Micellar Enhanced Spectrofluorimetric Determination of Diphenylpyraline HCl and Dual Detection Cyclodextrin Micellar Liquid Chromatographic Method for Simultaneous Determination of Diphenylpyraline HCl, Paracetamol and Caffeine in Dosage Form

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Abstract

Highly sensitive micellar spectrofluorimetric method (Method I) has been developed and validated for the determination of diphenylpyraline HCl in pharmaceutical tablets and in plasma. Sodium dodecyl sulfate at pH 5 enhances the fluorescence intensity of diphenylpyraline at 286 nm that allow its determination at nano-level in plasma with mean percent recovery ± S.D of 99.719 ± 0.338. In addition, Green cyclodextrin micellar liquid chromatographic method (Method II) has been developed and validated for simultaneous determination of diphenylpyraline, paracetamol and caffeine using cyclodextrin micellar mobile phase consisted of 30 mM Brij*35, 0.5 mM hydroxypropyl β-cyclodextrin and phosphate buffer pH 4: MeOH (95:5, %v/v) that allows their simultaneous determination with enhanced spectrofluorimetric detection of diphenylpyraline. Method II was successfully applied for the simultaneous determination of diphenylpyraline, paracetamol and caffeine in a ternary laboratory prepared mixture containing all possible excipients with mean percent recoveries ± S.D of 100.176 ± 1.008, 101.166 ± 0.415 and 100.708 ± 1.836, respectively. The methods are verified to have excellent greenness.

1. Introduction

Diphenylpyraline HCl, DPP, (4-benzhydryloxy-1-methylpiperidine hydrochloride) is histamine H_1-receptor antagonist; antihistamine [1, 2]. It is used to treat allergy symptoms. The chemical structure of DPP is shown in (Fig. 1). DPP contains a benzenoid chromophore. A methylene group separates the two aromatic rings in DPP leading to the formation of isolated chromophores which explains the weakness of molar absorptivity and the native fluorescence properties of the drug.

Reported methods for determination of DPP are based on spectrophotometry [3], liquid chromatography [4], potentiometry using ion selective electrode [5] and, differential pulse voltammetric and conductimetric determinations [6]. Simultaneous estimation of DPP with other drugs using liquid chromatography [7], gas chromatography [8, 9] were also reported in literature.

Paracetamol, PAR, (N-(4-hydroxyphenyl) acetamide) is analgesic; antipyretic (Fig. 1) [1, 2]. PAR was determined by spectrophotometry [10–17], HPLC [16, 18–20] and GC [21]. Other methods concerning the simultaneous determination of PAR with other drugs as UV spectrophotometry [22–26], HPLC [24, 27–32], reversed-phase capillary liquid chromatography[33], and potentiometric determination using carbon paste sensor modified with gold nanoparticles [34] were also reported.

Caffeine, CFF, (1,3,7-trimethyl-3,7-dihydro-1H-purine-2,6-dione) is central nervous system stimulant [1, 2]. The chemical structure of CFF is shown in (Fig. 1). Literature survey revealed different methods for determination CFF either alone as: UV spectrophotometry [35–38], colorimetry [39], spectrofluorimetry [40], HPLC[41–43], HPTLC[44], GC [45, 46] and, ELISA [47] or with other drugs using UV spectrophotometry [48, 49], HPTLC [50–52], HPLC [53–56], UPLC [57].

Figure 1

Spectrofluorimetry has a major role in analysis of biological, environmental, and pharmaceutical samples due to its high inherent sensitivity. The method also shows increased selectivity due to measuring at both excitation and emission wavelengths[58–59]. Some analytes have low native fluorescence, so application of direct spectrofluorimetry is not sufficient to determine these analytes with high sensitivity. Different approaches were applied to enhance the fluorescence of fluorophores having low inherent native fluorescence. Approaches are based on confinement of analytes in organized media, e.g., micelles and cyclodextrins, that produce several supramolecular interactions with analytes. Inclusion in cyclodextrins' cavities or interaction with micelles, either with charged head or hydrophobic core, may lead to fluorescence enhancement. This may be attributed to restricting the fluorophore molecules movement and increasing their rigidity, and so the energy transfer to the surrounding environment by non-radiative relaxation was diminished. So, the quantum yield is increased and the intensity of fluorescence of the guest molecule is enhanced [60]. This enhancement has been employed to increase the selectivity and the sensitivity in various luminescence techniques [61, 62]. Moreover, the incorporation of surfactants and/or cyclodextrins in the mobile phases in micellar liquid chromatography (MLC) enables the isocratic separation and quantitation of analytes that have different physicochemical properties [63].

Not only enhanced spectrofluorimetry allows the determination of analytes in low concentrations, but also it serves to solve challenges in the field of pharmaceutical analysis. Usually two or more therapeutic agents are combined in fixed doses to enable treatment of one disease by different mechanisms, or controlling multiple related diseases. The major challenge that face the analyst is that how to analyse these drugs in the ratio of the dosage form, especially if the minor component in the combination product is also a poorly absorbing/emitting species. In this situation, usually two traditional approaches may be used. The first depends on spiking the dosage

form assay solution with a standard amount of the minor component to enrich that component in the sample, the method is termed “Sample enrichment technique” [64]. After that the spiked amount is subtracted to enable calculating the actual concentration of that component. The second approach depends on measuring the assay solution of the pharmaceutical product at two dilutions. The first measurement at high concentration ratio enables the determination of low absorbing/emitting species, then a second dilution is performed to allow the determination of the second species in its linearity range. Both approaches may not be applied to all cases, due to either error in the calculated recoveries of the minor component especially in the presence of high spiked concentration in the first approach, or high interference produced by the major component in the second. Enhancing fluorescence of minor components in organized media represents a new solution for such problems.

To the best of our knowledge, that there is no spectrofluorometric method for assay of DPP. Moreover, there is no reported method for simultaneous determination of DPP, PAR and CFF. The present study aims to develop and validate two methods for determination of DPP. Method I is based on enhancing the native fluorescence of DPP using the anionic surfactant sodium dodecyl sulfate (SDS), so allowing the spectrofluorimetric determination of DPP in pharmaceutical product and in spiked human plasma. The second method (Method II) is based on using b-cyclodextrin based MLC for the simultaneous determination of DPP, PAR and CFF. The three drugs show great variability in lipophilicity and spectroscopic properties. Also, DPP which is the minor component, is also the poorest absorbing/fluorescent species. Dual detection is performed by timely programmed operation of both UV and fluorescence detectors. The use of non-ionic surfactant Brij*35 and hydroxypropyl b-cyclodextrin (HP β-CD) in the mobile phase with the dual detection improved the chromatographic separation and quantitation of the three drugs in the ratio of the dosage form. The use of micelles and macromolecules impart excellent greenness to the developed methods.

2. Experimental

2.1. Instrument

2.1.1. Method I

All fluorescence spectra were recorded on a JASCO (FP-6300) spectrofluorometer (Tokyo, Japan). Emission spectra at 286 nm after excitation at 225 nm were recorded using the following measurement parameters; slit width of 10 nm, scanning speed of 1000 nm/min, the response was medium, and the sensitivity was medium. Spectral analysis was made using Spectra Manager software V1.53.01.

2.1.2. Method II

CMLC was performed on Aglient Technologies 1260 Infinity instrument (Santa Clara, USA) equipped with a G1311C quaternary pump, G1329B automatic injector, G1314F UV variable wavelength detector (VWD) detector and G1321C fluorescence detector (FLD). Chromatographic data were processed using Agilent Open LAB CDS Chemstation Edition software for LC system (Germany).

pH measurements were made by HANNA pH 211 Microprocessor pH-meter with double junction glass electrode (Rhode Island, USA). Filtration of mobile phase was through a 0.45-μm pore nylon membrane filter (47-mm) (Gelman, Germany) by Wiggens® V300 oil-free piston vacuum pump (Chem. Vac, China) and then the mobile phase was sonicated using 3510 Bransonic® ultrasonic cleaner (Branson Ultrasonics Corporation, USA). Memmert® thermostatic controlled water bath (SchwabachGmbh Co., Germany) was also used.

2.2. Materials and reagents

DPP (95%, purity), PAR (99%, purity) and CFF (98%, purity) were kindly obtained from Sigma Company for Pharmaceutical Industries (Quesna, Egypt). Tablet excipients including hydroxypropyl methylcellulose (HPMC K-15, 99.5% purity) (Iso-chem, fine chemicals), polyvinyl pyrrolidine (PVP-40T) (Sigma-Aldrich, MO, USA). povidone, crospovidone, magnesium stearate, calcium carbonate and starch were kindly obtained from Sigma Company for Pharmaceutical Industries (Quesna, Egypt). Sodium acetate trihydrate, sodium hydroxide, glacial acetic acid and citric acid monohydrate were purchased from ADWIC Co, Cairo, Egypt. Orthophosphoric acid (85% w/w, density = 1.685 g. mL^{-1}) (Sigma-Aldrich, MO, USA); potassium dihydrogen orthophosphate (Fisher Scientific, Pittsburgh, USA); methanol (MeOH) HPLC grade (Fisher Scientific, Pittsburgh, USA); a-cyclodextrin (α-CD)(Sigma-Aldrich, MO, USA), carboxy methyl cellulose (CMC) (ADWIC Co., Cairo, Egypt), sodium dodecyl sulfate (SDS; 99% purity) (Sigma-Aldrich, MO, USA), cetyltrimethylammonium bromide (CTAB; 96% purity) (Sigma-Aldrich, MO, USA); tween-80 (ADWIC Co., Cairo, Egypt), and tween-20,

HPβ-CD (Sigma-Aldrich, MO, USA), Brij*35 (Mol. Wt. 1199.56) (Fischer Bioreagents, Belgium), triethylamine (TEA) (Merck Schuchardt, Germany), Human blank plasma was obtained from Tanta University Hospital (Tanta, Egypt).

Teorell and Stenhagen buffer consists of a mixture of 1.0 M sodium hydroxide, 0.1 M hydrochloric acid, 1.0M citric acid, and 1.0 M phosphoric acid [65].

2.3. Chromatographic conditions

Chromatographic separation was performed on X-Bridge™ C18 column (150 x 4.6 mm, 5µm). The column temperature was adjusted at 40°C. Cyclodextrin micellar mobile phase consisted of 30 mM Brij*35, 0.5 mM HP β-CD and phosphate buffer pH 4: MeOH (95:5, %v/v). For preparation of 100 mL of the mobile phase, 3.6 g Brij*35 was first melted in a water bath, mixed with 0.073 g HP β-CD, then a volume of 95 mL phosphate buffer pH 4 was added with stirring on a magnetic stirrer till the solution become homogenous. The solution is allowed to be cooled at room temperature, then 5 mL MeOH was added, and then the mobile phase was filtered and sonicated for 30 min. The mobile phase was prepared daily and delivered isocratically at a flow rate of 0.8 mL.min^{-1}. The column was equilibrated for 45 min before use. The injection volume was 20 µL. UV detector was programmed to operate at 273 nm and after 3 minutes of run time changed to 245 nm for determination of CFF and PAR, respectively while fluorescence detection of DPP was performed at excitation wavelength of 225 nm and emission wavelength of 286 nm.

2.4. Standard solutions

Stock standard solutions (1 mg. mL^{-1}) of each of DPP, PAR and CFF were prepared by dissolving 25 mg of each of them separately in distilled water in 25-mL volumetric flask. 10 mL of each stock standard solution was further diluted with distilled water to 100-mL to obtain working standard solutions I that contain 100 µg. mL^{-1} of each. Moreover, working standard solutions II were prepared by diluting 10 mL of the previously prepared working standard solutions I to 100 mL using distilled water. Solutions were stable for at least 1week in the refrigerator.

2.5. General analytical procedure and calibration curves

2.5.1. Method I: For determination of DPP in bulk

In a series of 10-mL volumetric flasks, measured volumes of DPP working standard solution II (10 µg. mL^{-1}) were accurately transferred, 1 mL Teorell and Stenhagen buffer pH 5 and 1mL of 1% SDS were also added, mixed thoroughly, and then, completed to volume with distilled water to prepare solutions covering the concentration range of 0.1–1 µg. mL^{-1}. A parallel blank experiment was performed. The relative fluorescence intensity (*RFI*) of the resulted solutions were measured at 286 nm after excitation at 225 nm. Calibration curve constructed by plotting RFI_{286nm} vs. concentration of DPP (µg. mL^{-1}) and regression equation was computed.

2.5.2. Method I: For determination of DPP in plasma

Aliquots of 0.5 mL of human blank plasma were spiked with different volumes of 1 mg. mL^{-1} DPP, transferred into centrifugation tubes, and then 2.5 mL of acetonitrile was added to each, vortex mixed and centrifuged at 3000 rpm for 15 min. One mL of the supernatant was transferred into a series of 10-mL volumetric flasks, and then procedures were completed according to section **2.5.1.** to prepare solutions covering the concentration range of 0.2 to 0.5 µg. mL^{-1}. Construction of calibration curve was done by plotting RFI_{286nm} against the corresponding concentrations of DPP (µg. mL^{-1}) and the regression equation was derived.

2.5.3. Method II

In a series of 10-mL volumetric flasks, measured volumes of DPP, PAR and CFF stock standard /working standard solutions were accurately transferred, completed to volume with the mobile phase to prepare solutions covering the concentration range of 0.5–50 µg. mL^{-1}, 0.3–350 µg. mL^{-1}, and 0.3–50 µg. mL^{-1}, for DPP, PAR, and CFF respectively. Peak areas were integrated at 273 nm for CFF, 245 nm for PAR using UV detection and at 286 nm for DPP using fluorescence detection. Calibration curves were constructed by plotting peak area vs. drug concentration (µg. mL^{-1}), and regression equations were computed.

2.6. Applications of the developed methods

2.6.1. Determination of DPP in Lab prepared mixture using method I.

Dosage form containing DPP (Lergobine®) is not available in Egypt. A Lab prepared mixture was prepared by mixing the active ingredient with all possible excipients to prepare a mixture equivalent to the content of one tablet. The following ingredients were mixed: 5 mg DPP, 30 mg hydroxy propyl methyl cellulose (HPMC K-15), and 2 mg polyvinyl pyrrolidine (PVP-40T). The mixture was transferred into 100-mL volumetric flask, dissolved in 50 mL distilled water, sonicated for 20 min, and then the solution was made up to the required volume using distilled water. The solution was filtered, and the first 10 mL of the filtrate was discarded. The previous stock tablet solution is claimed to contain 50 µg. mL^{-1} DPP. An aliquot equivalent to 10 mL of the filtrate was transferred to a 100-mL volumetric flask, diluted to 100 mL using distilled water to prepare a working tablet solution containing 5 µg. mL^{-1} DPP. 1 mL of the previous solution was transferred to a 10-mL volumetric flask and procedures were completed as mentioned in section **2.5.1**. The prepared tablet assay solution is considered to contain 0.5 µg/mL of DPP. The assay solution was prepared six times. The concentration of DPP in Lab prepared mixture was obtained from the corresponding regression equation, and the percent recoveries ± standard deviation were calculated.

2.6.2. Application of method II to pharmaceutical dosage form

The dosage form containing DPP, PAR, and CFF (Corytab®) is not available in Egypt. A Lab prepared mixture is prepared by mixing the active ingredients with all possible excipients to prepare a mixture equivalent to the content of one tablet. The following ingredients were mixed: 2 mg DPP, 300 mg PAR, 16 mg CFF, 12 mg povidone, 30 mg crospovidone, 6 mg magnesium stearate, 12 mg calcium carbonate and 222 mg starch. The mixture was dissolved in 50 mL of the mobile phase, transferred into a 100-mL volumetric flask, sonicated for 20 minutes, and then completed to volume using the mobile phase, mixed well and filtrated. Tablet assay solution is prepared by diluting 10 mL of the previously prepared filtrate to 100 mL by the mobile phase. The prepared solution is considered to contain: 2 µg. mL^{-1} of DPP, 300 µg. mL^{-1} PAR, and 16 µg. mL^{-1} CFF. The assay solution is prepared six times, and procedures are completed as mentioned in section **2.5.3**. Concentrations of the studied drugs in the Lab prepared mixture were obtained from the corresponding regression equations, and the percent recoveries ± standard deviation were calculated.

3. Results And Discussion

DPP has a weak native fluorescence with maximum fluorescence intensity at l_{em} 286 nm after excitation at l_{ex} 225 nm in water. SDS causes a great enhancement in the fluorescence spectrum of DPP (Fig. 2) which allows its determination in very diluted solutions, dosage form, and plasma (C_{max} of DPP equals 0.1 µg. mL^{-1}). Generally, the micellar system creates a highly viscous media that would limit the movement of the enclosed fluorophore so minimizing the possibility of non-radiative deactivation process [66].The electrostatic attraction between the negatively charged head of SDS, and the positively charged DPP molecules may produce an additional limitation to DPP molecules movement and increase the rigidity leading to a great enhancement in the fluorescence intensity.

Although all the studied drugs are fluorescent, their simultaneous determination using spectrofluorimetry could not be achieved due to a phenomenon called resonance energy transfer (RET) which describes the transfer of the electronic energy between molecules [67]. Both CFF and PAR absorbed radiation at l_{em} of DPP leading to fluorescence quenching and the appearance of only a single emission peak for the ternary mixture. HPLC offered a solution for this problem through allowing separation of the three drugs using a suitable chromatographic condition. However, assay of such combination using LC met two challenges. The first was the great differences in lipophilicity of the three drugs (log P values are − 0.07, 0.46 and 3.9 for CFF, PAR and DPP, respectively) which require gradient elution to allow their separation. The addition of surfactants to the mobile phase in micellar liquid chromatography (MLC) could be a solution for such problem. Micelles in mobile phases allows isocratic separation of drugs with varying lipophilic characters without the need for gradient elution. The second challenge was the inability to determine DPP as a minor component in the presence of high concentrations of strongly absorbing PAR and CFF in the ratio of the dosage form. Dual UV/fluorescence detection with the incorporation of HP β-CD in the micellar mobile phase could solved this problem. HP β-CD enhanced the fluorescence of DPP, so allowing its direct determination in the laboratory prepared tablet without needing to perform sample enrichment or double dilution measurements.

3.1. Optimization of the experimental conditions

3.1.1. Method I

Different factors that influence the fluorescence of DPP were studied, e.g., type and concentration of organized media, pH, time, temperature, and type of diluting solvent.

a. Effect of organized media

The effect of various organized media including micellar solutions such as: CTAB (cationic surfactant), SDS (anionic surfactant) and tween-80 (nonionic surfactant) and different macromolecules, e.g. HP β-CD, a-cyclodextrin (α-CD), carboxy methyl cellulose (CMC) on the relative fluorescence intensity (*RFI*) of DPP were tested. SDS gave a marked increase in the $RFI_{286\ nm}$ of DPP (Fig. 3-a). The reason of this behavior may be due to the electrostatic attraction between the negatively charged anionic surfactant, SDS, and the positively charged DPP molecules leading to decrease the repulsion between the surfactant molecule head groups and completion of the micellization process as shown in (Fig. 4). The fluorescence enhancement caused by HP β-CD was also large (but still smaller than SDS). This finding suggested that addition of HP β-CD to the micellar mobile phase (method II) may lead to increase the sensitivity of the method for DPP determination.

The effect of SDS concentration was tested through using different volumes of 1% SDS in the range of 0.25 to 2.5 mL. It was observed that increasing volume of SDS resulted in an increase in RFI_{286} up to 1 mL, after which RFI_{286} was nearly constant. Therefore, 1 mL of 1% SDS solution was chosen as the optimum volume for determination of DPP (Fig. 3-b).

b. Effect of pH, type, and concentration of buffer

The effect of pH on the enhanced fluorescence of DPP was studied using Teorell and Stenhagen buffer covering the pH range from 2 to 10 (Fig. 3-c). It was found that fluorescence enhancement usually achieved at acidic pH values (pKa of DPP = 8.87) where the drug presents in the ionized cationic form. Buffer with pH 5 gave the maximum $RFI_{286\ nm}$. The electrostatic attraction between the negatively charged SDS and the positively charged DPP molecules will produce an additional limitation of the DPP molecules movement and increase its rigidity leading to a great enhancement in the fluorescence intensity.

Different buffers including acetate buffer, citrate buffer, and Teorell and Stenhagen buffer at pH 5 were tried to evaluate the effect of buffer type. It was found that maximum and constant RFI was achieved by using Teorell and Stenhagen buffer (pH 5) (Fig. 3-d). The influence of Teorell and Stenhagen buffer volume on the $RFI_{286\ nm}$ of DPP was also studied (Fig. 3-e). It was observed that increasing volumes of buffer solution resulted in an increase in $RFI_{286\ nm}$ up to 1 mL, after that a decrease in $RFI_{286\ nm}$ was observed. Therefore, 1 mL of Teorell and Stenhagen buffer pH 5 was chosen as the optimum for determination of DPP.

c. Effects of time and temperature

The effect of time on the fluorescence of DPP was determined by monitoring $RFI_{286\ nm}$ for 60 minutes. It was observed that the enhanced fluorescence is developed immediately and is stable throughout the time of the study.

The influence of temperature was also assessed by varying reaction temperature in the range 10 to 60°C. It was observed that increasing the temperature had led to a marked decrease in $RFI_{286\ nm}$. This may be attributed to increasing the possibility of the internal/external conversion to occur, so allowing non-radiative deactivation of the excited singlet state to occur easily and an increase in the loss of energy through collision with other unexcited drug or matrix molecules, and so, fluorescence quenching [61]. Lower temperatures (< 25^{o}C) increased the $RFI_{286\ nm}$ but its application is difficult. Therefore, all experiments were carried out at room temperature (25**°C**).

d. Diluting solvent effect

The influence of different diluting solvents (ethanol, methanol, acetonitrile, and water) on the $RFI_{286\ nm}$ of DPP was also investigated. The results revealed that the best solvent for dilution was water, as it provided the highest $RFI_{286\ nm}$ and the lowest blank reading. Obvious decrease in $RFI_{286\ nm}$ was observed when dilution was made by other organic solvents. The primary reason for this effect could be attributed to the denaturizing effect of organic solvents on the formed micelle as they dissolve in water and change their properties leading to decreasing micelles formation. In addition, organic solvents may also result in a decrease in micellar size [68].

e. Order of Addition

Different addition orders were tried and $RFI_{286\ nm}$ were measured. The following order (drug→ buffer → SDS) achieved the maximum fluorescence intensity as the drug must be positively charged by the buffer to facilitate binding to micelles' surface and then reaching the core (Fig. 3-f).

3.1.2. Method II

Different factors were optimized regarding the system suitability parameters. The optimum condition gave shorter retention times (t_R) with best resolution and tailing factor while number of theoretical plates, capacity factor and selectivity factor were usually acceptable.

a. Effect of type and concentration of surfactant

Different type of surfactants were tried. Surfactants are either used alone in the mobile phase or in combinaion (SDS, tween 20, Brij*35 and mixture of SDS and Brij*35). SDS can separate CFF and PAR while DPP was retained on the column and cannot be eluted by SDS. Tween 20 can separate the 3 drugs but runtime was very long. Only Brij*35 can separate the 3 drugs within a suitable runtime (Fig. 5). Different concentrations of Brij*35 in the range of 20 to 40 mM were tried. 30 mM Brij*35 was chosen as the optimum concentration with respect to retention time and tailing factor as shown (Fig. 6).

b. Effect of pH and type of buffer

Micellar mobile phases containing TEA/phosphoric acid with different pH values were tried. Only acidic mobile phases in the range of pH 2.5-5 showed short retention times and more symmetric peaks. Mobile phases with pH values < 4 were excluded due to bad resolution (Rs < 1.5). Mobile phase with pH 4 was chosen as optimum for separation of the studied drugs regarding t_R and T (Fig. 7). Different buffers including TEA/phosphoric, citrate, acetate, and phosphate at pH 4 were tried. Phosphate buffer was chosen as optimum buffer with respect to the retention time and tailing factor (Fig. 8).

c. Effect of concentration of hydroxypropyl β-cyclodextrin

Different concentrations of HP β-CD (0.1 to 5.0 mM) in the mobile phase were tried and peak area of DPP using fluorescence detector were recorded. 0.5 mM HP β-CD was chosen as the optimum concentration that achieved the maximum degree of enhancement of DPP fluorescence, so enabling its determination in the dosage form simultaneously with PAR and CFF (Fig. 9).

d. Effect of type and concentration of organic modifier in the mobile phase

Different types of organic modifier were tried (methanol, ethanol, propanol, butanol, and acetonitrile). MeOH was chosen as the best organic solvent with respect to the retention time and tailing factor (Fig. 10). Moreover different ratios of methanol (MeOH) in the mobile phase (5–25%) were tried. Retention time, tailing factor and resolution values were compared. Although increasing the percent of MeOH showed improvements in retention time and tailing factor especially for CFF and PAR, concentrations higher than 10% had bad impact on resolution. MeOH concentration of 5% and 10% shows nearly similar results regarding t_R, T and R_s, however 5% MeOH was chosen as the best to decrease the percent of organic modifier consumed by the method and so increasing the greenness of the method (Fig. 11).

e. Effect of temperature

Different column temperatures were tried in the range of 25 to 40°C. Column temperature of 40°C was chosen as optimum as it decreased the back pressure of the mobile phase and prevent the precipitation of the mobile phase into the system.

f. Effect of flow rate

Flow rate was increased gradually to decrease run time, enhance peak shape with maintaining acceptable resolution reaching 0.8 mL. min^{-1}after which the back pressure was very high.

The following experimental parameters were set as the optimum: [HP β-CD] = 0.5mM, [Brij*35] = 30 mM, 5%MeOH and 95% phosphate buffer pH 4. The optimum conditions were tried on a ternary mixture containing 16 µg. mL^{-1} CFF and 300 µg. mL^{-1} PAR and 2 µg. mL^{-1} DPP (Fig. 12), which is the same ratio of drugs in their tablet, and results for system suitability tests were calculated as shown in **Table 1**.

Table 1: System suitability parameters for the simultaneous determination of CFF, PAR and DPP (method II)

Parameter	CFF	PAR	DPP	Reference value [71]
Retention time, t_R (min)	2.323 ± 0.05	4.21 ± 0.02	5.34 ± 0.06	-
Tailing factor, T	1.6	1.62	1.7	≤ 2
Capacity factor, k'	3.65	7.42	9.67	≥ 2
Selectivity factor, α	1.81	1.81	1.24	≥ 1
Theoretical plates, N	2370	2264	2120	≥ 2000
HETP, mm	0.633	0.663	0.708	-
Resolution, Rs	6.53	6.53	2.13	≥ 2

3.2. Methods Validation

The proposed methods were validated regarding the ICH guidelines [69].

3.2.1. Linearity

For Method I, calibration curves were constructed by plotting the RFI_{286nm} of DPP versus the corresponding concentrations (µg/mL). For Method II, calibration curves were constructed by plotting the peak area of CFF, PAR and DPP solutions versus their corresponding concentrations (µg. mL^{-1}). Linearity range for Method I was 0.1–1µg. mL^{-1} for DPP and for Method II was 0.3–50, 0.3–350, and 0.5–50 for CFF, PAR and DPP, respectively. Method I was also applied in spiked human plasma with linearity range 0.2–0.5 µg. mL^{-1}. The statistical parameters of both methods are summarized in Table 2. The good linearity of the calibration curves was indicated by the high values of correlation coefficients (r) and negligible intercepts.

3.2.2. Detection and Quantitation Limits

The ICH guidelines [69] for calculation of detection limit (DL) and quantitation limit (QL) were followed based on dyandard deviation of the intercept and the slope of calibration curves. Calculated values of DL and QL are given in Table 2.

Table 2
The quantitative statistical parameters for the determination of DPP (method I) and for the simultaneous determination of CFF, PAR and DPP (method II)

Parameter	Method I		Method II		
	DPP (Bulk)	DPP (Plasma)	CFF (Bulk)	PAR (Bulk)	DPP (Bulk)
Concentration range (µg. mL^{-1})	0.1–1.0	0.2–0.5	0.3–50	0.3–350	0.5–50
Intercept (a)	-7.641	18.749	19.071	51.072	31.190
Slope (b)	787.269	680.298	66.849	104.764	97.644
Correlation coefficient (r)	0.9992	0.9992	0.9998	0.9998	0.9997
SD of residuals ($S_{y/x}$)	12.437	3.482	23.579	265.960	49.521
SD of intercept (S_a)	8.761	5.605	10.109	91.118	26.216
SD of slope (S_b)	14.180	15.535	0.429	0.652	0.997
DL (µg/mL)	0.037	0.027	0.499	2.870	0.886
QL (µg/mL)	0.111	0.082	1.512	8.697	2.685

3.2.3. Accuracy

The methods accuracy was assessed by triplicate analysis of three different concentrations of the studied drugs within their linearity ranges. The percent recovery ± SD were shown in (**Table 3)**. The values of percent recovery ± SD for DPP using Method I were 100.539 ± 0.565 and 99.719 ± 0.338 in bulk and in spiked human plasma, respectively. For Method II, The values of percent recovery ± SD were 100.144 ± 0.455, 100.102 ± 0.381 and 100.106 ± 0.904 for CFF, PAR and DPP, respectively.

Table 3: Evaluation of the accuracy for the determination of the studied drugs by the developed methods

Method	Drug	Concentration taken ($\mu g.\ mL^{-1}$)	Concentration found ($\mu g.\ mL^{-1}$)			Mean concentration found* ($\mu g.\ mL^{-1}$)	%Recovery	Mean % Recovery ± SD
Method I	DPP (Bulk)	0.2	0.199	0.204	0.202	0.202	100.916	100.539 ± 0.565
		0.6	0.611	0.598	0.606	0.605	100.812	
		0.8	0.803	0.796	0.798	0.799	99.889	
	DPP (Plasma)	0.25	0.247	0.249	0.252	0.249	99.712	99.719 ± 0.338
		0.35	0.350	0.353	0.347	0.350	100.061	
		0.45	0.453	0.446	0.443	0.447	99.385	
Method II	CFF (Bulk)	1.5	1.499	1.5051	1.52	1.508	100.536	100.144 ± 0.455
		16	16.040	16.025	15.965	16.130	100.250	
		45	44.84	44.69	44.36	45.47	99.644	
	PAR (Bulk)	1.5	1.51	1.507	1.497	1.505	100.311	100.102 ± 0.381
		45	45.324	45	45.125	45.15	100.333	
		300	300	299.5	297.463	298.988	99.663	
	DPP (Bulk)	2	1.981	1.958	1.974	2.012	99.067	100.106 ± 0.904
		15	15.107	15.078	14.982	15.26	100.711	
		45	45.243	45.36	45.26	45.11	100.541	

3.2.4. Precision

Precision was evaluated by assaying three different concentrations of each drugs within its linearity range on the same day (intraday precision) and on three consecutive days (inter-day precision). The relative standard deviation values (% RSD) were not more than 1.407 for DPP using Method I and not more than 1.050, 1.344 and1.642 for CFF, PAR and DPP, respectively using Method II as shown in (Table 4**).**

Table 4: Evaluation of the intra-day and inter-day precision for the determination of the studied drugs by the developed methods

Method	Drug	Concentration taken ($\mu g.\ mL^{-1}$)	Intra-day			Inter-day		
			Mean Concentration found* ($\mu g.\ mL^{-1}$)	**SD**	**%RSD**	**Mean concentration found* ($\mu g.\ mL^{-1}$)**	**SD**	**%RSD**
Method I	**DPP (Bulk)**	**0.2**	0.202	0.003	1.407	0.202	0.001	0.461
		0.6	0.605	0.006	1.063	0.606	0.003	0.420
		0.8	0.799	0.004	0.448	0.798	0.001	0.155
	DPP (Plasma)	**0.25**	0.249	0.002	0.901	0.250	0.002	0.808
		0.35	0.350	0.003	0.839	0.348	0.003	0.829
		0.45	0.447	0.005	1.185	0.449	0.005	1.093
Method II	**CFF (Bulk)**	**1.5**	1.508	0.716	0.011	1.507	0.003	0.167
		16	16.040	0.521	0.084	15.850	0.166	1.050
		45	44.84	1.271	0.570	44.742	0.170	0.380
	PAR (Bulk)	**1.5**	1.505	0.452	0.007	1.493	0.0006	0.041
		45	45.150	0.362	0.164	45.446	0.151	0.332
		300	298.987	0.449	1.344	303.831	0.459	0.151
	DPP (Bulk)	**2**	1.981	1.400	0.028	1.997	0.033	1.642
		15	15.107	0.935	0.141	15.061	0.199	1.321
		45	45.243	0.278	0.126	45.210	0.233	0.516

N.B.: * n = 3.

3.2.5. Specificity

The percent recoveries of the studied drugs in Lab prepared mixtures containing the drug(s) with all expected excipients in dosage form were determined for testing specificity as shown in (**Table 5**). The ability of the developed method to determine each drug alone without any interference from other drugs/excipients proved the specificity of the method.

Method	**Drug**	**Concentration taken ($\mu g.\ mL^{-1}$)**	**Concentration found ($\mu g.\ mL^{-1}$)**						**Mean concentration found* ($\mu g.\ mL^{-1}$)**	**Mean % Recovery ± SD**
Method I	**DPP**	**0.5**	0.506	0.502	0.495	0.502	0.507	0.498	0.502	100.333 ± 0.918
Method II	**CFF**	**16**	16.015	16.045	15.989	16.237	16.128	15.755	16.028	100.176 ± 1.008
	PAR	**300**	302.596	301.99	303.478	302.965	304.99	304.969	303.498	101.166 ± 0.415
	DPP	**2**	2.087	1.995	1.987	1.997	2.010	2.009	2.015	100.708 ± 1.836

* n = 6.

Table 5

Recovery data of the studied drugs from Lab prepared mixtures by the developed methods

3.2.5. Robustness

The optimum conditions set for Method I have been slightly modified to test the method robustness. Small deliberate variations were made in the volume of SDS (1 ± 0.2 mL), pH of buffer solution (5 ± 0.2) and temperature (25 ± 2°C). For Method II, small deliberate variations were made in the concentration of Brij*35 (30 ± 1 mM), concentration of HP β-CD (0.5 ± 0.05 mM), % of MeOH (5% ± 2), pH of buffer solution (4 ± 0.2) and temperature (40 ± 2°C). The robustness of the methods was indicated by the accepted values of percent recoveries (100% ± 2%) and % RSD (values < 2) as shown in (Table 6**).**

Table 6
Robustness of the developed methods (***Optimum condition**)

Parameters	**Modification**	**% Recovery**			**Mean % Recovery**	**SD**	**%RSD**
Method I							
pH	4.8	99.17			100.33	1.329	1.325
	5*	100.04					
	5.2	101.78					
Volume of SDS (mL)	0.8	100.91			100.74	0.628	0.623
	1*	100.04					
	1.2	101.26					
Temperature (°C)	23	99.52			100.39	1.088	1.083
	25*	100.04					
	27	101.61					
Method II							
Parameters	**Modification**	**% Recovery**			**Mean %Recovery**	**SD**	**%RSD**
		CFF	**PAR**	**DPP**			
pH	3.8	101.5	100.11	100.25	100.12	1.032	1.030
	4*	99.86	101.28	99.06			
	4.2	98.25	100.85	99.91			
Temperature (°C)	38	100.35	100.57	99.80	101.04	0.636	0.635
	40*	99.86	101.28	99.06			
	42	100.4	100.04	100.75			
Concentration of Brij*35 (mM)	29	99.45	100.65	99.56	101.02	1.889	1.871
	30*	99.86	101.28	99.06			
	31	105.25	101.29	100.16			
Concentration of HP β-CD (mM)	0.45	102.45	99.995	99.78	100.44	0.981	0.977
	0.5*	99.86	101.28	99.06			
	0.55	102.39	100.79	99.68			
% MeOH	3%	100.40	100.53	99.92	100.32	0.652	0.650
	5%*	99.86	101.28	99.06			
	7%	100.49	100.65	100.80			

3.3. Assessments of the greenness of the developed methods

The developed methods greenness was assessed using the analytical Eco-Scale [70]. The ideal green method has a value of 100 and penalty points are assigned for each of the analytical procedure parameters e.g., reagents, hazards and waste that deviate from ideal green analysis. The calculated analytical Eco-Scale value of the developed methods were 78 and 82 for Method I and Method II, respectively which indicate excellent green analytical methods as shown in (Table 7**).**

Table 7
Calculation of greenness of the developed methods using analytical Eco-scale.

Method I		**Method II**	
Reagents	**Penalty points**	**Reagents**	**Penalty points**
SDS	0	Brij*35	0
Teorell and Stenhagen buffer	10	Phosphate buffer	0
Water	0	hydroxyl propyl β-cyclodextrin	0
		5%MeOH	6
∑	**10**	∑	**6**
Instrument	**Penalty points**	**Instrument**	**Penalty points**
Transport	1	Transport	1
Spectrofluorometer	2	HPLC/UV/fluoremetry	2
Occupational hazards	0	Occupational hazards	0
Waste	9	Waste	9
∑	**11**	∑	**12**
Total penalty points	**22**	**Total penalty points**	**18**
Score	**78**	**Score**	**82**

4. Conclusion

Two micelles mediated analytical methods were developed and validated for determination of DPP either alone or in combination with CFF and PAR. Method I is based on micelle enhanced spectrofluorimetry using SDS. The surfactant increases the fluorescence intensity of the drug allowing its determination in dosage form and in spiked human plasma. The method is simple, rapid, sensitive, and efficient in comparison with other spectrophotometric and chromatographic methods. Also a cyclodextrin based MLC method (Method II) was developed for simultaneous determination of CFF, PAR and DPP in their Lab prepared mixture with all possible excipients. Addition of organized media solved different challenges in analysis of the cited drugs in different samples. The greenness of the developed methods was judged. The presented methods are suitable for routine pharmaceutical analysis.

Abbreviations

DPP: diphenylpyraline HCl

PAR: paracetamol

CFF: caffeine

CMLC: Cyclodextrin micellar liquid chromatography

SDS: sodium dodecyl sulfate

HP β-CD: hydroxypropyl β cyclodextrin

DL: Detection limit

QL: Quantitation limit

%RSD: percentage relative standard deviation

S_a**:** standard deviation of the intercept

S_b: standard deviation of the slope

$S_{y/x}$**:** standard deviation of the residuals

S: slope

SD: standard deviation

T: tailing factor

t_R**:** retention time

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Figures

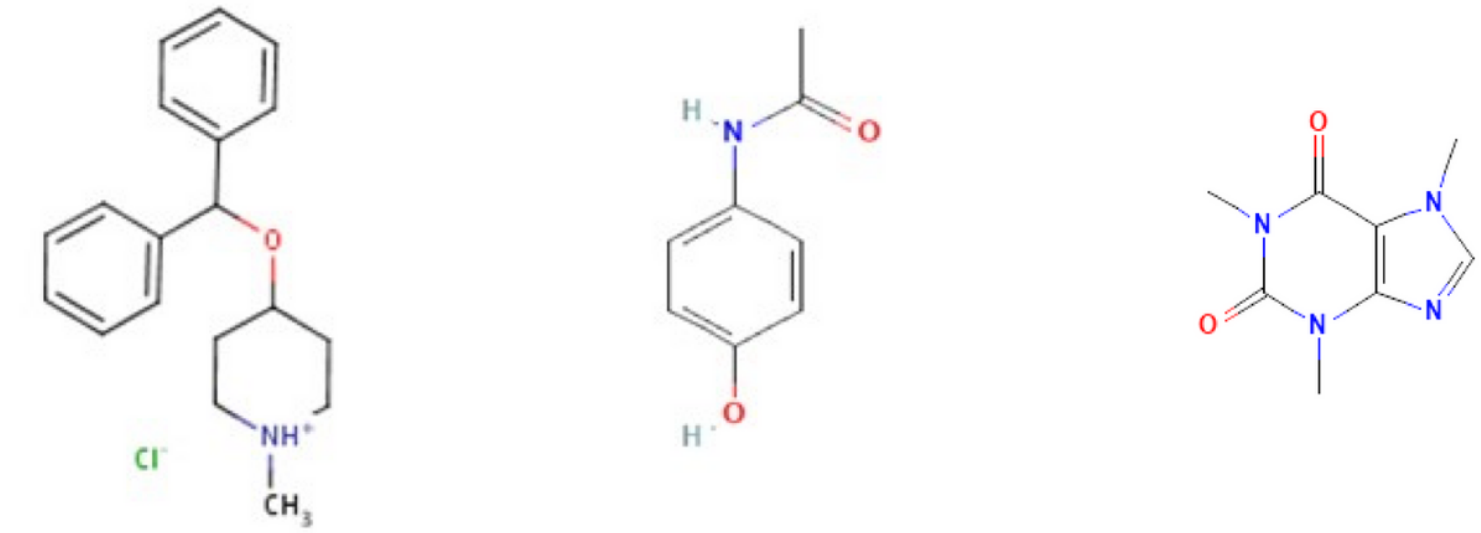

Figure 1

Chemical structures of DPP, PAR and CFF

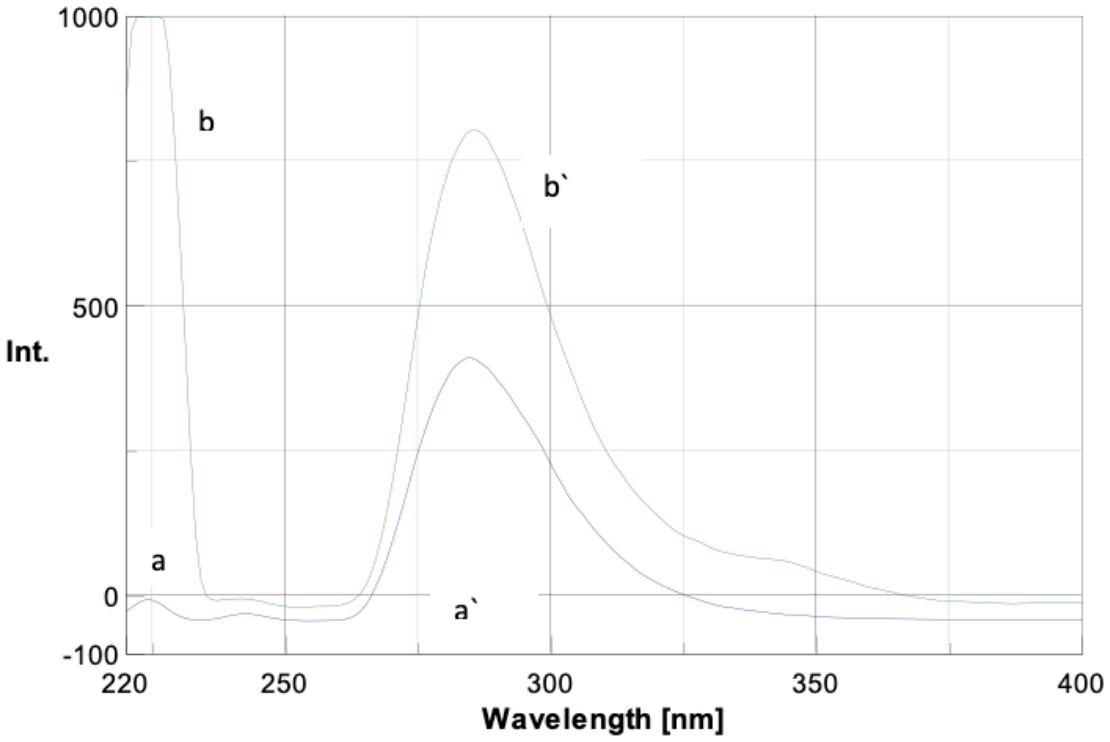

Figure 2

Excitation and emission spectra of DPP in distilled water in absence (a, a') and in the presence (b, b') of SDS .

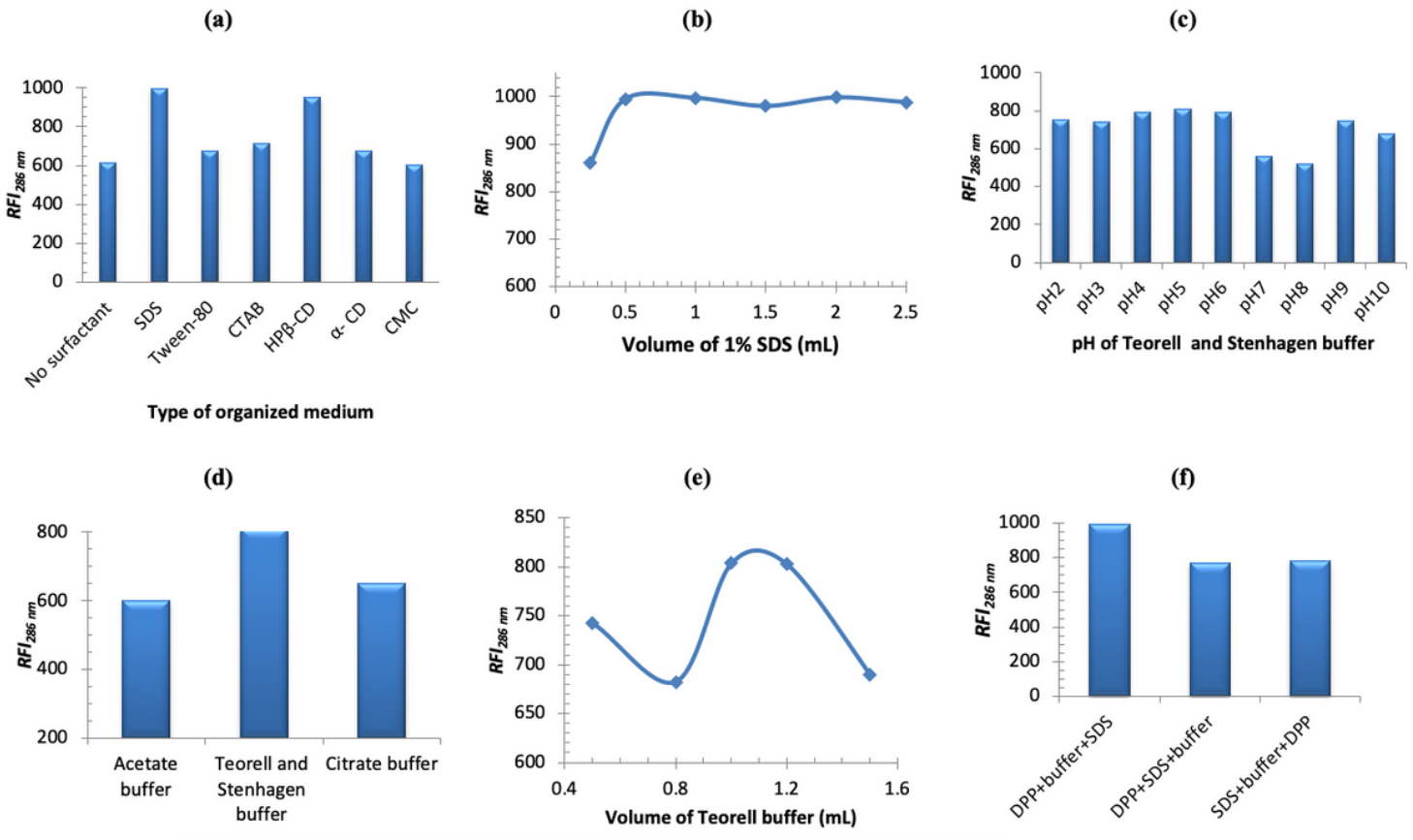

Figure 3

Graphical diagram showing reaction of DPP with SDS molecules.

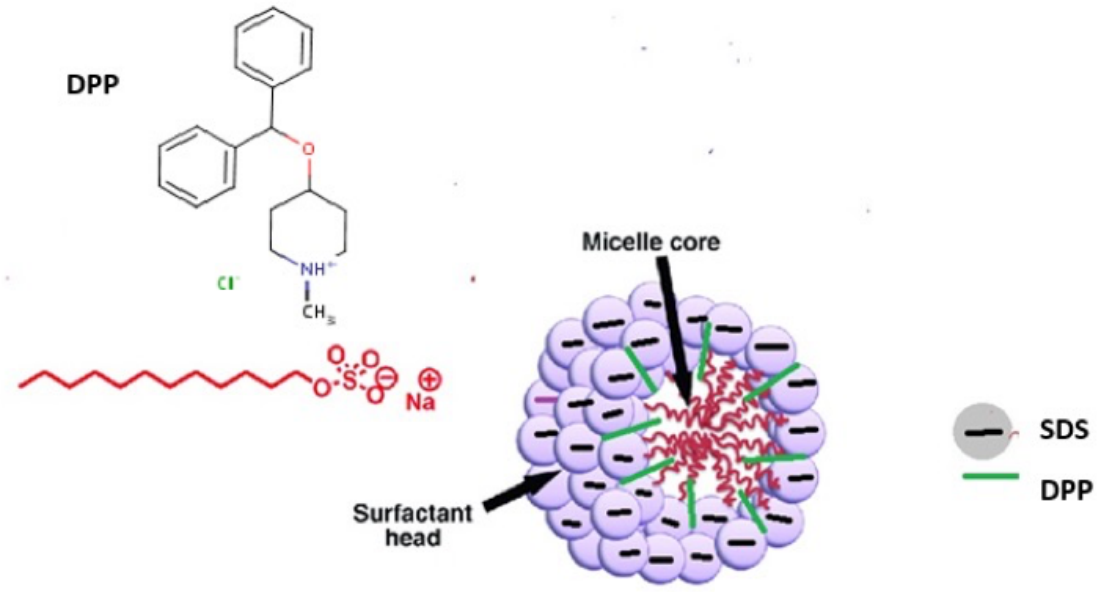

Figure 4

Chromatogram of DPP, PAR, and CFF in the same ratio of dosage form at the optimum chromatographic conditions.

Drug concentrations were: 2 µg. mL^{-1} DPP, 300 µg. mL^{-1} PAR, and 16 µg. mL^{-1} CFF. Detection at 273 nm and 245 nm using UV detector and at 286 nm using fluorescence detector.

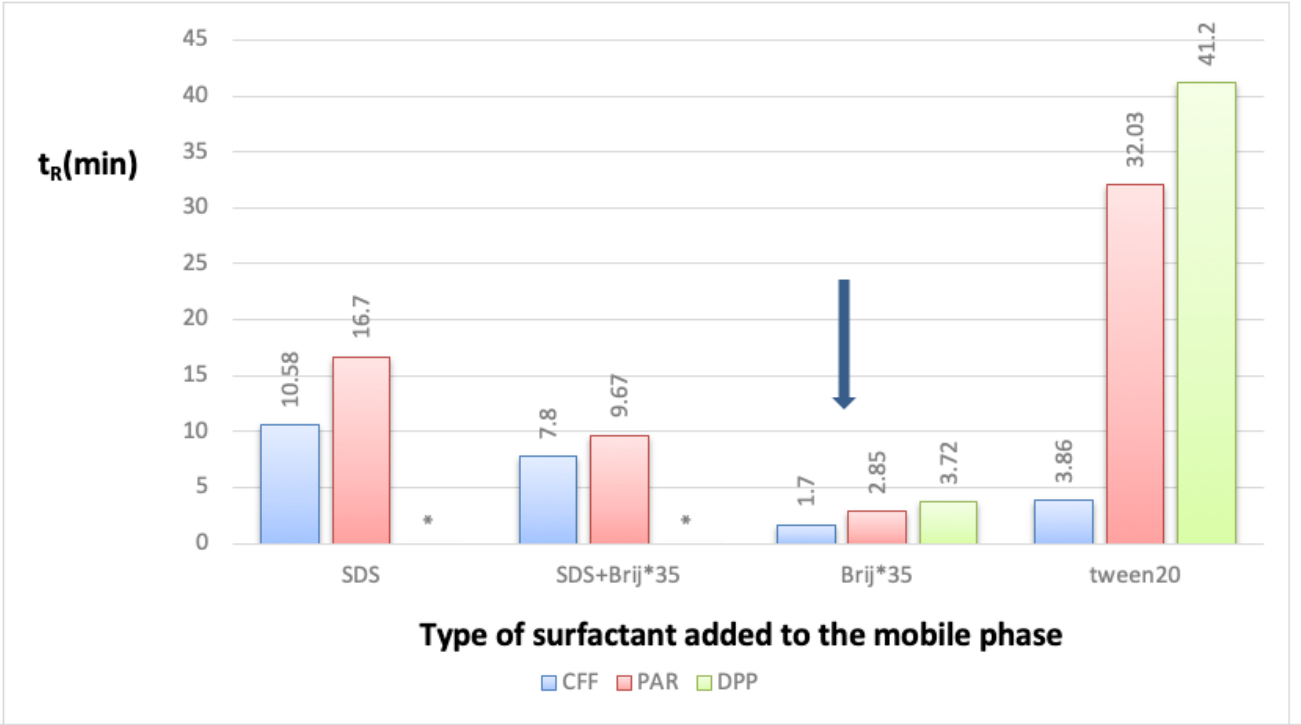

Figure 5

Effect of (a) type of organized media, (b) concentration of SDS, (c) buffer pH, (d) buffer type, (e) Teorell and Stenhagen buffer volume, (f) order of addition on the $RFI_{286\ nm}$ of DPP.

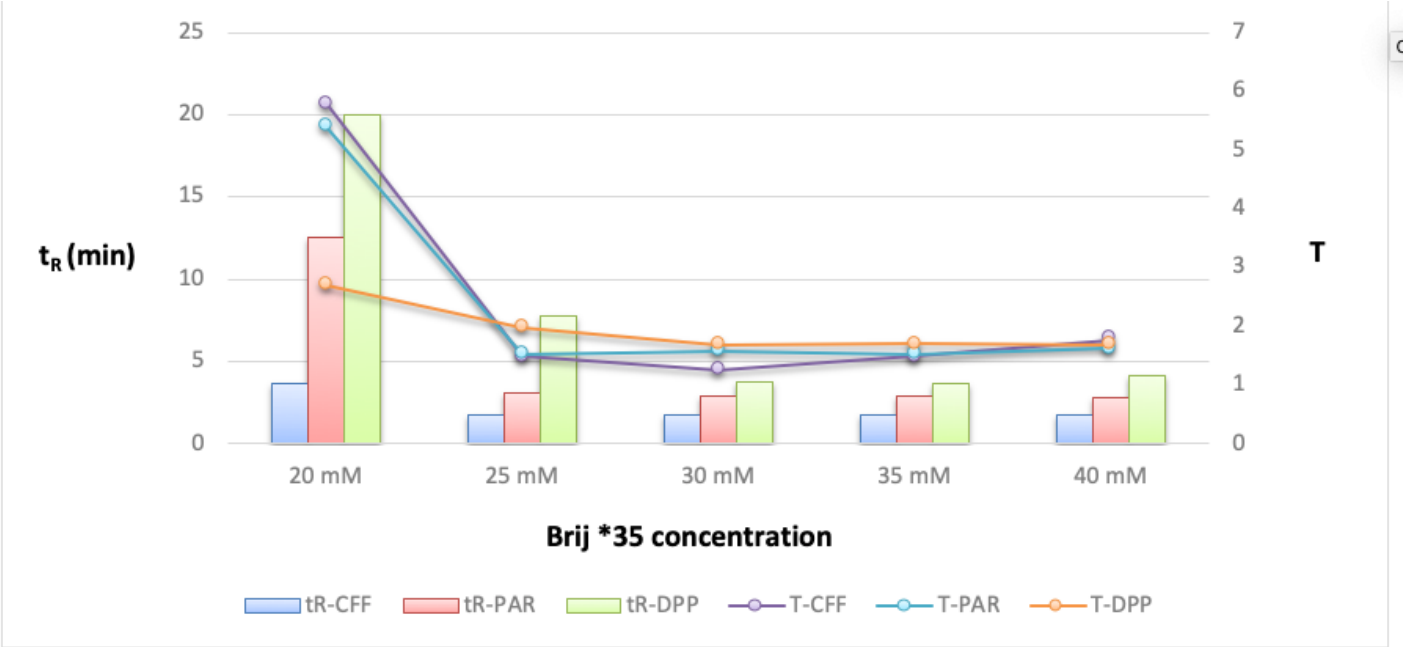

Figure 6

Effect of type of surfactants on the retention time (t_R) of CFF, PAR and DPP.

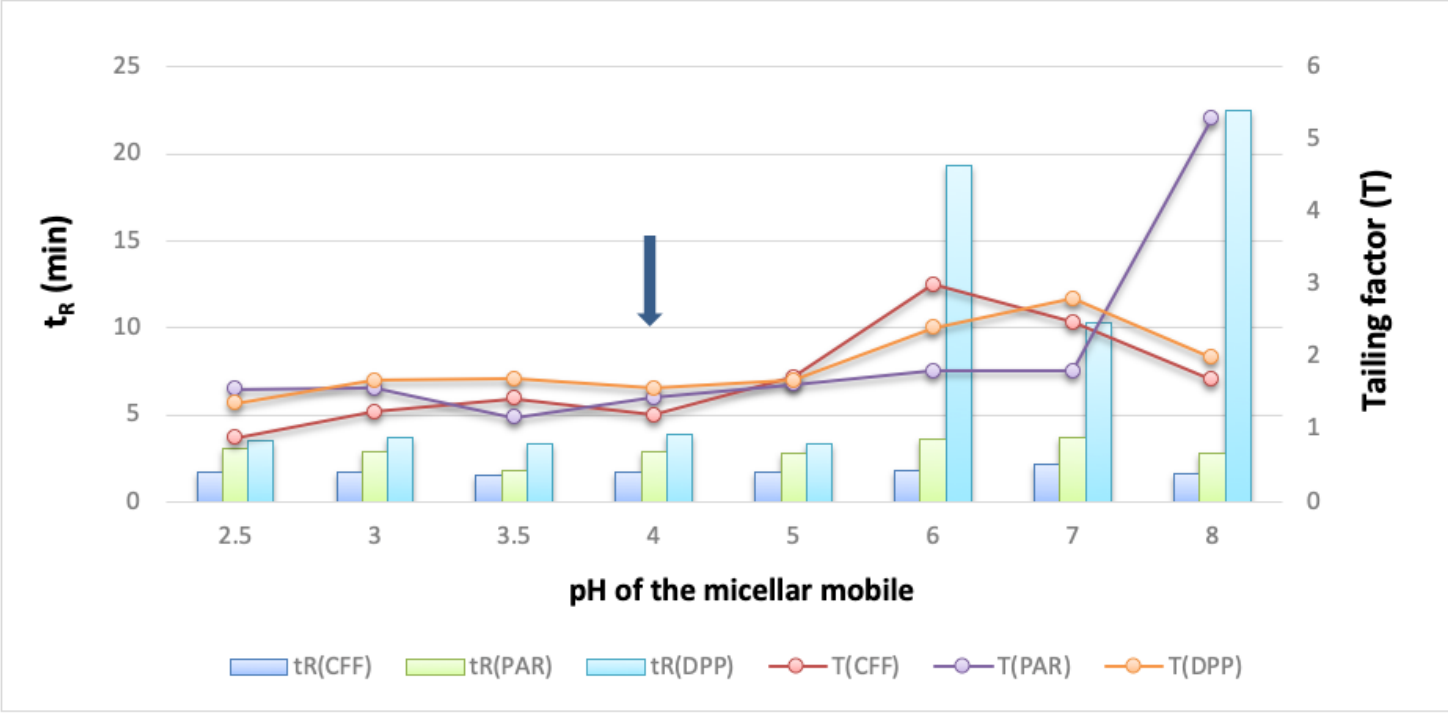

Figure 7

Effect of Brij*35 concentration on the retention time (t_R) and tailing factors (T) of CFF, PAR and DPP.

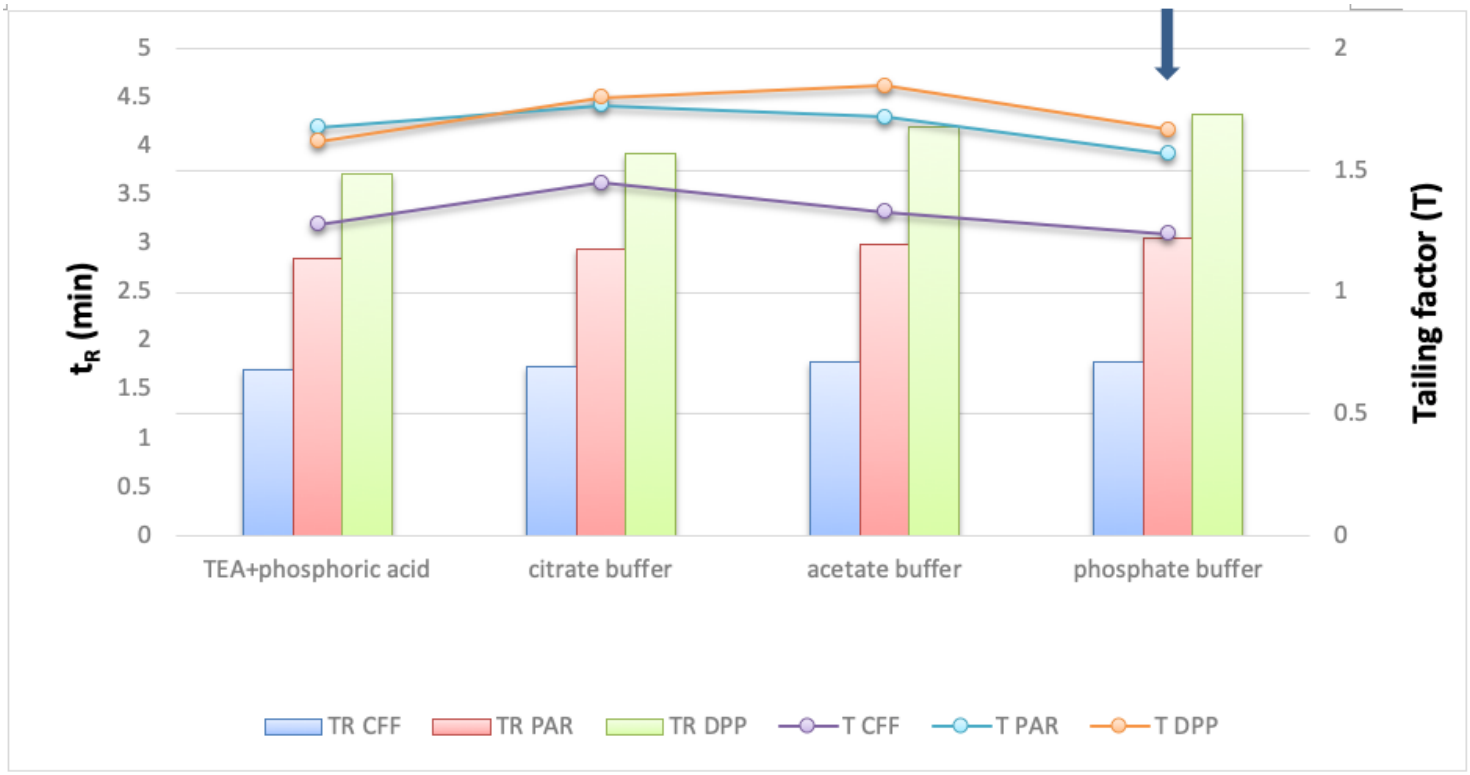

Figure 8

Effect of pH of the mobile phase on the retention times (t_R) and tailing factors (T) of CFF, PAR and DPP.

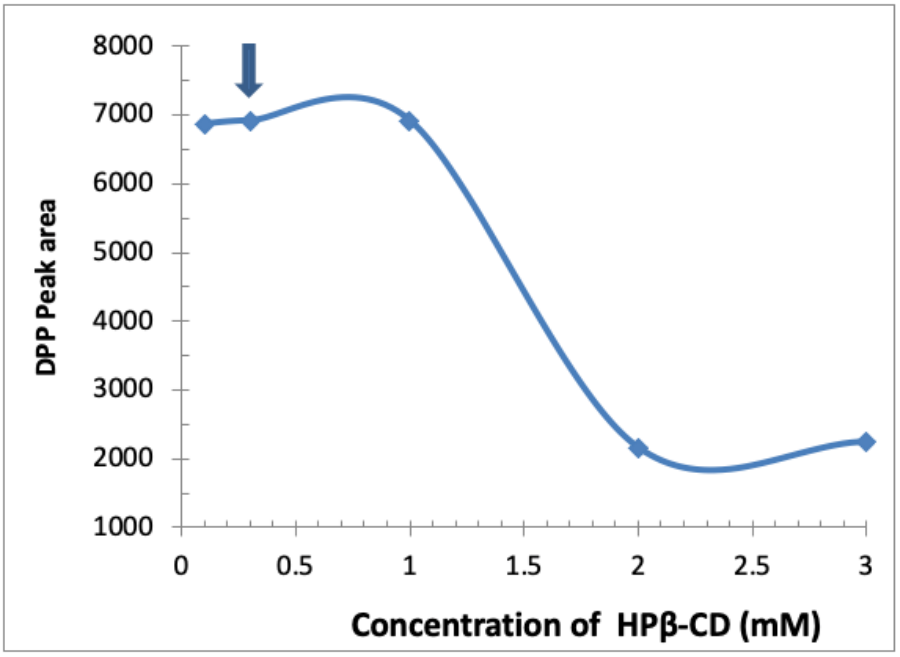

Figure 9

Effect of type of buffer on the retention times (t_R) and tailing factors (T) of CFF, PAR and DPP.

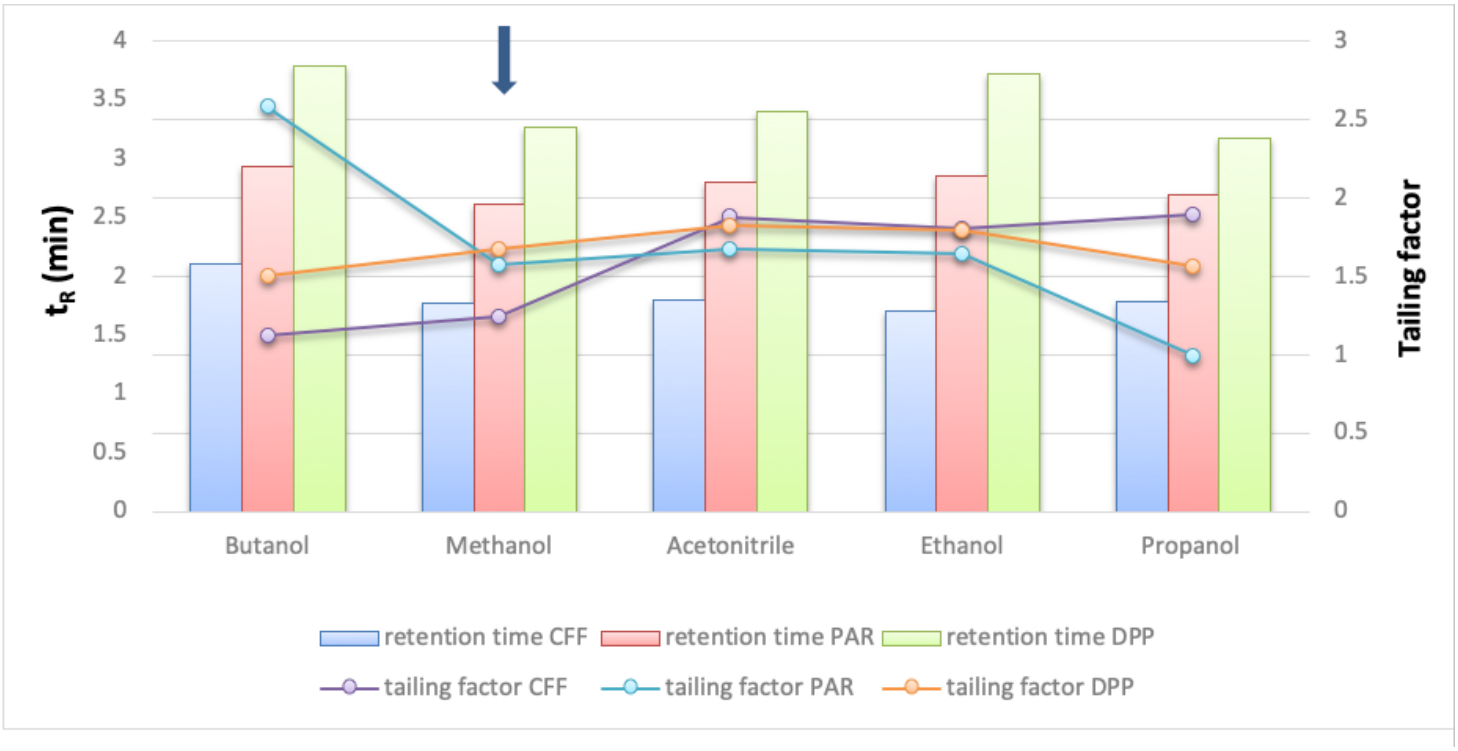

Figure 10

Effect of concentration of HP β-CD in the mobile phase on the peak area of DPP.

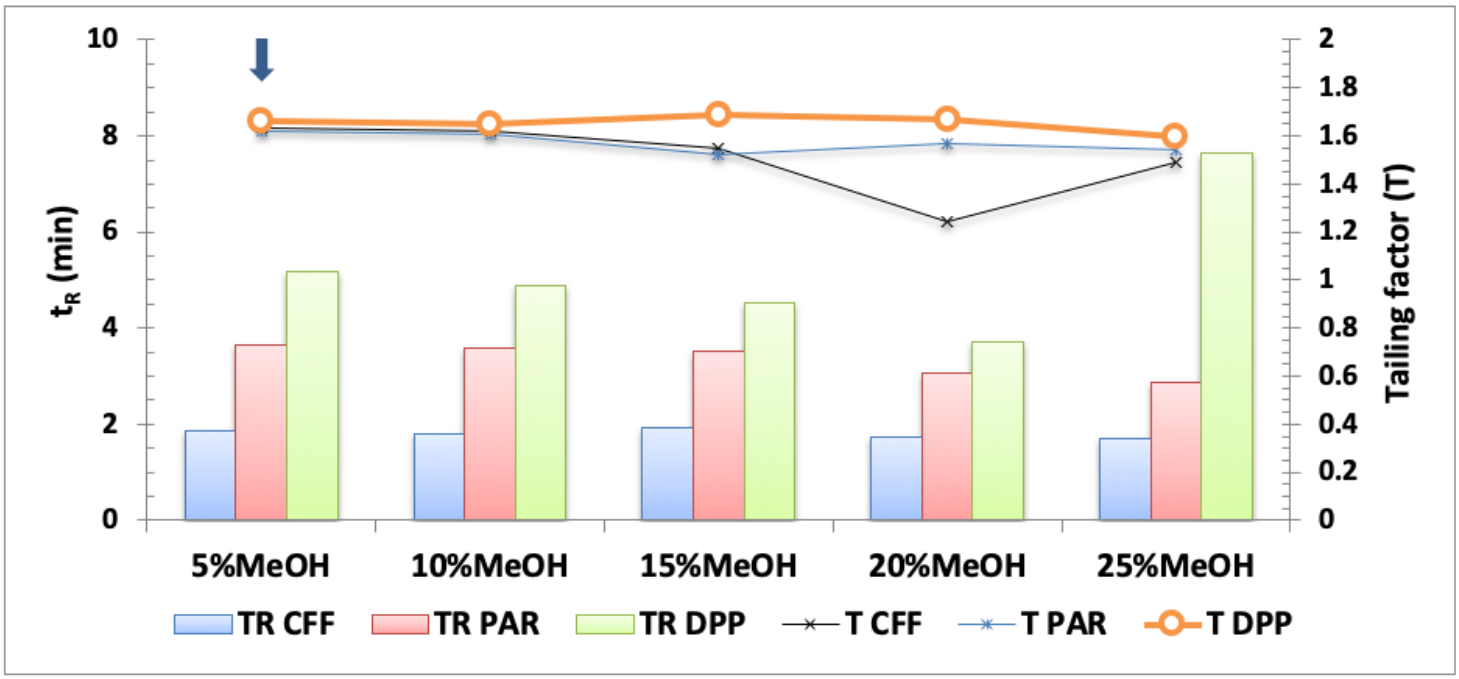

Figure 11

Effect of type of organic modifier on the retention times and tailing factors of CFF, PAR and DPP.

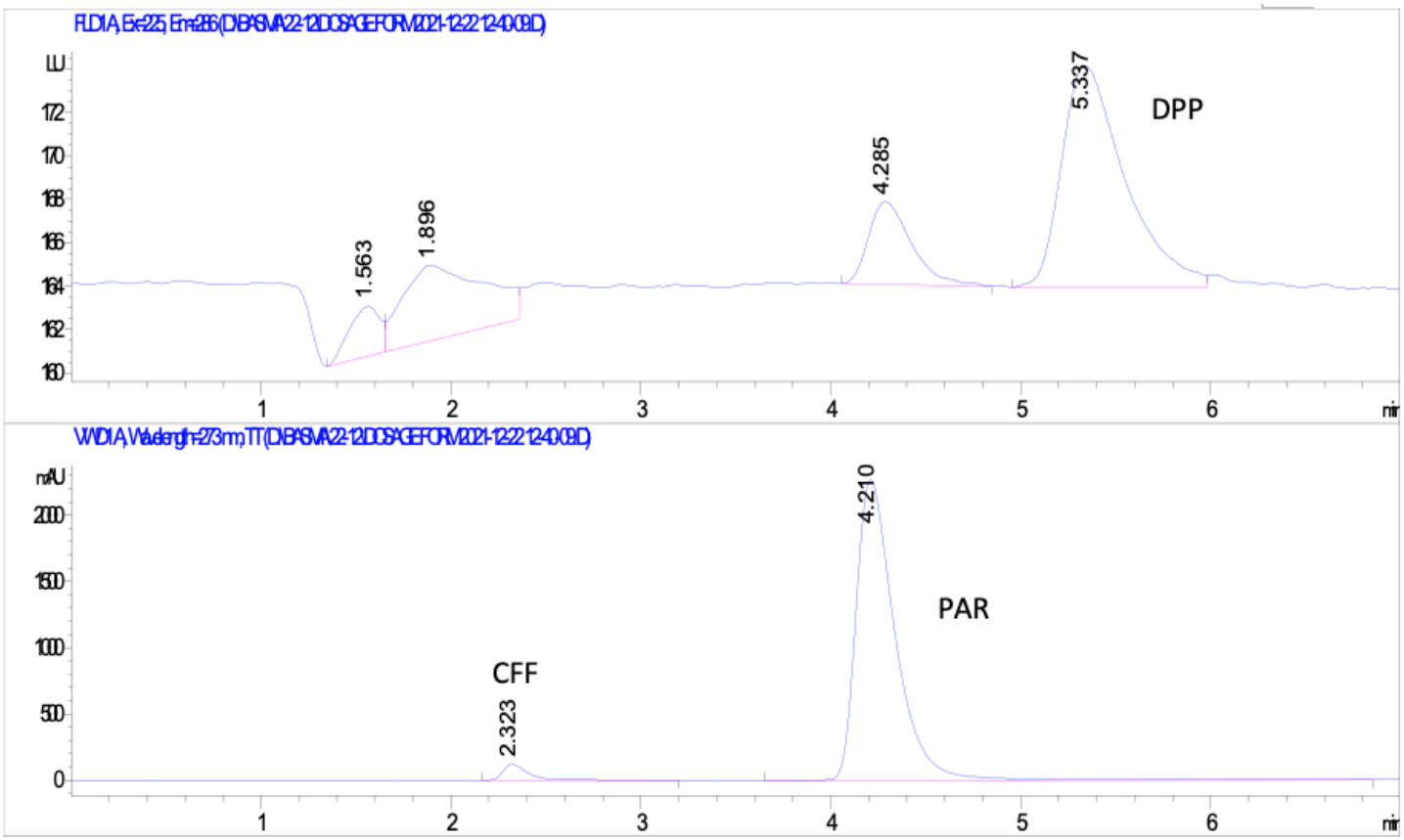

Figure 12

Effect of % of methanol on the retention times and tailing factors of CFF, PAR and DPP.