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Sustainable liquid chromatographic determination and purity assessment of a possible add-on triple-action over-the-counter pharmaceutical combination in COVID-19

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ARTICLE INFO

Keywords:

Over-the-counter medications
Triple-action formula
HPTLC-densitometry
HPLC-DAD
Greenness profiling
Dissolution monitoring, Purity testing

ABSTRACT

Nowadays, all researchers are focused on combating the pandemic COVID-19. According to recent statistics, most patients are managed at home. An over-the-counter (OTC) triple action formula containing paracetamol (PAR), aspirin (ASP), and diphenhydramine (DIPH) is widely prescribed for pain, fever and as night-time sleep aid. For COVID-19 patients, this combination is now suggested as part of symptomatic therapy and prophylaxis. In this work, two simple liquid chromatographic approaches were designed for simultaneous determination of PAR, ASP, and DIPH in Excedrin® PM caplets, beside three specified official toxic impurities, namely, *p*-aminophenol, *p*-nitrophenol, and salicylic acid. The first method comprised high-performance thin-layer chromatographic separation coupled with densitometric quantification, on silica gel HPTLC 60 F₂₅₄ aluminium sheets as the stationary phase, ethyl acetate–methanol–aqueous ammonium hydroxide (10.0: 2.0: 0.1, by volume) as the developing system and scanning was performed at 210.0 nm. The second one is a high-performance liquid chromatography coupled with diode array detector. Successful separation of the six components was performed on XTerra C₁₈ column with isocratic elution of mobile phase 0.1% triethylamine acidified water: methanol (70:30, v/v) adjusted with *o*-phosphoric acid to pH 3.0 and methanol (90:10, v/v) with flow rate programming and detection at 210.0 nm. Validation of the proposed methods was performed according to ICH guidelines. Both methods were successfully used for quality control of the cited drugs in their marketed formulation. Moreover, the in-vitro release study was monitored using the proposed HPLC-DAD method. The greenness profile of the proposed methods was assessed and comparatively evaluated through various assessment tools, specifically; the analytical eco-scale system, national environmental method index (NEMI), green analytical procedure index (GAPI) and analytical greenness (AGREE) metric.

1. Introduction

Over-the-counter (OTC) is currently a strongly competitive market and a prominent area of focus [1]. Oral pharmaceutical combinations of analgesics and antihistaminics are widely used as night-time headache relief and sleep aid, they are among the most important sectors of OTC medications.

Paracetamol (PAR) is an antipyretic and analgesic agent [2], that can be used in palliative care for COVID-19 symptoms control according to WHO guidance [3]. Among PAR pharmacopeial related substances are *p*-nitrophenol (PNP) and *p*-aminophenol (PAP), namely, impurity F and K, respectively [2]. PAP is PAR main hydrolytic degradation product [4,5],

in addition, it exhibits teratogenic [6] and nephrotoxic effects [7]. Similarly, PNP exhibits genotoxicity and carcinogenicity [8]. Aspirin (ASP) is a non-selective *cyclo*-oxygenase inhibitor with antipyretic, analgesic, and anti-inflammatory actions [2]. Because of its anti-platelet aggregation and lung damage prevention, as well as its viral replication suppression impact, ASP is also used as a prophylactic therapy in COVID-19 patients [9]. The main degradation product and pharmacopeial impurity C of ASP is salicylic acid (SAL) [2]. SAL is reported to cause toxic symptoms as gastric membrane, esophagus damage and ototoxicity [10]. Diphenhydramine (DIPH), a first-generation antihistamine [2], is recently reported to have direct antiviral action against SARS-CoV-2 [11]. Fig. 1 displays the chemical structures of the cited

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drugs and these related substances.

Co-formulated PAR, ASP and DIPH is an effective and highly tolerated analgesic therapy that is used to treat headache, insomnia, and pain. The significance of the cited drugs arises from the recent researches on the prospects of employing them as an add-on remedy for COVID-19 patients, specifically home-treated and mild cases [12,13]. The literature survey revealed the lack of a reported analytical method for the concurrent determination and purity testing of the three components in their newly introduced pharmaceutical formulation. Yet, all of the published work were focused on the determination of PAR and ASP combination along with other compounds using HPLC [14–18], UV-Spectrophotometry [19–21], and voltammetry [22,23]. Similarly, PAR and DIPH combination was analyzed by different spectrophotometric [24–26], HPLC [26,27], and capillary gas chromatographic [28] methods.

Chromatography is currently considered as a gold standard technique in pharmaceutical industry, owing to its outstanding advantages such as low operation cost, availability, good sensitivity, small sample size requirements and diversity of applications [29–33]. Recently, analysts aimed to apply a competent analytical methodology with improved eco-friendliness and sustainability to preserve our environment from chemical threats [31,34,35]. Accordingly, the aim of the current work is planned to introduce simple, selective, cost-effective, and eco-friendly HPTLC and HPLC-DAD methods for simultaneous analysis and purity testing of the cited drugs in their pure forms and pharmaceutical formulation. Ecological impact and analytical performance of the proposed methods are compared to bring a greener analytical approach to a close. In addition, the dissolution profiles of the cited active pharmaceutical ingredients (API) from the marketed product were monitored using the proposed HPLC-DAD method.

2. Experimental

2.1. Equipments

2.1.1. For HPTLC-densitometry

Chromatographic separation was carried out using silica gel 60 F₂₅₄ HPTLC plates (20 × 10 cm) (Merck, Darmstadt, Germany) as the stationary phase. Sample application is carried out using Camag Linomat-5 autosampler by means of Camag micro-syringe (Camag, Muttenz, Switzerland). Scanning and densitometric measurements were

performed by a Camag TLC scanner (model number 3S/N 1302319), operated with winCATS® software. The adjusted scan mode was reflectance-absorbance with slit of 3.0 × 0.45 mm dimension and scanning speed of 20 mm/s. Densitograms with recorded peak areas were obtained.

2.1.2. For HPLC-DAD method

The separation module was Waters Alliance 2695 LC equipped with a pump integrated with a mixing system, vacuum degasser, Waters photodiode array detector 996 (PDA), and an auto-sampler (Milford, United States). The system was controlled with Empower2 chromatography software for data processing and manipulation. The utilized column was XTerra C₁₈ (100 × 4.6 mm, 5 μm) manufactured by Waters™ Corporation (Milford, United States). A Jenway pH apparatus (model 3510, UK) was used to adjust the solvent's pH.

2.1.3. Dissolution test apparatus

The dissolution profiling was performed using VanKel VK 7000 (USA) apparatus fitted with standard USP type-II paddle and six vessels.

2.2. Materials and reagents

Pure PAR was provided by El Nasr Pharmaceutical Co., Cairo, Egypt, while ASP was supplied from Al-Gomhoria Chem. Co. (Cairo, Egypt). Purities were verified using their respective official methods and found to be 99.80% ± 0.50 for PAR [2] and 100.30% ± 1.87 for ASP [36]. Pure DIPH as hydrochloride salt was obtained from Wanbury Ltd. Co. (India). Its purity was evaluated and found to be 100.54% ± 0.83, using its BP method [2]. PNP, PAP and SAL were provided by Sigma-Aldrich (Darmstadt, Germany) with stated purity of ≥ 99%.

Excedrin® PM Headache caplets (B.N. 46172679), a product by GlaxoSmithKline, USA, were claimed to comprise PAR 250 mg, ASP 250 mg, and DIPH 38 mg as citrate salt per caplet.

HPLC-grade methanol was obtained from Sigma-Aldrich (Darmstadt, Germany). Also, analytical-grade solvents and reagents were used including o-phosphoric acid (El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt), triethylamine, ethyl acetate (S.D. Fine Chemicals Ltd., India), and aqueous ammonium hydroxide solution (25%) (Pioneer Chemical Co., Giza, Egypt). De-ionized distilled water was provided by Otsuka Pharmaceutical Co. (Cairo, Egypt).

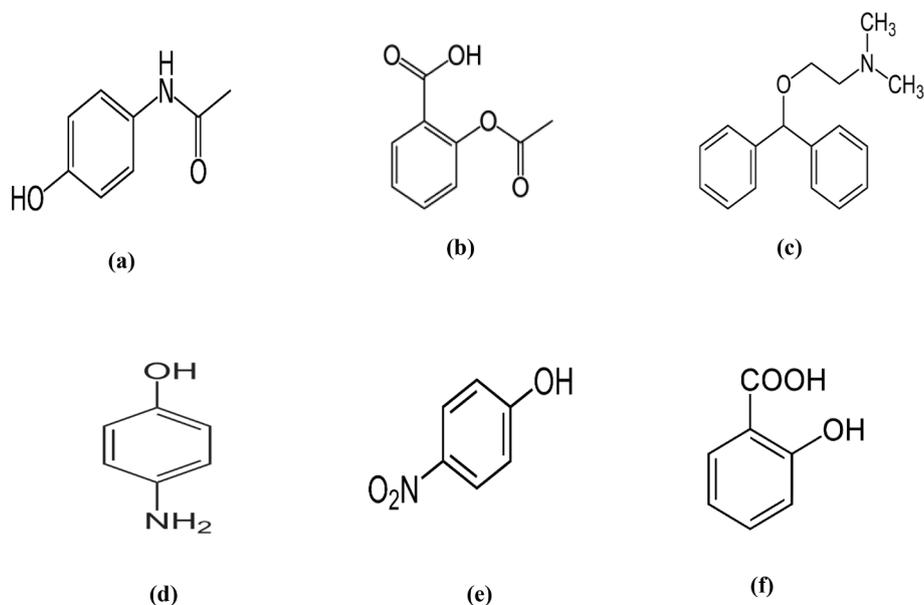


Fig. 1. Chemical structure of (a) Paracetamol, (b) Aspirin, (c) Diphenhydramine, (d) p-Aminophenol, (e) p-Nitrophenol, and (f) Salicylic acid.

2.3. Stock and working standard solutions

Stock standard solutions (1.0 mg/mL) of the studied drugs were prepared, separately, in methanol. Further, PAP, PNP, and SAL working standard solutions (100.0 µg/mL) were prepared by appropriate dilutions of their corresponding stock standard solutions. The prepared solutions were kept in the refrigerator and protected from light.

2.4. Procedures

2.4.1. Chromatographic conditions

2.4.1.1. For HPTLC-densitometry. Chromatographic separation was carried out on 20 × 10 cm Silica gel 60 F₂₅₄ HPTLC plates as stationary phase with a developing mixture system composed of ethyl acetate–methanol–aqueous ammonium hydroxide solution (10.0: 2.0: 0.1, by volume). Samples were applied in triplicates, using a Camag autosampler, as bands of 6 mm width, and 10 mm apart from the sides and bottom border of the plates. The development was performed in a pre-saturated Camag binary glass chamber for 45 min at room temperature. Ascending development, over 8 cm of the plates, was then allowed. The developed plates were removed, air dried and scanned at 210.0 nm using the previously specified instrumental conditions.

2.4.1.2. For HPLC-DAD method. The XTerra C₁₈ (100 mm × 4.6 mm, 5 µm) column was used for isocratic separation of studied analytes with a mixture of 0.1% triethylamine acidified water: methanol (70:30, v/v) adjusted with *o*-phosphoric acid to pH 3.0 and methanol (90:10, v/v) as mobile phase at room temperature. The flow rate was adjusted to 1.0 mL/min, and starting from 5.5 min was fixed at 1.5 mL/min. The mobile phase components were filtered using 0.45 µm Millipore membrane filter, degassed, and sonicated for 15 min in an ultrasonic bath prior to use. The samples were filtered and applied with 50 µL injection volume, in triplicates, into the LC system using an autosampler. The separation was successfully achieved within 12 min and detection was performed at 210.0 nm.

2.4.2. Construction of the calibration curves

2.4.2.1. For HPTLC-densitometry. Accurately measured volumes in the range of 1.00–15.0, 1.00–15.0, 0.20–9.00, and 0.10–3.00 µg/band of PAR, ASP, DIPH and studied impurities from their respective stock and working standard solutions were applied, separately, in triplicates as bands using the HPTLC system with the previously described chromatographic conditions. The scanning profiles were obtained, and the calibration plots were constructed relating the average peak area to the corresponding concentration of each drug. Finally, the polynomial regression equations were computed.

2.4.2.2. For HPLC-DAD method. Different volumes of each analyte were accurately transferred from their respective stock and working standard solutions to 10-mL volumetric flasks, to get solutions in concentration range of 1.00–14.0 µg/mL for PAP, 5.00–160.0 µg/mL for PAR, 2.00–160.0 µg/mL for ASP, 1.00–13.0 µg/mL for PNP, 1.00–14.0 µg/mL for SAL, and 2.00–55.0 µg/mL for DIPH. Volumes were brought to the mark with the mobile phase. Samples were prepared in mixtures and injected into the HPLC-DAD system, in triplicates, using the previously specified chromatographic conditions. The calibration curves were built by plotting the average peak areas against the respective drug concentrations.

2.4.3. Analysis of pharmaceutical formulation

Ten Excedrin® PM caplets, each contain labelled amounts of 250 mg PAR, 250 mg ASP, and 38 mg DIPH as citrate salt, were individually weighed, powdered and thoroughly mixed in a hand mortar. Then,

accurately weighted quantity, equal to one caplet, was subsequently transferred to a 100-mL volumetric flask, by 50-mL methanol, and left in the sonicator for 45 min. The volume was completed with methanol then filtered. The prepared solution was further diluted with methanol (for HPTLC) and mobile phase (for HPLC), to achieve the appropriate concentrations within PAR, ASP and DIPH linearity ranges, and the procedures were then carried out as described under each method. The found concentrations and the %recoveries of studied drugs were computed from the respective regression equations.

Definite portions of PAR, ASP and DIPH pure standards were added to an accurately weighted powdered caplets. Following that, the same extraction and dilution were carried out to perform the standard addition technique.

2.4.3.1. Dissolution testing. The aforementioned HPLC-DAD procedure was applied for monitoring the dissolution behaviour of Excedrin® PM caplets in water as per USP requirements [36]. Dissolution testing was performed by placing one caplet in a USP-dissolution apparatus II vessel, containing 900 mL water as a dissolution medium, equilibrated at 37 °C ± 0.5°C and operated at 100 rpm. Five mL samples were withdrawn at pre-determined time intervals of 10, 20, 25, 30, 45, and 60 min, and the volume was replenished with an equal volume of fresh medium. The collected samples were then filtered through a 0.22 µm syringe filter and analysed. The experiment was carried out in triplicate. The % dissolution was calculated, by means of the corresponding regression equation for each drug in the studied medium, and the in-vitro release profiles of PAR, ASP and DIPH were then plotted.

3. Results and discussion

For regular drug analysis, chromatography is a well-established and gold standard technique in quality control laboratories all over the world. Resolution and quantification of drugs in presence of pharmacopial impurities with extremely similar structures and thus predicted to have comparable physical and chemical characteristics is a key challenge in the development and validation of the analytical procedures. Moreover, designing analytical processes in such a manner that results in a considerable reduction or elimination of the use and/or generation of harmful substances is a current drive [31,37]. So, this work aims at developing and validating first sustainable, selective, and robust analytical methods for simultaneous estimation and purity assessment of PAR, ASP, and DIPH in their new OTC pharmaceutical combination. The suggested HPLC-DAD method was exploited for monitoring the dissolution profiles of the cited drugs in the combined formulation as well. Finally, the suggested approaches' ecological and health implications were assessed using several green evaluation indicators.

3.1. Method development and optimization

3.1.1. For HPTLC-densitometry

HPTLC-densitometric procedure was developed and optimized taking in account the use of green solvents and excluding toxic and hazardous ones (i.e., acetonitrile, toluene, and chloroform). Trials started using various eco-friendly developing systems such as ethyl acetate-ethanol, ethyl acetate-butanol and ethyl acetate–methanol in varying ratios (4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 v/v), respectively. However, the results were not acceptable due to observed tailing and incomplete separation of the studied compounds. Then, glacial acetic acid, aqueous ammonium hydroxide, and triethylamine were tried as pH modifiers to the developing mixture of ethyl acetate–methanol. DIPH was retained at the baseline in acidic pH. On the other hand, improved separation was attained upon using alkaline pH in the developing system. Aqueous ammonium hydroxide was preferred over triethylamine owing to its less environmental hazardous in addition to better bands symmetry.

Different volumes of aqueous ammonium hydroxide solution were tried, and the optimal ratio of ammonia was found to be (0.1, by volume) regarding both resolution and selectivity. Increasing the aqueous ammonium hydroxide ratio has led to increased retention of ASP at the baseline. Finally, successful separation of all eluted compounds with appropriate R_f values, sharp and symmetric peaks was attained upon using ethyl acetate–methanol and aqueous ammonium hydroxide in the ratio of 10.0: 2.0: 0.1, by volume as the developing system, Fig. 2a. Different scanning wavelengths were assessed for densitometric measurements (210.0, 220.0, 230.0, and 254.0 nm), taking in account the absorbance spectra of the studied compounds. Scanning at 210.0 nm provided best sensitivity and peak symmetry, as shown in Fig. 2a. For evaluating the chromatographic system's suitability, different parameters were calculated for the six components [38]. All of the calculated criteria were within the acceptable ranges, Table 1.

3.1.2. For HPLC-DAD method

The developed approach was designed with the chief goal of developing a robust, simple, green and efficient method for quantitative analysis of PAR, ASP and DIPH, simultaneously, along with three of their official impurities with optimum resolution in a reasonable analysis time. Method development was conducted using Waters XTerra C₁₈ (100 × 4.6 mm, 5 μm) column. Several organic solvents were tried namely, acetonitrile, methanol and ethanol, and acidified water with *o*-phosphoric acid as the aqueous phase with varying ratios, pH, and flow rates. For the sake of method greenness, acetonitrile was excluded as an organic modifier. Whereas, methanol was selected, as it gives promising separation with stable baseline. In addition, triethylamine was examined, as a mobile phase additive, for peak enhancement. Satisfactory resolution and symmetric peaks were obtained using isocratic elution of 0.1% triethylamine acidified water: methanol (70:30, v/v) adjusted with *o*-phosphoric acid to pH 3.0 and methanol (90:10, v/v). Flow rate programming have been tried. Adjusting flow rate at 1.0 mL/min and set at 1.5 mL/min starting from 5.5 min, resulted in sharp peaks with reduced analysis time and solvent consumption. Finally, detection wavelength was carefully chosen and assigned to be 210.0 nm. Finally, all six components were well resolved under the optimized chromatographic conditions as presented in Fig. 2b. In order to guarantee chromatographic system's performance, System suitability parameters were computed. Results were in good agreement to the acceptable limits [39].

3.2. Evaluation of analytical method greenness

In chemical laboratories, green analytical chemistry principles are well-known. To properly evaluate the environmental influence of chemical processes, specialized assessment tools are needed [40]. The desire to replace conventional pharmaceutical analytical methods, which depend on the usage of hazardous chemicals, with more eco-friendly green ones without affecting performance features has grown in light of the importance of environmental protection. [41]. Four tools were presented to verify and appraise the greenness of the proposed methods. We also provided a comprehensive benchmark to compare their environmental impacts. These tools are the analytical eco-scale, national environmental methods index (NEMI), green analytical procedure index (GAPI), and analytical greenness (AGREE) metric.

A semi-quantitative evaluation was achieved upon using analytical eco-scale by assigning penalty points based on the impact of different method parameters, including the utilized chemicals, instrumental energy consumption, waste generation, and occupational risk [42]. The analytical eco-score is computed by subtracting the total parameters penalty points from 100 as the perfect green method base value. Table 2 displays the analytical eco-scores >75, emphasizing the proposed methods as excellent ones.

The NEMI is a qualitative approach used to evaluate the total environmental safety of the analytical methodology [43]. The NEMI pictogram is represented by a simple circle divided into four quadrants: the first comprises persistent, bio-accumulative, and toxic chemicals, the second represents the hazardousness of chemicals, while the third and fourth embraces the corrosiveness of the mobile phase, and the amounts of waste generated, respectively [44]. Concerning the proposed HPTLC-densitometry, and HPLC-DAD methods, the US EPA Toxic Release Inventory (TRI) chemical list did not list ethyl acetate as PBT nor hazardous [45]. Conversely, methanol is present in the TRI list. The pH of the mobile phase used was ~ 9 and 3 for each method, respectively, so the pH is not corrosive (pH not less than 2 or > 12). The generated waste is less than 50 g per sample. All quarters of the proposed method pictogram are colored green except the hazardous quarter due to methanol, as illustrated in Table 2.

The GAPI tool has been newly introduced as an indicator for assessing the green characteristics of the entire analytical measures beginning from sample preparation and collection to final determination

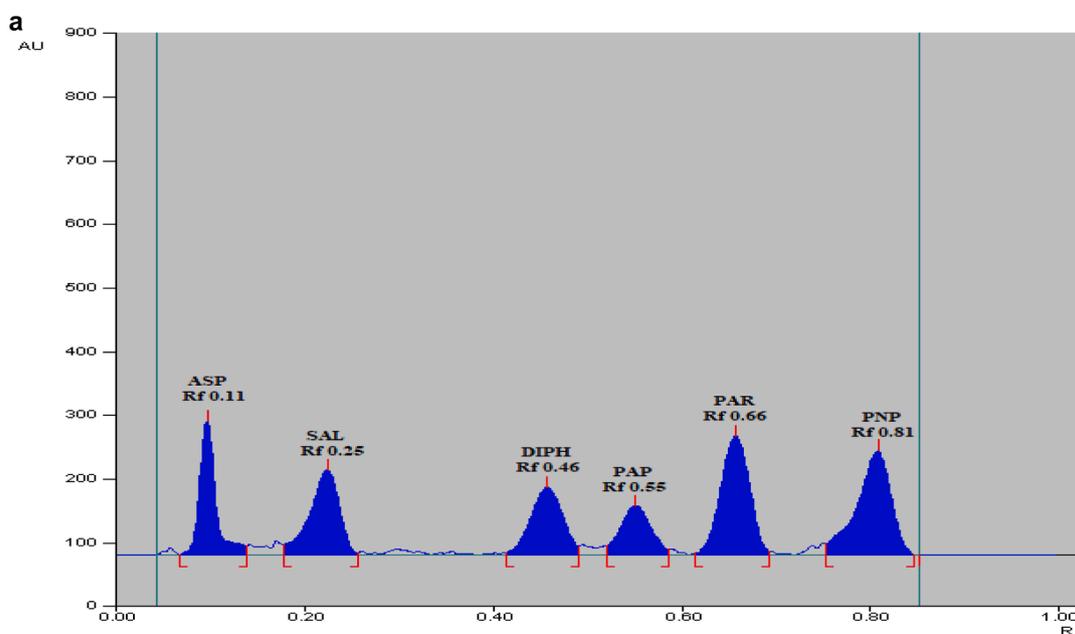


Fig. 2a. HPTLC-densitogram of a mixture of ASP (2.0 μg/band), SAL (0.1 μg/band), DIPH (2.0 μg/band), PAP (0.1 μg/band), PAR (2.0 μg/band), and PNP (0.1 μg/band), scanned at 210.0 nm using ethyl acetate–methanol–aqueous ammonium hydroxide (10.0: 2.0: 0.1, by volume) as the developing system.

Table 1
System suitability parameters of the proposed HPTLC-densitometric and HPLC-DAD methods.

Method	Parameter	ASP	SAL	DIPH	PAP	PAR	PNP	Reference value [38]
HPTLC-densitometry	Retardation factor (R_f) \pm 0.02 ^a	0.11	0.25	0.46	0.55	0.66	0.81	
	Capacity factor (k') ^b	8.09	3.00	1.17	0.81	0.52	0.23	
	Selectivity (α) ^c		2.69	2.56	1.46	1.55	2.24	> 1
	Resolution (R_s) ^d		2.68	3.53	1.54	1.63	3.74	$R_s > 1.5$
	Tailing factor (T)	1.00	0.95	1.00	1.00	1.00	0.95	$T \leq 2$
	Parameter	PAP	PAR	ASP	PNP	SAL	DIPH	Reference value [39]
HPLC-DAD	Retention time (min \pm 0.2)	1.127	1.690	4.820	5.729	7.217	9.754	
	Selectivity (α) ^c		2.07	4.08	1.22	1.29	1.39	> 1
	Resolution (R_s) ^d		2.82	8.94	2.14	3.50	3.17	$R_s > 1.5$
	Tailing factor (T)	1.00	1.14	1.00	1.00	1.10	1.12	$T \leq 2$
	Column efficiency (N)	324	784	1560	2885	2228	1264	$N > 2000$
	Height equivalent to theoretical plate HETP (cm/plate)	3.0×10^{-2}	1.2×10^{-2}	6.40×10^{-3}	3.47×10^{-3}	4.49×10^{-3}	7.91×10^{-3}	

^a Retardation factor (R_f) = distance travelled by the analyte/distance travelled by the solvent front.

^b Capacity factor (k') = $(1 - R_f) / R_f$.

^c Selectivity (α) = k'_2/k'_1 calculated for each of two successive peaks.

^d Resolution (R_s) = $(R_{f2} - R_{f1}) / 0.5 (w_1 + w_2)$, where R_f is the retardation factor, and w is the peak width calculated for each of two successive peaks for HPTLC and = $2(t_{RB} - t_{RA}) / (W_{B+} + W_A)$ for HPLC.

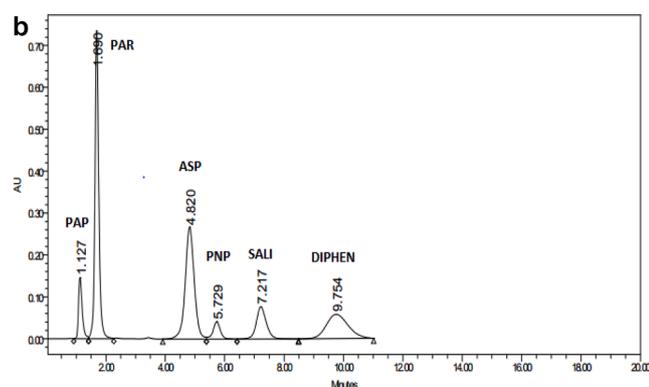


Fig. 2b. HPLC Chromatogram of standard PAP (8.0 μ g/mL), PAR (70.0 μ g/mL), ASP (70.0 μ g/mL), PNP (8.0 μ g/mL), SAL (8.0 μ g/mL), and DIPH (35.0 μ g/mL), using a C_{18} column and isocratic elution of 0.1% triethylamine acidified water: methanol (70:30, v/v) adjusted with *o*-phosphoric acid to pH 3.0 and methanol (90:10, v/v) as the mobile phase at 210.0 nm.

step [46]. A visual pictogram comprised of five pentagrams, with a color code ranging from green to yellow to red, assigned to low, medium, and high ecological impact for each step, is used. The proposed methods showed green covered sections with fewer red shaded ones, as presented in Table 2.

Finally, the recently dedicated AGREE tool is used for the comprehensive evaluation of the analytical methodology greenness. It is a free downloadable software that creates a colored pictogram, covering the GAC's twelve fundamentals that are transformed to a final zero-one score [47]. A characteristic circular pictogram appears in the final assessment report, automatically and is divided into 12 sections with the ability to adjust the width of each section based on its significance. The section color ranged from deep red (=0) to deep green (=1) [47]. Table 2 shows the obtained AGREE pictograms with an overall score of 0.69 and 0.65 for the developed HPTLC-densitometry and HPLC-DAD methods, in order.

3.3. Analytical method validation

Method validation parameters were assessed according to the ICH guidelines to assure the validity of the proposed analytical methods [48]. Working calibration range was established considering the correlation between the mean peak area and the corresponding concentration

of each drug. Table 3 displays the linearity ranges of the proposed methods and their computed regression parameters.

Accuracy of the developed methods was assured by the acceptable mean %recoveries obtained upon analyzing each drug at five different concentration levels covering the linearity ranges, Table 3.

Three different chosen concentrations of each drug were analyzed in triplicates, on the same day and on three consecutive days, to assess the method's intra-day and inter-day precision, respectively. The attained percentage relative standard deviation (RSD%) were less than 2, Table 3.

Good separation of the studied drugs along with cited impurities demonstrates the selectivity of the proposed methods, Fig. 2. In addition, the good % recoveries of PAR, ASP and DIPH in their co-formulation ensures the absence of chromatographic interference from common caplets excipients, Table 4. Specificity was further assured via winCATS spectral correlation tool (for HPTLC) or online by DAD (for HPLC) by monitoring the purity for each eluting drug peak.

LOD and LOQ were calculated for the official impurities using the slope of standard calibration curve and standard deviation of residuals. The achieved LOD and LOQ values reflect the proposed methods' good sensitivity, Table 3.

Robustness of the developed methods was ascertained by calculating RSD% upon performing minor changes in the optimized experimental conditions. System suitability parameters were not significantly influenced, and the % RSD values were within the accepted range, less than 2%, Table 3.

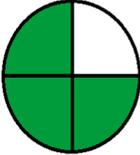
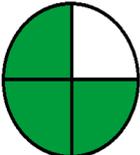
3.4. Analysis of the dosage form (Excedrin® PM caplets)

PAR, ASP, and DIPH were efficiently assayed in their co-formulation, Excedrin® PM caplets by the validated methods, keeping in consideration the wide disparities in their concentrations. Sample preparation was performed employing single extraction step with methanol, showing no interference from caplets excipients. Furthermore, the standard addition technique was performed, and the proposed methods validity was successfully implemented, Table 4. The good results and minimal sample manipulation steps as well turn the attention on the worth applicability of the proposed methods as routine and ecofriendly quality control protocols of the three drugs.

Pharmaceutical formulation was assayed by the developed methods near the expiry date. The samples showed traces of SAL (ASP synthetic precursor and main degradation product), Fig. S1. Accordingly, the suggested methodologies were found to be both selective and sensitive

Table 2

Greenness assessment of the proposed chromatographic methods according to Analytical Eco-Scale, NEMI, GAPI and AGREE tools.

For HPTLC-densitometry Method		NEMI Pictogram	GAPI Assessment ^c	AGREE Assessment ^d
Eco-Scale assessment				
Reagents	Penalty points (PPs)			
Ammonia (25%)	6			
Ethyl acetate	4			
Methanol	6			
Instrument				
Energy consumption ^a	1			
Occupational Hazard	3 (Not a closed system)			
Waste ^b	3			
Total PPs	23			
Analytical Eco-Scale total Score	77			
Comment	Excellent green analysis			
For HPLC-DAD Method				
Eco-Scale assessment				
Reagents	Penalty points (PPs)			
Methanol	6			
Water	0			
Triethylamine (TEA)	6			
Phosphoric acid (OPA)	2			
Instrument				
Energy consumption ^a	1			
Occupational Hazard	0			
Waste ^b	5			
Total PPs	20			
Analytical Eco-Scale total Score	80			
Comment	Excellent green analysis			

^a Score of '1' is given as for LC technique; the energy used is ≤ 1.5 kWh per sample.^b Calculated as: volume of developing system per run /number of samples on HPTLC plate for HPTLC method, while as run time \times flow rate for HPLC method.^c GAPI Assessment evaluated according to Green Analytical Procedure Index parameters description [46].^d AGREE Assessment evaluated by using Analytical GREENness Metric approach and Software [47].**Table 3**

Regression and validation parameters of the proposed HPTLC-densitometric and HPLC-DAD methods for the determination of a ternary mixture of Paracetamol, Aspirin, and Diphenhydramine along with impurities in pure form.

Method Parameter	HPTLC-densitometric method						HPLC-DAD method					
	ASP	SAL	DIPH	PAP	PAR	PNP	PAP	PAR	ASP	PNP	SAL	DIPH
Range	1.0–15.0 $\mu\text{g}/\text{band}$	0.1–3.0 $\mu\text{g}/\text{band}$	0.2–9.0 $\mu\text{g}/\text{band}$	0.1–3.0 $\mu\text{g}/\text{band}$	1.0–15.0 $\mu\text{g}/\text{band}$	0.1–3.0 $\mu\text{g}/\text{band}$	1.0–14.0 $\mu\text{g}/\text{mL}$	5.0–160.0 $\mu\text{g}/\text{mL}$	2.0–160.0 $\mu\text{g}/\text{mL}$	1.0–13.0 $\mu\text{g}/\text{mL}$	1.0–14.0 $\mu\text{g}/\text{mL}$	2.0–55.0 $\mu\text{g}/\text{mL}$
Regression equations parameters												
Slope (b) ^a	—	—	—	—	—	—	115,654	92,652	81,984	70,838	174,626	79,667
Coefficient 1 (b1) ^b	-57.454	-526.72	-149.91	-511.6	-46.208	-399.85	—	—	—	—	—	—
Coefficient 2 (b2) ^b	1933	6237.1	3853.7	4779	1384.6	4637.6	—	—	—	—	—	—
Intercept (a) ^{a, b}	2549	333.14	39.537	23.597	4490.7	39.508	-51579	43,093	-33780	—	—	—
Correlation	1.000	1.000	1.0000	1.000	0.9999	1.000	0.9999	0.9999	1.000	10,265	116,781	7834.9
Coefficient (r)	—	—	—	—	—	—	—	—	—	0.9998	0.9998	1.000
Accuracy (Mean \pm SD)	100.49 ± 1.061	100.69 ± 0.785	99.62 ± 0.649	99.95 ± 0.816	99.18 ± 0.504	99.57 ± 0.750	100.76 ± 0.347	100.22 ± 0.678	99.44 ± 0.868	99.42 ± 0.946	100.65 ± 0.619	100.00 ± 1.428
Precision												
(\pm %RSD) ^c	0.279	0.633	0.286	0.527	0.261	0.948	0.244	0.359	0.171	0.526	0.196	0.591
(\pm %RSD) ^d	1.132	1.158	0.587	1.151	1.243	1.439	0.842	1.077	1.066	1.567	0.946	1.265
LOD ^e	—	0.009	—	0.013	—	0.010	0.175	—	—	0.178	0.232	—
LOQ ^e	—	0.027	—	0.040	—	0.030	0.529	—	—	0.538	0.703	—
Robustness ^f	0.700	1.198	1.204	1.033	1.042	1.382	1.696	1.162	1.057	1.800	1.129	1.456

^a Regression equation for HPLC: $A = a + bc$, where 'A' is the average peak area and 'c' is the concentration ($\mu\text{g}/\text{mL}$).^b Coefficient 1 and 2 are the coefficients of X^2 and X , respectively. Following a polynomial regression: $A = b1x^2 + b2x + a$, where 'A' is the average peak area, 'c' is the concentration ($\mu\text{g}/\text{band}$), 'b1' and 'b2' are coefficients 1 and 2, respectively and 'a' is the intercept.^c Intra-day precision [average of three different concentration of three replicates each ($n = 9$) within the same day], for HPTLC the concentrations were (3.0, 5.0, 7.0 $\mu\text{g}/\text{band}$) for ASP & DIPH, (2.0, 4.0, 6.0 $\mu\text{g}/\text{band}$) for PAR, (0.5, 1.0, 2.0 $\mu\text{g}/\text{band}$) for SAL, PAP and PNP. For HPLC: the concentrations were (6.0, 8.0, 10.0 $\mu\text{g}/\text{mL}$) for PAP, PNP, and SALL, (30.0, 50.0, 70.0 $\mu\text{g}/\text{mL}$) for PAR & ASP and (15, 25, 35 $\mu\text{g}/\text{mL}$) for DIPH.^d Inter-day precision [average of three different concentration of three replicates each ($n = 9$) repeated on three successive days], the concentrations were the same as in intra-day precision.^e LOD and LOQ are calculated according to ICH, $3.3 \times \text{SD}$ of the residuals/slope and $10 \times \text{SD}$ of the residuals/slope, respectively.^f for HPTLC: average of the change in scanning wavelength (± 1 nm), ethyl acetate ratio (± 1 %) and saturation time (± 5 min). For HPLC: average for flow rate (± 0.1 mL/min) and pH (± 0.1).

Table 4

Results obtained by applying the proposed HPTLC-densitometric and HPLC-DAD methods for the determination of Paracetamol, Aspirin, and Diphenhydramine in Excedrin® PM Headache caplets and application of standard addition technique.

Pharmaceutical formulation	HPTLC-densitometry					HPLC-DAD method				
	Drug	%Found ± SD ^a	Standard Addition Technique Claimed (µg/ band)	Pure added (µg/ band)	%Recovery of the pure added ^b	Drug	% Found ± SD ^a	Standard Addition Technique Claimed (µg/ mL)	Pure added (µg/ mL)	%Recovery of the pure added amount ^b
Excedrin® PM Tablets (Each Tablet was Labelled to Contain 250 mg PAR, 250 mg ASP & 38 mg DIPH)	PAR	100.10 ± 1.403	5	2.5	98.94	PAR	99.46 ± 1.919	50	24.0	99.85
				5.0	101.33				52.0	101.57
				10.0	100.67				104.0	99.55
				Mean ± SD	100.32 ± 1.236				Mean ± SD	100.32 ± 1.094
				2.5	101.77				24.0	100.75
	ASP	100.55 ± 0.896	5	2.5	99.62	ASP	100.15 ± 1.751	50	24.0	100.42
				5.0	100.09				52.0	100.64
				10.0	100.49 ± 1.135				104.0	100.64
				Mean ± SD	100.49 ± 1.135				Mean ± SD	100.60 ± 0.169
				2.5	98.11				24.0	98.89
	DIPH	99.12 ± 0.964	0.76	0.38	98.12	DIPH	99.07 ± 1.028	7.60	3.2	98.89
				0.76	98.12				7.2	99.77
				1.52	99.06				15.6	101.55
				Mean ± SD	98.43 ± 0.541				Mean ± SD	100.07 ± 1.357
				0.38	98.12				7.2	99.77

^bAverage of three experiments.

^a Average of five determinations.

for detecting the likely found impurities and/or degradation product(s) in the pharmaceutical formulation.

3.5. Dissolution testing of Excedrin® PM caplets by the proposed HPLC method

The development of drug formulations should include the establishment of in-vitro dissolution profile to ensure batch-to-batch consistency and to correlate the in-vitro/in-vivo pattern. In-vitro release of the cited drugs was monitored in water as the dissolution medium [36]. Drug release profiles were constructed by plotting the percentage of drug dissolved versus time, Fig. 3. In the current study, the percentage of studied drugs' release from Excedrin® PM caplets in water were >75% after 45 min for PAR, ASP, and DIPH. Accordingly, the specified acceptance criteria expressed as quantity (Q) of active substance dissolved in definite time is fulfilled [36].

3.6. Statistical analysis

To ensure the results of the proposed methods, statistical comparison was carried out, after computing the *t* and *F*-values, between the obtained results and those resulted from applying the official methods [2,36]. The calculated *t* and *F*-values were found to be less than the respective theoretical ones, presenting no remarkable difference between the proposed and the applied official methods, Table S1.

4. Conclusion

Paracetamol, aspirin, and diphenhydramine are newly co-formulated in a novel triple action formula recently used as COVID-19 OTC remedy. Novel, sustainable, reliable, and cost-effective chromatographic methods were developed and validated for separation and estimation of the three cited drugs together with three of their potential official impurities. The use of safe and less hazardous solvents was greatly taken in consideration. The greenness profile was evaluated via four common assessment metrics; analytical eco-scale system, NEMI, GAPI and AGREE tools. Additionally, in-vitro dissolution profiles were successfully monitored for the cited drugs from caplet dosage form using the proposed HPLC method. The proposed methods' capacity to detect trace quantities of potential impurities also makes it a good choice for impurity profiling of the cited pharmaceutical formulation.

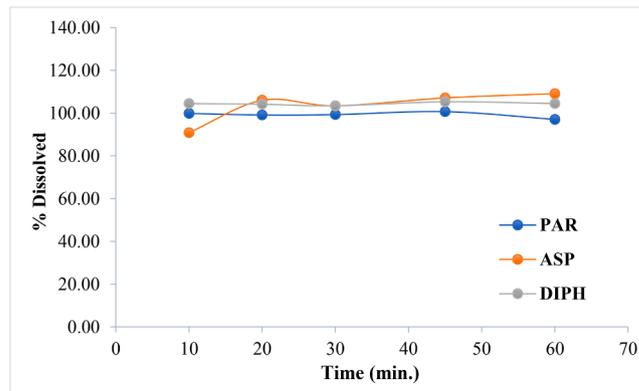


Fig. 3. Dissolution profiles of PAR, ASP, and DIPH from Excedrin® PM caplets in water as a dissolution medium.

CRediT authorship contribution statement

Hoda M. Marzouk: Conceptualization, Methodology, Writing – review & editing, Supervision. **Engy A. Ibrahim:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Maha A. Hegazy:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Samah S. Saad:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2022.107400>.

References

- [1] A. Nezhadali, M.R. Shapouri, M. Amoli-Diva, A.H. Hooshangi, F. Khodayari, Method development for simultaneous determination of active ingredients in

- cough and cold pharmaceuticals by high performance liquid chromatography, *Heliyon* 5 (12) (2019) e02871.
- [2] British Pharmacopoeia Commission, London, Her Majesty's Stationery Office, 2016.
 - [3] World Health Organization (WHO), Clinical Management of COVID-19, (2020).
 - [4] E. Wyszczka-Kaszuba, M. Warowna-Grzeskiewicz, Z. Fijalek, Determination of 4-aminophenol impurities in selected pharmaceutical preparations by HPLC method with amperometric detection, *Acta Pol. Pharm.* 58 (2001) 325–329.
 - [5] M. Bloomfield, A sensitive and rapid assay for 4-aminophenol in paracetamol drug and tablet formulation, by flow injection analysis with spectrophotometric detection, *Talanta* 58 (6) (2002) 1301–1310.
 - [6] T. Harada, E. Kimura, M. Hirata-Koizumi, A. Hirose, E. Kamata, M. Ema, Reproductive and Developmental Toxicity Screening Study of 4-Aminophenol in Rats 31 (2008) 473–486.
 - [7] R.C. Harmon, M.V. Terneus, K.K. Kinningham, M. Valentovic, Time-dependent effect of p-Aminophenol (PAP) toxicity in renal slices and development of oxidative stress, *Toxicol. Appl. Pharmacol.* 209 (2005) 86–94, <https://doi.org/10.1016/j.taap.2005.03.011>.
 - [8] G. Eichenbaum, M. Johnson, D. Kirkland, P. O'Neill, S. Stellar, J. Bielawne, R. DeWire, D. Areia, S. Bryant, S. Weiner, D. Desai-Krieger, P. Guzzie-Peck, D. C. Evans, A. Tonelli, Assessment of the genotoxic and carcinogenic risks of p-nitrophenol when it is present as an impurity in a drug product, *Regul. Toxicol. Pharmacol.* 55 (1) (2009) 33–42.
 - [9] T. Diaz, B.H. Trachtenberg, S.J.K. Abraham, R. KosagiSharaf, A.A. Durant-Archibold, Aspirin Bioactivity for Prevention of Cardiovascular Injury in COVID-19, *Front. Cardiovasc. Med.* (2020) 317.
 - [10] M. Tian, B. Adams, J. Wen, R. Matthew Asmussen, A. Chen, Photoelectrochemical oxidation of salicylic acid and salicylaldehyde on titanium dioxide nanotube arrays, *Electrochim. Acta.* 54 (14) (2009) 3799–3805.
 - [11] L.R. Reznikov, M.H. Norris, R. Vashisht, A.P. Bluhm, D. Li, Y.S.J. Liao, A. Brown, A. J. Butte, D.A. Ostrov, Identification of antiviral antihistamines for COVID-19 repurposing, *Biochem. Biophys. Res. Commun.* 538 (2021) 173–179, <https://doi.org/10.1016/j.bbrc.2020.11.095>.
 - [12] S. Pandolfi, V. Simonetti, G. Ricevuti, S. Chirumbolo, Paracetamol in the home treatment of early COVID-19 symptoms: A possible foe rather than a friend for elderly patients, *J. Med. Virol.* 93 (2021) 5704–5706.
 - [13] A.A.R. Mohamed-Hussein, K.M.E. Aly, M.-E. Ibrahim, Should aspirin be used for prophylaxis of COVID-19-induced coagulopathy? *Med. Hypotheses.* 144 (2020) 109975.
 - [14] G.M. Hadad, W.M.M. Mahmoud, The use of a monolithic column to improve the simultaneous determination of caffeine, paracetamol, pseudoephedrine, aspirin, dextromethorphan, chlorpheniramine in pharmaceutical formulations by HPLC—a comparison with a conventional reversed-phase silica-b, *J. Liq. Chromatogr. Relat. Technol.* 34 (2011) 2516–2532.
 - [15] Y. Yonggang, W. Zhenhong, C. Yinshan, Determination of Paracetamol, Caffeine and Aspirin in Compound Paracetamol Tablets by HPLC, *Chinese Pharm. Aff.* 6 (2008).
 - [16] H.A. Hashem, Chromatographic application on calixarene bonded stationary phases: A stability indicating method for simultaneous determination of paracetamol, caffeine and acetylsalicylic acid in exceedrin tablets, *Chromatographia* 71 (2010) 31–35.
 - [17] H.G. Cemal Akay, İ.T. Değim, A. Sayal, A. Aydin, Yalçın Özkan, Rapid and Simultaneous Determination of Acetylsalicylic Acid, Paracetamol, and Their Degradation and Toxic Impurity Products by HPLC in Pharmaceutical Dosage Forms, *Turkish, J. Med. Sci.* 38 (2008) 167–173.
 - [18] T. Dongala, N.K. Katari, A.K. Palakurthi, S.B. Jonnalagadda, Stability-indicating HPLC method for simultaneous quantification of 14 impurities in exceedrin tablet formulations and identification of new impurity by LC–MS in accelerated stability studies, *Biomed. Chromatogr.* 33 (10) (2019).
 - [19] G. Murtaza, I. Hussain, S.A. Khan, A. Shabbir, A. Mahmood, M.H.H. Bin Asad, K. Farzanal, N.S. Malik, Development of a UV-spectrophotometric method for the simultaneous determination of aspirin and paracetamol in tablets, *Sci. Res. Essays.* 6 (2011) 417–421.
 - [20] A.C. Moç, F. Sponar, A. Medvedovici, C. Sârbu, Simultaneous spectrophotometric determination of aspirin, paracetamol, caffeine, and chlorphenamine from pharmaceutical formulations using multivariate regression methods, *Anal. Lett.* 43 (5) (2010) 804–813.
 - [21] A. Özdemir, E. Dinç, F. Onur, Utilization of multivariate calibration techniques for the spectrophotometric simultaneous determination of paracetamol, aspirin and caffeine in a pharmaceutical formulation, *Turkish, J. Pharm. Sci.* 1 (2004) 139–151.
 - [22] A. Yiğit, Y. Yardım, M. Çelebi, A. Levent, Z. Şentürk, Graphene/Nafion composite film modified glassy carbon electrode for simultaneous determination of paracetamol, aspirin and caffeine in pharmaceutical formulations, *Talanta* 158 (2016) 21–29.
 - [23] A. Yiğit, Y. Yardım, Z. Sentürk, Voltammetric sensor based on boron-doped diamond electrode for simultaneous determination of paracetamol, caffeine, and aspirin in pharmaceutical formulations, *IEEE Sens. J.* 16 (6) (2016) 1674–1680.
 - [24] N. Ali, M. Abdelkawy, N.S. Abdelhamid, Simultaneous determination of paracetamol and diphenhydramine hydrochloride mixture in the presence of their degradation products, *IOSR J Pharm Biol Sci.* 6 (2013) 44–52.
 - [25] E. Souri, A. Rahimi, N.S. Ravari, M.B. Tehrani, Development of a rapid derivative spectrophotometric method for simultaneous determination of acetaminophen, diphenhydramine and pseudoephedrine in tablets, *Iran. J. Pharm. Res. IJPR.* 14 (2015) 435.
 - [26] K. Palur, S.C. Archakam, B. Koganti, Chemometric assisted UV spectrophotometric and RP-HPLC methods for simultaneous determination of paracetamol, diphenhydramine, caffeine and phenylephrine in tablet dosage form, *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* 243 (2020), 118801.
 - [27] G. Gao, X. Wang, Y. Dong, Z. Wang, S. Mu, Simultaneous determination of paracetamol, caffeine, ephedrine hydrochloride and diphenhydramine hydrochloride in compound paracetamol and diphenhydramine hydrochloride tablets by HPLC, *J. Shenyang Pharm. Univ.* 5 (2008).
 - [28] R. Yao, Q. Xu, L. Du, Direct determination of four components in compound paracetamol and diphenhydramine tablets by wide bore capillary gas chromatography, *Se Pu Chinese J. Chromatogr.* 25 (2007) 258–261.
 - [29] J. Plotka, M. Tobiszewski, A.M. Sulej, M. Kupka, T. Górecki, J. Namieśnik, Green chromatography, *J. Chromatogr. A.* 1307 (2013) 1–20.
 - [30] S.H. Youssef, D. Mohamed, M.A. Hegazy, A. Badawey, Green liquid chromatographic methods with ultraviolet and tandem mass spectrometry detection: an application to ternary mixture of paracetamol, pseudoephedrine, and cetirizine in capsules, *J. AOAC Int.* 103 (2020) 148–155.
 - [31] H.M. Marzouk, E.A. Ibrahim, M.A. Hegazy, S.S. Saad, Greenness profile assessment of selective liquid chromatographic methods for determination of a quaternary antimigraine combination along with three of their related official impurities, *Biomed. Chromatogr.* 35 (2021) 1–11.
 - [32] E. Darweish, M.S. Eissa, Y.M. Payez, H.M. Marzouk, Chromatographic estimation of a novel triple-therapy combination targeting *Helicobacter pylori* eradication in different matrices, *Bioanalysis* 13 (20) (2021) 1547–1557.
 - [33] H.M. Marzouk, M.R. Rezk, A.S. Gouda, A.M. Abdel-Megied, A Novel Stability-Indicating HPLC-DAD Method for Determination of Favipiravir, a Potential Antiviral Drug for COVID-19 Treatment; Application to Degradation Kinetic Studies and In-Vitro Dissolution Profiling, *Microchem. J.* 172 (2022) 106917.
 - [34] P. Anastas, N. Eghbali, Green chemistry: Principles and practice, *Chem. Soc. Rev.* 39 (2010) 301–312.
 - [35] D. Mohamed, M.A. Hegazy, G.M. El-Sayed, S.H. Youssef, Greenness evaluation of different chromatographic approaches for the determination of dextromethorphan, phenylephrine & brompheniramine in their pharmaceutical formulation, *Microchem. J.* 157 (2020), 104893.
 - [36] The United States Pharmacopeia and The National Formulary, Rockville MD, USA: USP 42-NF 37, U.S. Pharmacopeial Convention, 2019.
 - [37] A.M. Abou Al-Alamein, M.K. Abd El-Rahman, E.M. Abdel-Moety, E.M. Fawaz, Green HPTLC-densitometric approach for simultaneous determination and impurity-profiling of ebastine and phenylephrine hydrochloride, *Microchem. J.* 147 (2019) 1097–1102.
 - [38] P.S. Variyar, S. Chatterjee, A. Sharma, Fundamentals and theory of HPTLC-based separation, in, *High-Performance Thin-Layer Chromatogr.*, Springer (2011) 27–39.
 - [39] Reviewer Guidance, Validation of chromatographic methods. Center for Drug Evaluation and Research (CDER), (1994).
 - [40] M. Tobiszewski, M. Marć, A. Gałuszka, J. Namieśnik, Green chemistry metrics with special reference to green analytical chemistry, *Molecules* 20 (2015) 10928–10946.
 - [41] E.S. Elzanfaly, M.A. Hegazy, S.S. Saad, M.Y. Salem, L.E. Abd El Fattah, Validated green high-performance liquid chromatographic methods for the determination of coformulated pharmaceuticals: A comparison with reported conventional methods, *J. Sep. Sci.* 38 (2015) 757–763.
 - [42] A. Gałuszka, Z.M. Migaszewski, P. Konieczka, J. Namieśnik, Analytical Eco-Scale for assessing the greenness of analytical procedures, *TrAC, Trends Anal. Chem.* 37 (2012) 61–72.
 - [43] L.H. Keith, L.U. Gron, J.L. Young, Green analytical methodologies, *Chem. Rev.* 107 (6) (2007) 2695–2708.
 - [44] M. Tobiszewski, Metrics for green analytical chemistry, *Anal. Methods.* 8 (15) (2016) 2993–2999.
 - [45] Chemical List for Reporting Year (2019). https://www.epa.gov/sites/default/files/2020-02/documents/ry_2019_tri_chemical_list_0.pdf.
 - [46] J. Plotka-Wasyłka, A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index, *Talanta* 181 (2018) 204–209.
 - [47] F. Pena-Pereira, W. Wojnowski, M. Tobiszewski, AGREE - Analytical GREENness Metric Approach and Software, *Anal. Chem.* 92 (14) (2020) 10076–10082.
 - [48] ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (2005) R1.