

# Determination of Nitidine Chloride, Toddalolactone, and Chelerythrine Chloride by HPLC

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## Key Words

Zanthoxylum Nitidum (Roxb.) DC, Zanthoxylum Nitidum var. Fastuosum, Pharmacopeia of the People's Republic of China (PPRC), Traditional Chinese Medicine, Herbal Medicine, Alkaloids

## Introduction

Zanthoxylum nitidum (Roxb.) DC (Figure 1A) is an important traditional Chinese medicine. The Pharmacopeia of the People's Republic of China (PPRC) 2010 regulates this dried root as an herbal medicine.<sup>1</sup> Nitidine (structure of its chloride shown in Figure 2) is the specific active component in the dried root version of Zanthoxylum nitidum (Roxb.) DC,<sup>2</sup> and nitidine chloride is reported to be beneficial for both killing and constraining the growth of cancerous cells.<sup>3-6</sup> For example, it may inhibit the proliferation of human hepatocarcinoma cell line SMMC-7721 and renal carcinoma cell line Ketr-3,<sup>3,4</sup> induce the apoptosis of tumor cell lines SPC-A-1 and Tea8113,<sup>5</sup> and decrease the cell survival probability of human nasopharyngeal cancer cell line CNE1 and breast cancer cell line MDA-MB-231.<sup>6</sup>

Zanthoxylum nitidum var. fastuosum (Figure 1B) is another plant in the same genus as Zanthoxylum nitidum (Roxb.) DC. Although Zanthoxylum nitidum var. fastuosum is not regulated in the PPRC, its dried root is still used in Chinese folk medicine because some of its reported medical benefits, such as promotion of blood circulation, pain relief, treatment of gastric ulcers, and reduction of inflammation, are the same as those reported for Zanthoxylum nitidum (Roxb.) DC.<sup>7</sup>



Figure 1A. Zanthoxylum nitidum (Roxb.) DC and (b) root

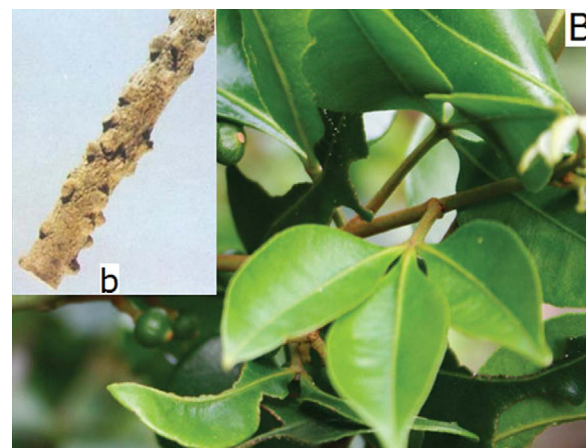


Figure 1B. Zanthoxylum nitidum var. fastuosum and (b) root

The major active components of *Zanthoxylum nitidum* (Roxb.) DC and *Zanthoxylum nitidum* var. *fastuosum* are alkaloids (e.g., chelerythrine chloride, structure shown in Figure 2). Nitidine and toddalolactone (structures shown in Figure 2) are the specific active components in *Zanthoxylum nitidum* (Roxb.) DC and *Zanthoxylum nitidum* var. *fastuosum*, respectively.<sup>2</sup> The PPRC 2010 method regulates the quality control of *Zanthoxylum nitidum* (Roxb.) DC using a high-performance liquid chromatography (HPLC) method for determination of nitidine chloride, but uses a thin-layer chromatography (TLC) method for detection of chelerythrine chloride and toddalolactone, where the presence of toddalolactone is not permitted.

## Goal

The purpose of this work is to develop an efficient and comprehensive HPLC method for the quality control analysis of *Zanthoxylum nitidum* (Roxb.) DC. This method must separate the main active components of *Zanthoxylum nitidum* (Roxb.) DC and *Zanthoxylum nitidum* var. *fastuosum*: nitidine chloride, chelerythrine chloride, and toddalolactone.

## Equipment

- Thermo Scientific Dionex UltiMate 3000 HPLC system including:
  - SR-3000 Solvent Rack without degasser
  - LPG-3400RS Pump
  - WPS-3000TRS Autosampler
  - TCC-3000RS Thermostatted Column Compartment
  - DAD-3000RS Diode Array Detector
- Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software version 6.80, SR9 or higher
- SK3200 KUDOS LHC Series Dual Frequency Ultrasonic Cleaner, KUDOS, Shanghai, China
- Thermo Scientific Orion 2-Star Benchtop pH Meter

## Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Methanol (CH<sub>3</sub>OH), HPLC Grade (Fisher Chemical P/N AC610090040)
- Ethanol (CH<sub>3</sub>CH<sub>2</sub>OH), Analytical Grade, SCRC, China
- Acetonitrile (CH<sub>3</sub>CN), HPLC Grade (Fisher Chemical P/N AC610010040)
- Ammonium Acetate (CH<sub>3</sub>COONH<sub>4</sub>), Analytical Grade, SCRC, China
- Acetic Acid (CH<sub>3</sub>COOH), Analytical Grade, SCRC, China

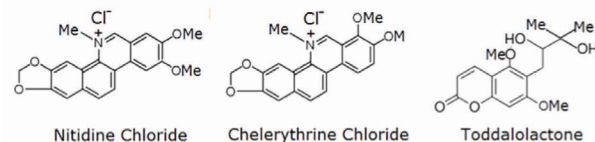


Figure 2. Structures of nitidine chloride, chelerythrine chloride, and toddalolactone

## Standards

Nitidine chloride, chelerythrine chloride, and toddalolactone (purity ≥95%) were purchased from the Research Center of Standardization of Chinese Traditional Medicine, Shanghai, China. Procedures for the preparation of working standard solutions were based on those specified in the PPRC monograph.<sup>1</sup>

### Stock Standard Solutions

Weigh ~10 mg of nitidine chloride, chelerythrine chloride, and toddalolactone standards and dilute in three 100 mL volumetric flasks with methanol/water (7:3, v/v). The concentration of these standards is 100 µg/mL.

### Working Standard Solutions for Calibration

Prepare five working standard solutions for calibration with 0.25, 0.5, 1.0, 2.5, and 10 µg/mL concentrations by adding the proper amount of stock standard solution and making dilutions with methanol/water (7:3, v/v).

## Samples

*Zanthoxylum nitidum* (Roxb.) DC was donated by Guangdong Institute for Drug Control, Guangzhou, China. Nitidum toothpaste was purchased from a local supermarket.

### Preparation of *Zanthoxylum Nitidum* (Roxb.) DC Sample

Procedures for the preparation are based on specifications in the PPRC monograph.<sup>1</sup> Weigh 1.0 g of ground sample, place in a conical beaker, and add 20 mL methanol/H<sub>2</sub>O (7:3, v/v). Sonicate for 30 min at 200 W, 59 KHz and cool to room temperature. Filter the sonicated sample through a 0.22 µm membrane (Millex™-LH) and transfer the filtrate to a 50 mL volumetric flask. Add 20 mL methanol/H<sub>2</sub>O (7:3, v/v) to the filtered residue, then repeat the procedures of extraction and filter once. Move the filtrate to the 50 mL volumetric flask and dilute with methanol/H<sub>2</sub>O (70:30, v/v).

### Preparation of Nitidum Toothpaste Sample

Weigh 2 g of toothpaste sample and mix it with a suitable amount (~1 g) of Diatomaceous Earth Dispersant for ASE (P/N 062819). The remainder of the procedure is the same as that for the *Zanthoxylum nitidum* (Roxb.) DC sample.

## Conditions

Column:	Thermo Scientific Acclaim PA, 3 $\mu$ m Analytical (2.1 $\times$ 150 mm) (P/N 061317)
Mobile Phase:	25 mM ammonium acetate (pH 4.5 with acetic acid)/acetonitrile
Gradient:	Acetonitrile, 0 min, 20%; 8 min, 30%; 15 min, 70%, curve 4; 15.5–18 min, 20%
Flow Rate:	0.6 mL/min
Inj. Volume:	5 $\mu$ L
Temperature:	30 $^{\circ}$ C
Detection:	UV absorbance at 273 nm

## Results and Discussion

### Separation of Extract of *Zanthoxylum Nitidum* (Roxb.) DC on Different Acclaim Columns

The HPLC method for nitidine chloride in the PPRC 2010 uses a C18 stationary phase. Three Acclaim™ columns—a C18 stationary phase and two polar-embedded stationary phases—were evaluated for separation of nitidine chloride, chelerythrine chloride, and toddalolactone, as well as the other components in *Zanthoxylum nitidum* (Roxb.) DC.

The major active components in *Zanthoxylum nitidum* (Roxb.) DC are alkaloids of nitidine compounds (e.g., dihydronitidine, nitidine chloride, and oxynitidine) and chelerythrine compounds (e.g., chelerythrine, 6-methoxy-5,6-dihydrochelerythrine, oxychelerythrine, 6-ethoxy-5,6-dihydrochelerythrine, des-*N*-methylchelerythrine, and 7-demethyl-6-methoxy-5,6-dihydrochelerythrine).<sup>8</sup> A small quantity of lignanoids (e.g., *L*-sesamin and *L*-asarinin) and coumarins are also present.<sup>8</sup>

The PPRC method recommends a C18 stationary phase to determine nitidine chloride using a formic acid/triethylamine/acetonitrile mobile phase in a 35 min gradient. The use of triethylamine may improve the peak shape of basic compounds on the C18 stationary phase; however, it can damage the C18 stationary phase. Therefore, an Acclaim 120 C18 column and two kinds of polar-embedded reversed-phase columns—the Acclaim PA and PA2 columns—were evaluated using an ammonium acetate buffer mobile phase. Table 1 lists the calculated asymmetry of peaks of nitidine chloride and chelerythrine chloride separated from a sample extract. Good peak shapes were obtained on the Acclaim PA column, making it the best choice for the analysis.

Table 1. Calculated asymmetry of nitidine chloride and chelerythrine chloride

Analyte	Nitidine Chloride			Chelerythrine Chloride		
	C18	PA	PA2	C18	PA	PA2
Asymmetry	1.32	1.11	1.18	1.46	1.23	1.37

### Influence of the pH Value of the Mobile Phase Buffer

The influence of the pH of the ammonium acetate buffer mobile phase on the separation of the *Zanthoxylum nitidum* (Roxb.) DC extract using the Acclaim PA column was investigated. The buffer pH value was adjusted by changing the amount of added acetic acid. As shown in Figure 3A, the retention times of peaks 1–4 (peak 3 is nitidine chloride and peak 4 is chelerythrine chloride) increase as buffer pH increases. Comparison of the UV spectra of these peaks (Figure 4) shows that peaks 1 and 2 are likely nitidine compounds.

Figure 3B shows the chromatograms with detection by UV absorbance at 250 nm. Compared to Figure 3A with detection by UV absorbance at 273 nm, there are two unknown compounds (peaks 5 and 6) that elute between peaks 2 and 3 (nitidine chloride). Their UV spectra match neither nitidine chloride nor chelerythrine chloride. At pH 5.5, a small peak following chelerythrine chloride (peak 4) may interfere with the determination of peak 4; at pH 4.0, the resolution ( $R_s$ ) between peaks 6 and 3 (nitidine chloride) is 1.41 and that can be improved to 2.5 when the buffer pH value increases to 4.5. The spectra of analytes and scales of the chromatograms suggest that the detection at 273 nm using pH 4.5 mobile phase buffer is more sensitive. Therefore, a detection wavelength of 273 nm and a pH value of 4.5 were chosen for further analysis.

Column: Acclaim LC PA, 3  $\mu$ m, Analytical, 2.1  $\times$  150 mm  
 Mobile Phase: 25 mM ammonium acetate (pH 4.5 with acetic acid)/acetonitrile  
 Gradient: Acetonitrile: 0 min, 20%; 8 min, 30%; 15 min, 70%; 15.5–18 min, 20%  
 Flow Rate: 0.6 mL/min  
 Injection Volume: 5  $\mu$ L  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV absorbance at (A) 273 nm, (B) 250 nm  
 Chromatograms: (a) pH 4.0, (b) pH 4.5 and (c) pH 5.5

Peaks: 1, 2, 5 and 6: unknown  
 3: nitidine chloride  
 4: chelerythrine chloride

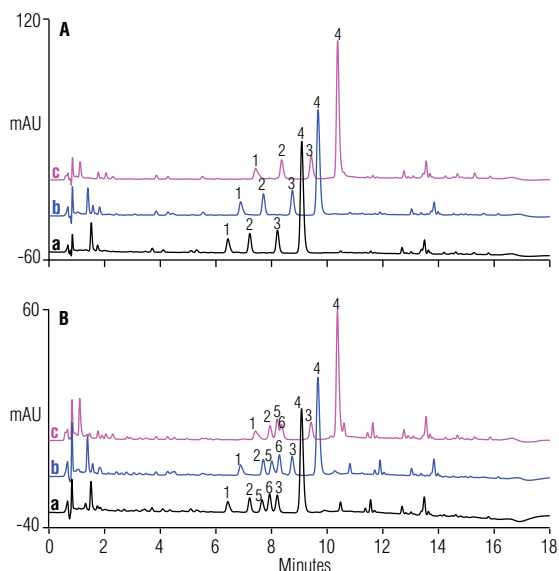


Figure 3. Chromatograms of a *Zanthoxylum nitidum* (Roxb.) DC sample detected at (A) 273 nm and (B) 250 nm at different buffer pH values

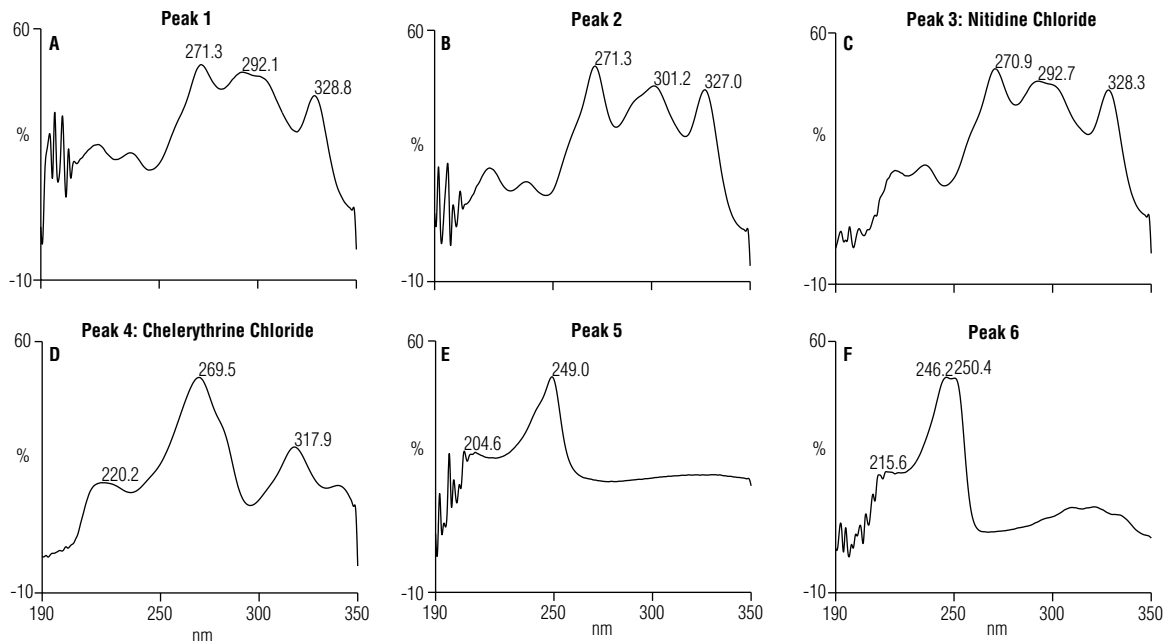


Figure 4. UV spectra of some compounds separated from a *Zanthoxylum nitidum* (Roxb.) DC sample; Spectrum A: peak 1; B: peak 2; C: peak 3 (nitidine chloride); D: peak 4 (chelerythrine chloride); E: peak 5; and F: peak 6

### Reproducibility, Linearity, and Detection Limits

Figure 5 illustrates good separation of nitidine chloride, chelerythrine chloride, and toddalolactone under the specified chromatographic conditions. Method precision was estimated by making seven consecutive injections of a calibration standard with a concentration of 2.5 µg/mL for each. Retention time and peak area reproducibilities are summarized in Table 2 and show good precision.

Calibration linearity of nitidine chloride, chelerythrine chloride, and toddalolactone was investigated by making six consecutive injections of a mixed standard prepared at five different concentrations (30 total injections). The external standard method was used to establish the calibration curve and quantify nitidine chloride, chelerythrine chloride, and toddalolactone in the *Zanthoxylum nitidum* (Roxb.) DC and nitidum toothpaste samples. Excellent linearity was observed from 0.25 to 10 µg/mL when plotting the concentration versus the peak area, and the correlation coefficients were  $\geq 0.998$  for all (Table 3).

The method detection limits (MDLs) of nitidine chloride, chelerythrine chloride, and toddalolactone were calculated using the equation:

$$\text{Detection Limit} = St_{(n-1, 1-\alpha=0.99)}$$

The symbol  $S$  represents standard deviation of replicate analyses,  $n$  represents number of replicates,  $t_{(n-1, 1-\alpha=0.99)}$  represents Student's value for the 99% confidence level with  $n - 1$  degrees of freedom. Eleven replicate injections of a *Zanthoxylum nitidum* (Roxb.) DC sample spiked with 5 µg/mL of standards mixture were used to determine the MDL. Table 3 summarizes the MDL data, which show good method sensitivity.

Table 2. Reproducibility for peak retention time and area

Analyte	Retention Time RSD	Peak Area RSD
Toddalolactone	0.045	0.865
Nitidine chloride	0.048	1.433
Chelerythrine chloride	0.043	1.320

Column: Acclaim LC PA, 3 µm, Analytical, 2.1 × 150 mm  
 Mobile Phase: 25 mM ammonium acetate (pH 4.5 with acetic acid)/acetonitrile  
 Gradient: Acetonitrile: 0 min, 20%; 8 min, 30%; 15 min, 70%; 15.5–18 min, 20%  
 Flow Rate: 0.6 mL/min  
 Injection Volume: 5 µL  
 Temperature: 30 °C  
 Detection: UV absorbance at 273 nm

Peaks: 1: toddalolactone  
 2: nitidine chloride  
 3: chelerythrine chloride

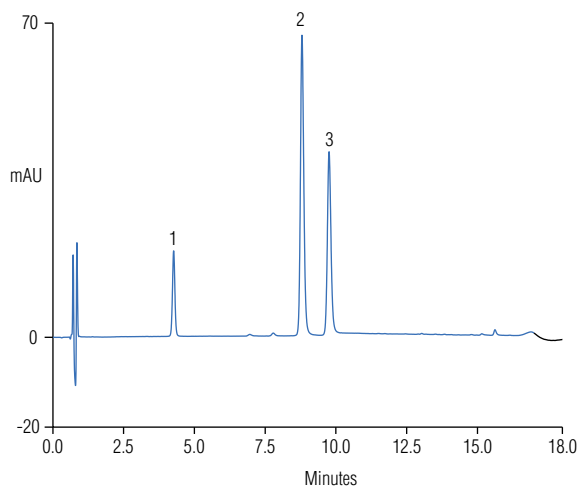


Figure 5. Chromatogram of a mix of nitidine chloride, chelerythrine chloride, and toddalolactone standards (10 µg/mL each)

Analyte	Regression Equation	<i>r</i>	Range (µg/mL)	MDL (µg/mL)
Toddalolactone	$Y = 0.1921 X + 0.0049$	1.0000	0.25–10	0.08
Nitidine chloride	$Y = 0.9791 X - 0.0504$	0.9982		0.07
Chelerythrine chloride	$Y = 0.6362 X - 0.0313$	0.9986		0.09

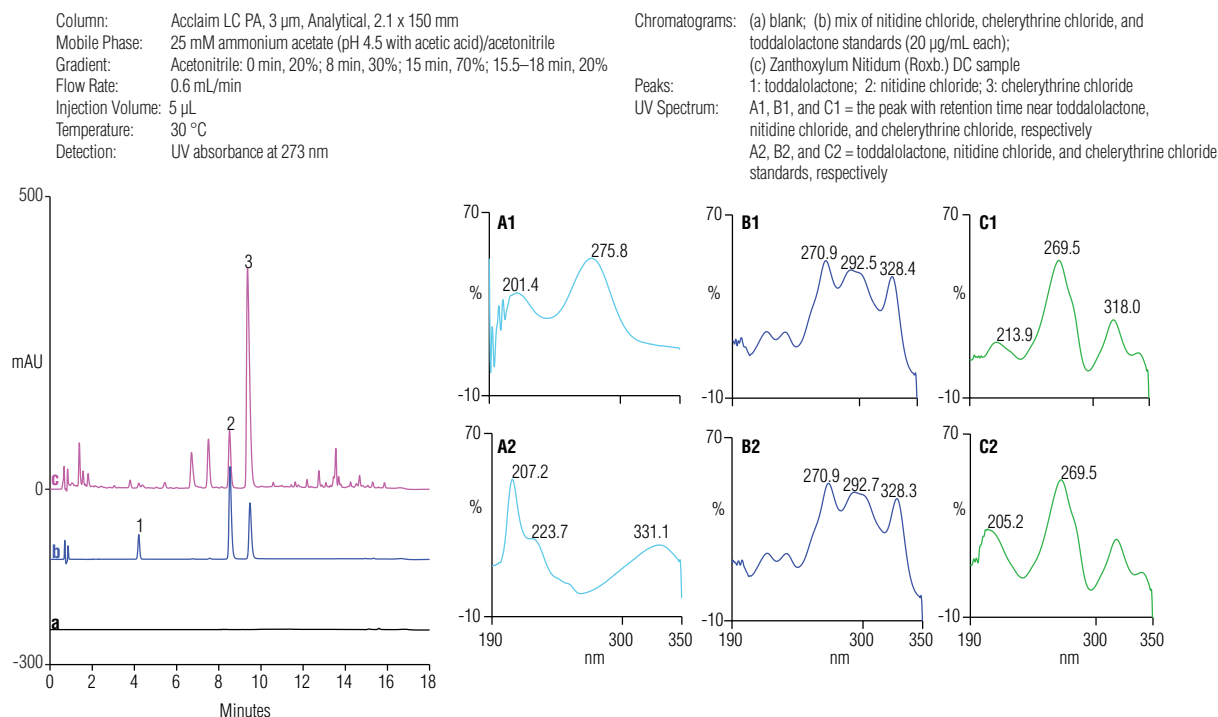


Figure 6. Chromatogram of a Zanthoxylum nitidum (Roxb.) DC sample

### Sample Analysis

Figure 6 shows the chromatograms of a Zanthoxylum nitidum (Roxb.) DC sample as well as a mixture of nitidine chloride, chelerythrine chloride, and toddalolactone standards. A small peak with retention time near that of toddalolactone was found. However, comparison of the UV spectra, also shown in Figure 6, reveals that the peak was not toddalolactone (Figure 6, Spectra A1 and A2). The two peaks were labeled as nitidine chloride and chelerythrine chloride in the sample because their UV spectra were consistent with that of nitidine chloride and chelerythrine chloride standards (Figure 6, Spectra B1, B2, C1, and C2), indicating their presence in the Zanthoxylum nitidum (Roxb.) DC sample. The calculated peak purity match factors for nitidine chloride and chelerythrine chloride separated from the Zanthoxylum nitidum (Roxb.) DC sample are all  $\geq 991$  (the corresponding value for 100% purity is 1000), demonstrating the good separation of nitidine chloride and chelerythrine chloride.

Figure 7 shows the chromatograms of a nitidum toothpaste sample. Three small peaks with retention times near those of toddalolactone, nitidine chloride, and chelerythrine chloride were found. However, as the UV spectra in Figure 7 show, two of them match neither

toddalolactone (Spectrum A1 and A2) nor nitidine chloride (Spectrum B1 and B2), thus indicating the absence of toddalolactone and nitidine chloride in the nitidum toothpaste sample. Despite the low UV absorbance of the small peak with retention time near that of chelerythrine chloride (Figure 7, Spectrum C1), its UV spectrum is similar to that of chelerythrine chloride (Figure 7, Spectrum C2), indicating the possible presence of chelerythrine chloride in the nitidum toothpaste sample. However, the determined amount is lower than the MDL. Therefore, nitidum toothpaste is an inaccurate name.

The analysis results and related data are summarized in Table 4. These data show excellent spike recovery for nitidine chloride, chelerythrine chloride, and toddalolactone, thereby indicating method accuracy and demonstrating that this HPLC method provides good selectivity and suitability for the rapid analysis of nitidine chloride, chelerythrine chloride, and toddalolactone in Zanthoxylum nitidum (Roxb.) DC and related products.

Column: Acclaim LC PA, 3  $\mu$ m, Analytical, 2.1  $\times$  150 mm  
 Mobile Phase: 25 mM ammonium acetate (pH 4.5 with acetic acid)/acetonitrile  
 Gradient: Acetonitrile: 0 min, 20%; 8 min, 30%; 15 min, 70%; 15.5–18 min, 20%  
 Flow Rate: 0.6 mL/min  
 Injection Volume: 5  $\mu$ L  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV absorbance at 273 nm

Chromatograms: (a) blank; (b) mix of nitidine chloride, chelerythrine chloride, and toddalolactone standards (20  $\mu$ g/mL each); (c) nitidum toothpaste sample  
 Peaks: 1: toddalolactone, 2: nitidine chloride, 3: chelerythrine chloride  
 UV Spectrum: A1, B1, and C1 = the peak with retention time near toddalolactone, nitidine chloride, and chelerythrine chloride, respectively; A2, B2, and C2 = toddalolactone, nitidine chloride, and chelerythrine chloride standards, respectively

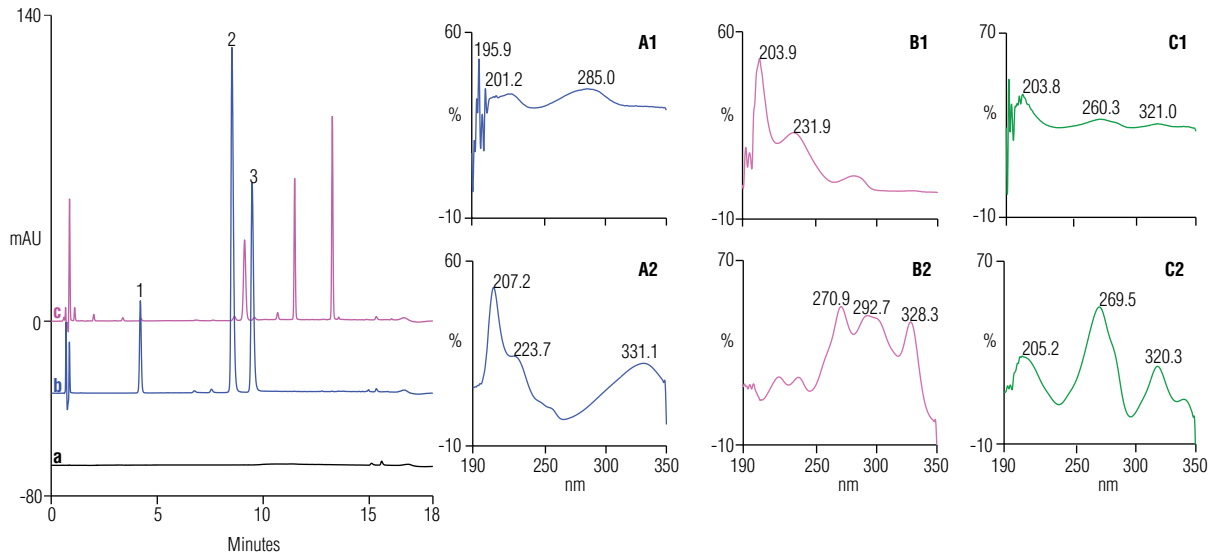


Figure 7. Analysis of a nitidum toothpaste sample

Table 4. Analysis results of the active components of *Zanthoxylum nitidum* (Roxb.) DC

Sample	Analyte	Detected ( $\mu$ g/g)	Added ( $\mu$ g/g)	Found ( $\mu$ g/g)	Recovery (%)
Zanthoxylum nitidum (Roxb.) DC	Toddalolactone	Not Found	5	5.6	112
	Nitidine chloride	70		4.5	90
	Chelerythrine chloride	3600		5.5	110
Nitidum toothpaste	Toddalolactone	Not Found	—	—	—
	Nitidine chloride	Not Found			
	Chelerythrine chloride	< MDL >			

## Conclusion

This work describes an HPLC method that uses UV absorbance detection to rapidly determine nitidine chloride, chelerythrine chloride, and toddalolactone in *Zanthoxylum nitidum* (Roxb.) DC and related products. The determination was performed on the UltiMate™ 3000 HPLC system controlled by Chromeleon™ CDS software. The Acclaim PA column delivers good peak shape for nitidine chloride and chelerythrine chloride, using an ammonium acetate buffer/acetonitrile mobile phase without triethylamine. This method can be used for quality control of *Zanthoxylum nitidum* (Roxb.) DC and related products. This approach is superior to both the PPRC method that measures the purported active components of *Zanthoxylum nitidum* (Roxb.) DC using two different methods—HPLC and TLC—and the HPLC method that requires a long separation time.

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