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Development and Validation of a Gradient RP-HPLC–DAD Method for the Simultaneous Determination of p-Hydroxybenzoic Acid, Benzoic Acid and Salicylic Acid in Gymnema Sylvestre Leaves and Extract

Kumaraswami Salimath, Ariraman Sevugarajan, Tamil Amuthan

Department of Quality Control, Bioextract, Tumkur, Karnataka, India.

Abstract

The use and monitoring of preservatives such as p-hydroxybenzoic acid (pHBA), benzoic acid (BA), and salicylic acid (SA) in food and pharmaceutical products require reliable and sensitive analytical procedures. HPLC-based methods have been widely reported for the determination of benzoic acid, salicylic acid, and related preservatives in food and pharmaceutical matrices. In herbal extracts, particularly those prepared using methanolic or alcoholic solvents, trace-level determination of these compounds can be analytically challenging because of the complexity of plant matrices and the presence of naturally occurring phenolic compounds. The present study aimed to develop and validate a simple, precise, and robust reverse-phase high-performance liquid chromatographic (RP-HPLC) method with diode-array detection (DAD) for the simultaneous determination of pHBA, BA, and SA in Gymnema sylvestre leaves and their corresponding extracts, and to evaluate their occurrence in the raw material and extracts.

Chromatographic separation was achieved on a C18 column using a binary gradient system consisting of 0.1% orthophosphoric acid and a mixture of acetonitrile–methanol, with detection at 241 nm. The analytical method was validated according to ICH Q2(R1) guidelines for specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). The method demonstrated good linearity ($r^2 > 0.99$) over the tested concentration range, acceptable precision (RSD < 2%), and satisfactory recoveries. These validation characteristics demonstrate the suitability of the method for quantitative determination of the selected analytes in the investigated botanical matrix.

Application of the validated method to G. sylvestre samples obtained from different geographical regions demonstrated that pHBA, BA, and SA were present at low concentrations in both the leaves and corresponding extracts. The detection of phenolic compounds in plant-derived materials is consistent with the established literature on the occurrence and analysis of plant phenolics. Furthermore, comparison of the raw materials with their corresponding extracts showed no consistent or substantial enrichment during extraction. These findings indicate and confirm that the detected preservatives are naturally present G. sylvestre Leaves.

Keywords: *Gymnema Sylvestre; HPLC–DAD; P-Hydroxybenzoic Acid; Benzoic Acid; Salicylic Acid; Method Validation; Phenolic Compounds; Herbal Extract.*

INTRODUCTION

Benzoic acid, salicylic acid and p-hydroxybenzoic acid are analytically important compounds because they can occur in food and plant materials and may also be encountered in products in which preservative use is regulated. Reliable determination is therefore important for quality control and regulatory assessment. HPLC methods have been widely applied to benzoic acid and salicylic acid in food matrices [1, 2], while validated chromatographic methods have also been reported for preservatives such as benzoic and sorbic acids [3, 4].

Gymnema sylvestre is a medicinal plant widely used in traditional and modern herbal formulation. herbal preparations and is chemically characterized by a diverse phytochemical profile. Phenolic compounds are common constituents of plant-derived materials, and their extraction and chromatographic behavior have been discussed extensively in the literature. Because extraction can alter

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the relative distribution of soluble constituents, trace analytes detected in an extract should be interpreted with consideration of both the raw material and the extraction process [3, 5].

In complex herbal matrices, matrix-derived compounds may overlap chromatographically with target analytes. A gradient RP-HPLC approach can provide greater control of retention and elution strength than a simple isocratic system, which is particularly relevant when several analytes with different chromatographic properties are determined simultaneously.

Although analytical procedures for preservatives have been reported for food and pharmaceutical matrices, the present study addresses a specific analytical question in *G. sylvestre*: whether pHBA, BA and SA can be selectively determined at low levels in leaves and extracts, and whether the levels observed in extracts are markedly increased relative to the corresponding raw materials.

RESEARCH OBJECTIVE:

The objective of this study was to develop and validate a gradient RP-HPLC–DAD method for simultaneous determination of pHBA, BA and SA in G. sylvestre leaves and extract in accordance with ICH Q2(R1) [6], and to evaluate whether these compounds are naturally present in the leaves and carried over into the extract without enrichment during the process.

MATERIALS AND METHODS

Chemicals and Reagents

p-Hydroxybenzoic acid (>99%) was procured from Loba Chemie Pvt. Ltd., (Mumbai, India). Benzoic acid and salicylic acid (>99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC gradient grade) were purchased from Thomas Baker (Mumbai, India). Orthophosphoric acid (LR grade) was obtained from Thomas Baker (Mumbai, India). HPLC-grade water was supplied by Rankem (India).

Instrumentation

Chromatographic analysis was performed using a Shimadzu Prominence-I series HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with binary gradient pump, autosampler, column oven and diode-array detector. Data acquisition and processing were performed using Shimadzu Lab Solutions software. Separation was achieved using a Phenomenex Luna C18 column (250 mm × 4.6 mm i.d.,) 5 µm particle size; Phenomenex Inc., CA, USA).

Chromatographic Conditions

Mobile phase A was 0.1% v/v orthophosphoric acid in water and mobile phase B was acetonitrile:methanol (80:20, v/v). The mobile phases were filtered through a 0.45 µm membrane filter. The below mentioned binary gradient was set for testing. The flow rate was 1.0 mL/min, injection volume 20 µL, detection wavelength 241 nm and total run time 50 min.

Gradient program for mobile phase:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.01	100	0
5.00	100	0
30.00	0	100
40.00	0	100
45.00	100	0
50.00	100	0
50.01	Stop	Stop

Preparation of standards and samples:

- **Preparation of Diluent**

0.1% Ortho phosphoric acid: mixed acetonitrile and methanol (25:75, v/v).

- **Preparation of Standard solutions**

50 mg of each individual reference standard was weighed separately into a 100 mL volumetric flask, dissolved and diluted to volume with diluent. Filter using what man filter paper. From this stock, 1 mL of standard from each, is transferred to a 100 mL volumetric flask and diluted to volume with diluent to obtain 0.005 mg/mL.

- **Preparation of G. sylvestre leaves solution**

2 g of powdered leaves was weighed and transferred to a 50 mL volumetric flask, dissolved and diluted with diluent, filtered through whatman filter paper, and 1 mL of the resulting solution was further diluted to 100 mL to obtain a nominal sample concentration of 0.4 mg/mL.

- **Preparation of G. sylvestre extract**

2 g of extract was treated in the same manner as the leaf sample to obtain a nominal concentration of 0.4 mg/mL.

Method Validation and Statistical Analysis

Validation was conducted for specificity, linearity, accuracy, precision (System precision and intermediate precision), robustness, LOD, LOQ. The validation framework followed ICH Q2(R1) [6]. Statistical evaluation was performed using mean value, standard deviation (SD), and relative standards deviation (RSD). Linearity was assessed by least-squares regression analysis, and correlation coefficients were calculated.

Routine Sampling and Extraction Comparison

G. sylvestre leaves were obtained from three geographical locations in India: Southern India (RM1; All Season Herbs Pvt. Ltd., Bangalore), Central India (RM2; Jeeva Nutrilabs, Raipur) and Western India (RM3; Silver Roots Agro, Mumbai).

Extracts prepared from RM1, RM2 and RM3 were designated GE1, GE2 and GE3, respectively. All six materials were tested for pHBA, BA and SA.5. Results

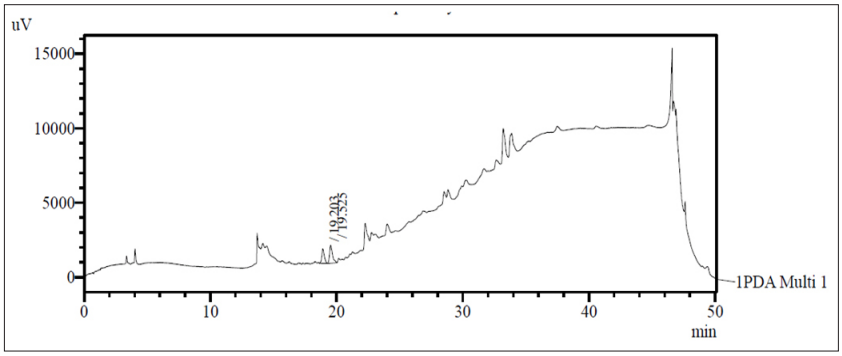
RESULT

Specificity and Chromatographic Resolution

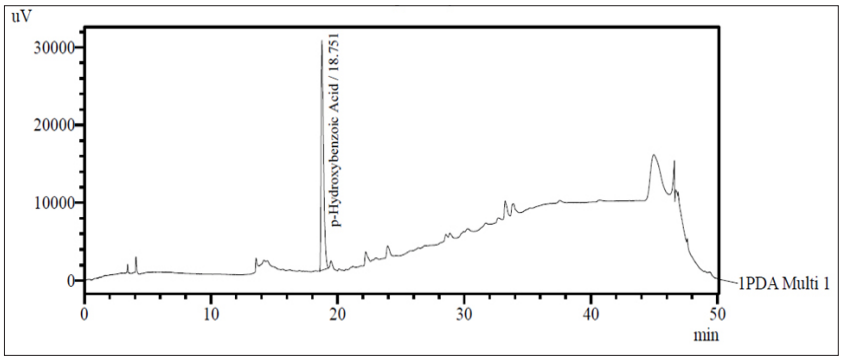
No interfering peak was observed at the retention times of the target analytes in the diluents/blank. Two diluent peaks were observed at approximately 19.2 and 19.5 min, but they did not interfere with the target analytes. Retention times in individual and mixed standards were comparable. pHBA, BA and SA showed retention times of approximately 18.75, 22.62 and 23.90 min, respectively. Resolution was 9.9 between pHBA and BA and 2.86 between BA and SA; the latter exceeded the standard acceptance criteria NLT 2.0 (Refer Pic 1 to Pic 5).

Table 1. Specificity data for the identification of Retention times standards and evaluation of System suitability parameters.

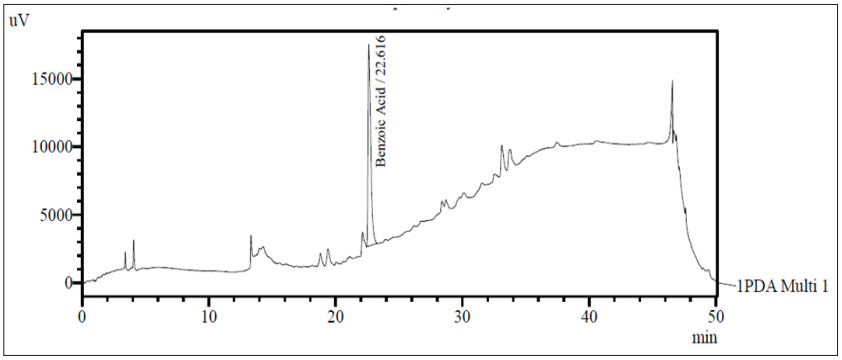
Specificity:				
Diluent (Blank)	No interference of peaks observed			
Standard name	**Retention time in Individual standard solutions (minutes)**	**Retention time in mixed standard solution (minutes)**	**Resolution**	**Acceptance criteria**
P-HBA	18.751	18.642	0	0
Benzoic acid	22.616	22.54	9.9	NLT 6.0
Salicylic acid	23.903	23.75	2.86	NLT 2.0



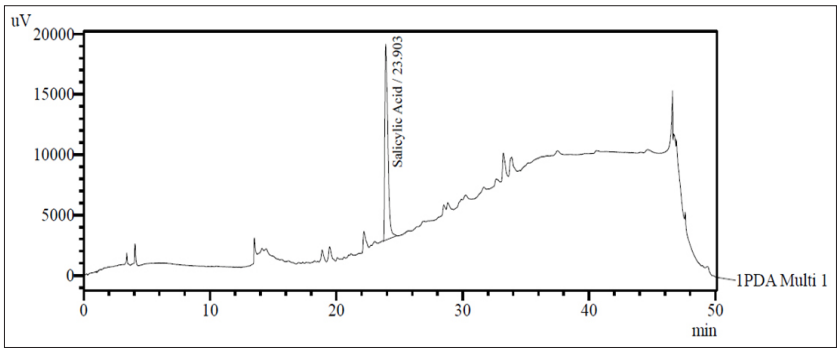
Pic 1. Chromatogram of blank (diluent): 2 peaks observed at 19.2 and 19.5 minutes but are not interfering with any other analyte peaks.



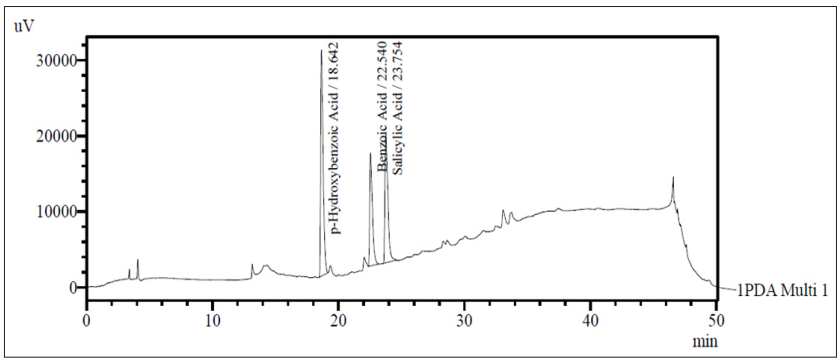
Pic 2. P-Hydroxy Benzoic acid standard chromatogram (Retention time-18.751 min)



Pic 3. Benzoic acid standard chromatogram (Retention time-22.616 min)



Pic 4. Salicylic acid standard chromatogram (retention time-23.903)



Pic 5. Mixed Standard chromatogram shows the retention times of individual analyte is matching to each standard in mixed solution.

Precision

Precision is a Degree of agreement among individual test results (repeatability, intermediate precision, reproducibility).

System Precision: Mixed standard solution of concentration of 0.005mg/mL of each analyte is injected in 6 replicates. Relative Standard Deviation is calculated for each analyte and found that the RSD is within the limits. Refer table 2.

Table 2. System precision study: Area responses of Individual analytes in replicate injections

System precision:			
Standard	**pHBA (µV)**	**Benzoic acid (µV)**	**Salicylic acid (µV)**
Injection 1	412738	228400	312073
Injection 2	404616	222900	305041
Injection 3	404452	222882	305421
Injection 4	402738	226400	314073
Injection 5	414452	225882	315421
Injection 6	401364	224386	312572
Average	406726.67	225141.67	310766.83
Stdev	5479.30	2165.66	4447.92
RSD (<2.0%)	**1.35**	**0.96**	**1.43**

Intermediate Precision: Intermediate Precision is studied by preparing and injecting *Gymnema Sylvestre* leaves powder solution and *Gymnema Sylvestre* Extract Solution. The contents of pHBA, BA and SA are calculated in all 6 preparations and a Relative Standard Deviation is reported. All the RSD found are within the set limits and details are tabulated as in table 3 and 4.

Also, Robustness of the method is studied with the same solutions on different day using different HPLC and varying the flow rate for around 10% (0.9 to 1.1 mL per minute). No variations were found in the results and details are tabulated as in table 3 and 4.

Table 3. Method precision study: Results in ppm of Individual analytes in 6 different preparations of *Gymnema Sylvestre* leaves.

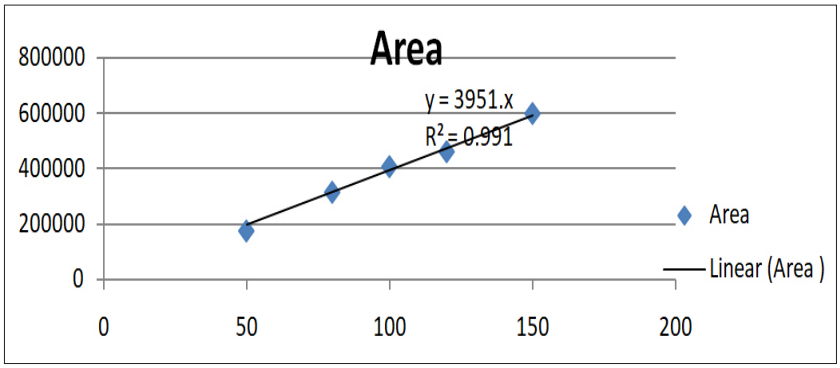
Intermediate precision-Leaves				**Robustness-Leaves**		
Results in ppm	**pHBA**	**BA**	**SA**	**pHBA**	**BA**	**SA**
Preparation 1	10	74	55	9	69	51
Preparation 2	11	78	58	8	68	49
Preparation 3	10	81	61	9	67	48
Preparation 4	12	75	61	10	69	53
Preparation 5	11	76	62	9	74	58
Preparation 6	12	79	68	10	71	55
Average	11.00	77.17	60.83	9.17	69.67	52.33
Stdev	0.89	2.64	4.36	0.75	2.50	3.78
RSD (NMT 20.0%)	8.13	3.42	7.16	8.21	3.59	7.2

Table 4. Method precision study: Results in ppm of Individual analytes in 6 different preparations of *Gymnema Sylvestre* extract.

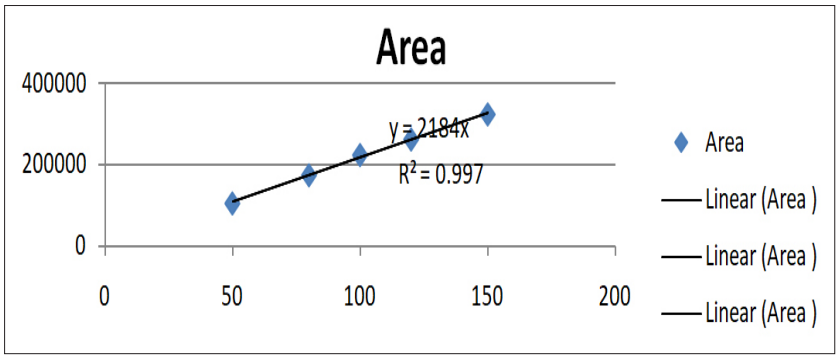
Intermediate precision-Extract				**Robustness-Extract**		
Results in ppm	**pHBA**	**BA**	**SA**	**pHBA**	**BA**	**SA**
Preparation 1	11	78	52	10	70	52
Preparation 2	10	75	52	9	70	53
Preparation 3	9	75	48	9	72	51
Preparation 4	11	74	46	9	71	56
Preparation 5	11	76	51	10	70	58
Preparation 6	10	71	58	11	69	54
Average	10.33	74.83	51.17	9.67	70.33	54.00
Stdev	0.82	2.32	4.12	0.82	1.03	2.61
RSD (NMT 20.0)	7.90	3.10	8.05	8.45	1.47	4.8

Linearity

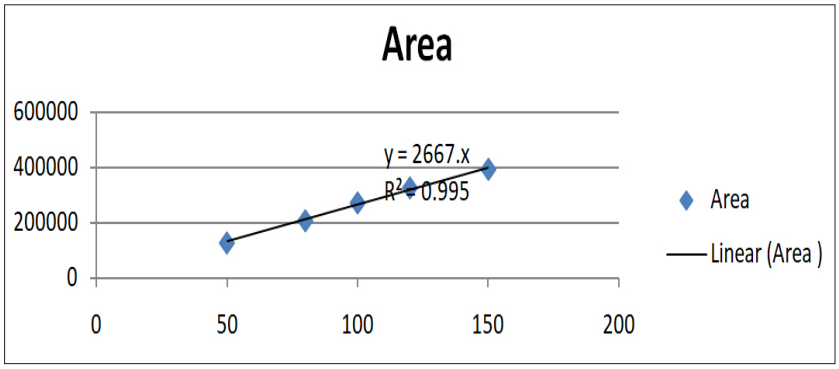
Linearity is an ability to obtain results directly proportional to the analyte's concentration over a given range. Linearity of the method is studied by preparing and injecting the standard solution at concentrations of 50%, 80%, 100%, 120% and 150% w.r.t standard concentration. The area response is graphed against the standard concentration and co relation coefficient for the Linear curve is calculated which is more than 0.99 in all 3 standards at different concentrations. Refer below graphs 1, 2 and 3.



Graph 1. Linearity of area responses in µV (Y-axis) of p-Hydroxy Benzoic acid at different concentration (X-axis) w.r.t Standard preparation (50, 80, 100, 120 & 150%)



Graph 2. Linearity of area responses in µV (Y-axis) of Benzoic acid at different concentration (X-axis) w.r.t Standard preparation (50, 80, 100, 120 & 150%)



Graph 3. Linearity of area responses in µV (Y-axis) of Salicylic acid at different concentration (X-axis) w.r.t Standard preparation (50, 80, 100,120 & 150%)

Accuracy

Accuracy is the Closeness of test results to the true value. Accuracy of the method is studied by preparing and spiking a known concentration of the standard solution at a concentration of 50%, 100% and 150% w.r.t standard concentration. The area response is recorded for each analyte and recovery % is calculated against the spiked standard concentration. The results are tabulated as in and co relation coefficient for the Linear curve is calculated which is more than 0.99 in all 3 standards at different concentrations. Refer below tables 5, 6 and 7.

Table 5. Accuracy is performed by spiking the known standard of p-Hydroxy Benzoic acid and area responses in µV are recorded and recovery percentage is calculated.

p-Hydroxy Benzoic Acid						
Spike Concentration	**Results in %**					
	Stated values in µV	**Actual values in µV**	**Recovery difference**	**Recovery %**	**Result**	**Limit**
50	598378	404963	47.76	95.5	94.0	80-120%
	591423	404963	46.04	92.1		
	596245	404963	47.23	94.5		
100	838416	404963	107.04	107.0	107.1	
	837308	404963	106.76	106.8		
	840586	404963	107.57	107.6		
150	1003942	404963	147.91	98.6	100.4	
	1015676	404963	150.81	100.5		
	1025468	404963	153.23	102.2		

Table 6. Accuracy is performed by spiking the known standard of Benzoic acid and area responses in µV are recorded and recovery percentage is calculated.

Benzoic Acid						
Spike Concentration	**Results in %**					
	Stated values in µV	**Actual values in µV**	**Recovery difference**	**Recovery %**	**Result**	**Limit**
50	352476	223958	57.38	114.8	103.6	80-120%
	338126	223958	50.98	102.0		
	329254	223958	47.02	94.0		
100	474105	223958	111.69	111.7	111.5	
	475574	223958	112.35	112.3		
	471286	223958	110.43	110.4		
150	573704	223958	156.17	104.1	103.8	
	571694	223958	155.27	103.5		
	572832	223958	155.78	103.9		

Table 7. Accuracy is performed by spiking the known standard of Salicylic acid and area responses in µV are recorded and recovery percentage is calculated.

Salicylic Acid						
Spike Concentration	**Results in %**					
	Stated values in µV	**Actual values in µV**	**Recovery difference**	**Recovery %**	**Result**	**Limit**
50	433193	272659	58.88	117.8	114.3	80-120%
	428879	272659	57.30	114.6		
	423315	272659	55.25	110.5		
100	569077	272659	108.71	108.7	106.6	
	560106	272659	105.42	105.4		
	560423	272659	105.54	105.5		
150	690356	272659	153.19	102.1	104.3	
	706160	272659	158.99	106.0		
	701278	272659	157.20	104.8		

Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of detection is calculated based on the signal to noise ratio for each analyte as, LOD=3.3*(S/N). Limit of Quantification is calculated based on the signal to noise ratio for each analyte as, LOQ=10*(S/N).

The calibration curve for p-Hydroxy Benzoic acid, Benzoic acid and Salicylic acid are found to be linear in the concentration range of 0.0025 mg/mL to 0.0075 mg/mL (r^2 is >0.99).

The values of Limit of Detection (LOD) and Limit of Quantification (LOQ) and LOQ were 0.18 and 0.54 mg/kg for p-Hydroxy Benzoic acid, 0.25 and 0.75mg/kg for Benzoic acid and 0.20 and 0.60mg/kg for Salicylic acid respectively.

From the data obtained by LOD and LOQ, it is concluded that this method is suitable for

the detection of preservatives in the trace level as below:

p-Hydroxy Benzoic acid: 0.5mg/kg

Benzoic acid: 0.75mg/kg

Salicylic acid: 0.6mg/kg

Stability of Solutions

The mixed standard solution was evaluated at 24, 48 and 72 h at room temperature in closed conditions. At 24 h, RSD values were 0.65%, 1.59% and 1.20% for pHBA, BA and SA, respectively. At 48 h, the corresponding values were 0.95%, 1.38% and 0.61%. At 72 h, pHBA and SA remained below 2.0%, while BA showed an RSD of 2.18%. Thus, the supplied data support a controlled solution-use period of up to 48 h under the evaluated conditions. Refer below table 8, 9 and 10,

Table 8. Area responses in µV after keeping the solution for 24 hours of after initial preparation

Standard	P-Hydroxy Benzoic acid (in µV)	Benzoic acid (in µV)	Salicylic acid (in µV)
Injection 1	404896	229365	308562
Injection 2	408567	231589	309457
Injection 3	412581	232158	304564
Injection 4	408896	227581	308561
Injection 5	411258	226275	307789
Injection 6	408254	222456	315892
Average	**409075.33**	**228237.33**	**309137.50**
Stdev	**2664.05**	**3621.66**	**3718.18**
RSD (NMT 2.0%)	**0.65**	**1.59**	**1.20**

Table 9. Area responses in µV after keeping the solution for 48 hours of after initial preparation

Standard	P-Hydroxy Benzoic acid (in µV)	Benzoic acid (in µV)	Salicylic acid (in µV)
Injection 1	399258	224589	306548
Injection 2	401237	224578	305555
Injection 3	402158	221891	306897
Injection 4	401856	218455	304625
Injection 5	408962	217562	304545
Injection 6	407458	219586	309568
Average	**403488.17**	**221110.17**	**306289.67**
Stdev	**3824.08**	**3056.15**	**1872.85**
RSD (NMT 2.0%)	**0.95**	**1.38**	**0.61**

Table 10. Area responses in µV after keeping the solution for 72 hours of after initial preparation

Standard	P-Hydroxy Benzoic acid (in µV)	Benzoic acid (in µV)	Salicylic acid (in µV)
Injection 1	395689	209564	302222
Injection 2	395468	215894	301892
Injection 3	392547	221542	299582
Injection 4	389685	218794	298456
Injection 5	396857	211258	294568
Injection 6	399912	218975	295689
Average	**395026.33**	**216004.50**	**298734.83**
Stdev	**3536.44**	**4717.85**	**3148.43**
RSD (NMT 2.0%)	**0.90**	**2.18**	**1.05**

ROUTINE TESTING

Gymnema Sylvestre Leaves are procured from 3 different geographical locations in India viz, Southern India (from All Season herbs Pvt. Ltd, Bangalore, India-Coded as RM1), Central India (Jeeva Nutrilabs, Raipur, India-Coded as RM2) and Western India (Silver Roots Agro, Mumbai, India-Coded as RM3). Using these 3 raw materials, an extract has been prepared from each and labeled as GE1 (prepared from RM1), GE2 (prepared from RM2) and GE 3(Prepared from RM3) respectively.

All the 3 raw materials and 3 extracts are tested for the above said preservatives and the results are tabulated as below in table 11:

Table11. All results in mg/kg or ppm.

Analyte	RM1	RM2	RM3	GE1	GE2	GE3
pHBA (ppm)	4	3	3	5	4	4
BA (ppm)	26	25	34	27	21	32
SA (ppm)	36	25	35	36	27	34

DISCUSSION

The principal analytical challenge in the present study was the simultaneous determination of three low-level acidic compounds in a complex botanical matrix. Food-matrix HPLC methods for benzoic and salicylic acids and validated preservative methods have demonstrated the usefulness of reversed-phase chromatography for these compounds [3, 4, 7, 8, 9]. However, the present application involves *G. sylvestre* leaves and extract, where multiple endogenous constituents can complicate chromatographic selectivity. The gradient program used here provided clear separation of the target analytes, particularly the 2.86 resolution between BA and SA, satisfying the standard criteria.

The precision results indicate that the chromatographic system itself was reproducible. System precision RSDs remained below 2% for all three analytes, consistent with the intended use of the method for routine quantitative analysis. Intermediate precision values were higher than system precision values, as expected when independent sample preparations and matrix effects are introduced. Nevertheless, the reported RSDs remained within the study's stated NMT 20% criterion. The robustness experiment also showed that changing the flow rate from 0.9 to 1.1 mL/min did not produce a material loss of analytical performance.

The linearity data showed $r^2 > 0.99$ across 0.0025–0.0075 mg/mL. This is important because trace-level measurements depend not only on chromatographic separation but also on proportional detector response. The accuracy results, with recoveries within 80–120%, further support the suitability of the method for quantification at the evaluated levels. These validation characteristics are consistent with the general analytical-validation principles described in ICH Q2(R1) [6].

The method's LOD and LOQ values indicate that the procedure can detect concentrations below the levels observed in the routine samples. This is analytically important because all routine samples contained measurable quantities of the three analytes. The use of chromatographic methods for phenolic compounds in plant-derived matrices is supported by established literature on phenolic-acid determination and extraction [3, 4, 5, 10, 11].

A key observation was the similarity of analyte concentrations between raw materials and their corresponding extracts. For example, RM1 contained 4 ppm pHBA, 26 ppm BA and 36 ppm SA, while GE1 contained 5, 27 and 36 ppm, respectively. RM2 contained 3, 25 and 25 ppm compared with 4, 21 and 27 ppm in GE2. RM3 contained 3, 34 and 35 ppm compared with 4, 32 and 34 ppm in GE3. These results do not show a consistent or substantial increase of the three analytes following extraction. The pattern is therefore compatible with carryover of compounds already present in the botanical raw material.

The geographical comparison also demonstrates that the absolute concentrations varied among raw materials. BA was highest in RM3 (34 ppm), whereas SA was highest in RM1 and RM3 (36 and 35 ppm, respectively). Such variability is plausible for plant-derived materials because phytochemical composition can vary with origin and other raw-material factors [3, 4, 10, 11]. The current data therefore support a raw-material-associated interpretation, but they should not be over-interpreted as definitive proof of biosynthesis.

The distinction between 'naturally present' and 'not intentionally added' is scientifically important. The present HPLC–DAD results demonstrate analytical presence and show that extraction did not result in consistent enrichment. They do not, by themselves, establish the biochemical pathway by which each compound arose, nor do they completely exclude contamination introduced before sampling or during processing. Literature on phenolic acids in plants supports the occurrence of related compounds in plant matrices [3, 4, 10, 11], while conventional HPLC methods establish the broader analytical basis for determining benzoic, salicylic and related acids [1, 2, 7, 8, 9, 12]. Therefore, the most scientifically defensible conclusion from the present dataset is that the detected compounds are associated with the investigated plant materials and are carried into the extracts without a consistent concentration increase.

The solution-stability results further contribute to method practicality. The standard remained within the stated 2% RSD criterion through 48 h, whereas the BA result at 72 h exceeded the criterion slightly (2.18%). A 48-h controlled use period is therefore better supported by the available evidence than a 72-h period. This type of operational limitation is relevant to routine laboratory implementation because it links validation data directly to sample and standard handling.

Overall, the validation and application data indicate that

the gradient RP-HPLC–DAD procedure is suitable for routine monitoring of pHBA, BA and SA in *G. sylvestre* leaves and extracts. Its principal strength is the combination of chromatographic resolution, acceptable precision, linearity, recovery, sensitivity and demonstrated applicability to multiple geographical raw materials.

Earlier work has described HPLC determination of benzoic acid and salicylic acid in food matrices [7], validation of HPLC procedures for benzoic and sorbic acids [1], and official liquid-chromatographic determination of benzoic acid in orange juice [2]. Methods for phenolic acids in plant-derived foods and broader studies of phenolic compounds have also established HPLC as a useful analytical platform for these constituents [3, 4, 10, 11]. Additional reports address benzoic and sorbic acid determination [8], salicylic acid in pharmaceutical products [12], and simultaneous preservative analysis [9].

Compared with these broader applications, the present method is tailored to the botanical matrix of *G. sylvestre* and uses gradient elution to manage separation of pHBA, BA and SA. The reported resolution and validation characteristics demonstrate that the method is capable of distinguishing the three target analytes in the investigated herbal matrix. The routine-sample data further extend the application from method validation to comparative assessment of raw materials and corresponding extracts.

Overall, the validation and application data indicate that the gradient RP-HPLC–DAD procedure is suitable for routine monitoring of pHBA, BA and SA in *G. sylvestre* leaves and extracts. Its principal strength is the combination of chromatographic resolution, acceptable precision, linearity, recovery, sensitivity and demonstrated applicability to multiple geographical raw materials

CONCLUSION

A robust and sensitive RP-HPLC–DAD method was successfully developed and validated for the simultaneous determination of pHBA, BA, and SA in *Gymnema sylvestre* leaves and extracts. Statistical validation confirmed the reliability of the method for routine analysis. The study demonstrates that the detected preservatives are naturally present in the raw material and are not enriched during extraction, addressing regulatory concerns regarding preservative contamination in herbal extracts.

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