



Validation of an HPLC-RI method for the quantification of D-pinitol from *Mesembryanthemum crystallinum*

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Abstract A high-performance liquid chromatography with refractive index detection (HPLC-RI) method was developed and validated to quantify D-pinitol in *Mesembryanthemum crystallinum*. Effective separation was achieved with a retention time of 8.7 min. The proposed method demonstrated excellent linearity ($r^2 = 0.9999$) over a 0.125–2 mg/mL range, with a calibration curve equation of $Y = 102.37X - 181.97$. The limits of detection and quantification were 19.2 and 58.2 $\mu\text{g/mL}$, respectively. Accuracy, assessed by recovery tests at three spiked concentrations, showed recovery rates between 103.24 and 112.69% with relative standard deviations (RSDs) ranging from 0.84 to 4.19%. Precision analyses revealed intra- and inter-day RSDs of 0.77 and 1.26%, respectively, at a concentration of 20.0 mg/mL. This study presents the first validated HPLC-RI method for D-pinitol quantification in *M. crystallinum*, providing a reliable and cost-effective analytical tool that fills a gap in existing research, as well as a methodological basis for future pharmacological and phytochemical studies.

Keywords High-performance liquid chromatography · *Mesembryanthemum crystallinum* · Method validation · D-Pinitol · Refractive index detector

Introduction

The ice plant (*Mesembryanthemum crystallinum*) is an annual plant belonging to the Aizoaceae family, which is native to the Namib Desert in southern Africa and cultivated in arid regions such as Western Australia, the southwestern United States, and the Pacific coasts of Mexico and Chile [1,2]. This plant derives its name from the salt crystals that form on its bladder cells, which resemble ice crystals. These bladder cells, located on the surface of the stems and leaves, contain beneficial compounds such as inositol and β -carotene, as well as various minerals. Traditionally consumed in Africa to replenish water and salt and to prevent dehydration, its salty, savory flavor has made it a popular functional vegetable in Europe and Japan, where it has been added to a variety of foods for decades [3]. The ice plant possesses a wide range of bioactive properties, including antioxidant, hypoglycemic, and antimicrobial effects [4,5]. Its extracts have been reported to ameliorate hyperglycemia and exhibit antihypertensive, hypoglycemic, hypolipidemic, nootropic, and memory-enhancing activities [6–8]. Several studies have demonstrated that the bladder cells of this plant contain polyols such as D-pinitol, that have blood sugar level-lowering properties [9–11]. D-Pinitol, extracted from various plants, has been the focus of many scientific investigations on its multifunctional properties and its potential as a therapeutic agent. The specificity of D-pinitol is said to be responsible for its various bioactivities [12]. Numerous research papers show that it effectively mimics insulin's properties, which makes it useful for increasing glucose transport and making the tissue more insulin-sensitive [13,14].

Given the increasing recognition of *M. crystallinum*'s benefits, its cultivation has significantly increased in Korea. However, despite its potential to treat diabetes, hypertension, and obesity by preventing fat production and increasing lipolysis, very few studies have characterized the potential therapeutic applications of the ice plant [15]. Given its potential health benefits, further research on this highly functional vegetable is needed. Despite

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promising evidence for D-pinitol as a therapeutic agent, there are no studies on the method validation of D-pinitol in *M. crystallinum* by high-performance liquid chromatography with refractive index detection (HPLC-RI) method, which only confirms the necessity of new research to clear the question of the precise reaction of D-pinitol and its long-term effectiveness.

Previous studies employed gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the D-pinitol content of pharmaceutical substances and their products, including carob syrup, *Desmodium adscendens*, and *Ceratonia siliqua* L. (carob tree) [16–18]. These techniques have also been used for analytical method validation. However, what makes the RI detector in HPLC more accessible in validating methods lies in its universal detection capability, allowing analysis of non-ultraviolet (UV) absorbing compounds like sugars and lipids, which are invisible to standard UV detectors, making it a common choice for method validation studies [19,20]. In the field of functional food development, method validation standards are crucial [21]. Accurate assessment of bioactive compounds is essential to ensure the efficacy, safety, and overall quality of these products [22,23]. Method validation serves as the central checkpoint to ensure that analytical procedures fit their designated purpose and provide consistent, reproducible results [24,25]. This validation process includes critical parameters such as accuracy, precision, specificity, sensitivity, and reproducibility, all of which are essential in establishing the reliability of the employed analytical methods [26].

Although several studies have reported method validations for GC-MS, LC-MS/MS, and HPLC-RI for D-pinitol [27–30], no studies have focused on method validation for *M. crystallinum*. Therefore, this study sought to fill this gap by presenting a validated HPLC-RI method specifically for *M. crystallinum*. This provides a reliable analytical approach for the quantification and study of D-pinitol in this species, facilitating further research and potential applications in the pharmaceutical and nutritional fields.

Materials and Methods

Plant Materials

Ice plants (*M. crystallinum*) were acquired from Hyundai Food & Beverage Co., Ltd., Gwangju, Korea. The ice plants used in this experiment were grown in March 2024 in Goryeong, Korea. All experiments were conducted using dried *M. crystallinum* (DMC). A voucher specimen was deposited at Department of Plant Science and Technology, Chung-Ang University, Anseong, Korea.

Instruments and Reagents

A Waters Alliance 2695 system (Milford, MA, USA) equipped with a pump, autosampler, and Waters 410 RI detector was used for the analysis. HPLC-grade acetonitrile (ACN) and water were purchased from Honeywell (Burdick and Jackson, Muskegon, MI,

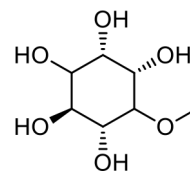


Fig. 1 Chemical structure of D-pinitol

USA) and Scharlau (Barcelona, Spain), respectively. HPLC-grade trifluoroacetic acid (TFA) was provided by Thermo Scientific (Waltham, MA, USA). D-pinitol was supplied by the Natural Product Institute of Science and Technology (www.nist.re.kr) (Fig. 1).

Preparation of Samples and Standard Solutions for HPLC-RI

The DMC (10 g) was extracted with ethanol (300 mL) under reflux at 80–85 °C for 3 h and then evaporated *in vacuo*. The resulting extract was dissolved in a solvent mixture of water and ACN (3:7) at a concentration of 20 mg/mL and filtered using a polyvinylidene fluoride 0.20 µm syringe filter. To create the standard calibration curve, a stock solution of D-pinitol was prepared by dissolving it in the solvent mixture (water and ACN at a 3:7 ratio). Working solutions were then prepared by diluting the stock solution to concentrations ranging from 0.125 to 2.0 mg/mL.

HPLC-RI Conditions

The HPLC-RI analysis was performed on a reversed-phase HPLC system equipped with an RI detector using a YMC-Triart Diol-HILIC column (4.6×250 mm, 5 µm, Kyoto, Japan). Analyses were performed at a flow rate of 0.8 mL/min in an isocratic elution system at a column oven temperature of 50 °C. The injection volume was 10 µL. Detection was performed with the RI detector set at an internal temperature of 50 °C. The mobile phase consisted of 0.1% TFA in water and 0.1% TFA in ACN (10:90) over 30 min.

Method Validation

The newly proposed HPLC-RI method was rigorously validated for specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and linearity, following the guidelines set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (Guideline IHT, 2015). To assess specificity, evaluations were conducted with previous research methods to identify any potential interference in the retention times and RI chromatogram of spike test between the D-pinitol standard and DMC samples, ensuring that the peaks corresponded to the same compound [31]. Accuracy was evaluated through recovery tests, where sample extracts were spiked with three different standard mixtures at varying concentrations, and the percentage recovery of the compound in the extract was calculated. Precision validation involved analyzing one sample

extract concentration for intra-day (repeatability) and inter-day (intermediate precision) assessments, each performed five times. Intra- and inter-day precision were measured by calculating the standards' relative standard deviation (RSD; %) on the same day and two different days, respectively. Linearity was assessed using five concentrations of the standard solution (ranging from 0.125 to 2.0 mg/mL) and performing three injections for each concentration. A calibration curve was constructed by plotting the known concentrations of the standard solutions against the peak areas. Linearity was confirmed through linear least-squares regression analysis, with the correlation coefficient (r^2) reflecting the degree of linearity. After fitting the calibration curve, the LOD (the lowest detectable amount or concentration of the analyte) and LOQ (the minimum quantifiable amount of the analyte with acceptable repeatability and accuracy) were calculated based on the standard deviation values of the intercept (σ) and the slope (S) using the following formulas: $\text{LOD} = 3.3 (\sigma/S)$ and $\text{LOQ} = 10 (\sigma/S)$.

Calibration Curve

The concentrations of the standard solution were plotted against the corresponding peak areas to create a calibration curve. The correlation coefficient (r^2) was used to establish the linearity of the calibration curve, which was then used to calculate the standard concentrations in the samples. Calibration functions were

established according to the peak area (Y), concentration (X , $\mu\text{g/mL}$), and mean $\pm$ standard deviation ($n = 3$).

Results and Discussion

The results of the development and validation of the proposed HPLC-RI method for D-pinitol quantification are illustrated in Fig. 2. Specificity of the method was confirmed in the chromatograms of pinitol standard, DMC samples, and spiked DMC samples (Fig. 2). Effective separation was achieved with a retention time of 8.7 min throughout the 30-min analytical procedure. The high accuracy of the validated method is demonstrated by the correct peak resolution of D-pinitol and the absence of interference in retention time detection. The presence of D-pinitol in DMC was confirmed via the spike test, which accurately detected the presence of this compound, and the chromatogram in Figure 2 shows a clear separation. These results indicate that the HPLC-RI method was successfully validated for the specific analysis of D-pinitol in DMC.

A calibration curve with a concentration range of 0.125–2 mg/mL of D-pinitol was constructed (Table 1). The equation of the calibration curve was determined to be $Y = 102.37X - 181.97$, where Y represents the peak area and X represents the concentration of the standard ($\mu\text{g/mL}$). The correlation coefficient for the five

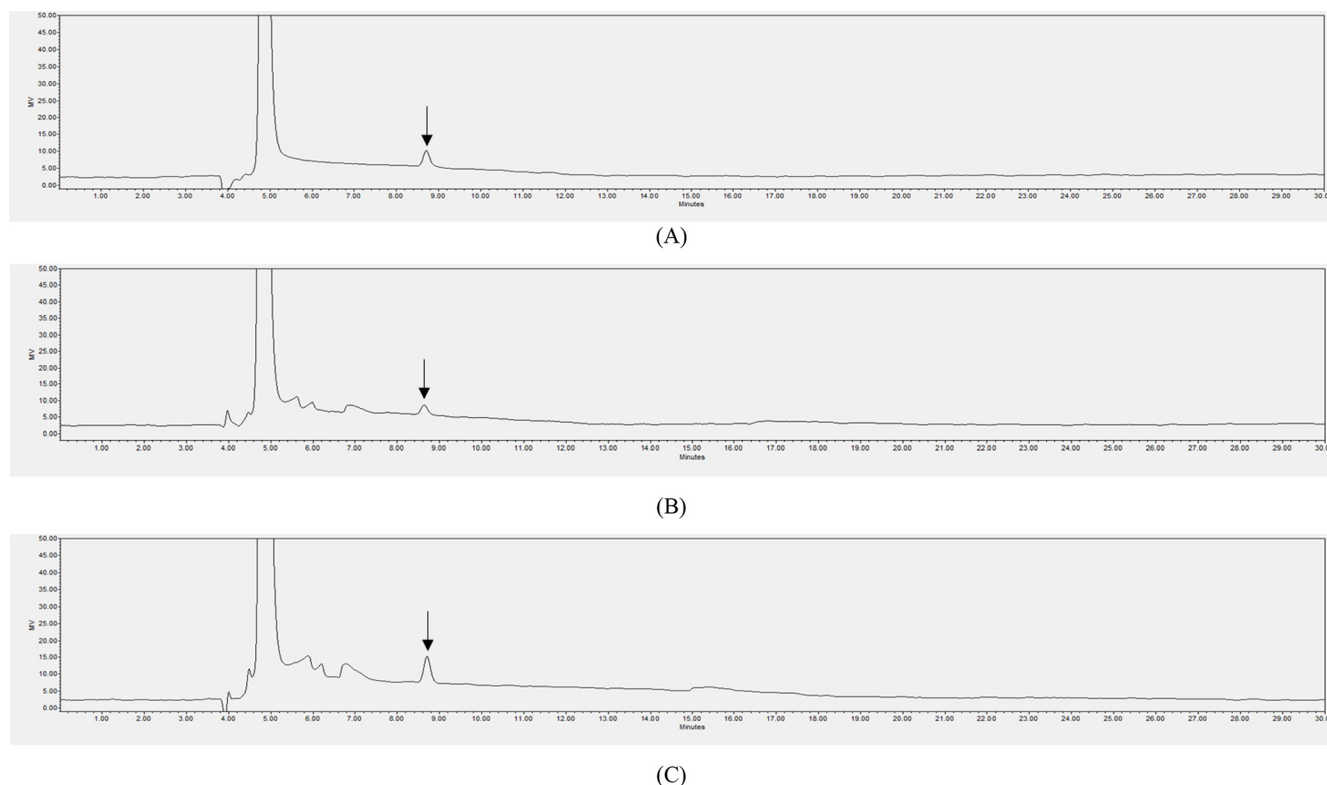


Fig. 2 HPLC-RI chromatograms and specificity of D-pinitol standard (A), D-pinitol in DMC (B), and spike test (C)

Table 1 Linearity and related statistics for the calibration curve data of D-pinitol

Compound	t_R	Range (mg/mL)	Calibration equation	r^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
D-pinitol	8.7	0.125-2	$Y = 102.37X - 181.97$	0.9999	19.2	58.2

t_R = retention time

Y = peak area, X = concentration of the standard ($\mu\text{g/mL}$)

r^2 = correlation coefficient for the five data points in the calibration curve ($n = 3$)

Table 2 Accuracy analysis statistics for the determination of D-pinitol

Compound	Spike amount (mg/mL)		Recovery (%)			Average (%)	RSD (%)
	Sample	D-pinitol	1 st	2 nd	3 rd		
D-pinitol	20.0	0.25	111.70	112.69	110.58	111.70	0.95
		0.5	103.24	110.32	111.71	108.42	4.19
		1.0	109.33	110.87	111.00	110.40	0.84

data points in the calibration curve was determined to be $r^2 = 0.9999$. The LOD and LOQ were calculated as 19.2 $\mu\text{g/mL}$ and 58.2 $\mu\text{g/mL}$, respectively. The accuracy analysis for the determination of D-pinitol involved spiking known amounts of the compound into samples. The recovery percentages for different spike amounts (0.25, 0.5, and 1.0 mg/mL) were then determined. The mean recovery percentages for D-pinitol ranged from 103.24 to 112.69%, with RSD values ranging from 0.84 to 4.19% (Table 2). Both intra- and inter-day precision were evaluated for the determination of D-pinitol, and D-pinitol contents were measured at a concentration of 20.0 mg/mL. Intra-day precision, determined by analyzing the samples five times within a single day, yielded an average content of 49.61 mg/g with an RSD of 0.77%. Inter-day precision, determined by analyzing the samples on five consecutive days, resulted in an average content of 49.96 mg/g with an RSD of 1.26% (Table 3).

This research constitutes the first documented validation of a method for detecting D-pinitol specifically in *M. crystallinum*. The

validation parameters, including specificity, linearity, accuracy, precision, LOD, and LOQ, demonstrated that the method is reliable and reproducible. Although previous studies have validated methods for D-pinitol using advanced techniques such as GC-MS and LC-MS/MS [32-35], these validations were conducted in other plant species and were not specific to DMC. This highlights the gap addressed by this study, which focuses specifically on the detection and quantification of D-pinitol in DMC. Additionally, unlike UV/Vis detectors, RI detectors can detect compounds that are not UV absorbers, such as D-pinitol [19,20]. The successful validation of the HPLC-RI method for D-pinitol in DMC has significant implications for both research and industrial applications. Accurate quantification of D-pinitol, which possesses promising health-promoting properties, is now achievable. This advancement will facilitate further pharmacological and phytochemical studies on this compound. Moreover, the method can be incorporated into quality control processes for DMC-derived products, ensuring their consistency and efficacy. This study also sets a precedent for the validation of analytical methods for other understudied phytochemicals in DMC and related species. The methodological framework and validation protocols established herein can serve as a reference for future analytical developments in this field.

In summary, this study introduced and validated an analytical method based on HPLC-RI for the quantification of D-pinitol in DMC. As detailed in the Results section, this method demonstrated efficient and effective analytical capabilities, showing satisfactory accuracy and precision for routine analytical use. D-Pinitol has potential applications in various fields such as the pharmaceutical, cosmetic, and food industries. The use of plant-derived bioactive compounds, including D-pinitol, can provide a variety of health and beauty benefits with a high level of safety. Therefore, developing a method for the quantification of D-pinitol as a marker compound in DMC is of great importance, and this study confirms the reliability of the HPLC-RI method for this purpose.

Table 3 Intra- and inter-day precision for the determination of D-pinitol

Sample concentration (mg/mL)	D-pinitol content (mg/g)	
	Intra-day (n = 5)	Inter-day (n = 5)
20.0	50.00	50.65
	49.83	49.41
	49.80	50.27
	49.12	50.30
	49.27	49.19
Average content (mg/g)	49.61	49.96
Standard deviation	0.384	0.63
RSD (%)	0.77	1.26
Average content (mg/g)	49.78	
Standard deviation	0.25	
RSD (%)	0.51	

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