



Research Article

Disperse Solid-Phase Extraction Cleanup for the Determination of 1-Deoxynojirimycin in Mulberry Leaves with Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry

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A new determination method of 1-deoxynojirimycin (1-DNJ) in mulberry leaves based on ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has been developed. Dried and crushed mulberry leaves' sample was extracted by MeCN-water solvent, purified by graphitized carbon black (GCB) and primary secondary amine (PSA) to remove organic acids and pigments, and then analyzed after attenuation and filtration. The calibration curve showed linearity in the concentration range of 10–500 ng/mL, with the correlation coefficient of 0.998. Recoveries of spiked 1-DNJ at three fortification levels ranged from 94.6% to 96.4%, with relative standard derivation below 1.2%. Additionally, the matrix effect was assessed as negligible. Compared with methods by gas chromatography (GC) and liquid chromatography (LC) via real sample detection, the proposed method acquired better stability and detection efficiency. These results proved that this method has advantages of simple operation, complete purification, small pretreatment loss, good precision and accuracy, and high determination specificity, which is suitable for massive monitoring and precise quantitation of 1-DNJ in mulberry leaves.

1. Introduction

Mulberry branch (*Ramulus mori*) is a traditional Chinese medicinal herb, and its leaves are the main food of silkworm. It has been demonstrated that the intake of mulberry leaves or their extracts could treat diabetes, and the main active component is 1-deoxynojirimycin (1-DNJ) [1, 2], which is a kind of alkaloid compound with nitrogen atoms. It was found to be a potential α -glucosidase inhibitor by effectively suppressing the transformation of carbohydrate in the human body, reducing the sugar content in blood, and inhibiting the rapid rise of blood glucose and insulin secretion after eating [3].

At present, the detection methods of 1-DNJ are mainly based on gas chromatography (GC) and high-performance liquid chromatography (HPLC) [4–6]. It is reported that 1-DNJ concentrations in mature leaves varied from 0.1341 to

1.472 mg/g of dry leaves among 132 mulberry varieties [7]. However, due to the characteristics of high water solubility and weak absorbance, the methods based on GC and HPLC need suitable derivatization processes [4–9], as well as the method employed gas chromatography-tandem mass spectrometry (GC-MS) [10]. Therefore, the complex pre-treatment operation will lead to a long preprocessing time and poor stability.

In recent years, ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has been widely used in the determination of trace amounts, such as pesticides, veterinary drugs, and mycotoxins [11, 12]. It has also been employed for the determination of 1-DNJ in mulberry leaves, which can overcome the problem of derivatization in the methods based on GC or HPLC and could acquire lower detection limits [13–15]. However, considering the accuracy and repeatability of the method, how to

effectively remove pigments such as chlorophyll, organic acids, and other interfering substances which lead to the matrix effect and instrument pollution is an important problem for the detection of pesticide residues in vegetables with dark green color [16], which is also applied to the pretreatment process of mulberry leaves. Some materials for purification were synthesized and found to be effective in removing these matrix interferences. Organic acids could be removed by silica microspheres bonded with amidogen, which was upgraded and replaced by primary secondary amine (PSA), while graphitized carbon black (GCB) has better adsorbing capacity on chlorophyll [16]. Some solid-phase extraction (SPE) methods based on these materials have been widely used in the analysis of pesticide residues and pollutants in vegetables and herbal plants [17–19], especially the quick, easy, cheap, efficient, rugged, and safe (QuEChERS) method which was the most popular in this field in recent years [20–22].

In this study, a massive monitoring and precise quantitation method of 1-DNJ in mulberry leaves based on LC-MS/MS was established, including (1) determining a suitable column and mobile phase proportion and acquiring optimal instrument parameters for the LC-MS system; (2) determining the optimal extraction, purification, and other pretreatment processes; (3) carrying out the methodology validation and comparing with the existing GC and HPLC methods. It is the first development for the determination of 1-DNJ in mulberry leaves with both LC-MS/MS system and matrix interference purification.

2. Materials and Methods

2.1. Chemicals and Solvents. Mulberry leaves were collected from Tai'an (Shandong Province, China). The leaves were harvested, cleaned, air-dried at 80°C for 12 hours, ground into powder, and then passed through a 100-mesh sieve and stored in a drying dish until used.

The reference material of 1-deoxynojirimycin (1-DNJ) was purchased from DASF Biological Co., Ltd. (Nanjing, China) with the purity of 98%. Acetonitrile (MeCN) and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany), while graphitized carbon black (GCB) and primary secondary amine (PSA) purification powder were purchased from Agela Technologies (Tianjin, China).

2.2. Standard and Sample Pretreatment. Stock standard solutions (100 µg/mL): 10 mg of 1-deoxynojirimycin standard was accurately weighted (accurate to 0.1 mg) into a 100 mL brown volumetric flask and diluted to volume with methanol. The working solution was prepared in the initial mobile phase (MeCN: water = 1:1) and diluted to prepare the calibration curves at six concentration points of 500, 200, 100, 50, 20, and 10 ng/mL. The stock solution was kept at 4°C before being used.

200 milligrams of the dried mulberry leaf powder was weighed and put into a 50 mL centrifuge tube. After 50 mL of 30% MeCN/water solution was added, the sample was

sonicated in an ultrasonic bath for 5 min, following centrifugation at 7000 r/min for 5 min at 4°C. Subsequently, 5 mL of the supernatant was collected into a centrifuge tube with 45 mg PSA and 30 mg GCB added and then centrifuged at 10,000 r/min for 5 min after being vortexed for 1 min. Finally, 1.0 mL of purified supernatant was transferred to a 50 mL volumetric flask and then diluted to volume with the initial mobile phase solvent. The solution was filtered using a nylon syringe filter (0.2 µm) before being injected into the LC-MS system.

It should be mentioned that because negative samples with no 1-DNJ were unavailable, in the tests for pretreatment discussion, samples detected by the published method [6] were employed as quality control (QC) samples; therefore, the parameter “accuracy” was selected to exhibit the relative detection accuracy in the comparing groups. We thought it was a reasonable solution for this test.

2.3. LC-MS/MS Instrumentation. Samples were analyzed by a set of liquid chromatography-tandem mass spectrometry, equipped with the electrospray ionization source (ESI) and triple quadrupole mass analyzer (ExionLC-Triple Quad 3500, USA). The chromatographic separation was performed with the SunShell C18 (4.6 × 100 mm, 2.6 µm) (ChromaNik, Japan) column using a flow rate of 0.35 mL/min at 40°C. Mobile phases A and B of HPLC were deionized water and MeCN, respectively, and a 5 min gradient program was employed: proportion of B was kept at 50% for 2 min, then increased to 90% within 0.8 min and kept for 1.1 min, then suddenly decreased to 50% within 0.01 min, and kept to the end. The injection volume was 2 µL. ESI was performed in the positive ion mode with the 550°C interface temperature. The gas of curtain and ion sources 1 and 2 was set at 10, 50, and 50 psi, respectively. The capillary voltage was set at 5,500 V and the declustering potential of the parent ion (m/z = 164) as 40 eV. Four ions with m/z as 146.0, 109.9, 128.0, and 69.0 were adopted as product ions, in which the first one was employed as a quantitative ion, and their collision voltages were 19, 22, 19, and 26 eV, respectively, while the dwell time of each ion was set as 200 ms.

3. Results and Discussion

3.1. Optimization for Instrument Analysis

3.1.1. Comparison of the Separation Column. Theoretically, 1-DNJ would be better separated by a column bonded with amidogen because it was employed for the separation of monosaccharides such as glucose and fructose with similar structures in the HPLC system [23, 24], while it was used for 1-DNJ analysis and showed a good result in the previous study [13]. In this study, some types of column were employed and tested, including SunShell C18 (4.6 × 100 mm, 2.6 µm), ZORBAX Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm), ACQUITY UPLC® BEH HILIC (2.1 × 50 mm, 1.7 µm), and Pinnacle II Amino (4.6 × 150 mm, 3 µm). As shown in Figure 1, it was found that the SunShell C18 column can lead to the retention time near 2 min, earlier than the Pinnacle II Amino column but later than other columns, while it could

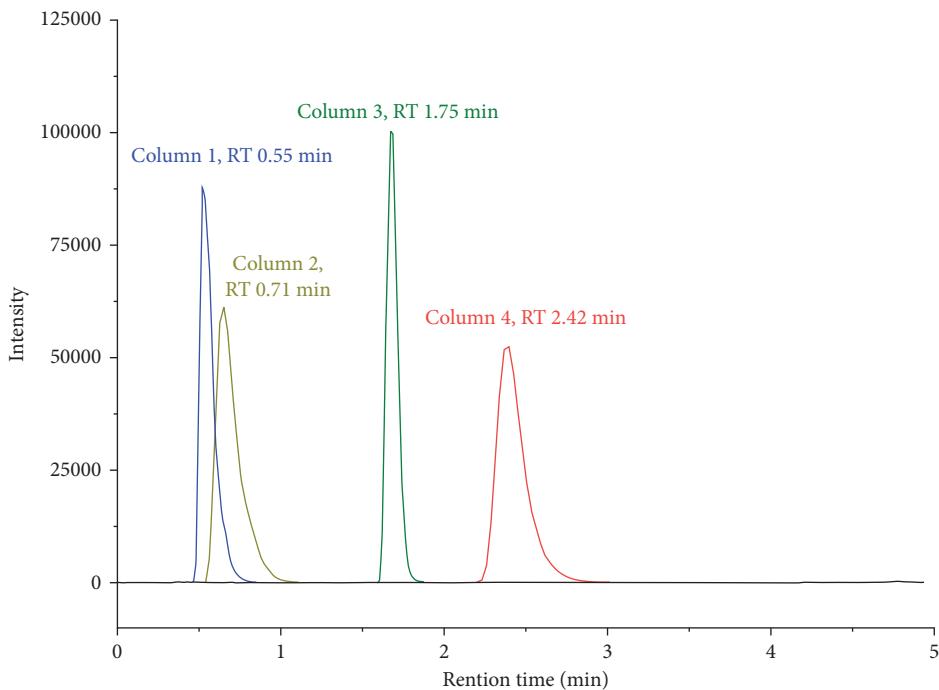


FIGURE 1: Real sample test chromatograms for the quantitation ion of 1-DNJ with the four types of column ($c = 500 \text{ ng/mL}$). Column 1: Agilent ZORBAX Eclipse Plus C18, $2.1 \times 50 \text{ mm } 1.8 \mu\text{m}$, column 2: Waters ACQUITY UPLC® BEH HILIC, $2.1 \times 50 \text{ mm } 1.7 \mu\text{m}$, column 3: SunShell C18, $4.6 \times 100 \text{ mm } 2.6 \mu\text{m}$, and column 4: Pinnacle II Amino, $4.6 \times 150 \text{ mm, } 3 \mu\text{m}$.

acquire a satisfactory peak shape and separation. Thus, SunShell C18 was chosen for the HPLC system in this study.

3.1.2. Mobile Phase and Solvents. Good separation performance and perfect distribution status of target compounds between the stationary and mobile phase would be achieved by appropriate mobile phase and solvents, as well as influence on peak width [25, 26]. Due to the strong water solubility of 1-DNJ, water would help eluting, while the organic solvent would lead to 1-DNJ retaining on the stationary phase, which differ from distribution characters of other substances. Therefore, the mobile phase proportion and gradient elution procedures must be considered. For the organic solvent, methanol and MeCN were chosen for the test, and we found that using MeCN would acquire a better peak shape, smaller column pressure, and better stability of injection. Thus, MeCN was selected.

Furthermore, the proportion of the initial mobile phase and gradient elution procedure were optimized. It was found that a higher proportion of water at initial would lead to retention time earlier than 1 min and tend to bring matrix effect, which was unsatisfactory. However, higher ratio of the organic solvent would make matrix interferences be eluted more earlier. Thus, we set up several combinations of them to test the same samples, employing retention time, peak area, and full width at half maximum (FWHM) as parameters. It is shown in Table S1 that, with the increase of the proportion of the organic phase, the retention time decreased, and the peak area value of the target increased, while the FWHM that is closely related to column efficiency showed a gradual decrease. Considering these factors, the

mobile phase of water and MeCN with equal proportion was selected for this method. Moreover, the imino group in the 1-DNJ structure would lead to the cationic characteristic, so it is necessary to provide hydrogen ions for the ionization process by adding formic acid. We also verified the performance while adding 0.1% formic acid, finding that the response (peak area) increased by 22.6%, and the stability difference was also obvious. Finally, optimal initial of the mobile phase, elution gradient, and solvent for determination were confirmed.

3.1.3. Optimization of Instrument Parameters. Standard solution of 1-DNJ was injected directly into mass spectrometry by using the syringe pump and detected by the ESI source, acquiring a higher response in the positive mode than the negative mode. Strong response of the precursor ion appeared with m/z of 164.0, while product ions of m/z 146.0, 109.9, 128.0, and 69.0 showed relatively higher response. Thus, in the multiple reaction mode (MRM), based on the difference of response, m/z 146.0 was reasonable to be applied as the quantitation ion, while the other 3 ions were employed as qualification ions. From the overlaid ion chromatogram shown in Figure 2, we can see that the monitoring parameters can achieve an ideal absolute response value and relative response ratio.

3.2. Optimization on the Pretreatment Process and Parameters

3.2.1. Extraction Efficiency of Different Solvents. Mixed MeCN-water was employed as the extraction solvent in this study. We compared the extraction effect of different

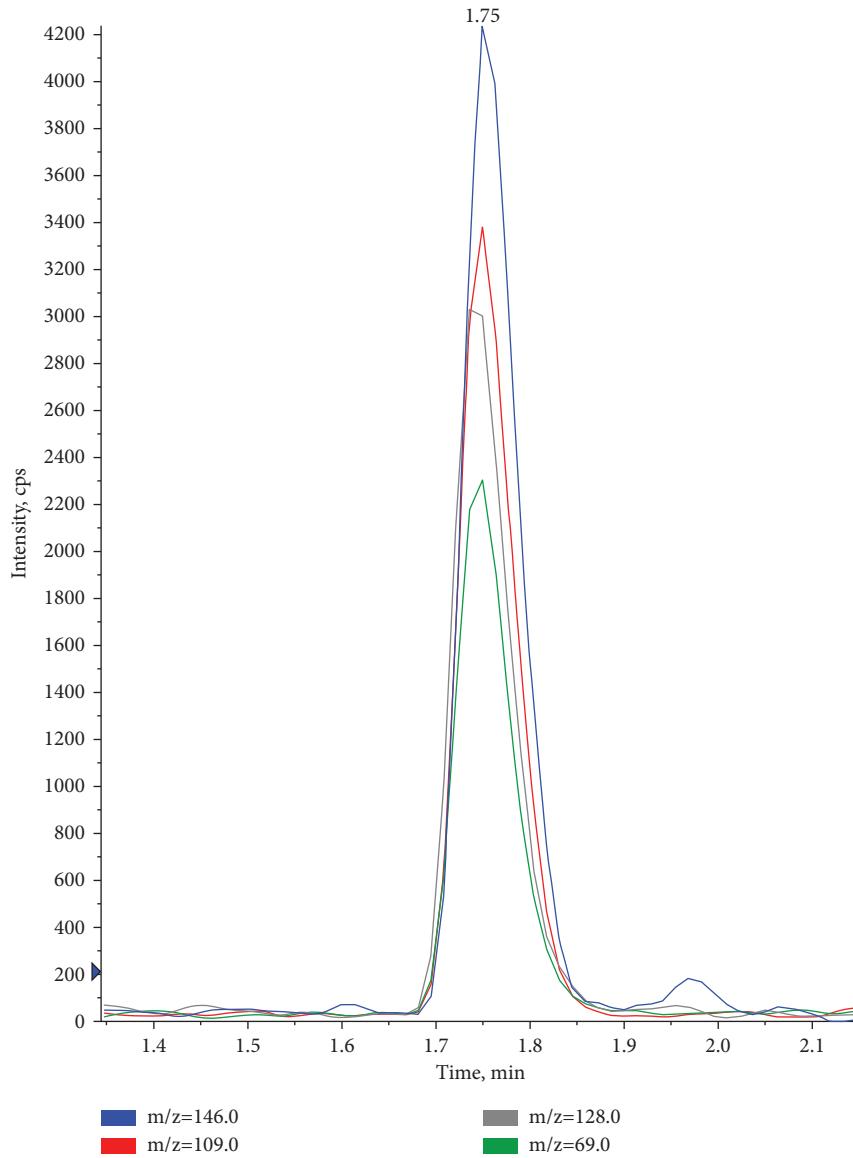


FIGURE 2: Overlaid chromatogram of four selective ions ($c = 50 \text{ ng/mL}$).

proportions of MeCN in the extraction solvent (0%, 10%, 30%, 50%, 80%, and 100%), as shown in Figure 3. The highest extraction efficiency was achieved, while the MeCN content was 30%. Because of the strong water solubility of 1-DNJ, a higher proportion of water in the extraction solvent was needed, but it still needs some organic solvent to assist extraction.

3.2.2. Research on Purification. There are many coextracts in plant foods, including organic acids and chlorophyll [16]. These substances brought into the LC-MS system will not only cause the contamination of the HPLC system, ion source, and quadrupole mass analyzer but also lead to matrix effect and matrix interference, which will affect the accuracy of determination [27, 28]. Thus, purification for the extraction solvent was set as a key point of this study. Some processes applied in pesticide residue determination were

referred [29, 30], including disperse solid-phase extraction (d-SPE) based on GCB and PSA, as well as solid-phase extraction (SPE) based on cation exchange, and comparison was also taken among different cleanup procedures about the performance and efficiency. From Figure 4, we can see that average accuracies by d-SPE, SPE, and no cleanup process were 89.6%, 62.4%, and 106.3%, respectively. Although the accuracy of the no cleanup group seems higher, there was an interference peak that did not completely separate with the target peak, which seriously affected quantitation. For the group of SPE, strong water solubility of 1-DNJ leads to considerable loss in the eluting process, and the repeatability was not satisfactory.

Furthermore, dosages of GCB and PSA were discussed. We set the adding amount of both as 10 mg, 20 mg, 40 mg, and 80 mg in the orthogonal test to compare the parameters of accuracy, stability, and visual purification effect. It was found that accuracy of 1-DNJ improved with increasing

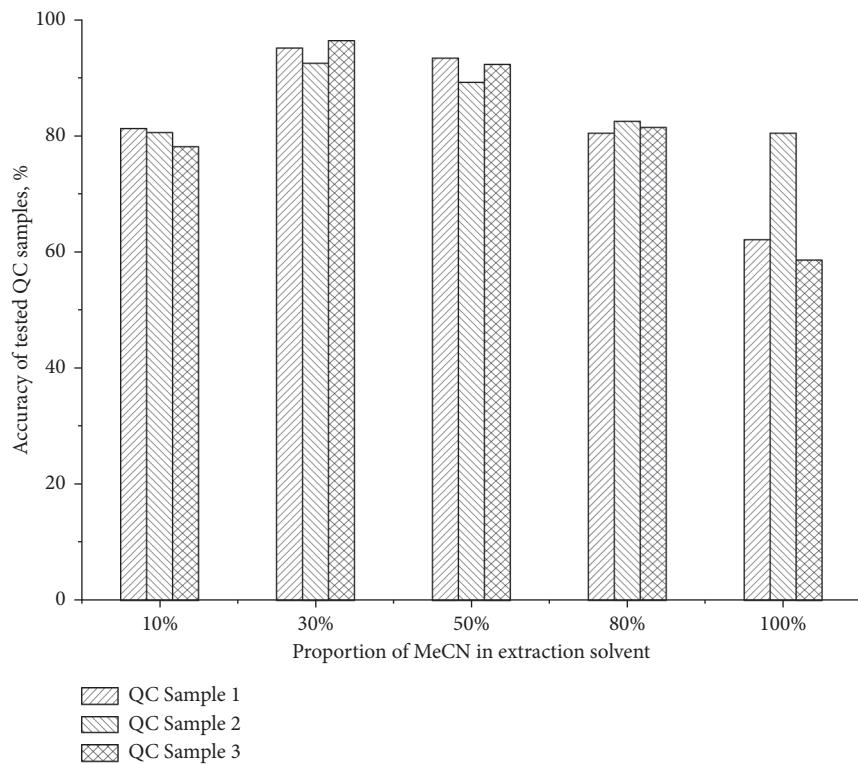


FIGURE 3: Influence of acetonitrile proportion in the solvent on 1-DNJ extraction efficiency.

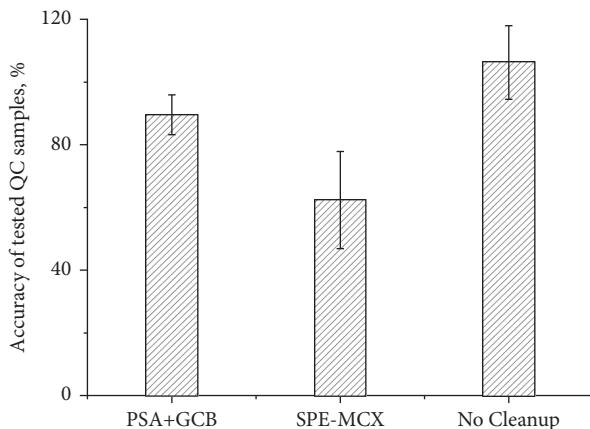


FIGURE 4: Influence of different purification processes on 1-DNJ detection accuracy.

adding amount of PSA, yet it showed little decrease when the adding amount of PSA was more than 40 mg. This may be because PSA could effectively adsorb interfering organic acids rather than alkaline compounds such as 1-DNJ, thus reducing matrix interference in mass spectrometry analysis. For GCB, similar regularity was found, but the accuracy significantly decreased while adding more than 40 mg. Due to the cyclic planar structure in 1-DNJ molecules, the target would be more or less adsorbed by GCB though there is no conjugated double bond. In terms of visible color, it is shown in Figure 5 that adding more than 20 mg GCB had little effect, indicating that 20–30 mg GCB added could adsorb most chlorophyll in the matrix extraction solvent.

Combining with the accuracy reference of the orthogonal test data in Table S2 and Figure 6, the amount of 45 mg PSA and 30 mg GCB was determined.

Purification for the extraction solvent will lead to results, the obvious one of which is the change of the acquired ion chromatogram. The extraction ion chromatograms are shown in Figure 7, including purified sample extraction solvent and unpurified one for two samples. It can be seen that the signal of interferences following the peak of 1-DNJ is markedly improved, which would help for the accuracy of quantitation. This is the important reason for employing the purification process.

3.2.3. Dilution Ratio. The contents of 1-DNJ in mulberry leaves were at the level of thousandth (w/w) [13, 31]. For LC-MS analysis, the optimal linear range was 10–500 ng/mL in this study. Thus, the dilution ratio of the extraction process was set at about 100 times, which means 0.5 g mulberry leaves were extracted with 50 mL extraction solvent and diluted by 50 times during purification and dilution.

3.3. Method Validation. Although the content of 1-DNJ in mulberry leaves was relatively high, the validation for limits of detection and quantitation (LOD and LOQ) was carried out completely in this study. Based on 3 times and 10 times of signal-to-noise ratio, they were acquired as 4 ng/mL and 10 ng/mL, respectively, and the corresponding method detection limit (MDL) and method quantitation limit (MQ) were 20 mg/kg and 50 mg/kg, respectively. For standard solvents with concentration higher than 500 ng/mL,

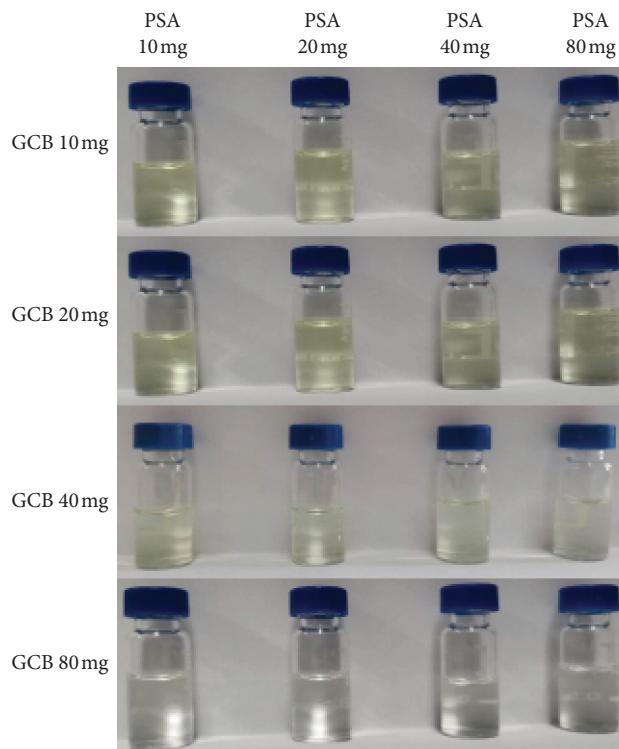


FIGURE 5: Visual color comparison of the orthogonal test for adding amount of GCB and PSA.

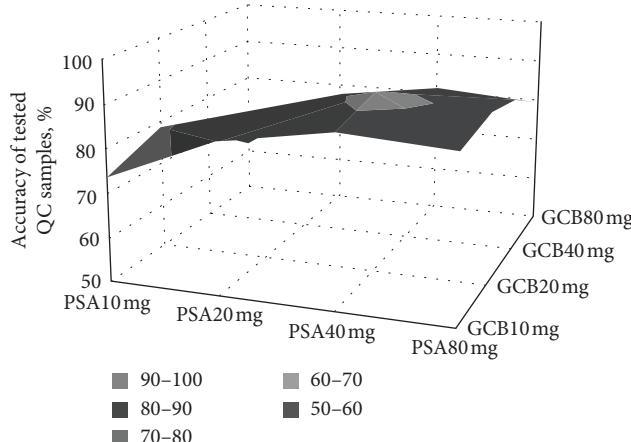


FIGURE 6: Response surface diagram of the data in Table S2.

considerable response attenuation was found, which may be because of competitive soft ionization of 1-DNJ in the ESI source, so the linear range of 10–500 ng/mL was determined. Six concentration points of 10, 20, 50, 100, 200, and 500 ng/mL were chosen for the calibration curve, and the linear equation was calculated as $Y = 1.3342e5X$, with correlation coefficient as 0.998. Precision and accuracy were also investigated by spiking in the positive QC sample, the 1-DNJ concentration of which was detected by this method as 0.78 mg/g. Spiked concentrations of 1-DNJ were 0.1, 0.5, and 2 mg/g, respectively, with six parallel samples for each concentration. Recoveries for the three levels were 96.4%, 95.7%, and 94.6%, respectively, while the relative standard

deviations (RSDs) were 0.9%, 1.2%, and 0.9%, respectively. The above validation data were satisfactory, indicating good method applicability.

Considering that negative samples of the mulberry leaf were hard to acquire, much less to prepare matrix standard solution, we investigated the matrix effect. 100 and 500 ng/mL levels of 1-DNJ were spiked into the test solution of positive samples that had already acquired response peak area to prepare matrix standard solution. The difference value of peak area before spiking and after spiking was employed as the peak area of spiked matrix standard solution. The matrix effect (ME) value was calculated by the equation $ME = A_{\text{Matrix}}/A_s$, where A_{Matrix} is the peak area of

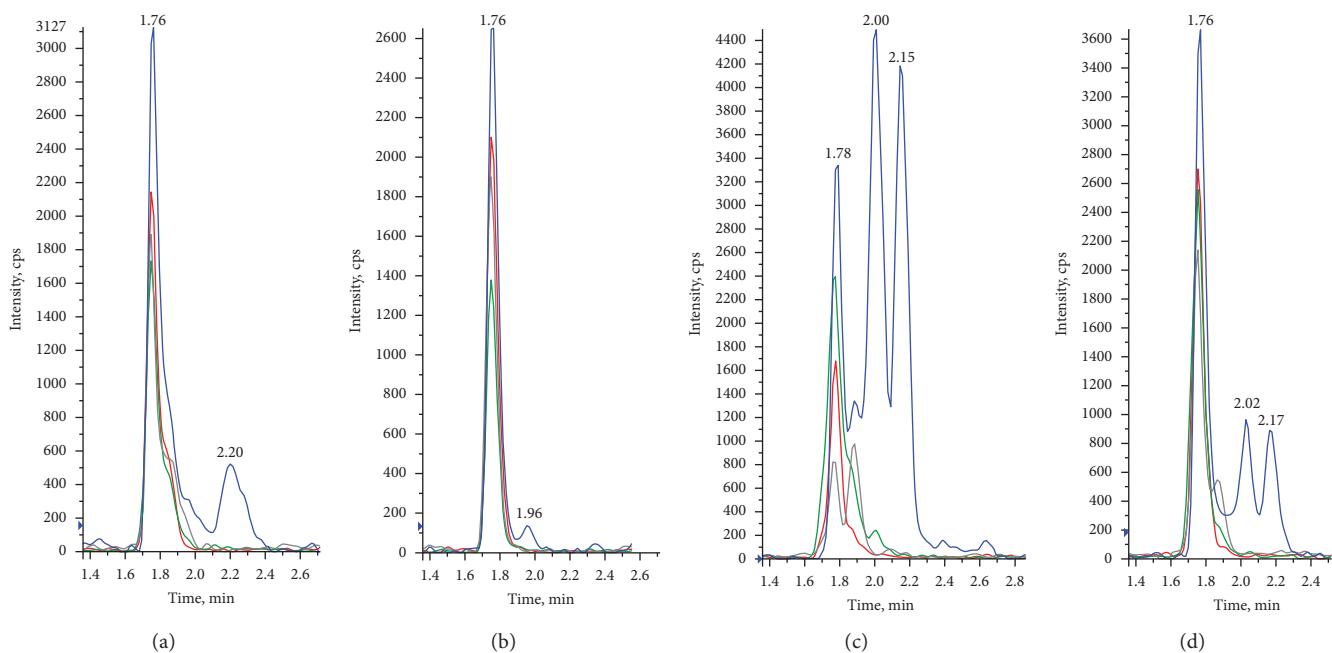


FIGURE 7: Comparison of chromatograms for purified and unpurified samples ($c=50 \text{ ng/mL}$). (a) Sample 1, unpurified. (b) Sample 1, purified. (c) Sample 2, unpurified. (d) Sample 2, purified.

spiked matrix standard solution and A_s is the peak area of pure solvent standard solution [19].

The ME was 0.91 and 0.93, respectively, at the concentration level of 100 and 500 ng/g, which shows little difference. The matrix effect value was considered to be negligible effect at the range of 0.9–1.1 in the previous study [32]. So, preparing standard solution by initial mobile phase solvent is suitable for this method.

3.4. Investigation of Method Applicability. In this study, thirteen mulberry leaf samples were collected from eight areas of China, and the information is shown in Table S3. All the samples were detected by this method and other two methods based on GC and HPLC, respectively [4, 5]. Every sample was set three parallel tests. The results are also listed in Table S3. There are some different analysis results between the three detection methods. Data of the GC group seem generally lower, and data of the HPLC group seem partially higher and with bad repeatability. The analysis results by this method showed best repeatability and most of them distributed fall in between the other two groups. This is mainly because both methods based on GC and HPLC required derivatization of 1-DNJ; thus, the uncertain efficiency and stability of derivatization lead to the test performance worse than the present study method. For this method, the RSDs of 13 samples ranged from 0.5% to 1.5%, indicating that this method is relatively mature and stable.

4. Conclusion

In this study, a new analysis method for the determination of 1-DNJ in mulberry leaves based on LC-MS was developed. Optimal parameters were determined by testing the

instrument analysis condition, while matrix interference was effectively reduced and satisfactory cleanup performance was acquired by studying the procedure process, especially disperse purification based on GCB and PSA. MDL and MQL were verified at 20 and 50 mg/kg, respectively, and this method exhibited good accuracy and precision based on different levels of spiked recovery test, while the matrix effect was found to be negligible. Comparing with the published methods detected by GC or HPLC, this developed method has solved the problems such as poor repeatability and quantitative inaccuracy brought by derivatization and improved the detection efficiency obviously. Furthermore, the developed approach can be used for massive monitoring and precise quantitation of 1-DNJ in mulberry leaves, and it has important application value and innovation significance.

Data Availability

The data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Table S1: effect of different mobile phase compositions on the chromatographic peak ($c = 100 \text{ ng/mL}$). Table S2: data of the orthogonal test for the adding amount of GCB and PSA ($n = 6$). Table S3: detection results of 1-DNJ from different kinds of mulberry leaf samples via three analysis methods. (Supplementary Materials)

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