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Research Article

Drug Quantification by Simultaneous HPLC Analysis

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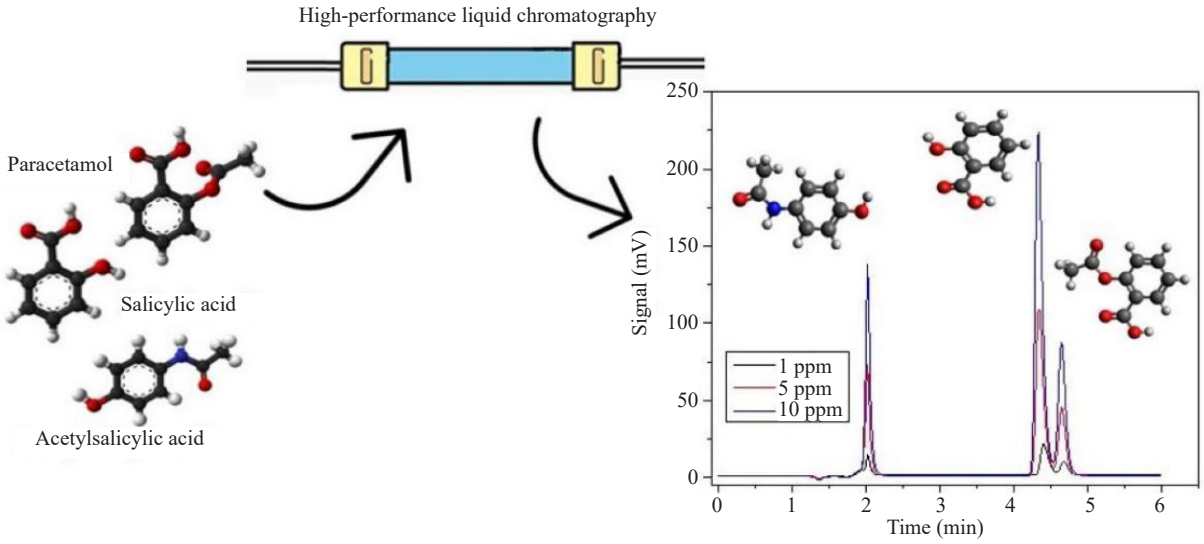
Graphical Abstract:



Abstract: High-Performance Liquid Chromatography (HPLC) analysis has been commonly used to monitor (separate, identify, and quantify) potentially toxic compounds in the environment. In this context, a large amount of research has investigated the so-called Emerging Contaminants (ECs), which are new pollutants that, until a few years ago, were not detected or were considered to pose a low risk to the environment and were not regulated. In this work, in particular, the technique was employed for the simultaneous determination of three emerging pollutants: Paracetamol (PAR), Salicylic Acid (SA), and Acetylsalicylic Acid (ASA) in the sample matrix. To study the effect of a set of factors on the responses, a factorial design was used. The responses (chromatographic parameters) considered in the experimental design took

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into account asymmetry, tailing factor, and resolution. The following factors were investigated: buffer solution pH, mobile phase flow rate, and the proportion of buffer in this mobile phase. The results indicated that optimized control of mobile phase pH, facilitated by judicious use of buffer solutions, forms the basis for achieving optimal HPLC separations. In addition, tests were performed to assess the interference from the matrix in the analysis.

***Keywords*:** Emerging Contaminants (ECs), compounds separation, chromatographic parameters, High-Performance Liquid Chromatography (HPLC)

1. Introduction

The growing dependence on pharmaceuticals to maintain human health poses an unintended consequence: the contamination of aquatic and terrestrial environments with these Emerging Contaminants (ECs). Among these, Paracetamol (acetaminophen, PAR), Salicylic Acid (SA), and Acetylsalicylic Acid (aspirin, ASA) are prevalent due to their widespread use as analgesics and antipyretics. While individually recognized for their therapeutic benefits, their presence in the environment raises considerable concerns for ecological and human health.

Paracetamol was identified as the most frequently detected pharmaceutical in surface water across 70 countries, highlighting the difficulty in removing this contaminant.[1,2] Its persistence in waterways stems from incomplete removal during wastewater treatment processes, leading to chronic exposure for aquatic organisms, which have been linked to detrimental effects like endocrine disruption in fish and genotoxicity in invertebrates.[3,4]

Aspirin, although less frequently detected than paracetamol, also presents environmental concerns. Its presence in surface water and sediments has been reported,[5,6] highlighting potential harm to aquatic populations. Its transformation products, including salicylic acid, further contribute to the broader ecological effects associated with its presence.

Salicylic acid, the primary metabolite of aspirin, is similarly detected in various environmental compartments, including wastewater, surface water, and groundwater.[7] Its presence can potentially alter microbial communities.[8]

High-Performance Liquid Chromatography (HPLC) stands out as an invaluable technique in the investigation of ECs due to its exceptional precision, sensitivity, and versatility. Almost 90% of all analyses of low molecular weight samples are carried out using Reverse Phase-HPLC (RP-HPLC). In RP-HPLC, the stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water, methanol, acetonitrile (or) mixtures of these. It works on the principle of hydrophobic interactions; hence, the more nonpolar the material is, the longer it will be retained. Reversed-phase HPLC is by far the most popular mode of chromatography.[9]

In particular, HPLC is optimal for the separation of chemical and biological compounds that are non-volatile. Among these, we can mention more specifically drugs such as aspirin, ibuprofen, or acetaminophen (Tylenol).[10]

In this context, the development of HPLC methods is important in the general use of this technique and in a wide range of applications. In pharmaceutical analysis, it is used to determine the purity and potency of medicinal substances and products, as well as to evaluate their stability and degradation. Compounds are studied under various conditions.[11]

ECs, often associated with newly recognized environmental threats, can be present in trace amounts, requiring analytical methods with high sensitivity for detection.[12] HPLC's capability to analyze complex mixtures and provide detailed information about individual components is crucial for identifying and characterizing these contaminants in environmental samples.[13,14] In this sense, the safety and efficacy of pharmaceutical products are fundamental requirements in drug therapy. The safety of a medicine is determined by its pharmacological-toxicological profile, as well as the adverse effects caused by impurities in bulk and pharmaceutical forms.[15]

Some authors have discussed the advantages and disadvantages of HPLC methods. Stojanović et al.[16] presented several modifications of HPLC methods that increase their environmental compatibility, as well as ways to assess environmental compatibility; Boukhobza and Crans[17] compared HPLC methods and modes by studying sample preparation, chelating reagents, mobile phase, and detection methods. This study indicated that the HPLC technique, which is a non-trivial technique due to its various methods and modes, showed good results in the characterization, separation, and speciation of vanadium compounds in a variety of matrices.

In particular, the pH value of the mobile phase holds significant importance as it strongly influences separation efficiency. This impact is due to its ability to control the ionization state of analytes, especially for compounds that can

ionize, such as pharmaceuticals, pesticides, and environmental contaminants.[13,18] Analysts manipulate the pH using buffer solutions to control interactions between analytes and the stationary phase, which in turn affects retention time, resolution, and peak shape.[19]

Beyond influencing basic separations, pH optimization allows overcoming specific analytical challenges. Analyzing weakly acidic analytes prone to tailing due to strong interaction with the stationary phase benefits from a slightly basic mobile phase (pH > pKa). This reduces analyte ionization, weakening interactions and minimizing tailing effects. Buffer solutions further enhance peak shape by maintaining a consistent pH throughout the analysis.[13,14] On the other hand, some salt buffers are hygroscopic, which can cause chromatographic alterations such as enhanced tailing of basic chemicals and possibly selectivity discrepancies.[20]

The evolution in the techniques employed to identify and quantify those contaminants is very important in order to help assessment and control the contamination levels. In this context, the contribution of this work is to investigate a technique capable of quantifying these contaminants (PAR, SA, ASA) in the same matrix using an HPLC system. A central factorial design of experiments was applied to investigate 3 factors: the buffer solutions (pH value), the flow of the mobile phase, and the amount of buffer in the mobile phase.

2. Materials and methods

2.1 *Chemicals*

The solutions used in this work were prepared with ultrapure water, and the chemicals were of analytical grade. Acetylsalicylic acid ($C_9H_8O_4$, ASA ≥ 98%, Biotec, São Paulo, Brazil), Salicylic Acid ($C_7H_6O_3$, SA ≥ 98%, Biotec), Paracetamol ($C_8H_9NO_2$, PAR ≥ 98%, Sigma-Aldrich, St Louis, USA), Potassium dihydrogen phosphate anhydrous (KH_2PO_4, Química Moderna, São Paulo, Brazil), Dipotassium hydrogen phosphate (K_2HPO_4, Química Moderna), Phosphoric acid (PA, Biotec), Acetonitrile (C_2H_3N, HPLC grade-J.T. Baker, Cuidad de México, Mexico) and Caffeine (99%) supplied by Química Fina LTDA.

2.2 *Equipment*

The HPLC model used was a YL Clarity 9100, equipped with a Security Guard Phenomenex pre-column (KJO-4282), C-18 column (5 µm, 150 × 4.6 mm, Kromasil) and Ultraviolet-Visible (UV-Vis) detector, monitoring the wavelength of 210 nm. The mobile phase consisted of mixtures of acetonitrile and phosphate buffers, varying the proportion of the mixture and the buffer used according to the experimental design. The methodology was based on the methods described by Kounaris Fuziki et al.[19,21] The column temperature was 30 °C and the injection volume was 20 µL.

2.3 *Design of experiments*

A 3^3 factorial design was chosen for the initial screening of the experimental conditions, opting for α points with values -1, 0, and +1. As one of the factors investigated was the pH from the buffers, a design that used different values of α was avoided. In all the tests, the concentration of all three contaminants was 10 ppm. Therefore, the flow rates tested were 0.8, 1.0, and 1.2 $mL \cdot min^{-1}$; phosphate buffers ranged in pH values from 2.8, 4.3, and 5.8; and the eluent proportions were 50:50 (acetonitrile:buffer); 40 : 60, and 30 : 70 (% buffer, 50, 60, and 70), respectively. A total of 27 screening tests were performed (Table 1).

The scanning test results showed that only the pH 2.8 buffer allowed the separation of peaks in the chromatogram. A new 2^3 factorial design was developed for the pH value of 2.8, considering variations in flow rate (Q) and buffer percentage, as described in Table 2.

Table 1. Screening tests conditions -3^3 design

Run	pH	Q (mL·min^{-1})	% Buffer
1	2.8	0.8	50
2	2.8	0.8	60
3	2.8	0.8	70
4	2.8	1	50
5	2.8	1	60
6	2.8	1	70
7	2.8	1.2	50
8	2.8	1.2	60
9	2.8	1.2	70
10	4.3	0.8	50
11	4.3	0.8	60
12	4.3	0.8	70
13	4.3	1	50
14	4.3	1	60
15	4.3	1	70
16	4.3	1.2	50
17	4.3	1.2	60
18	4.3	1.2	70
19	5.8	0.8	50
20	5.8	0.8	60
21	5.8	0.8	70
22	5.8	1	50
23	5.8	1	60
24	5.8	1	70
25	5.8	1.2	50
26	5.8	1.2	60
27	5.8	1.2	70

Table 2. 2^3 factorial design experimental matrix

Run	Q (mL·min^{-1})	% Buffer
1	0.8 (-1)	50 (-1)
2	0.8 (-1)	60 (0)
3	0.8 (-1)	70 (+1)
4	1 (0)	50 (-1)
5	1 (0)	60 (0)
6	1 (0)	70 (+1)
7	1.2 (+1)	50 (-1)
8	1.2 (+1)	60 (0)
9	1.2 (+1)	70 (+1)

In response, a function was created to consider 3 chromatographic parameters: Asymmetry, Tailing, and Resolution. Thus, the influences for the quantification of these species could be studied at the same time. Asymmetry and Tailing, the closer to 1, the better the result; Resolution, the higher the better. The calculations for each of these parameters were based on the work of Sankar.[9] In this way, a function V was created, which encompasses the 3 parameters, according to Equation (1):

$$V(A,B,C) = \left(\frac{1}{\exp^{|(A-1)|}}\right) * \left(\frac{1}{\exp^{|(B-1)|}}\right) * C \quad (1)$$

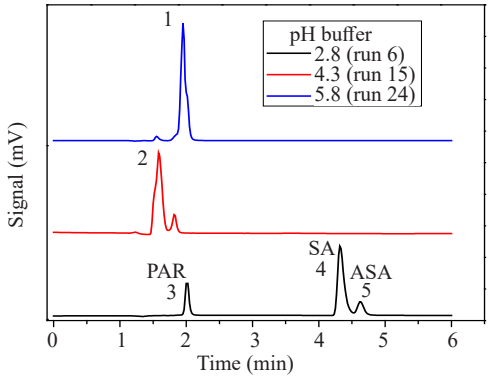
Where: A Asymmetry; B Tailing; C Resolution and V Quantitative response (Higher means a result with more resolution, less asymmetric and tailing). For each chromatogram, a value was calculated and considered as the response variable in the response surface methodology together with the input data.

2.4 *Matrix influence*

Additional tests were performed to assess the matrix interference in the analysis. A solution was prepared with the same concentrations of PAR, SA, and ASA (10 ppm) in water from the public supply (tap water), which, due to the nature of the water treatment process (chlorination process), contains chloride ions. Since the conditions of this water are not fully known, there may be other interfering agents, but in minimal quantities compared to Cl^-. In another solution, in addition to using tap water, caffeine was added as an interfering agent in the quantification process of the other compounds. Caffeine is present in the population's medicines and beverages, such as coffee, tea, soft drinks, and energy drinks. Thus, caffeine is a compound that can be considered a "tracer" in water since, if the presence of caffeine is detected, it is most likely that this caffeine is present there due to anthropogenic action, either through the discharge of sanitary effluent or the discharge of industrial effluent. The tests were performed at a 1.0 $mL \cdot min^{-1}$ flow rate, using the three buffer solutions mentioned in section 2.3.

3. Results

3.1 *Factors influence*



Peak	Ret. time (min)	Resolution	Height (mV)	Area (mV.s)
1	1.95	2.575	504.838	2888.43
2	1.158	-	350	3336.483
3	2.017	0.862	157.257	1151.335
4	4.317	13.57	298.601	2102.73
5	4.633	1.495	60.105	508.553

Figure 1. Comparative chromatograms from runs 6, 15, and 24 [1 $mL \cdot min^{-1}$; 30 : 70 (acetonitrile: buffer)]

Figure 1 shows the results obtained under flow conditions of 1 mL·min^{-1}; 30 : 70 (acetonitrile: buffer) for pHs 2.8, 4.3, and 5.8. As shown in Figure 1, at pHs 4.3 and 5.8, it is not possible to identify the separation of three distinct peaks or with adequate resolution for the three drugs studied (ASA, SA, and PAR). Based on this result, it was necessary to develop a second experimental design considering only pH 2.8 and variations in flow rate and buffer percentage in the eluent.

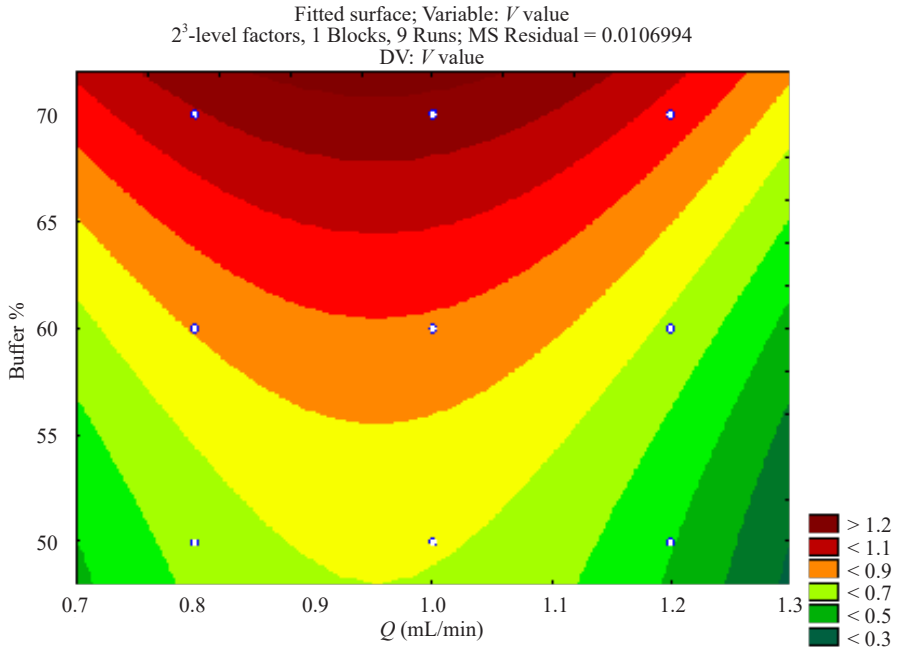


Figure 2. Fitted contour surface for *V* value

Table 3. Experimental design matrix and calculated *V* value used as response

Run	*Q* (mL·min^{-1})	% Buffer	*V* (value)
1	0.8 (-1)	50 (-1)	0.6471
2	0.8 (-1)	60 (0)	0.9053
3	0.8 (-1)	70 (+1)	0.9872
4	1 (0)	50 (-1)	0.7456
5	1 (0)	60 (0)	0.8580
6	1 (0)	70 (+1)	1.1537
7	1.2 (+1)	50 (-1)	0.4657
8	1.2 (+1)	60 (0)	0.6036
9	1.2 (+1)	70 (+1)	1.0723

Table 3 presents the results (*V*) of the 2^3 factorial design: eluent flow rate and acetonitrile:buffer ratio. The answer is given by the calculated value of *V*. The contour surface obtained is indicated in Figure 2.

The Analysis of Variance (ANOVA) results are shown in Table 4.

Table 4. ANOVA generated as response for the Design of Experiments (DoE)

Factor	SS	df	MS	*F*	*p*
(1) *Q* (mL·min^{-1}) *L* + *Q*	0.06481	2	0.03241	1.7884	0.27871
(2) % Buffer *L* + *Q*	0.28038	2	0.14019	7.7368	0.04219
Error	0.07248	4	0.01812		
Total SS	0.41767	8			

3.2 *Method validation*

To validate the analysis method, the calibration curves for the pollutants indicated in Figure 3 were obtained. The chromatographic analysis is presented in Figure 4.

The limit of detection (LOD) of an individual procedure is the smallest amount of analyte in a sample that can be detected; however, it is not necessarily quantified as an exact number. The limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be measured with acceptable accuracy and precision under the declared operating conditions of the method.[20] Additionally, the LOD and LOQ can alternatively be calculated using the standard deviation of response (SD) and calibration slope(s) of the curve(s) at values close to the LOD.

Table 5. LOD and LOQ calculated for each drug

Drug	LOD (mg·L^{-1})	LOQ (mg·L^{-1})
PAR	0.31	0.76
SA	0.52	0.89
ASA	0.29	0.64

The LOD and LOQ of each of the components studied were determined using equations (2) and (3). The results are shown in Table 5.

$$\mathrm{LOD} = \frac{3.3 * \mathrm{SD}}{S} \tag{2}$$

$$\mathrm{LOQ} = \frac{10 * \mathrm{SD}}{S} \tag{3}$$

SD: Standard deviation of the intercept; *S*: Slope of the calibration curve.

The method validation also considered the accuracy based on the analyte recovery (Table 6).

Table 6. Calibration curves theoretical and measured concentrations. Calculated accuracy based on the analyte recovery (AR%)

Theoretical concentration (ppm)	Measured concentration (ppm)			AR %		
	PAR	SA	ASA	PAR	SA	ASA
1	1.00	0.96	0.97	99.57	95.93	97.50
2.5	2.49	2.51	2.43	99.56	100.27	97.14
5	5.02	5.03	5.13	100.49	100.61	102.68
7.5	7.50	7.57	7.54	100.01	100.87	100.48
10	9.99	9.94	9.93	99.91	99.38	99.26

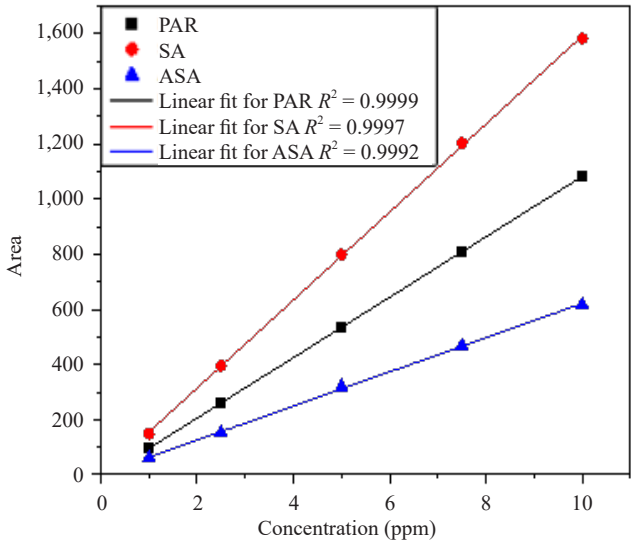


Figure 3. Calibration curves for the pollutants

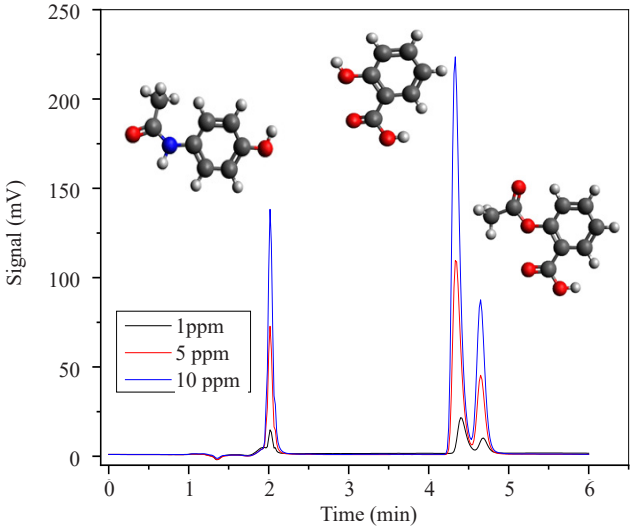


Figure 4. Chromatograms for the samples containing all the pollutants

3.3 *Matrix influence*

Sample matrices can interfere with separation and detection, leading to reduced sensitivity and selectivity. In addition, column degradation can occur over time due to sample matrix effects, column overloading, and other factors. Periodic column maintenance and replacement can help address this challenge. Table 7 presents the characterization of tap water (Cl^-).

Table 7. Tap water parameters

Parameter	pH	Color	Fluoride	Turbidity	Free residual chlorine	Total aluminum	Total iron	Total manganese
	7	3uH	0.8 mg/L	1 NTU	1.2 $mg \cdot L^{-1}$	0.1 $mg \cdot L^{-1}$	0.1 $mg \cdot L^{-1}$	0.0 $mg \cdot L^{-1}$

Figure 5 shows the results obtained under flow conditions of 1 mL·min^{-1}; 30 : 70 (acetonitrile:buffer), pH 2.8; matrix: Ultrapure, Cl^-, and Caffeine.

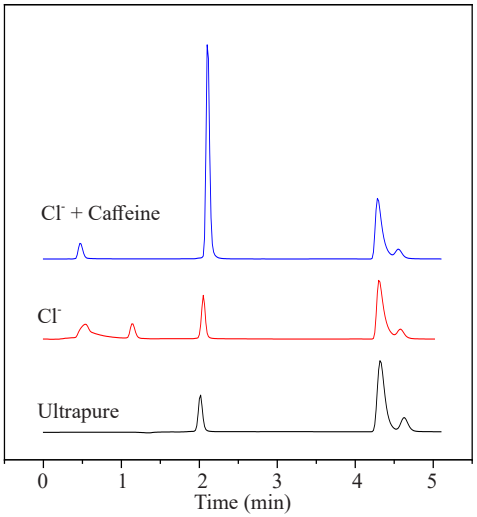


Figure 5. Chromatograms for the samples in different matrices

4. Discussion

Analyzing the preliminary results obtained from the 27 screening tests, with the help of calculating the *V* value, it was possible to quantify which of the analyzed methods are more efficient. However, before calculating the results, a qualitative check was carried out, first seeking to identify 3 distinct peaks in the chromatograms. The experiments that used the buffer with pH 2.8 were the only ones that managed, although not perfectly, to separate the three contaminants and generate the three respective peaks, as seen in Figure 1. This can be explained by the fact that buffer composition directly determines the ionic strength, pH, and electrostatic interactions within the separation medium. These parameters critically influence analyte ionization state, retention, and resolution.

The chromatograms are from runs 6, 15, and 24 (conditions see Table 1). The experimental run 6 was the one that presented the best *V* value, among which they were able to separate the 3 contaminants effectively. Both tests were performed with a flow rate of 1 mL·min^{-1} and a ratio of 30 : 70 (acetonitrile:buffer); differing only in the use of buffer, pH 2.8, 4.3, and 5.8 for tests 6, 15, and 24, respectively.

Observing the pKa values of the contaminants, SA: 2.97;[22] ASA: 3.70;[23] PAR: 9.38;[24] It can be seen that using the pH 2.8 buffer, the ASA is protonated, as well as part of the SA, favoring a greater interaction with the stationary phase and also interacting with each other, being able to separate the peaks and identify them separately in the chromatograms.[9,13] In other buffers, these interactions are less favored, meaning that, with the pH 4.3 buffer, 2 peaks overlap and with the pH 5.8 buffer, the peaks overlap completely, losing the ability to identify each of the pollutants studied separately.[10,14]

Due to this, tests with buffers of pH 4.3 and 5.8 were not considered to carry out the experiments. Thus, an adjustment was made for a face-centered factorial design with only 2 factors: eluent flow rate and acetonitrile:buffer ratio (Table 1). The experiments were then analyzed, generating the results in Figure 2 and Table 3.

The ANOVA table was generated with $R^2 = 0.8975$, with the buffer proportion as a significant factor, at a confidence level of 95%, according to the results. As shown in Figure 2, the fitted surface indicates that the higher the buffer proportion, and a flow of 1 mL·min^{-1} are the best conditions to separate the peaks to an acceptable level. The condition of 30 : 70 (acetonitrile:buffer) and a flow equal to 1 mL·min^{-1} was identified as the optimal condition investigated.

The optimal conditions were applied to validate the method and calculate the LOD and LOQ. This analysis was carried out for each pollutant within its respective quantification range. The concentration and analytical signal relationship was evaluated based on the three pharmaceutical calibration curves. The results indicated good linearity

in the confidence curves. Across all pharmaceutical products, strong correlations were observed, with correlation coefficients greater than 0.999 (Figure 3). It can also be observed that all pollutants could be observed separately in the chromatogram (Figure 4). The calculated concentration (Table 6) indicated considerable accuracy of the method, with recovery (AR%) higher than 95%.

Table 8. Comparison of some of the previously reported methods for simultaneous determinations of compounds

Compounds	Column	Mobile phase	pH	Detection λ	LOD/LOQ	Other information	Refences
ASA, PAR, caffeine, and phenobarbital in tablets	Bio SiL HL C18, 5 µm, 250 × 4.6 mm	Acetonitrile-water (25 : 75 v/v)	2.5	207 nm	LOD: 9×10^{-5} – 1.7×10^{-4} mg·ml^{-1}; LOQ: 2.5×10^{-4} – 5.6×10^{-4} mg·ml^{-1}		25
Determination of Bisoprolol (BIS), Amlodipine besylate (AML), Telmisartan (TEL), and Atorvastatin (ATV) within human plasma	Thermo Hypersil BDS C18 column (150×4.6 mm, 5.0 µm)	Mobile phase comprised of ethanol and 0.03 M potassium phosphate buffer (40 : 60) ratio	5.2	210-260 nm	-	Fluorescence detector was set to 227ex/298em for BIS, 294ex/365em for TEL, 274ex/378em for ATV, and 361ex/442em for amlodipine	26
Hydrochlorothiazide (HCZ), Amlodipine (AMD), Olmesartan (OLM), Telmisartan (TEL), and Irbesartan (IRB) in binary and ternary coformulations	Zorbax C18	Acetonitrile, methanol, and 20 mM phosphate buffer in a 45 : 20 : 35 (v/v/v) ratio	3.5	230 nm	LOQ (µg/mL) 4.31 (HCZ); 0.68 (AMD); 4.07 (OLM); 7.06 (IRB); 4.14 (TEL)		27
PAR, SA and ASA in serum b	25 cm × 4.5 mm I.D. Spherisorb 5 µm ODS (Jones Chromatography)	Acetonitrile-methanol-water (25 : 10 : 65)	3.0	234 nm	-		28
Determining the content of SA and individual unknown impurities in a new pharmaceutical product	Waters Symmetry C18 (4.6 × 250 mm, 5 µm)	Mixture of 85% orthophosphoric acid, acetonitrile, and purified water (2 : 400 : 600)		237 nm	LOQ was established as a concentration of 0.0005 mg·mL^{-1}		29
Determination of ascorbic acid, phenylephrine, PAR, and caffeine	Onyx Monolithic C18 (100 × 4.6 mm)	Acetonitrile and phosphate buffer (pH 6.50) 10 : 90 (v/v)	6.50	210 nm (phenylephrine, PAR, and SA) and 235 nm (ascorbic acid and caffeine)			30
Emerging pollutants: PAR, SA, and ASA	C-18 column (5 µm, 150 × 4.6 mm, Kromasil)	30 : 70 (acetonitrile: buffer)	2.8, 4.3, and 5.8		LOD (mg·L^{-1}): 0.31 (PAR); 0.52 (SA); 0.29 (ASA). LOQ (mg·L^{-1}): 0.76 (PAR); 0.89 (SA); 0.64 (ASA)	Design experiments Matrix Influence	Present work

Based on the conditions indicated by the DoE as the best and the calibration curves for each of the medicines, the LOD and LOQ of each pollutant were determined as indicated in Table 5. The results indicated LOD and LOQ levels are considered acceptable, and comprise the limits of the calibration curve, presenting a good working range. A table comparing some of the previously reported methods for simultaneous determinations of compounds is presented (Table 8).

The use of water with Cl^- as a matrix had a reduced influence on the results obtained for PAR, SA, and ASA. However, the addition of caffeine led to a considerable increase in the peak associated with PAR, affecting the measurement of this drug. This result highlighted the relevant interference that other organic molecules can present in the results measured from the present methodology.

5. Conclusions

The HPLC method was studied and validated for the analysis of the drugs: PAR, ASA, and SA. Mobile phase pH manipulation, facilitated by the judicious use of buffer solutions, emerges as a cornerstone for achieving optimal HPLC separations. From fine-tuning retention and resolution to tackling specific analytical challenges, understanding and controlling pH enables the full potential of this versatile technique. In addition, the influence of the matrix on the analysis of caffeine was evaluated, influencing the measurement of this drug.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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