Determination of Ceftazidime-avibactam Content Employing Green Analytical Chemistry Principles Combined with Response Surface Methodology

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Abstract [**Objectives**] To quantify the content of ceftazidime-avibactam (CAZ-AVI) employing green analytical chemistry principles and assess the environmental sustainability of the proposed method. [**Methods**] The concentrations of disodium hydrogen phosphate and potassium dihydrogen phosphate, as well as the pH of the buffer solution, were optimized using a Box-Behnken design. The evaluation method's performance was validated through methodological assessment, and its environmental sustainability was appraised employing the AGREE, BAGI, and Complex GAPI tools. [**Results**] The optimal conditions were determined to be 27.79 mmol/L disodium hydrogen phosphate, 20.27 mmol/L potassium dihydrogen phosphate, and a pH of 3.52. The linear ranges for ceftazidime (CAZ) and avibactam (AVI) were 0.09 - 2.25 and 0.1 - 3 μg/mL, respectively, with correlation coefficients exceeding 0.999. The respective contents were 87.61% and 9.90%. Recovery rates were 98.56% for CAZ and 97.57% for AVI, while the detection limits were as low as 0.44 and 0.75 ng/mL, respectively. The green chemistry evaluation yielded a favorable result, with an AGREE score of 0.85. [**Conclusions**] The method is sensitive, accurate, and environmentally sustainable, making it suitable for the quality inspection of CAZ-AVI.

Key words Ceftazidime-avibactam (CAZ-AVI), Response surface methodology, Green analytical chemistry (GAC), Content determination, High performance liquid chromatography (HPLC)

1 Introduction

Ceftazidime-avibactam (CAZ-AVI) is a compound preparation of novel β-lactamase inhibitor, primarily consisting of ceftazidime (CAZ) and avibactam (AVI). Since its introduction in China in 2019, it has been extensively utilized for the treatment of infections caused by Gram-negative bacteria[1]. Recent studies have demonstrated that CAZ-AVI exhibits significant therapeutic efficacy in treating central nervous system infections and nontuberculous mycobacterial infections associated with organ transplantation[2-3]. Currently, there are relatively few analytical methods available for quantifying CAZ-AVI content, and these methods possess limited sensitivity. Consequently, it is imperative to optimize content determination techniques to satisfy quality control requirements[4].

Green analytical chemistry (GAC) seeks to safeguard the environment and ensure the safety of analysts. Its fundamental principle is to minimize or eliminate the use of toxic organic solvents during drug analysis[5]. The Box-Behnken response surface methodology offers advantages such as requiring fewer experiments and reducing associated costs[6]. In this experiment, the response surface methodology was employed to optimize the chromatographic conditions of high performance liquid chromatography (HPLC). The analytical method was evaluated according to the principles of GAC using appropriate assessment tools, with the aim of developing an efficient, accurate, and environmentally sustainable method for the quantification of CAZ-AVI.

2 Instruments and reagents

2.1 Instruments The instruments employed in this study comprised a high-performance liquid chromatograph (Shimadzu LC-20ADXR, Shimadzu Enterprise Management Co., Ltd.), a C_{18} chromatographic column (4.6 mm × 250 mm, 5 μm, BORRAIY), an electronic balance (BSA124S-CW, Sartorius Scientific Instruments Co., Ltd.), and an ultrasonic cleaner (KQ-500E, Kunshan Ultrasonic Instrument Co., Ltd.).

2.2 Reagents CAZ-AVI (batch No.: FJ240801), CAZ reference substance (batch No.: TDGBKJ240502CP), and AVI reference substance (batch No.: WS-PH2404004) were all procured from Guangxi Kelun Pharmaceutical Co., Ltd. Acetonitrile (chromatographic grade) was obtained from Tianjin Kemio Chemical Reagent Co., Ltd. Potassium dihydrogen phosphate and 85% orthophosphoric acid were sourced from Shanghai Aladdin Biochemical Technology Co., Ltd. Disodium hydrogen phosphate was purchased from Shanghai Macklin Biochemical Co., Ltd. Ultra-pure water was also utilized in the study.

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3 Methods and results

3.1 Chromatographic conditions Mobile phase A consisted of a phosphate buffer solution, while Mobile phase B was acetonitrile. The gradient elution program was as follows: 0 – 4 min, 3% – 6% B; 4 – 6 min, 6% – 10% B; 6 – 10 min, 10% – 20% B; 10 – 12 min, 20% – 10% B; and 12 – 15 min, 10% – 3% B. The sample solvent was a 3% acetonitrile aqueous solution. Detection was performed at a wavelength of 205 nm. The injection volume was 10 μL, the column temperature was maintained at 40 ℃, and the flow rate was set to 1 mL/min.

3.2 Preparation of test solution 5.00 mg of CAZ-AVI was transferred into a 10 mL volumetric flask, dissolved in 3% acetonitrile aqueous solution, and diluted to constant volume to prepare a 500 μg/mL stock solution. Subsequently, 1.00 mL of this stock solution was precisely measured and diluted with the same solvent to a final volume of 10 mL, yielding a 50 μg/mL test solution.

3.3 Chromatographic parameter scoring A comprehensive scoring system was implemented, with a total score of 100 points. The weights assigned to resolution and retention time were each 20%, while peak area, tailing factor, theoretical plate number, and signal-to-noise ratio were each weighted at 15%. The scoring criteria were as follows: resolution ≥ 1.5; retention time minimized; peak area and theoretical plate number maximized; tailing factor approaching 1; and signal-to-noise ratio minimized. Chromatograms were independently evaluated by three experimenters, and the average of their scores was calculated.

3.4 Response surface methodology

3.4.1 Single-factor test. Using the chromatographic parameter score as the evaluation index, this study investigated the effects of disodium hydrogen phosphate concentration (10 – 30 mmol/L), potassium dihydrogen phosphate concentration (10 – 30 mmol/L), and buffer pH (3.5 – 4.5) in the mobile phase on chromatographic parameters.

The results indicated that the chromatographic parameters were optimal at a disodium hydrogen phosphate concentration of 25 mmol/L. Therefore, the response surface was designed with three concentration levels: 20, 25, and 30 mmol/L. Similarly, the chromatographic parameters reached optimal values at a potassium dihydrogen phosphate concentration of 20 mmol/L, which was selected as the intermediate point for the response surface design. Furthermore, the chromatographic parameters were optimal at a buffer solution pH of 3.5, with the response surface analysis conducted across three pH levels ranging from 3.0 to 4.0.

3.4.2 Interaction test. The experiment was designed using Design Expert 13 software, with disodium hydrogen phosphate concentration (A), potassium dihydrogen phosphate concentration (B), and buffer pH (C) as independent variables, and the chromatographic parameter score as the dependent variable. The levels of each factor are presented in Table 1.

Table 1 Factors and levels in the design

Level	Disodium hydrogen phosphate concentration (A) // mmol/L	Potassium dihydrogen phosphate concentration (B) // mmol/L	Buffer pH (C)
–1	20	15	3
0	25	20	3.5
1	30	25	4

Design Expert 13 was utilized to design 15 experimental groups based on the factors and levels presented in Table 1, with the corresponding results summarized in Table 2. The regression equations were subsequently obtained. Specifically, the CAZ parameter score was modeled as: $Y = -215.59 + 12.81A + 4.26B + 56.00C + 0.009AB + 0.15AC - 0.075BC - 0.27A^2 - 0.1B^2 - 8.34C^2$; and the AVI parameter score was expressed as: $Y = -171.72 + 4.63A + 6.85B + 73.58C + 0.003AB - 0.01AC - 0.02BC - 0.09A^2 - 0.17B^2 - 10.30C^2$.

Table 2 Experimental design and results

No.	A	B	C	CAZ parameter score	AVI parameter score
1	–1	–1	0	76.10	77.40
2	1	–1	0	75.14	78.90
3	–1	1	0	76.70	78.16
4	1	1	0	76.64	76.80
5	–1	0	–1	77.33	82.12
6	1	0	–1	76.16	76.88
7	–1	0	1	76.33	75.80
8	1	0	1	76.70	76.11
9	0	–1	–1	80.06	83.86
10	0	1	–1	81.50	76.13
11	0	–1	1	80.57	77.00
12	0	1	1	81.26	78.00
13	0	0	0	85.60	75.94
14	0	0	0	84.77	83.20
15	0	0	0	86.14	75.60

3.4.3 Analysis of variance. An analysis of variance was performed on the experimental data, with the results presented in Table 3. The model demonstrated an exceptionally high degree of fit with the scoring outcomes of the chromatographic parameters of CAZ and AVI. Significance testing revealed that the factors influencing the chromatographic parameters of CAZ ranked in the following order: A > C > B. Conversely, the factors affecting the chromatographic parameters of AVI were ranked as: B > C > A. In summary, the concentration of disodium hydrogen phosphate exerted a significant effect on the chromatographic parameters of CAZ, whereas the concentration of potassium dihydrogen phosphate and the pH of the buffer solution significantly influenced the chromatographic parameters of AVI.

Table 3 Analysis of variance in regression models

Dependent variable	Source of variance	Sum of squares	Degree of freedom	Mean square	*F*	*P*
CAZ	Model	196.80	9	21.87	106.64	<0.000 1
	A	0.41	1	0.41	2.02	0.21
	B	2.24	1	2.24	10.91	0.02
	C	0.01	1	0.01	0.02	0.89
	AB	0.20	1	0.20	0.99	0.37
	AC	0.59	1	0.59	2.89	0.15
	BC	0.14	1	0.14	0.69	0.45
	A^2	170.13	1	170.13	829.69	<0.000 1
	B^2	24.40	1	24.40	118.97	0.001 1
	C^2	16.06	1	16.06	78.31	0.000 3
	Residue	1.03	5	0.21		
	Lack of fit	0.07	3	0.02	0.05	0.91
	Pure error	0.95	2	0.48		
	Sum	197.82	14			
	R^2	0.994 8				
	R_{adj}	0.985 5				
AVI	Model	103.16	9	11.46	32.63	0.000 6
	A	1.73	1	1.73	4.92	0.08
	B	0.23	1	0.23	0.66	0.45
	C	0.85	1	0.85	2.41	0.18
	AB	0.03	1	0.03	0.08	0.79
	AC	0.01	1	0.01	0.01	0.91
	BC	20.74	1	0.01	0.04	0.85
	A^2	68.43	1	20.74	59.05	0.000 6
	B^2	24.48	1	68.43	194.82	<0.001
	C^2	1.76	1	24.48	69.70	0.001
	Residue	0.21	5	0.35		
	Lack of fit	1.54	3	0.07	0.09	0.91
	Pure error	104.92	2	0.77	32.63	
	Sum	103.16	14			
	R^2	0.983 3				
	R_{adj}	0.953 1				

3.4.4 Effects of various factors on chromatographic parameters of CAZ. As illustrated in Fig. 1, the slope associated with the concentration of disodium hydrogen phosphate was steeper compared to other factors, indicating a more pronounced effect on the chromatographic parameters of CAZ. The contour plot further revealed that the color variation corresponding to the interaction between potassium dihydrogen phosphate concentration and buffer pH was more gradual than that observed in the other two groups. This suggests that the influence of these two factors on the chromatographic parameters of CAZ is relatively minor, a finding that aligns with the results of the analysis of variance.

3.4.5 Effects of various factors on chromatographic parameters of AVI. As illustrated in Fig. 2, during the interaction experiment with the concentration of disodium hydrogen phosphate, both the concentration of potassium dihydrogen phosphate and the pH of the buffer solution exhibited steeper slopes. This observation indicates that their effects on the chromatographic parameters were more pronounced than that of disodium hydrogen phosphate concentration. Furthermore, in the interaction between the concentration of potassium dihydrogen phosphate and the buffer solution pH, the former exerted a slightly greater influence on the chromatographic parameters than the latter. In summary, the concentration of potassium dihydrogen phosphate and the pH of the buffer solution had a more substantial impact on the chromatographic parameters of AVI.

3.4.6 Verification of optimization results. The optimized response surface analysis yielded the following conditions: a disodium hydrogen phosphate concentration of 27.79 mmol/L, a potassium dihydrogen phosphate concentration of 20.27 mmol/L, and a buffer solution pH of 3.52. Based on these parameters, three parallel experiments were performed. The chromatographic results obtained fell within the 95% confidence interval, demonstrating that the response surface model effectively and reliably optimizes the chromatographic conditions and is suitable for content determination.

3.5 Validation of HPLC methodology

3.5.1 Exclusivity. Under the optimized chromatographic conditions, the injection results are presented in Fig. 3. The peak shapes and resolution of the two drugs were satisfactory, with no evidence of tailing observed.

3.5.2 Linearity. Appropriate amounts of CAZ and AVI reference substances were accurately weighed and dissolved in a 3% acetonitrile aqueous solution to prepare seven reference solutions with varying concentrations. The CAZ concentrations ranged from 0.09 to 2.25 μg/mL, while the AVI concentrations ranged from 0.1 to 3 μg/mL. A 10 μL aliquot of each solution was injected for analysis. The peak area (Y) was plotted against the concentration of the reference substance (X) to assess the linear relationship. The equation for the peak area of CAZ was determined as $Y = 32\,033X + 1\,724.5$ ($r = 0.999\,8$), while the regression equation for AVI was established as $Y = 31\,726X - 214.76$ ($r = 0.999\,6$). Both compounds exhibited a strong linear relationship within the tested concentration ranges. The calculated contents of CAZ and AVI were 87.61% and 9.90%, respectively.

3.5.3 Recovery rate. A reference solution containing 50 μg/mL of CAZ and AVI was prepared as the sample addition solution. The test solution was combined with the reference substances of both drugs to yield solutions with addition levels of 50%, 100%, and 200%, respectively. Recovery rates were determined through triplicate experiments for each addition level. The mean recovery rate for CAZ was 98.56%, while that for AVI was 97.57%, with relative standard deviation (RSD) values of 0.8% and 0.9%, respectively. These results indicate satisfactory recovery rates.

3.5.4 Detection limit and quantification limit. The detection limit and quantification limit were established based on signal-to-noise ratios of 3 and 10, respectively. Specifically, the detection and quantification limits for CAZ were calculated as 0.44 and 1.5 ng/mL, respectively, while those for AVI were determined to be 0.75 and 2.5 ng/mL, respectively. This method demonstrates high sensitivity and is capable of accurately quantifying samples with relatively low concentrations.

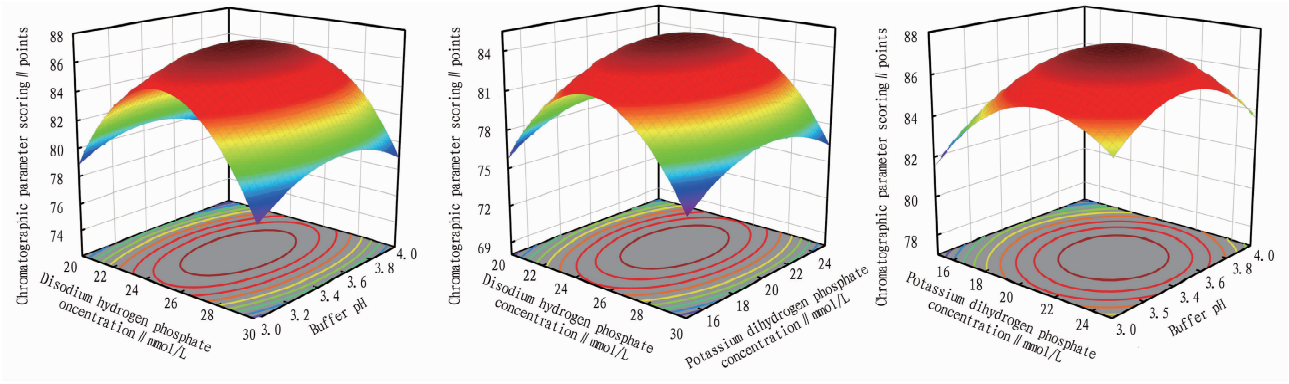


Fig. 1 Effects of various factors on the chromatographic parameters of CAZ

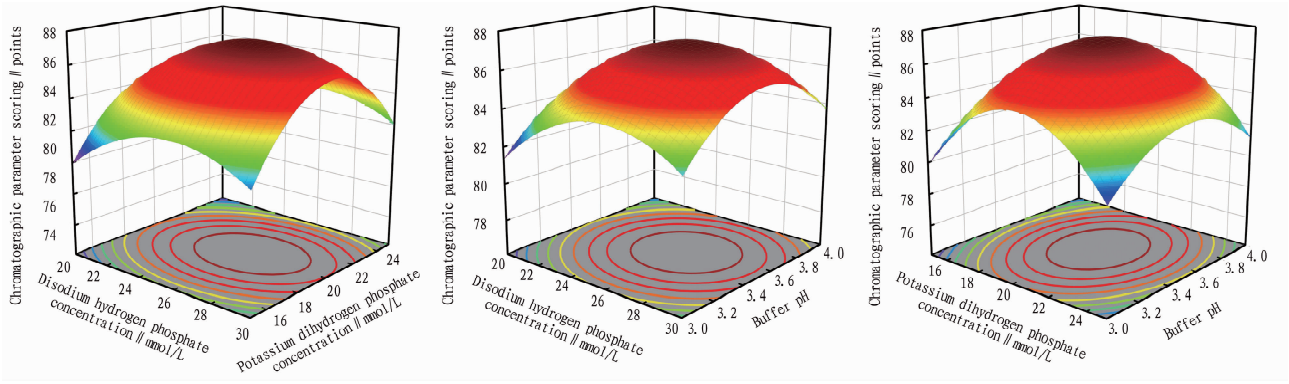
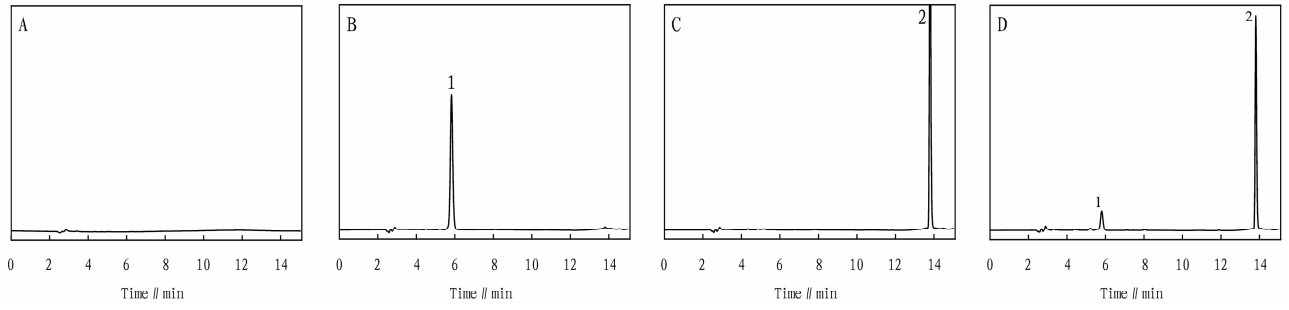


Fig. 2 Effects of various factors on the chromatographic parameters of AVI



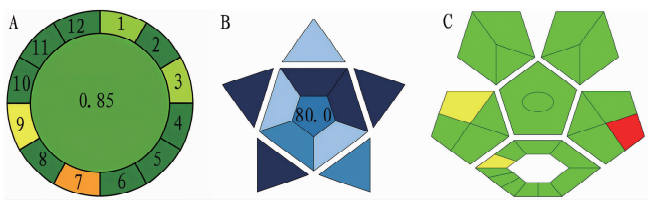
NOTE A. Blank reference solution; B. AVI reference solution; C. CAZ reference solution; D. Test solution; 1. Avibactam; 2. Ceftazidime.

Fig. 3 HPLC chromatograms of test samples

3.5.5 Repeatability. A test solution with a concentration of 50 μg/mL was prepared, and six consecutive injections were performed to determine the *RSD* of the peak area. The results indicated that the *RSD* values for CAZ and AVI were 1.53% and 1.40%, respectively, demonstrating good repeatability.

3.5.6 Stability. The test solution was stored in a refrigerator maintained at 3 ℃ for durations of 2, 6, 10, 14, 18, and 24 h prior to injection. The *RSD* values of the peak areas for CAZ and AVI were determined to be 1.24% and 1.22%, respectively. These results indicate that the test solution remained stable for up to 24 h.

3.6 GAC assessment The results are presented in Fig. 4. The GAC assessment primarily comprises the analytical greenness calculator (AGREE), the blue applicability grade index (BAGI), and the complex green analytical procedure index (Complex GAPI). Among these, AGREE evaluates analytical methods from the perspective of environmental sustainability[7], based on the 12 guiding principles of GAC, with each principle scored on a scale from 0 to 1. The average greenness score obtained for this method was 0.85, indicating overall greenness and a high level of environmental friendliness of the analytical procedure.



NOTE A. AGREE; B. BAGI; C. Complex GAPI.

Fig. 4 Results of three GAC assessment tools

BAGI is a tool designed to assess the practicality of analytical

methods, presenting outcomes through scores accompanied by graphical representations[8]. It comprises 10 analytical sections, each corresponding to a specific criterion, with darker colors indicating greater practicality. The evaluation of this method yielded a score of 80 points and a relatively dark color, suggesting that the analytical method is highly practical.

Complex GAPI primarily evaluates the health and environmental safety of experimenters[9] and comprises six main components. The low, medium, and high levels of impact of the analytical method on human health and the environment are represented by green, yellow, and red colors, respectively. The overall assessment result was green, indicating that this method exerts minimal impact on the health of experimenters and demonstrates excellent environmental compatibility.

In conclusion, an evaluation of the analytical method from three distinct perspectives, employing three green analytical tools, indicates that this method is environmentally sustainable, highly practical, and exerts minimal impact on personnel health.

4 Discussion

The regulation of chromatographic conditions constitutes a fundamental aspect of content determination. In the single-factor tests conducted within the response surface methodology, the buffer salt concentration was maintained between 20 and 30 mmol/L, as deviations beyond this range adversely affected elution efficiency and compromised column pressure stability. Interaction analysis revealed that the concentration of disodium hydrogen phosphate exerted a more pronounced influence on the chromatographic parameters of CAZ, whereas the concentration of potassium dihydrogen phosphate and the pH of the buffer solution more significantly affected the chromatographic parameters of AVI. This differential effect can be attributed to the chemical nature of the analytes: CAZ contains carboxyl groups and is acidic, while AVI is classified as an alkaline diazacyclic compound. Upon hydrolysis, disodium hydrogen phosphate exhibits alkaline properties, thereby exerting a greater effect on the elution of the acidic compound CAZ. Conversely, potassium dihydrogen phosphate becomes acidic after hydrolysis and, in conjunction with buffer pH, significantly influences the elution of the alkaline compound AVI.

HPLC analysis was performed under optimized chromatographic conditions, yielding well-defined peak shapes and satisfactory resolution. The results were consistent with those reported in the literature[4]. Notably, this method employed gradient elution, which proved more suitable for analyzing samples with significantly differing component concentrations compared to the isocratic elution approach described in the literature. Furthermore, the detection limits for the two drugs determined by this method were 0.44 and 0.75 ng/mL, respectively, substantially lower than the 2 and 1 μg/mL detection limits reported previously[4]. Consequently, this method offers advantages in reducing costs and minimizing waste during drug analysis and detection.

The GAC assessment employed three analytical tools, AGREE, BAGI, and Complex GAPI, to evaluate the method from three perspectives: environmental impact, practicality of analytical techniques, and the health and safety of experimenters, respectively. The method demonstrated favorable performance. During the elution process, the organic phase proportion reached a maximum of 20%, with a duration of less than 5 min, effectively minimizing the use of organic solvents. This aligns with the GAC principles of pollution reduction and the rational utilization of resources. The phosphate buffer solution utilized in this method comprised disodium hydrogen phosphate and potassium dihydrogen phosphate. Compared to the ammonium dihydrogen phosphate employed in the analysis of CAZ in the 2025 edition of the *Chinese Pharmacopoeia*[10], this substitution further mitigates environmental impact.

In this study, the response surface methodology was employed. Through single-factor and multi-factor interaction tests, chromatographic analysis conditions were optimized and subsequently validated, resulting in the development of an analytical method for determining the content of CAZ-AVI. To assess its compliance with the principles of GAC, three GAC assessment tools were utilized. The assessment outcomes were favorable, indicating that the method developed for the quantification of CAZ-AVI is both environmentally sustainable and reliable, and is suitable for the quality analysis and detection of pharmaceutical compounds. The findings provide a reference framework for future investigations aimed at replacing organic solvents with more environmentally benign alternatives.

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(To page 10)

mediate substrate and requiring Mg^{2+} or Mn^{2+} as cofactors, this enzyme orchestrates complex catalytic reactions—including ionization, cyclization, and rearrangement—to generate diverse sesquiterpene carbon skeletons[22-24]. In summary, DXS3 functions upstream in sesquiterpenoid biosynthesis, whereas TPS22 operates downstream. Both are predominantly expressed in roots and synergistically regulate bioactive compound accumulation. This investigation elucidated their spatiotemporal expression patterns in relation to nardosinone accumulation, demonstrating that monitoring *NjDXS3* and *NjTPS22* expression levels in leaves enables non-invasive assessment of bioactive compound accumulation in subterranean medicinal organs.

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