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Accurate and Precise Method Validation for Quantifying Ascorbic Acid in COVID-Related Dietary Supplements

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Abstract

In recent years, increased scrutiny of food products and dietary supplements has highlighted the need for precise and accurate analytical methods to ensure component safety from production to consumption. Thus, the present study was aimed to develop a rapid, sensitive and green liquid chromatographic method for measuring ascorbic acid (ASA). The experiment was carried out by using a simplest HPLC, employing an isocratic elution setup, and lowering the amount of organic modifier, with a ZORBAX Eclipse C18 column (4.6 x 150 mm, 5 µm) at room temperature. The mobile phase consisted of methanol and phosphate buffer, adjusted to pH 3 ± 0.1 in a 10:90 (v/v) ratio. Ultraviolet detection at 254.00 nm was used for monitoring the eluent. Accuracy was found to have an RSD of 1.255%. Intra- and inter-day precision were both under 2.0% RSD, with values of 1.494% and 1.236%, respectively, and recovery ranged between 100-102%. The limit of detection was 1.080 µg/mL, and the limit of quantitation was 3.260 µg/mL. The developed method was effectively applied to the ASA dosage form. The evaluation of the developed method's greenness yielded a high rating of 0.89, demonstrating its practical reliability and effectiveness.

Keywords: Ascorbic Acid, High-Performance Liquid Chromatography, Dietary Supplements.

1. Introduction

Ascorbic acid (ASA), commonly known as Vitamin C, stands as one of the most important nutrients required for maintaining human health. It plays a pivotal role in various physiological functions, including collagen synthesis, antioxidant defense, immune response modulation, and wound healing. This water-soluble vitamin cannot be synthesized by the human body, necessitating regular dietary intake to prevent deficiency-related conditions such as scurvy (Nikolin B,et.al, 2004). In recent times, the role of ASA has garnered significant attention, particularly amidst the COVID-19 pandemic. While not a direct cure for the virus, studies have explored its potential benefits in supporting immune function and reducing the severity of respiratory infections. Research suggests that adequate levels of ASA may help mitigate inflammation and oxidative stress, factors often exacerbated during viral infections like COVID-19. Furthermore, its antioxidant properties are thought to contribute to the protection of cells from damage caused by free radicals, potentially aiding in the recovery process from various illnesses (Sorice A. et,al, 2014). Many

assays were applied to analyze ASA alone and with other compounds starting with the most famous High-Performance Liquid Chromatography (HPLC) in dosage form, food products, and dietary supplements. (**Table 1)** shows a comparison between the developed method and previously published HPLC methods for the analysis of ASA. Other recent thin-layer chromatography methods were reported to be sufficient in separating ASA in dosage forms (Akasaka K,et,al, 2013 and Pyka-Pająk A, et,al, 2018), Besides a lot of spectrophotometric methods applied (Sharaa ie, et al. 2019, Razzaka, et al., Fadhel dh, et al. 2012).

In the current study, a simple HPLC setup was developed and proved to be one of the best reliable methods for separations and is environmentally friendly as it presents several environmental advantages over traditional methods for analyzing It has reduced solvent usage, high sensitivity, and selectivity, shorter analysis time, and efficiency, compatibility with green chemistry, versatility, and flexibility. These improvements not only enhance environmental sustainability but also maintain high analytical performance.

Table 1: HPLC reported Methods:

Column	Mobile phase	Detection	Flow rate	Application	Ref
Reversed-phase high-performance liquid chromatographic method	mixture of the aqueous phase –methanol (80:20, v/v, pH 6.0 adjusted by phosphoric acid).	245nm	0.9 mL/min.	Oral solutions	(Hu L,et,al 2012)
C18 (symmetry® 4.6 µm × 280 mm, particle size 5 µm)	5 mM HTAB + 50 mM KH2PO4.	254 nm and 265 nm	1.2 mL/min.	Human plasma	(M. G. Kim,et, al, 2016)
C18, spherisorb column (150 mm 4.6 mm i.d. and 3 mm particle size)	0.01 mol/L dihydrogen ammonium phosphate, maintained at pH 2.6	254 nm	1 mL/min	Fruits and vegetables	**(**Chebrolu KK,et,al 2012)
C18 column (100 × 3 mm, 3 µm)	As eluent A, 15 mM phosphate buffer at pH 2.7, and eluent B, methanol		0.4 mL/min	Spice Plants	(Stan M,et,al, 2014)
C-18 (250 mm x 4.6)	acetic acid (500 ml) to 1.5g of 1-hexanesulfonic acid sodium salt and mixing well (pH 2.6)	280 nm	0.7 mL/ min	Dosage form	Mitic SS 2011
C18 (4.6 x 250 mm, 5 µm)	25 % methanol and 75 % water containing 0.05 mol/L monobasic sodium phosphate, 0.05 mol/L sodium acetate trihydrate, 189 µmol/L dodecyl trimethylammonium chloride and 36.6µmol/L tetraethylammonium bromide, adjusted to pH 4.8 using orthophosphoric acid.	Electrode 1 was set to -175 mV, detector range 500 nA, and electrode 2 was set at 550 mV, detection range 50 µA	0.8 ml/min	Plasma	Robitaille L 2015

C18 (Zorbax, 250 mm × 4.6 mm, 5.0 µm).	methanol (solvent B) and mixed aqueous solutions of 50 mM potassium dihydrogen phosphate and 2.5 mM hexadecyl trimethyl ammonium bromide (solvent A	242 nm	0.7 mL/min	Honey samples and food products	(Wu F, et al. 2023)
C18 (4.6 × 250 mm,)	acetonitrile-NaH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95,v/v) with a	245 nm	0.8 /mL min	Dosage form	(Long X,et al. 2020)
C18 column	HPLC grade water is brought to pH 2.2 with sulphuric acid: methanol (80:20, v/v) for the mobile phase.	243 nm	1 mL/min		(Ahmida MH, et al. 2009)
C18 (250 x 4.6 mm , 5µm)	isocratic elution mobile phase 0.1% orthophosphoric acid and acetonitrile (95:5, v/v)	254nm	1 mL/min	citrullus lanatus red flesh juice b	(Hashim NNA, et al. 2017)
C18 (ZOBRAX 4.6 µm × 150 mm, particle size 5 µm)	Methanol: phosphate buffer(10:90 v/v)	254 nm	1mL/min	Dosage form	**Current method**

2. Experimental:

2.1. Instruments and Software:

HPLC Agilent 1260 Infinity Series Liquid chromatography (Waldbornn, Germany). It consists of a quaternary pump-model VL G7111A, a variable wavelength detector (VWD)-model G7114A and a ZOBRAX Eclipse XDB C18 column- (4.6 * 150 mm), particle size (5 µm), IKA T10 B ultra turrax homogenizer (Germany), Jenway 3510 pH meter (UK by Cole-Parmer) with a combination glass electrode served to carry out pH measurements and D-78224 Singen/ Htw. transonic TI-H-5 sonicator (Elma, Germany) for degassing purposes.

2.2. Samples

2.2.1. Pure samples:

Ascorbic acid was bought from EL. NASR PHARMACEUTICAL CHEMICALS CO, EGYPT, ABUZAABAL, its purity was assessed to be 99.94 ± 1.58.

2.2.2. Market samples:

Vitamin C and zinc tablets IMMUNO-MASH® B.NO:M1067423, brought from Egyptian pharmacies.

2.2.3. Chemicals and reagents:

All reagents were of analytical grade and solvents were of HPLC grade: Methanol: HPLC grade (Merck, Germany); Phosphate buffer pH 3 ± 0.1: Prepared by dissolving 6.8 g of sodium dihydrogen phosphate (Adwic, Cairo, Egypt) in distilled water and was diluted to 1000.0 mL with water. The pH

Standard Solutions:

(a) Standard stock solutions of ASA(1mg/mL):

Stock solutions of ASA were prepared by accurately weighing 100 mg of each into four separate 100- mL volumetric flasks, 70 mL water was added, sonicated, and then completed to the mark using the same solvent to obtain 1 mg/mL.

(b) Standard working solutions of ASA (100µg/mL):

An accurate volume of 5 mL of the corresponding standard stock solution of ASA (1.0 mg/mL) was accurately transferred into a 100-mL volumetric flask and was completed to the mark with the used mobile phase consisting of methanol: 0.05 M phosphate buffer pH 3 ± 0.1 (10:90, v/v) to obtain a final concentration of 100 µg/mL working solution of each. All standard solutions were kept in bottles in the refrigerator.

2.3. Procedures:

2.3.1. Chromatographic conditions:

The proposed method was carried out at ambient temperature on ZOBRAXeclipse column – C18 (4.6 x 150 mm, 5µm). The mobile phase consisted

was adjusted with 1N phosphoric acid.

of methanol: phosphate buffer pH = 3 ± 0.1 at a ratio (10:90, v/v). The mobile phase was filtered using 0.45 mm membrane filters and degassed by a sonicator for 30 mins before use. The flow rate was kept at 1 mL/min throughout the separation process and the column was conditioned with the mobile phase for 30 mins. UV detection was performed at 254.0 nm

2.3.2. Construction of calibration curve:

Aliquots of ASA equivalent to 40–400 µg were separately and accurately transferred from their standard working solutions (100 µg/mL) into sets of separate 10- mL volumetric flasks and the volume was completed to the mark using the stated mobile phase to prepare solutions with concentrations in the range of 4-40 µg/mL for ASA. Triplicates for each concentration of each solution were injected into the HPLC under the previously mentioned chromatographic conditions. The linear calibration curve was constructed the ASA relating the area under the curve to its corresponding concentrations and the regression equation was computed.

2.3.3. Application of the proposed HPLC for the determination of ASA in Dosage Form by standard addition:

A sample consisting of 5 tablets of Vitamin C (IMMUNO-MASH®), each containing 500 mg/tablet, was weighed and pulverized. A portion of the resulting powder, equivalent to 1000 µg/mL, was accurately weighed and transferred to a 100 mL volumetric flask using the specified mobile phase. The flask was filled to the mark, then sonicated in an ultrasonic bath for 30 minutes and filtered using a syringe filter. Subsequently, solutions of 5, 10, and 30 µg/mL were prepared from this sample in separate 100 mL volumetric flasks, with each flask containing ASA standard solution of 10 µg/mL. After standard additions to the drug samples, concentrations of 15, 20, and 40 µg/mL were obtained, respectively. These solutions were adjusted to volume using the specified mobile phase and analyzed by the developed HPLC method. The entire process followed the outlined procedure for constructing a calibration curve.

3. Results and Discussion:

The pharmaceutical industry has accepted HPLC as separation power and sensitivity to quantification. HPLC can be found to be an integral part of the modern pharmacopeia. The powerful separation of HPLC and the ability to combine with a variety of detectors solved many problems and enabled the selective detection and sensitive determination of compounds in mixtures and complex matrices; the fact that let HPLC take its place in pharmacopeias and led HPLC to the frontline of the analysis techniques in the pharmaceutical industry. Among the huge variety of the present and continuously uprising stationary phases; still, the reversed-phase (RP) chromatography got the greatest share of more than75% in pharmaceutical applications; which could be attributed to the availability of several solvents that enable wider selectivity ranges and its advantages are numerous, the most outstanding being the extremely simple operating conditions.

3.1. Method Optimization:

The simultaneous determination and separation of ASA were achieved after several trials. Different mobile phase compositions and different ways of elution were studied of ratios; methanol: phosphate buffer (50:50, 25:75, 15:85, 12:88, 10:90 v/v) respectively, different buffer pH values were applied at 4 and PH 3, where asymmetry and resolution were functions of judgment as well as reducing the retention time **(Table 2) and (Figure 1)**. The optimum elution procedure was found to be an isocratic mobile phase consisting of methanol: 0.05 M phosphate buffer pH 3 ± 0.2 at a ratio (10:90, v/v). The retention time was found to be fast at 1.7 minutes for ASA. The pH of the phosphate buffer was of great importance as it critically affected the retention time and peak symmetry, pH 3 ± 0.2 was found to be suitable. Also, the usage of ZOBRAX eclipse XBD C18 column (4.6 x 150 mm, 5µm) improves the peak shapes and resolution of ASA. The mobile phase was introduced at a flow rate of 1 mL/min with UV detection at 254.0 nm. The usage of an isocratic system was sufficient for the quantification of ASA as shown in (**Figure 2).**

3.2. Method validation:

Validation of the proposed chromatographic method was done according to the ICH guidelines (kameyama y, et al. 2019) including linearity,

a reliable analytical technique because of its proven accuracy, specificity, precision, and robustness.

Table 2: Method Optimization to choose the most suitable system for the current method to determine ASA.

Organic Solvent	Retention Time at PH 4	Retention Time at PH 3
50	-	1.63
25	1.3	-
15	1.1	-
12	-	1.67
10	-	1.7
5	-	1.7

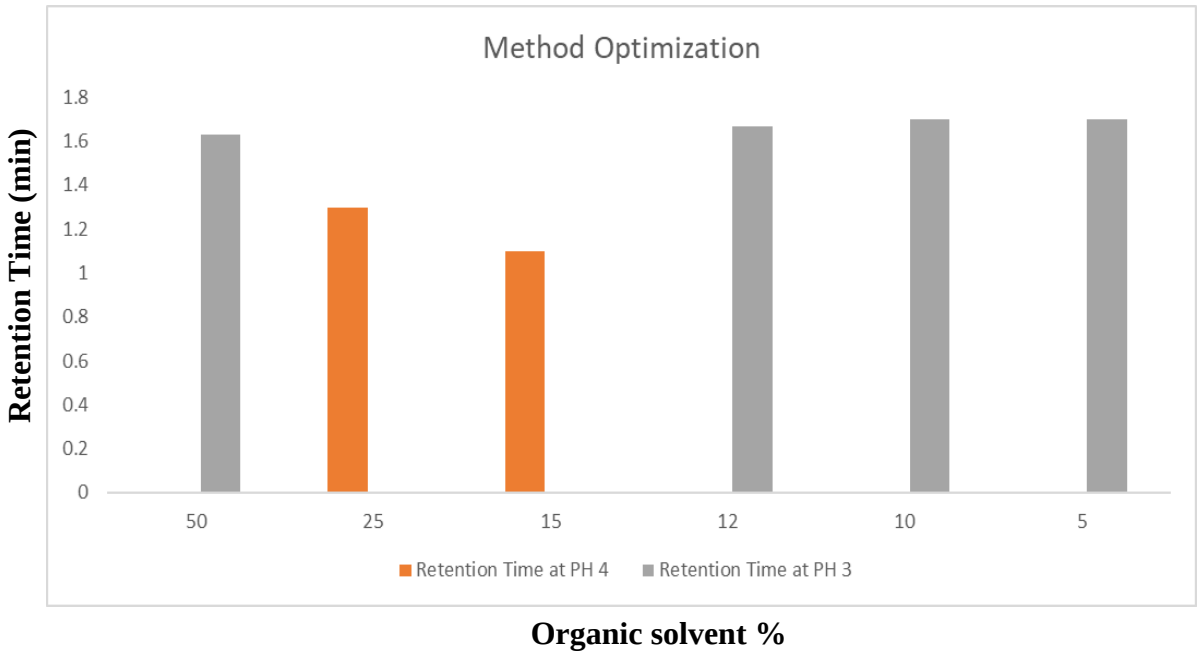

Figure 1: Method optimization according to changeable data in organic solvent % and buffer pH.

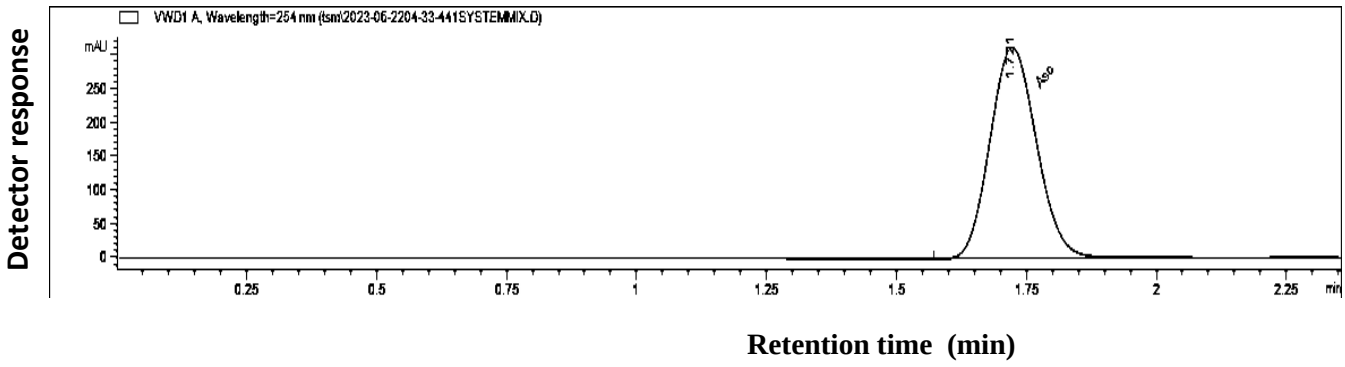

Figure 2: Chromatogram showing sufficient peak of ASA standard.

Table 3: Regression and validation parameters of the proposed HPLC method for the determination of pure ASA samples

Parameters	**HPLC**
	ASA
Range µg/mL	4-40
Slope	56.280
Intercept	122.760
Correlation coefficient (r)	0.9998
aLOD µg/mL	1.080
aLOQ µg/mL	3.260

[a] Calculated from equation [LOD =3.3 (SD / S), LOQ = 10 (SD / S); where SD is the standard deviation of intercept and S is the slope of the calibration-curve.

Table 4: System Suitability Parameters HPLC method developed for the determination of ASA.

Parameters	**HPLC**
	ASA
Capacity factor (K)	0.185
Number of theoretical plates	3568.071
HETP (cm/plate)	0.00718

Table 5: Accuracy calculations.

Concentration taken µg/mL	**Accuracy**	
	Mean concentration found* µg/mL	**Recovery %**
6	5.983	99.720
17.5	17.754	101.452
20	20.258	101.288
30	29.523	98.408
35	34.940	99.828
Mean %recovery ± SD		100.139± 1.256
RSD %		1.255
N.B.: * n = 5		

Table 6: Precision Calculations

Concentration taken µg/mL	**Intraday precision**		**Interday precision**
	Mean concentration found* µg/mL	**Recovery %**	**Recovery %**
15	15.110	100.740	100.312
20	19.645	98.223	99.262
30	29.440	98.133	98.759
Mean %recovery ± SD		99.032± 1.480	99.444 ± 1.229
RSD		1.494	1.236
N.B.: * n = 3			

Table 7: Standard Addition for application of ASA Dietary Supplement IMMUNO-MASH® of the proposed HPLC method for determination of ASA

Claimed drug	**Dietary Supplement**	**Found% ± SD[a]**	**Standard Addition[a]**		
			Added µg/mL	**Found µg/mL**	**Recovery%**
IMMUNO-MASH® 500mg	**10**	15.291	5	15.290	101.940
		19.545	10	19.545	97.726
		39.591	30	39.590	98.977
			Mean ± SD		99.548 ± 2.164
			%RSD		2.174

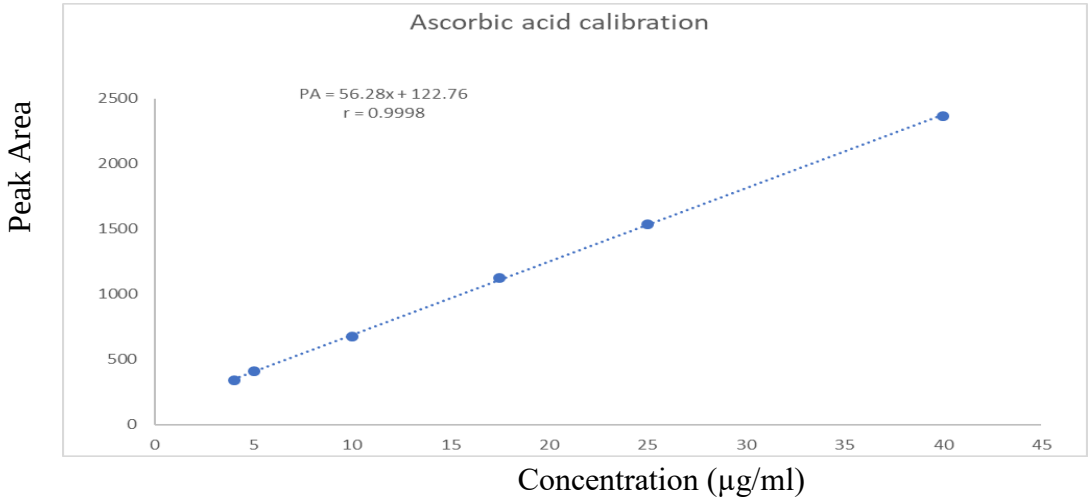

Figure 3: Calibration curve showing ASA Linearity.

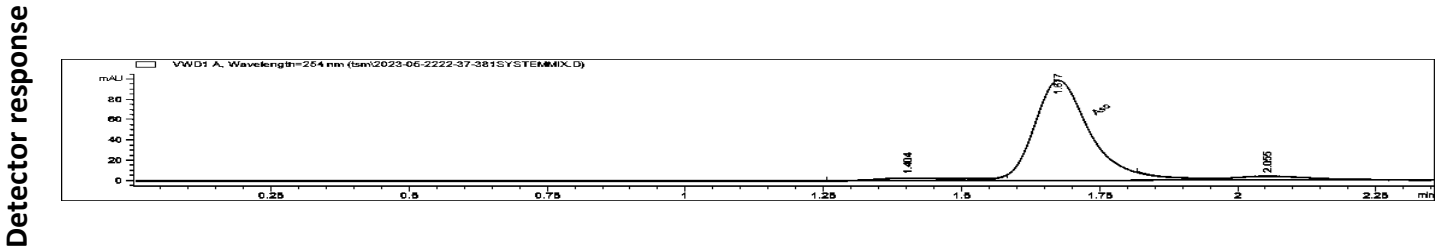

Figure 4: Chromatogram of a sample of IMMUNO-MASH® (ASA, Dietary supplement).

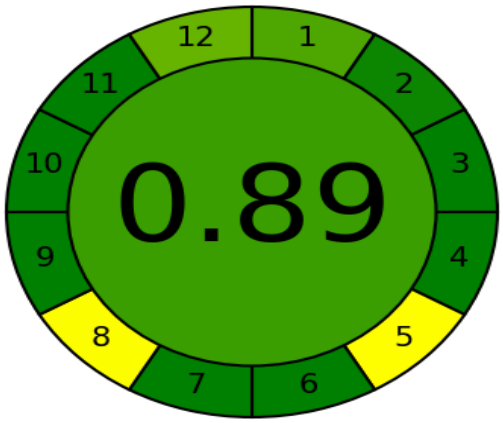

Figure 5: AGREEprep Prove that the applied method is green (Agree Index App)**.**

Table 8: Statistical comparison between the results obtained by the proposed HPLC method and those obtained by the reported method for the determination of pure ASA.

Parameter	ASA	Reported Method
		ASA
Mean	100.139	99.977
SD	1.256	1.714
Variance	1.579	2.937
n	5	5
Student's t-test[b] (2.306)	0.170	
F- value[b] (6.39)	1.860	

[a]HPLC method was done on the C18 column (250 x 4.6 mm i.d., 5µm), using a mobile phase of acetonitrile: water with 0.1% orthophosphoric acid (5: 95, v/v), and detection was done at 254nm. (Hashim NNA, et al.2017)
[b]The values represent the corresponding tabulated values of t and F at *p*=0.05.

3.2.1. Linearity and Range:

Linear correlations were obtained between the peak areas and the corresponding concentrations of ASA in the range of 2-24 µg/mL **(Figure 3)** the regression equations were computed and found to be:

PA=56.28C+122.76

r=0.9998 for ASA

Where PA is the peak area, C is the concentration of the drug in µg/mL and r is the correlation coefficient **(Table 3).**

3.2.2. Accuracy and precision:

The accuracy of the proposed method was checked by applying it to five different concentrations of ASA (6, 17.5, 20, 30, 35 µg/mL) then their corresponding concentrations were calculated from the regression equations proving a high degree of accuracy of the proposed method over the linearity range. The precision of the proposed method was determined by the analysis of three different concentrations of ASA (15, 20, 30 µg/m) in triplicates on a single day (repeatability or intraday) and on three consecutive days (intermediate or inter-day precision) as shown in **(Table 5 ,6).**

3.2.3. Selectivity:

The specificity of the suggested techniques was achieved by the ability to determine ASA without interference from the excipients or additives commonly present, where excellent recoveries were obtained (**Figure 4**).

3.2.4. Robustness:

The method proved to be robust by analysis of the cited drug under small variations of different experimental conditions such as adjusting the flow rate to 1.1 and 0.9 mL/min., changing the buffer pH within the ranges of 2.9 to 3.1 and altering the mobile phase (methanol: phosphate buffer) ratios to (9:91 v/v) and (11:89 v/v) respectively. Despite these modifications, no significant changes were detected, affirming the method's validity and consistent outcome.

3.3. System Suitability Parameters:

To ensure maximum performance, system suitability parameters were determined as shown in (**Table 4**) by calculating the capacity factor (k̀), and number of theoretical plates (N) for HPLC method and the system was proved to be suitable relative to

the reference values according to USP 39.

3.4. Reported Method;

The results obtained for the analysis of ASA in their pure form by the suggested methods were statistically compared to those obtained by the reported method for ASA. The calculated t and F values were less than the tabulated ones, demonstrating no significant difference concerning accuracy and precision as shown in **(Table 7).** Also, the standard addition method was applied to confirm the applicability of the developed method showing good results (**Table 8**).

3.5. Greenness of the method:

In recent years, the push towards environmentally sustainable practices has permeated various fields, including scientific research and industrial processes. Green methods—techniques that aim to minimize environmental impact while maintaining or enhancing efficacy—have become pivotal in advancing both ecological and economic goals. Among these innovative approaches, the use of AgreePrep has emerged as a notable advancement. The high precision of AgreePrep not only underscores its potential to deliver consistent results but also highlights its alignment with the principles of green chemistry and sustainability. The results revealed that the developed method is particularly remarkable for its high score rate of 0.89, showcasing its reliability and effectiveness in practical applications (**Figure 5**).

4. Conclusion:

The suggested validated chromatographic method was proved to be selective, fast, accurate, precise, green, and reproducible for the quantitative analysis and separation of ASA in its pure form and pharmaceutical formulations.

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