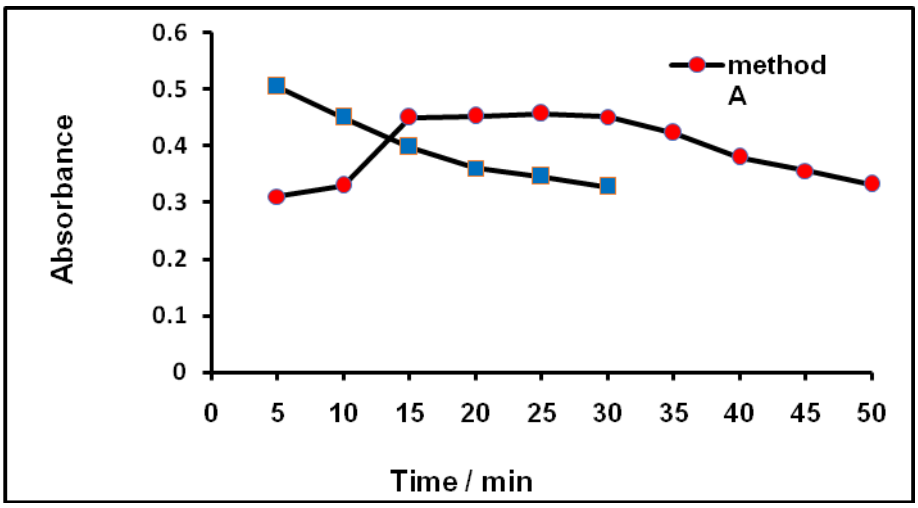

Figure 9: Effect of incubation time on the formation the colored products [conditions: method A; AMXT (1.0 µg mL^{-1}); 2,4 DNPH (0.04 mM); $NaIO_4$ (0.03 mM); NaOH (10 mM) reaction time (10 min); TX-114 (0.8%); CP temperature (60 ^{o}C). Method B: AMXT (3.0 µg mL^{-1}) ;4-AAP (0.4 mM); $NaIO_4$(0.8 mM); (NH_4OH(1.0 mM) ; reaction time (15 min); TX-114 (1.6%); CP temperature (60 ^{o}C)]

Table 1 shows a summary of the best values of the experimental variables for the direct determination of AMX drug spectrophotometrically at λ_{max} of 554 and 479 nm by the two proposed methods after CPE method.

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Table 1: The summary of optimum experimental conditions for the extraction of colored products by CPE for method A and B

Variable	Method A	Method A
$NaIO_4$	0.03 mM	0.8 mM
2,4 DNPH	0.04 mM	-
4-AAP		0.4 m M
NaOH	10 mM	-
NH_4OH		1.0 mM
TX-114	0.8%	1.6%
Temperature	60 ºC	60 ºC
Incubation time	25 min	5 min
λ_{max}	554	479

*Final concentrations in 10 mL solution that carried out by CPE

Stiochoimetry of the reaction

Using the aforementioned optimum conditions, the stoichiometry of the reaction between AMX and 2,4-DNPH (method A) and 4-AAP (method B) was assessed by using the limiting logarithmic method**[25].** In this method, two sets of experiments were performed followed the recommended CPE procedures of both methods. The first set of experiments was done by varying the concentration of the each reagent with fixed concentration of AMX, while the second set of experiments was conducted by varying the concentration of AMX with fixed concentration of each reagent. The logarithms of the obtained absorbance were plotted as a function of the logarithms of the concentrations of each reagent and AMX in the first and second sets of experiments, respectively. The slopes of the fitting lines in both sets of experiments were calculated. In each method, two straight lines were obtained. The values of the slopes of these lines were of 1.372 and 0.9443 by method A (Figure 10), indicating the molar ratio of reaction with DNPH/AMX was considered to be 2:1. Whilst in method B, the values of the slopes of these lines were 1.183 and 0.9476 (Figure 11), indicating the molar ratio of reaction with AAP/AMX was considered to be 1:1.

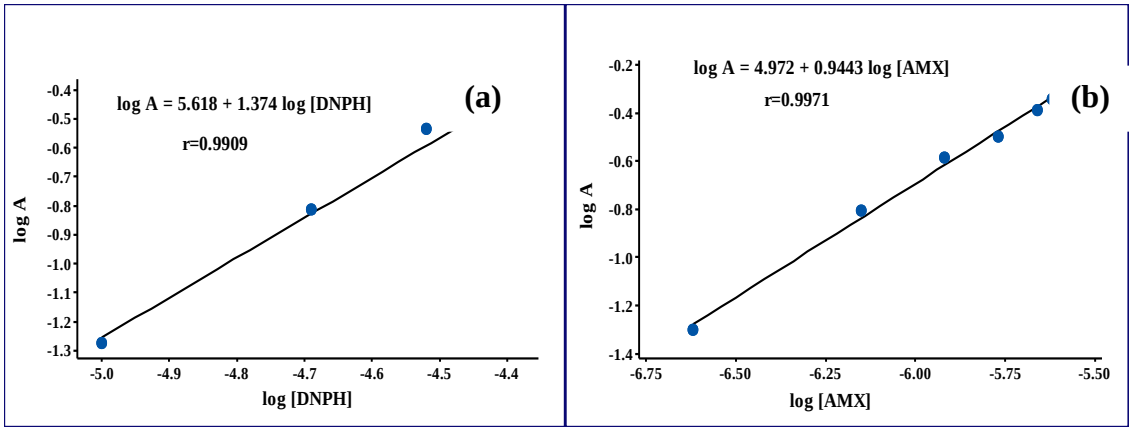

Figure 10: (a) Limiting logarithmic plots for the molar reactivity of DNPH with AMX: logarithm of absorbance *vs*. log [DNPH] at which [AMX] is kept constant; (b). logarithm absorbance *vs*. log [AMX] at which [DNPH] is kept constant.

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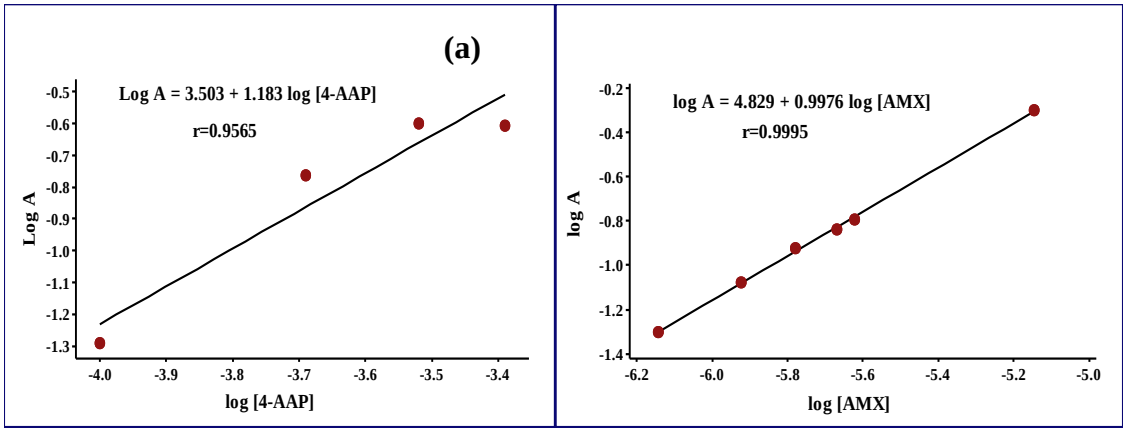
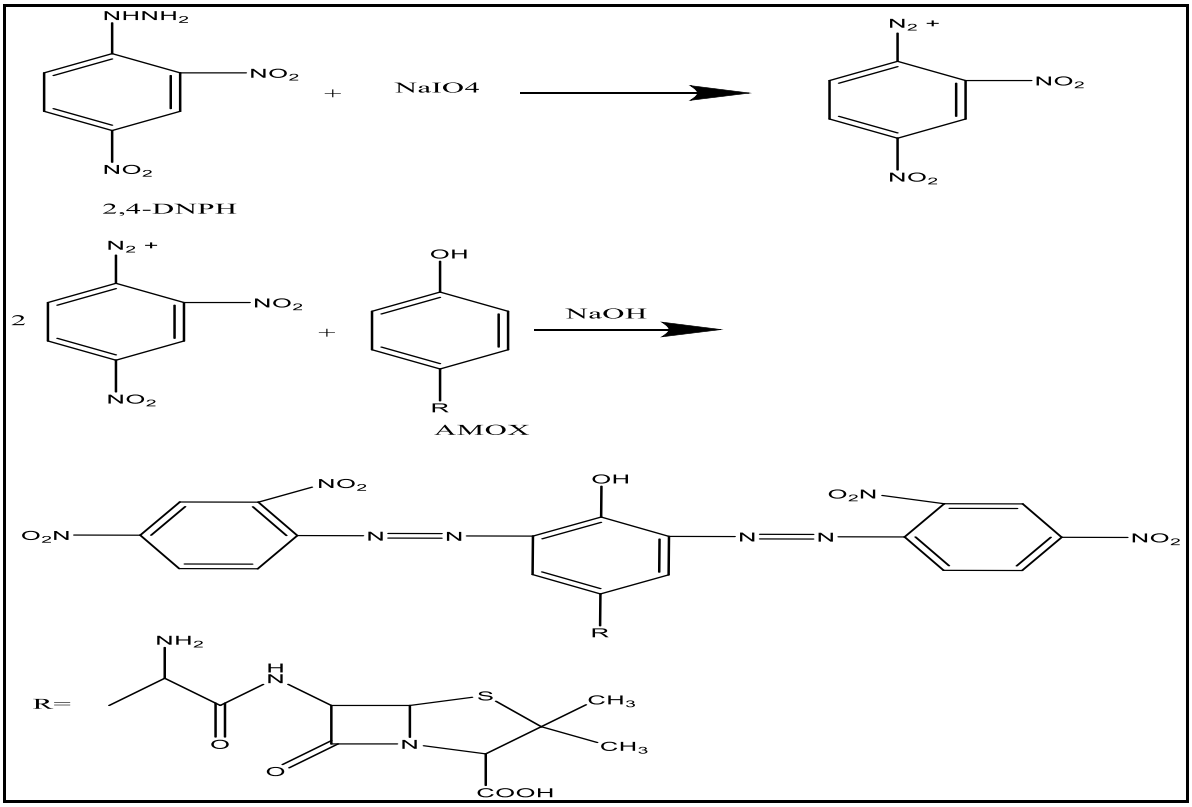


Figure11: (a) Limiting logarithmic plots for the molar reactivity of 4-AAP with AMX: logarithm of absorbance *vs*. log [AAP] at which [AMX] is kept constant; (b). logarithm absorbance *vs*. log [AMX] at which [AAP] is kept constant

Based on the above results and upon the oxidation of 2,4-DNPH by $NaIO_4$ to give diazonium cation **[12],** a reagent reacts with AMX in alkaline medium by electrophilic substitution to give deep a dark–purple color, so the proposed reaction path for method A can postulated as shown in scheme 1.



Scheme 1: A hypothetical reaction mechanism path between AMX and 2, 4 DNPH in alkaline medium.

Also, the loss of two protons from 4-AAP reagent upon its oxidation with $NaIO_4$ to form a nucleophilic intermediate which reacts with phenolic moieties of AMX in an alkaline medium by nucleophilic substitution reaction to give orange coloured quinonoid-type product **[26]** as shown in scheme 2 **[13]**.

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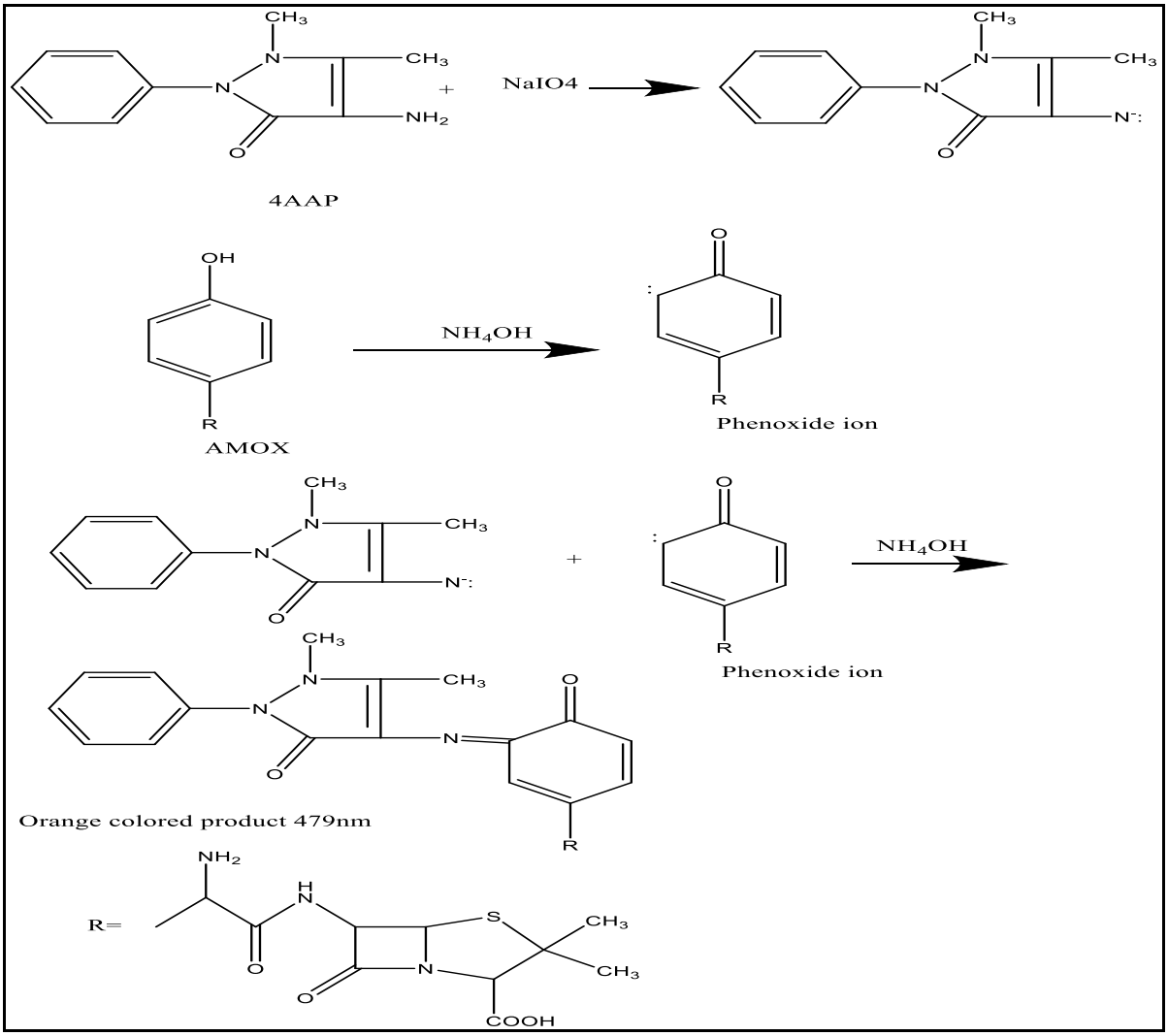

Scheme 2: A hypothetical reaction mechanism path between AMX and 4-AAP in alkaline medium

Stability of the colored products

Under the optimum conditions established (Table 1), the effect of time on the stability of both reaction products was examined through the following up the absorption signal measurements at different time intervals for the colored products separated after CPE. The results showed that the absorbance of the colored products separated remain relatively stable at least up to 3 hours (Figure 12). In addition, a negligible decrease in the absorbance was also noted for 24 hours. This will allow dealing with large samples analysis and their measurement with comfortable and appropriate times. This will make the proposed methods are applicable to a large number of samples and thus can be used for the purposes of quality control in the pharmaceutical analysis.

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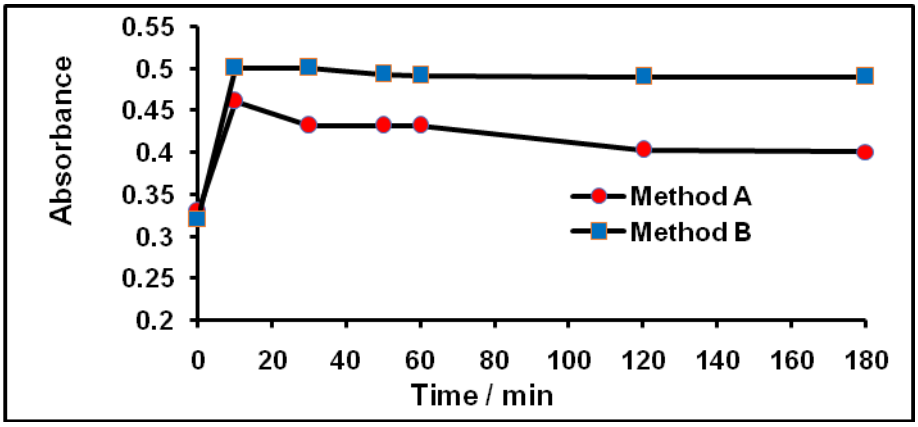

Figure 12: Effect of time on the colored products of AMX (1.0 µgml^{-1}) with DNPH at 554 nm and AMX (3.0 µgml^{-1}) with AAP at 479 nm.

Analytical methods performance

Under optimum conditions (Table 1), calibration curves for the assay of AMXT drug by its reaction with DNPH (method A) and AAP (method B) reagents were constructed by plotting the absorbance (at λ_{max} of 554 and 479nm) as a function of the corresponding concentrations as showed in Figure 13. Good regression lines were obtained in the concentration range of 0.2-2.0 µg mL^{-1} (method A) and 0.3-7.0 µg mL^{-1} (method B) for AMXT with percent linearity (R^2) of 99.84% and 99.94% which suggests a statistically valid fit. Analysis of variance (ANOVA) for the two regression lines was also conducted as showed in Table 2. ANOVA analysis supports that there is a strong significant relationship between the concentration of AMXT and absorbance units as F_{cal} >> F_{crit} (4131.61>> 8.81, dof 1, 6) and (9949.27>> 10.01), dof 1, 5) at α=0.05 (two-tailed), indicating that the predication based on the regression line is in conformity. These regression lines were used to estimate the AMXT concentration in the samples selected which appears justified on statistical basis.

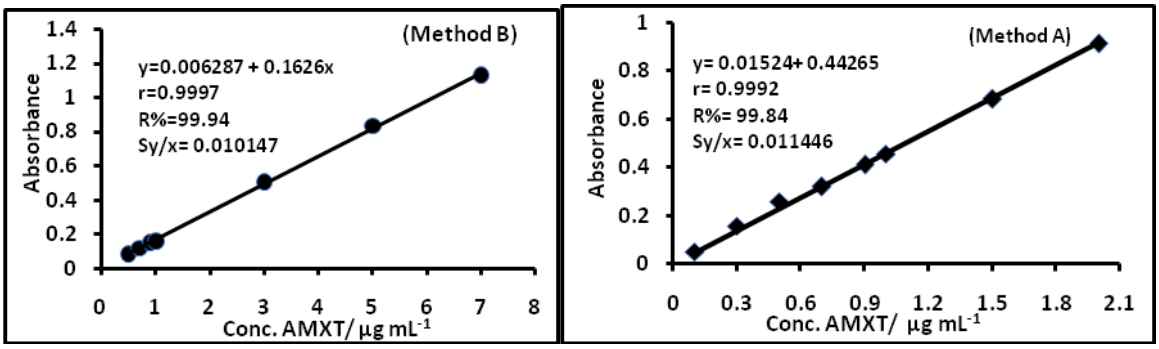

Figure 13: Calibration curves for AMX by the proposed method A and B.

Table 2: Analysis of Variance of regression line for AMX in method A and B

Method	**Source**	**dof**	**SS**	**MS**	**F -value**	Significance p-value
A	Regression	1	0.554135	0.554135	4131.61	**0.000**
	Residual (Error)	6	0.000805	0.000134		
	Total	7	0.554939			
B	Regression	1	1.024420	1.02442	9949.27	**0.000**
	Residual (Error)	5	0.000510	0.00010		
	Total	**6**	**1.024930**			

Statistical analytical figures of merit of the proposed methods are summarized in Table 3. The enrichment factors were calculated as the ratio between the slope of a curve obtained using aqueous solutions submitted

to the CPE procedure (y=0.44265 x+0.01524) or (y= 0.1626x+ 0.00628)) and to that obtained without CPE (y=0.003 x+0.002) and found to be of 147.55 and 54.20-fold for method A and B respectively. These enrichment factors allow to detect the drug in aqueous solutions spectrophotometrically at low detection limit of 0.077 (method A) and 0.170 µg mL^{-1}(method B) which based on the standard deviation of the response (residual standard deviation; σy/x) and the slope (s) of the calibration curve using the equation; LOD = $3\sigma_{y/x}/s$. The detection limits obtained by the proposed methods was in harmony with a few but much better than obtained with most reported methods in literature using different diazotization reactions with various chromogenic reagents as listed in Table 4. Further, the molar absorptivity (ε) of AMXT was found to be of $3.57x10^5$ and $1.00 x10^5$ $L.mol^{-1}cm^{-1}$ for method A and B respectively, indicating the excellent sensitivity of the proposed methods.

Table 3: Statistical data and analytical figures of merits for AMX by. Method A and B

Parameter	Method A	Method B
Product colour	Dark purple	Orange
λ_{max} (nm)	554	479
Regression equation (8 and 7 points)	y=0.01524+0.44265x	y= 0.00628+0.1626x
Standard deviation of regession line($S_{y/x}$)	0.011446	0.0110147
Correlation coefficient (r)	0.9992	0.9997
Coefficient of determination (R^2)	99.84	99.94
C.L. for the slope (b± ts_b) at 95%	0.44265±0.00734	0.1626±.0.01511
C.L. for the intercept (a± ts_a) at 95%	0.01524±0.06272	0.00628±0.00598
Beer's law range (µg mL^{-1})	0.2-2.0	0.3-7.0
Limit of Detection (µg mL^{-1})	0.077	0.17
Limit of Quantitation (µg mL^{-1})	0.28	0.63
Sandell's sensitivity (µg cm^{-2})	0.00226	0.0619
Molar absorptivity ($L.mol^{-1}.cm^{-1}$)	$3.57x10^5$	$1.00x10^5$
Compsition of the colored product*	1:2	1:1
RSD% (n=5)	3.21 at 0.7 µg mL^{-1}	1.34 at 1.0 µg mL^{-1}
RSD% (n=5)	0.10 at 1.5 µg mL^{-1}	0.16 at 7.0µg mL^{-1}
Preconcentration factor**	33.3	33.3
Enrichment factor	147.55	54.20
Recovery (%)	99.77±0.21	100.62±2.21
Extraction efficiency (%E)	99.30	99.60

* limiting logarithmic method ** Preconcentration factor was calculated the ratio of the original sample volume to that extracted volume of surfactant-rich phase.

Table 4: Reported methods for the determination of AMX by spectrophotometry after diazotization, oxidative coupling and charge transfer reactions.

Coupling Reagent Used/ Reaction Type	λ_{max} (nm)	Linearity (µg m^{l-1})	LOD (µg m^{l-1})	Ref.
MetocjhlopramideHCl/diazotization CPE	479	0.3-3.0	0.083	1
p-amino benzoic acid	435	0.4-10	0.187	9
procain / diazotization	450	0.4-14	0.192	
p-nitroaniline/ diazotization	478	0.5-100	0.104	10
2, 4- dinitrophenylhydrazine/ Oxidative Coupling	520	4-33	0.090	12
4-Aminoantipyrine /Oxidative coupling	510	1-60	0.173	13
o-nitroaniline /diazotization	435	25-400	5.100	14
N,N-dimethyl-p-phenylenediamine and potassium hexacyanoferrate (III)/ Oxidative coupling	600	2-40 10-70	0.637 4.900	15

Benzocain /diazotization	455	2-16	0.240	17
Metol / Charge transfer	620	5-60	1.494	18
bromocresol green/ ion pairing	630	1-13	0.060	19
$FeCl_3$+1,10-phenanthroline	510	2-20	-	27
o-nitroaniline/ diazotization	435	1-5	0.125	28
Ninhydrin	578	1-80	2.410	29
Sulphanilic acid/ diazotization	455	0.3-30	0.150	30
N-bromosuccinamid (NBS)+ methylene blue/ oxidation	663	5-50	-	31
2,4- dinitrophenylhydrazine (DNPH)/CPE	545	0.2-2.0	0.077	This
4-Aminoantipyrine /Oxidative coupling/CPE	479	0.3-7.0	0.170	work

Accuracy and Precision Study

Table 5: Accuracy and precision test for AMX in pharmaceutical and urine samples by proposed method (A)

sample	AMXT Taken (µg mL^{-1})	AMXT Found (µg mL^{-1})	Rec. (%)	Mean Rec%±C.L. at α=0.05	Accuracy (%E_{rel})	Precision (%RSD) (n=5)
Amoxicillin	Sample	1.000	-	99.07± 3.72	-	-
vial	0.7	1.703	100.40		0.40	0.783
(France)	1.0	1.973	97.30		-2.70	0.592
	2.0	2.990	99.50		-0.50	0.285
Urine sample	0.7	0.679	97.00	97.76±2.83	-3.00	1.020
	1.0	0.972	97.20		-2.80	0.250
	1.5	1.486	99.07		-0.93	0.067

Since the certificate reference materials (CRM's) that define exactly the true value of AMXT drug are not available, the validity of the proposed methods was evaluated via accuracy test in terms of recovery percentage. Standard additions method was conducted by spiking different concentrations of AMXT standard solutions (Table 5 and 6) to the drug sample prepared from vial produced from PANPHARMA S.A.(France) containing 1.0 µg mL^{-1} AMXT followed the recommended CPE procedures (A and B) and each solution was measured five times. The data in Table 5 and 6 revealed that the accuracies of the proposed methods were within the acceptable confidence limits at α=0.05, indicative the suggested methods are unbiased and confirmed that they are relatively interferences-free, from drug excipients that might be added during pharmaceutical formulation. Hence, the study of interferences from the drug matrix is almost an undue. Meanwhile, each spiked sample was repeated five times for precision test in terms of %RSD and found in the range of 0.28-0.78% and 0.8-4.5% for method A and B respectively, indicative a good precision. Moreover, the trueness of the developed methods was evaluated in the blank urine sample taken from a normal volunteer (method A) to test their applicability in this matrix. Each sample was spiked with different concentrations of AMXT standard solutions, followed the recommended CPE procedure (A) and each solution was measured five times as shown in Table 5. The results revealed that the proposed methods have once again demonstrated that they are relatively free from systemic and random errors when applying these methods in determining this medication in these matrices. Thus the proposed methods can be used in assay of AMXT in biological samples.

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Table 6: Accuracy and precision test for AMX in pharmaceutical and water samples by proposed method (B)

sample	AMX Taken ($\mu g\ mL^{-1}$)	AMX Found ($\mu g\ mL^{-1}$)	Rec. (%)	Mean Rec%±C.L at α=0.05	Accuracy (%E_{rel})	Precision (%RSD) (n=5)
Amoxicillin vial (France)	Sample	1.000	-	99.25± 2.06		
	1.0	1.984	98.40		-1.60	4.50
	3.0	4.018	100.06		0.04	1.67
	5.0	5.965	99.30		-0.70	0.80

Applications

In light of the above results that obtained from the proposed methods with standard solutions of the drug, which gave satisfactory analytical features, the methods were applied to determine the content of AMXT drug in various matrices to test its merit and reliability in routine chemical analysis**.**

Determination of AMXT drug in pharmaceuticals

The proposed methods A and B were applied to the determination of AMXT drug in three selected pharmaceutical vials produced in different countries containing 500 mg amoxicillin as an active ingredient. The samples were prepared as stated in the experimental section from which each sample was subjected to the recommended CPE and AMX drug estimated spectrophotometrically at λ_{max} of 554 and 479 nm. The results are presented in Table 7 and 8. The results revealed that the calculated t-values for AMX determination in different pharmaceuticals using DNPH and AAP as coupling reagents are less than t-tabulated (4.303) at 95% confidence interval and (n-1) degrees of freedom, so the null hypothesis Ho is maintained, concluding there is no evidence for systematic and random errors at the 95% confidence level and accordingly manufacturer's claims can be accepted.

Table 7: Determination of AMX drug in vial samples by the proposed method (A) and statistical comparison with quoted values.

Commercial name, and content	Practical Content(mg) (proposed method)	t=(x-μ)√n/s proposed method Vs. Claimed value at 95% C.I.	Mean %E_{rel}	%RSD (n=3)
Amoxicillin- AMITRON (Barcelona)LDP Laboratorios TORLAN S.A (Spain), vial 500mg	502.5 490.0 498.3 496.93±6.36	t_{cal}=0.84 0.84<4.303 p=0.492	-0.61	1.28
Amoxicillin (PANPHARMA S.A., France), vial 500 mg	502.0 495.7 497.5 498.40±3.24	t_{cal}=0.85 0.85<4.303 P=0.483	-0.32	0.65
Amoxicillin Pharma Roth (Germany), vial 500 mg	502.0 482.5 498.3 494.27±10.36	t_{cal}=0.96 0.96<4.303 P=0. 439	-1.15	2.10

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Table 8: Determination of AMX drug in vial samples by the proposed method (B) and statistical comparison with quoted values

Commercial name, and content	Practical Content(mg) (proposed method)	t=(x-μ)√n/s proposed method Vs. Claimed value at 95% C.I.	Mean $\%E_{rel}$	%RSD (n=3)
Amoxicillin- AMITRON (Barcelona)LDP Laboratorios TORLAN S.A (Spain), vial 500mg	471.0 500.3 497.3 489.53±10.12	t_{cal}=1.12 1.12<4.303 p=0.378	-2.09	3.29
Amoxicillin (PANPHARMA S.A., France), vial 500 mg	492.0 500.3 496.5 496.27±2.40	t_{cal}=1.56 1.56<4.303 P=0.260	-0.75	0.84
Amoxicillin Pharma Roth (Germany), vial 500 mg	507.0 500.8 493.3 500.37±6.68	t_{cal}=0.09 0.09<4.303 P=0. 935	0.74	1.37

Determination of AMXT drug in urine

Several pharmacokinetics studies indicated that the drug amoxicillin is ultimately excreted from the body through the urine and feces in significant quantities after its absorption into the blood stream, depending on the patient's status and pathological condition and excretion times [32] . Consequently , the application of the method (A) was directed to the possibility of detection the drug AMXT in urine samples in four normal volunteers to whom a single vial of Amoxicillin Pharma Roth (Germany) containing 500 mg AMXT was orally administered as described in the experimental section. The results are presented in Table 9. The results showed that the content of AMXT in urine was approximately at the same levels in all urine samples for up to 6 h and decreased thereafter. These results may not be consistent from the pharmacological point of view which indicates an increase in the secretion of the drug in the urine with time and be at its peak after 24 hours. However, these results can be accepted as a vital application for the proposed method to the case of non-transformation of amoxicillin to other metabolites as a result of metabolism.

Table 9: Determination of AMXT in urine samples by the proposed method A

Sample No.	Conc.AMX (μg mL^{-1}) after 2 hours	Con.AMX (μg mL^{-1}) after 4 hours	Con.AMX (μg mL^{-1}) after 6 hours	Con.AMX (μg mL^{-1}) after 8 hours
1	1.268	1.367	1.164	0.508
2	1.232	1.369	1.205	0.446
3	1.270	1.369	1.166	0.510
4	1.266	1.365	1.162	0.506
mean	1.259	1.367	1.174	0.4925
SEM*	0.00904	0.000957	0.0103	0.0155
C.L at 95%	±0.0290	±0.0035	0.0328	0.0485
t_4=2.776				

*SEM=standard error of mean (σ/√n)

Determination of AMXT drug in spiked Serum

The developed method (A) was used to check its applicability for the determination of AMXT in blood serum sample taken from one of the normal volunteer. The blood sample treated as described in the experimental section was divided into three (0.5 mL) portions and spiked with 0.7, 1.0 and 1.5 µg mL^{-1} of AMXT standard solutions. All the spiked serum samples were subjected to the recommended CPE procedure (A) and the drug AMXT determined spectrophotometrically at 554 nm for five replicate measurements. The results displayed in Table (10) revealed that good recoveries of the drug were achieved in the presence of serum matrix, suggesting the established method is unbiased, confirming it is relatively free from matrix interferences and therefore it can be adopted in routine analysis of AMXT in the clinical quality control laboratories.

Table 10: Assay of AMXT drug in serum sample by the method A

AMXT Taken (µg mL^{-1})	AMXT Found (µg mL^{-1})	Recovery (%)	Mean Rec%±C.L (t_{cri}=4.303 ,ϕ=2, 95% C.I)	Er%	RSD% (n=5)
0.7	0.681	97.29	97.57±4.27	-2.71	1.41
1.0	0.961	96.10		-3.90	0.82
1.5	1.490	99.33		-0.67	0.67

Determination of AMXT drug in water

Due to the lack of waste water from drug factories during the study, a tap water sample was taken in an attempt to apply the method (B) for estimating the drug in this matrix. Water sample was spiked with different concentration of AMXT standard solutions and followed the recommended CPE procedure B. The results presented in Table 11 revealed that the percent recovery of pure AMXT added was in the range of 97.08–102.44% with standard deviation of 0.220–0.952, indicating that the recovery was good, and that the water constituents did not interfere in the determination, concluding that the proposed method can be applied in environmental samples with high precision and accuracy.

Table 11: Determination of AMXT in tap water by the method B

Sample	AMX Taken (µg mL^{-1})	AMX Found (µg mL^{-1})	Recovery (%)	Mean Rec%±C.L at α=0.05	Accuracy (%E_{rel})	Precision (%RSD) (n=5)
Tap water	1.0	1.01	101.00	99.76±2.68	1.00	0.952
	3.0	2.97	99.00		-1.00	0.336
	7.0	6.95	99.29		-0.71	0.220

Statistical Comparisons of the Developed Methods

Table 12: Content of AMX (mg) in different samples by three suggested methods

item	sample	Method (A) mg/vial	Method (B) mg/ vial	Reported method (C) mg/ vial
1	AMX vial	502.5	471.0	488.0
	(Spain)	490.0	500.3	492.0
	500 mg	498.3	497.3	487.0
2	AMX vial	502.0	492.0	498.0
	(France)	495.7	500.3	483.7
	500 mg	497.5	496.5	493.8
3	AMX vial (Germany)	502.0	507.0	484.0
	500 mg	482.5	500.8	489.3
		498.3	493.3	498.2

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One factor experimental design (OFED) at one variable namely the content of active AMX in the analyzed pharmaceutical samples is used to find out the significance of obtained results in the two proposed methods (A and B) and one reported method (C) [1] via using ANalysis Of VAriance (ANOVA). The samples chosen for this experimental design are described in Table 12 for AMX in the selected samples measured in triplicate by each method.

The following hypotheses have been specified:

1. Null hypothesis (Ho) which specified that the mean content in all methods is equal, i.e.

$$\mu_A = \mu_B = \mu_C$$

2. Alternative hypothesis (Ha): which specified that at least one mean content in all methods is unequal, i.e.

$$\mu_A \neq \mu_B \neq \mu_C$$

This test was carried out at significance level (α=0.05) i.e. at 95% confidence level and it is assumed that the variances are equal in ANOVA. After analysis by using the statistical program (Minitab version 17), the ANOVA Tables of AMX content in the selected pharmaceutical samples is shown in the Table 13.

Table 13: ANOVA tables for AMX in the three selected pharmaceutical samples

Table	Sample	Source	dof	SS	MS	F	P-value
1	AMX vial	Method	2	37.98	18.99	0.17	**0.844**
	(Spain)	Error	6	653.99	109.0		
	500 mg	Total	8	691.97			
2	AMX vial	Method	2	67.33	33.66	1.23	**0.356**
	(Spain)	Error	6	163.63	27.27		
	500 mg	Total	8	230.96			
3	AMX vial	Method	2	148.7	74.37	1.08	**0.396**
	(Germany)	Error	6	411.6	68.61		
	500 mg	Total	8	560.4			

It can be noted from Table 13 that the calculated F^2_6-values were equal to 0.17, 1.23 and 1.08 for all samples selected. Since the critical F^2_6 -value at α=0.05 equals to 5.14 which higher than the calculated F-values, the null hypothesis (Ho) is accepted, indicative there is no significant difference in the drug content among the three methods in estimating AMXT content at 95% confidence. In addition, probability values were of high in all cases, indicating again the acceptance of the null hypothesis and no significant difference between the three methods. Moreover, the linear regression model in this design of ANOVA showed that the true value of drug content in the selected samples was equal to (490.71), (495.50) and (495.04) mg with the presence of random errors of each method through the coefficient values that appear beside each method as displayed in Table 14.

Table 14: Linear regression models in ANOVA design

Method	Linear regression equation
A	AMX content = 490.71+ 2.89 Method A - 1.18 Method B - 1.71 Method C
B	AMX content = 495.50+2.90 Method A + 0.77 Method B - 3.67 Method C
C	AMX content = 495.04 -0.78 Method A + 5.32 Method B - 4.54 Method C

CONCLUSION

Two simple and sensitive spectrophotometric methods after cloud point extraction were described for the determination of amoxicillin trihydrate in various matrices. The methods are based on oxidative coupling reactions of two reagents (2, 4 DNPH and 4-AAP) with AMXT drug in alkaline medium to form an

intense water-soluble and stable colored products which can be extracted into a nonionic surfactant as an eco-friendly mediating solvent. The developed methods have achieved characteristic features related to the analytical figures of merit and a good reliability in comparison to the other methods published (Table 4). Furthermore, the statistical comparison of the current established methods with our previous published paper have revealed that there is no significant difference in the drug content among the three methods in estimating AMXT content at 95% confidence, indicative the compatibility of these methods in determining of this medicament in the drug formulations. Therefore, these methods can be recommended for the routine analysis of AMXT in quality control laboratories. Also, these methods gave another additional advantage is their applicability to determine the drug in human urine, spiked serum and water samples. These merits make these methods a valuable alternative to many existing methods for the determination of AMXT in dosage forms, body fluid and environmental samples.

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