

# HPLC Method Development and Validation for Quantification of Berberine from *Berberis aristata* and *Berberis tinctoria*

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**Abstract:** An RP-HPLC method with photodiode array detection has been developed for the determination of major constituent berberine from *Berberis aristata* (Barberry) and *Berberis tinctoria*. Berberine was isolated from the plant extract on semi-preparative HPLC and separated on HPLC by using an isocratic mode consisting of 0.1% trifluoroacetic acid: acetonitrile (60:40, v/v) at a flow rate of 1 mL/min. Under these conditions, a plot of integrated peak area versus concentration of berberine was found to be linear over the concentration range of 0.2 µg/mL to 150 µg/mL. The limit of detection was 1 ng on column and limit of quantification was 2 ng on column for berberine. The berberine content in *B. aristata* and *B. tinctoria* was found to be 3.18% and 1.46% respectively.

**Keywords:** RP-HPLC; Photo Diode Array detector; Berberine.

## 1. Introduction

Berberine, an isoquinoline alkaloid, is the major constituent of *Berberis* species like *Berberis aristata* and *Berberis tinctoria* belonging to family Berberidaceae. *B. aristata* occurs in northern and *B. tinctoria* in southern region of India. Berberine is mainly present in roots, rhizomes and stem bark of plants [1, 2, 3]. Berberine extracts have been reported to possess hepatoprotective and anti-inflammatory actions [4, 5, 6]. This alkaloid has multiple therapeutic actions. The use of berberine has been described for almost all disorders of the body. The drug has been used in Indian and Chinese medicines for treatment of bacterial diarrhea, intestinal parasitic infections, and ocular trachoma infections. The root extract of the plant was used as a purgative and blood purifier. Besides its significant antimicrobial activity, berberine is also effective (200 mg/kg/day) in ameliorating diabetic nephropathy in rats [7, 8, 9]. Various clinical trials have established its therapeutic action in cardiovascular disorders, such as coronary artery disease, congestive heart failure, arrhythmia and hypertension [10, 11, 12, 13].

Literature survey reveals few HPLC methods for the estimation of berberine in plants [14, 15, 16, 17, 18] and in plasma [19] but these methods suffered from drawbacks like use of non-organic solvents, use of buffers which may deteriorate efficiency of column or HPLC

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instrument [14, 15, 16, 17, 18, 19] and time consuming [14, 15, 16, 18] for HPLC separation or less sensitive method.

Thus objective of current study was to develop a simple, rapid, precise and accurate HPLC method for routine analysis for quantification of berberine from *B. aristata*, *B. tinctoria* and formulation.

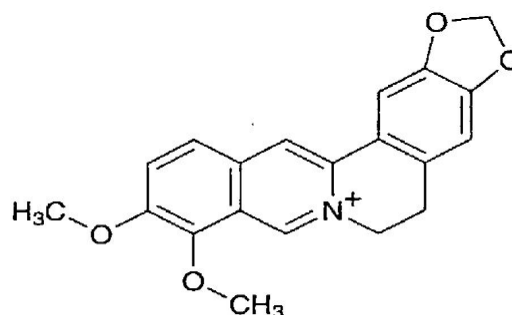
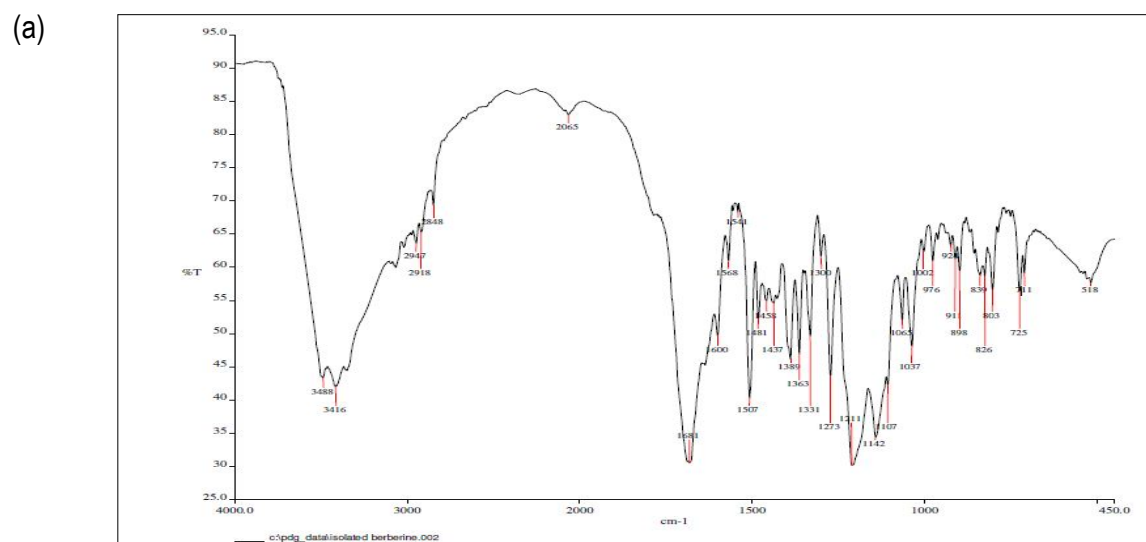


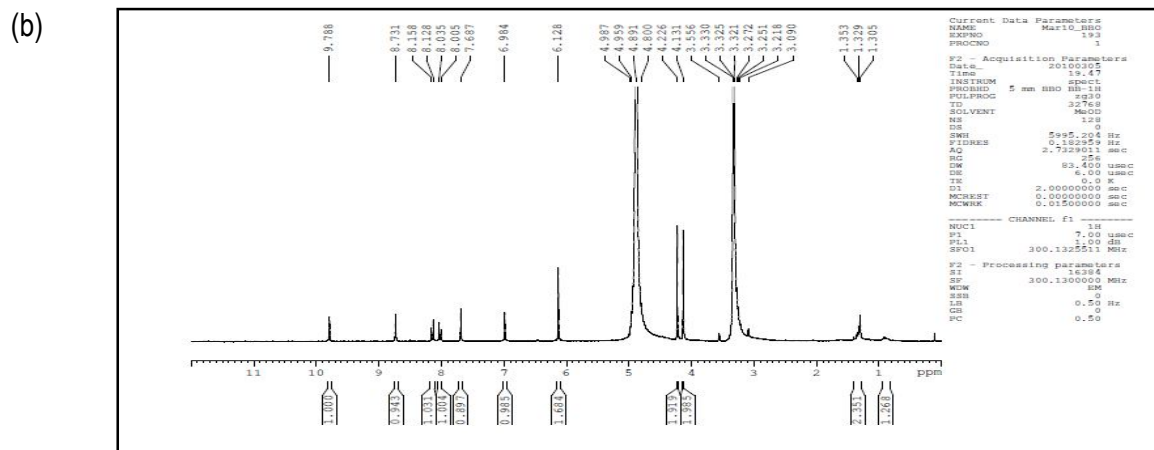
Figure 1. Structure of berberine

## 2. Materials and methods

Berberine was isolated using Semi-Preparative High Performance Liquid Chromatography. Purity and structure of isolated constituent (berberine) was confirmed by melting point, HPTLC, HPLC and spectral analysis like NMR, IR and MS (Figure 2). The purity of berberine was found to be more than 99.8% by HPLC and hence, was considered as working standard for the analysis purpose. A molecular ion peak can be seen at  $m/z$  335.8 ( $M+1$ ), that corresponds to the molecular weight of the compound 336.



Wavenumber (cm <sup>-1</sup> )	Group
2848	C-H Stretch
1681	C=C, C=N Stretch
1065	C-O Stretch



Sr No.	Position	ppm	Nature	Assignment	No. of protons
1	8	3.31	Triplet	-CH <sub>2</sub>	2
2	25	4.12	Singlet	-CH <sub>3</sub>	3
3	23	4.22	Singlet	-CH <sub>3</sub>	2
4	9	4.94	Triplet	-CH <sub>2</sub> -	2
5	2	6.17	Singlet	-CH <sub>2</sub> -	2
6	6	6.98	Singlet	-CH-	1
7	13	7.68	Singlet	-CH-	1
8	19	7.68	Doublet	-CH-	1
9	18	8.03	Doublet	-CH-	1
10	21	7.68, 8.72	Singlet	-CH-	1
11	14	9.79	Singlet	-CH-	1

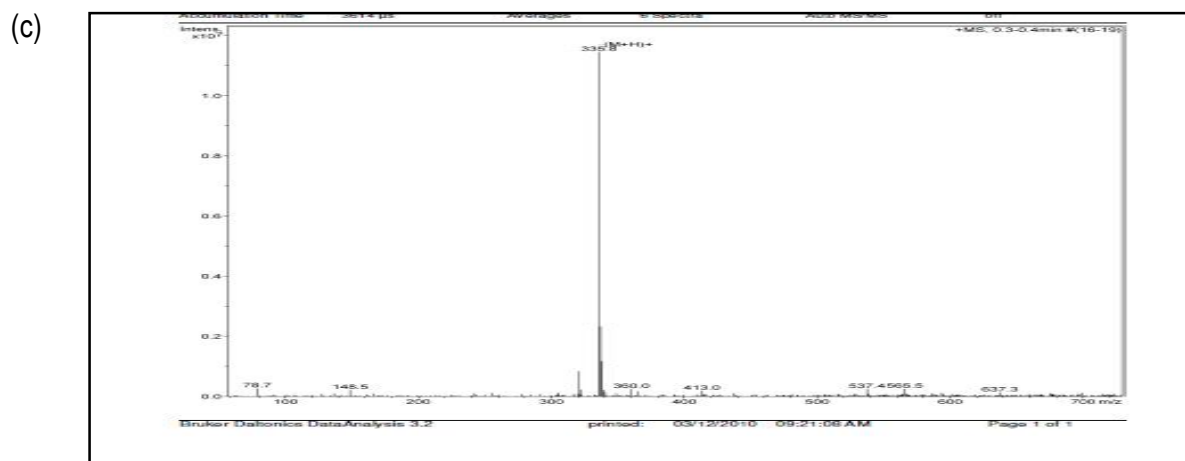


Figure 2. Spectral analysis of isolated berberine by: (a) IR; (b) NMR; (c) Mass

## 2.1. Chemicals

Acetonitrile and trifluoroacetic acid (TFA) of HPLC grade were procured from Merck (Darmstadt, Germany). Deionized water was obtained with an in-house Milli-Q Nanopure (Millipore, Bedford, MA, USA).

## 2.2. Collection and authentication of plant

Species of *Berberis* i.e. *B. aristata* and *B. tinctoria* were collected from local market of Mumbai and Ooty respectively. Both the plant species were authenticated at Piramal Life Sciences Ltd, Botany Department, Mumbai and the voucher specimens are deposited in herbarium. Collected plant material (stem bark) were dried under shade and grounded.

## 2.3. Extraction of plant material for analysis

Powdered stem bark of both species of berberis (800 g each) were extracted separately with 2.5 L methanol by stirring at 50°C for 3 hrs. The extraction was performed two times with the above mentioned protocol. The extract was obtained by drying the concentrated pooled extract under vacuum. Resulted extracts were used for estimation and comparison of Berberine content.

## 2.4. Equipment

The instrument used for the chromatographic separation was Dionex Ultimate 3000 equipped with a Ultimate 3000 pump, a Ultimate 3000 Autosampler, Ultimate 3000 Column compartment, Ultimate 3000 photo diode array detector (PDA) and column (Unisphere-C18, 5 $\mu$ m, 100A; 4.6 $\times$ 150 mm, Cat.No.U5515059-0, S/N: P9510515BJ0153 ). The absorption was measured in a full spectrum (200-400 nm) or at 350 nm for berberine. The chromatographic data was recorded and processed with chromeleon software.

## 2.5. Optimized chromatographic conditions

Separation was achieved on a Unisphere column (C<sub>18</sub>, 5  $\mu$ m, 100A; 4.6 $\times$ 150 mm, Cat.No.U5515059-0, S/N: P9510515BJ0153) at 30°C. The mobile phases consisted of 0.1 % TFA (A) and Acetonitrile (B) (in proportion of 60:40, v/v) was degassed before used. The flow rate was kept at 1.0 mL/min, temperature of column was set at 30 $\pm$ 2°C and the injection volume was 10  $\mu$ L. Quantification of Berberine was done at 350 nm. The peak in the HPLC chromatogram of *Berberis* species was tentatively identified by comparing the retention time and UV spectra of berberine in samples with working standard of berberine. The peak purity was checked by PDA software routines.

## 2.6. Standard solutions

Berberine working standard was accurately weighed and transferred to 5 mL volumetric flask and dissolved in methanol to obtain a solution concentration of 0.5 mg/mL. This solution was then further diluted to obtain the concentrations ranging from 0.2 to 150  $\mu$ g/mL. In the same way, three sets of control for Berberine were prepared from a separate stock, so as to lie in the lowest, middle and highest regions of the calibration curves. Further standard solution was prepared freshly each day by appropriate dilution of stock solution with methanol for intraday as well as interday analysis.

## 2.7. Test sample preparation

Plant extracts (100 mg) were exactly weighed into 100 mL volumetric flask fitted with a glass

stopper (Borosil cat. # 14-962-26F) and volume is made by methanol and extracted for 5 mins using a sonicator and allowed to stand for 5 mins. The mixture was then filtered through Whatmann no.42 filter paper and the desired concentration (0.1 mg/mL) is obtained. Then 10  $\mu$ L of the resulting solution was subjected to HPLC analysis and the concentration of the major constituent berberine, in two different *Berberis* extracts were calculated based on the equations for the calibration curves.

## 2.8. Linearity and range

For a long-term use of the analytical method a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve the stock solution was freshly diluted with methanol to obtain a set of 6 calibration standards. These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the injected standards. The complete procedure was repeated on three consecutive days. The so obtained three calibration curves were used to calculate a mean calibration graph.

## 2.9. Precision studies for berberine

Method precision (Intraday study) was determined by analyzing the berberine extract at the target concentration (100  $\mu$ g/mL) in six replicates preparations.

Intermediate precision (Interday precision) was carried out as described in intraday precision. A different analyst carried out the analysis on a different day, using a different HPLC (Agilent) and different Lot number of column.

## 2.10. Stability

Berberine extract (10 mg) was transferred into a 10 mL volumetric flask and diluted with methanol. The stock solution (1 mL) was diluted to 10 mL with methanol. The resulting sample solution were subjected to 30°C and analyzed on 16 and 24 hrs to observe the stability of sample solutions.

## 2.11. Robustness

Robustness was applied by making small deliberate changes in method parameters (mobile phase composition, column temperature, different lot of stationary phase, analyst and equipment) to validate the method.

# 3. Results and discussion

## 3.1. Chromatography

Under optimized conditions, Berberine along with other phytoconstituents from *Berberis* extract were eluted within 5 minutes. Figure 3 shows the typical LC chromatogram of working standard of berberine along with UV spectrum. Figure 4 shows chromatograms of berberine extracts of *B. aristata* and *B. tinctoria* at 350 nm respectively.

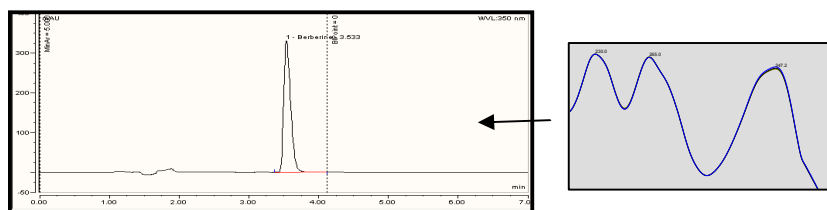


Figure 3. LC chromatogram of working standard berberine along with its UV spectrum

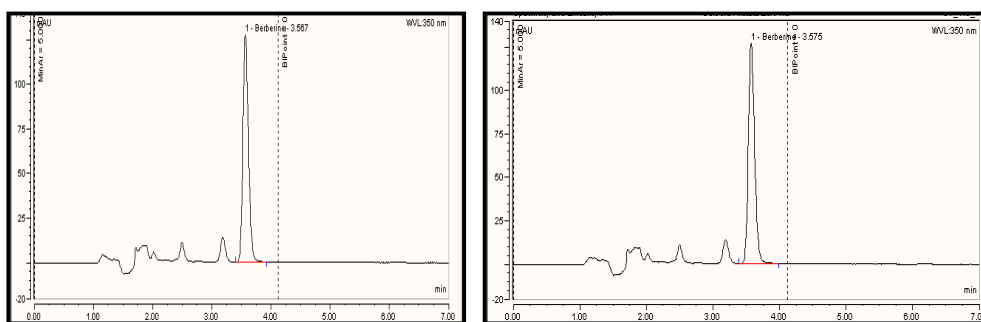


Figure 4. LC chromatograms of *B. aristata* and *B. tinctoria* extract

### 3.2. Limit of detection and limit of quantitation

The limit of detection (LOD) was determined by successively decreasing the concentration of berberine as long as a signal-to-noise ratio of 3:1 appeared. The LOD was found to be 1 ng on column (volume of injection is 10  $\mu$ L; corresponding to a concentration of 0.1  $\mu$ g / mL). The limit of quantitation (LOQ) was found to be 2 ng on column (volume of injection is 10  $\mu$ L; corresponding to a concentration of 0.2  $\mu$ g/mL) of berberine.

### 3.3. Linearity

The calibration was based on the analysis of working solutions at six concentration levels on 3 consecutive days for berberine standard (0.2-150  $\mu$ g/mL) with regression ( $R^2$ ) 0.9982 as shown in Figure 5.

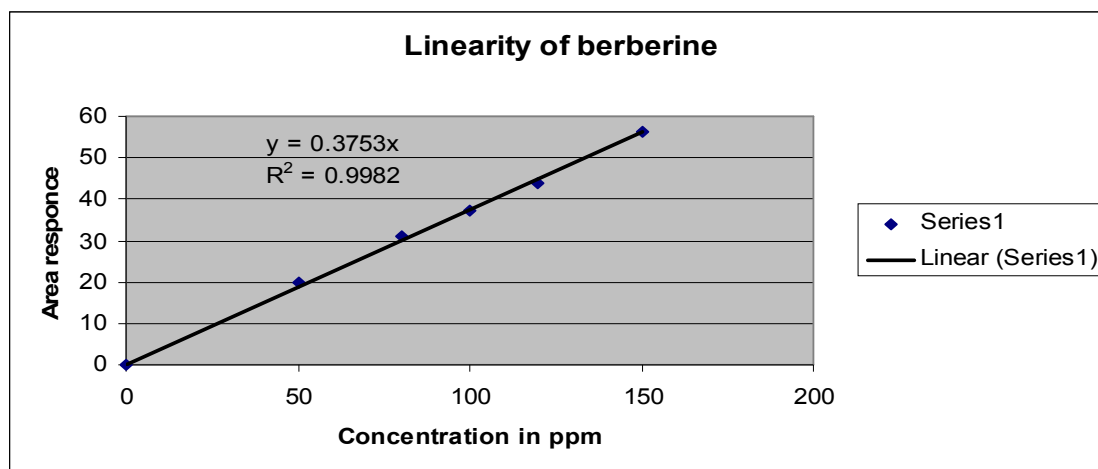


Figure 5. Calibration curve of berberine

### 3.4. Intraday and interday analysis for berberine

Furthermore the intraday and interday precisions were investigated by analyzing a target concentration in six replicates preparations of berberine extract.

**Table 1.** Precision and accuracy data for berberine

Concentration	Intraday		Interday	
	Precision (%R.S.D)	Accuracy (%)	Precision (%R.S.D)	Accuracy(%)
100 µg/mL	1.6223	99.3859	1.7320	99.5510

### 3.5. Stability

Analysis of stability samples in methanol on regular interval (16 and 24 hrs) revealed that the berberine, a major constituents in the extract of berberis species was stable in solution form with relative standard deviation (RSD, %) 1.47 (n = 3) for berberine at 30°C respectively.

**Table 2.** Stability data for berberine

Hours	Area of 100 µg/mL
0	36.66
	36.56
16	37.20
	37.59
24	37.86
	37.69
<b>Mean</b>	<b>37.26</b>
<b>S.D. (+/-)</b>	<b>0.5490</b>
<b>RSD (%)</b>	<b>1.47</b>

### 3.6. Robustness

The method was found to be re-producible from one analyst to another. The low values of R.S.D. obtained after small deliberate changes of the conditions (mobile phase composition, column temperature, different lot of stationary phase, analyst and equipment) used for the method indicated its robustness.

### 3.7. Sample analysis

Two sets of samples were analyzed for berberine in *B. aristata* and *B.s tinctoria*, according to the method described above. The average content of berberine in *B. aristata* and *B. tinctoria* was found be 3.18% (w/w) and 1.46% (w/w) on dry basis respectively.

#### 4. Conclusion

The study shows that the developed HPLC method is precise, specific and accurate for quantitation of berberine from *B. aristata* and *B. tinctoria* extracts using a photodiode array detector. The method is fully validated with satisfactory results. The method was successfully applied for routine analysis because of its simplicity and reproducibility.

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