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Improved High-Performance Liquid Chromatography (HPLC) Method for Qualitative and Quantitative Analysis of Allantoin in *Zea mays*

GHASEM HAGHI,* ROHOLLAH ARSHI, AND ALIREZA SAFAEI

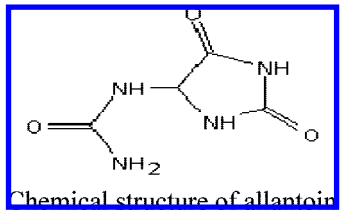
Research Center of Barij Essence Pharmaceutical Company, 87135-1178 Kashan, Iran

A high-performance liquid chromatography (HPLC) method for the qualitative and quantitative analysis of allantoin in silk and seed of *Zea mays* has been developed. Allantoin separation in crude extract was achieved using a C_{18} column and phosphate buffer solution (pH 3.0) as a mobile phase at ambient temperature at a flow rate of 1.0 mL/min and detected at 210 nm. The results showed that the amount of allantoin in samples was between 14 and 271 mg/100 g of dry plant material. A comprehensive validation of the method including sensitivity, linearity, repeatability, and recovery was conducted. The calibration curve was linear over the range of 0.2–200 μg/mL with a correlation coefficient of $r^2 > 0.999$. Limit of detection (LOD, S/N = 3) and limit of quantification (LOQ) values of the allantoin were 0.05 and 0.2 μg/mL (1.0 and 4.0 ng) respectively. The relative standard deviation (RSD) value of the repeatability was reported within 1.2%. The average recovery of allantoin added to samples was 100.6% with RSD of 1.5%.

KEYWORDS: ***Zea mays*; allantoin; HPLC; corn silk**

INTRODUCTION

The corn silk (*Zea mays*, Poaceae or Gramineae family) is used in the treatment of urinary troubles, such as cystitis, urethritis, enuresis, and prostatitis, especifically for acute or chronic inflammation of the urinary system. Corn silk is listed as a natural source of food flavoring, and its oil and flour are commonly used in cooking. In the U.S.A., corn silk is listed as generally regarded as safe (GRAS). The seed is classified as category N1, with no restriction on its use (*1*). Extracts are used as flavor components in major food products, such as alcoholic and nonalcoholic beverages, frozen dairy desserts, candy, and baked goods (*2*). Allantoin [(2,5-dioxo-4-imidazolidinyl)-urea] a product of purine metabolism is prepared synthetically by the oxidation of uric acid with alkaline potassium permanganate (*3*) and exists in corn silk (*1, 4*). The structure of allantoin is presented below.



Chemical structure of allantoin

Allantoin is an astringent and keratolytic. It is frequently used topically as a vulnerary to stimulate tissue repair in suppurating wounds, resistant ulcers, acne, seborrhea, cold sores, psoriasis, hemorrhoid, and other anorectal disorders (*5, 6*). Accomplishing satisfactory separation of this compound with similar polar constituents in biological samples by high-performance liquid chromatography (HPLC) is difficult because of overlapping of peaks (*7–11*). Further improvement in the simultaneous identification of allantoin, uric acid and creatinine in cattle urine was obtained by HPLC (*12*). We intended to find a HPLC method for quantification of allantoin in corn silk. A literature search revealed allantoin analysis only in the silk of 15 *Z. mays* hybrids by reversed-phase (RP)-HPLC (*13*). We observed basic problems in the reproducibility of this methodology. Therefore, the purpose of this study was to improve an analytical method for quantification of allantoin in corn silk using RP-HPLC. Herein, we report a simple and accurate method for the determination of the allantoin in silk and seed of *Z. mays*. In this paper, a RP-HPLC method with a comprehensive validation protocol for the qualitative and quantitative analysis of allantoin in corn silk is presented.

MATERIALS AND METHODS

Solvent and Chemicals. Methanol, acetone, potassium dihydrogen phosphate (all from Merck), and allantoin (Sigma) with purity ≥98% were used.

Plant Material. Silk of two *Z. mays* samples and their seeds were gathered from the Hoseinabad and Saifiabad of Kashan, Iran, in 2006. After collection, plant samples were immediately dried in a shaded and ventilated place at room temperature for 36 h.

Standard Solution. The chromatographic purity of allantoin was simultaneously checked at multiple wavelengths by means of RP-HPLC, UV spectroscopy, and other physical properties. The stock standard

* To whom correspondence should be addressed. Telephone: +98-866436-2112. Fax: +98-866436-2187. E-mail: g.haghi@barijessence.com.

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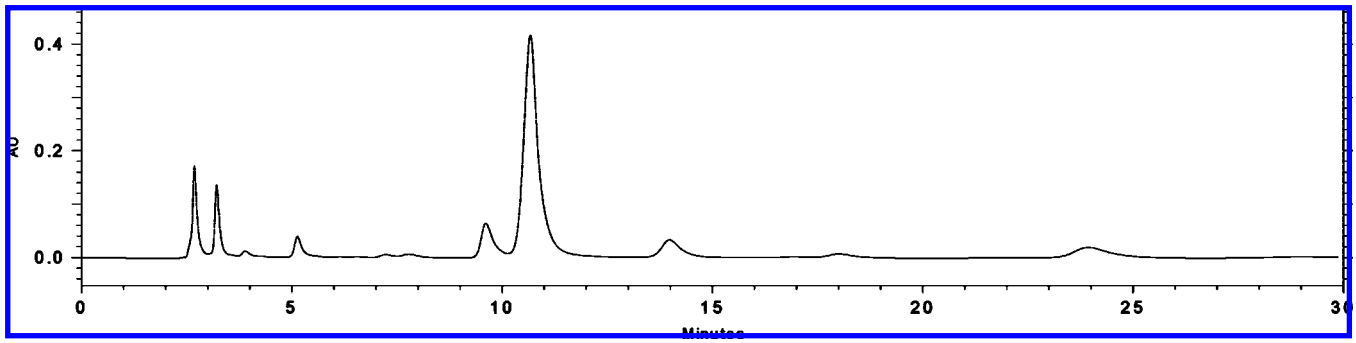

Figure 1. Chromatogram of *Z. mays* silk extract analyzed following the current method on an Eurospher column. Allantoin is at 3.2 min.

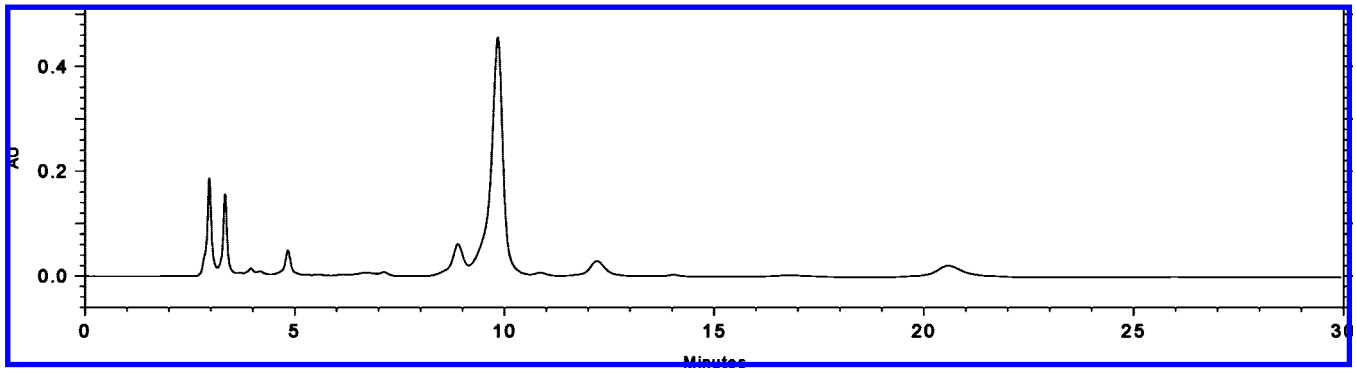

Figure 2. Chromatogram of *Z. mays* silk extract analyzed following the current method on a Nucleosil column. The retention time of allantoin peak is at 3.3 min.

solution (1.0 mg/mL) of allantoin was freshly prepared in water. A series of working standards (0.2–200 μg/mL) were prepared with water before injection. A 20 μL volume of each concentration was injected 3 times into the HPLC system for preparation of the calibration curve.

Sample Preparation. A 0.25 g amount of powdered silk or a 1 g amount of pulverized seed was extracted with 25 mL of methanol using a mixer vessel at ambient temperature for 30 min. The solution was vacuum-filtered through a glass filter covered with a paper filter (4.4 μm pore size). The residue was rinsed with 10 mL of methanol and added to a previous solution. The filtrate obtained was evaporated to dryness under vacuum. The dried extract was dissolved in 10 mL of water. The solution was centrifuged and filtered through a Chromafil filter (0.45 μm pore size). A 20 μL volume of this solution was injected 3 times into the HPLC system.

Chromatographic Conditions. The HPLC of allantoin in corn silk was achieved both by the earlier method (*13*) and our new procedure (current method).

Separation by the current method was performed for acquiring HPLC chromatogram and UV spectra using a Knauer instrument (WellChrom, pump K-1001, fast scanning UV detector K-2600, analytical degasser K-5004, Injector 2301 with a 20 μL loop) at ambient temperature, flow rate of 1 mL/min, and run time of 30 min. The mobile phase was prepared by dissolving 3.40 g of potassium dihydrogen phosphate in 900 mL of deionized water, adjusted to pH 3.0 with 10% orthophosphoric acid and diluted to 1000 mL with water (25 mM KH_2PO_4 solution). The buffer solution was filtered through a Chromafil filter (0.20 μm pore size). The column effluent was simultaneously monitored at four wavelengths between 210 and 235 nm and detection-affected at 210 nm.

Columns. The analytical columns tested were Nucleosil 100 C_{18} (25 × 4.6 mm i.d., 5 μm particle size), Eurospher 100 C_{18} (25 × 4.6 mm i.d., 5 μm particle size), and Grace Econosil C_{18} (previous Alltech; 25 × 4.6 mm i.d., 5 μm) without a precolumn.

Method Validation. Linearity test solutions for the assay method prepared from stock solution at seven concentration levels, from 0.2 to 200 μg/mL of analyte. The linearity was evaluated by linear regression analysis, which was calculated by the least-squares regression method. Recovery is reported as the percent salvage in relation to the known amount of analyte added to the sample. Recovery of the assay method was evaluated in triplicate at four different mass values (250–1000 μg) of allantoin using silk N1 in the intraday. Relative standard deviation was calculated.

RESULTS AND DISCUSSION

When attempting to duplicate the work of Maksimovic et al. in our laboratory, we observed only the peak of acetone. The allantoin peak was not detected in both the extract and standard solutions.

To optimize the chromatographic conditions of allantoin identification and to provide an acceptable separation the following parameters were surveyed: mobile phase, detection wavelength, column type, extraction solvent. Initially, for identification and separation of allantoin in silk extract, several solvents, such as water and water–methanol mixture with different ratios, were considered as the mobile phase under isocratic and gradient elution. Under these conditions, the peaks and retention times of silk extract were variable and overlapping. For separation of the peaks of analyte and other interfering compounds in the extract, water was used as the mobile phase, with pH in the range of 2.5–7.0 adjusted with orthophosphoric acid. A clear and good separation of the peaks was provided with phosphate buffer at pH 3.0. Our study confirmed that the peak shape and retention time of allantoin is pH-independent (*12*) but pH altered retention times of the other unknown compounds. Unknown peaks and their chemical structures will be elucidated in a future paper. Many nitrogen-containing compounds, such as basic drugs, are protonated at acidic or neutral pH and exhibit poor retention on a C_{18} column. For acidic analytes, it is exactly in inverse proportion that maximum retention can be attained at low pH. Allantoin is a neutral molecule, and its retention time is unaffected by the pH of the mobile phase.

To obtain better sensitivity and detection, silk extract in an effluent was simultaneously monitored at four wavelengths in

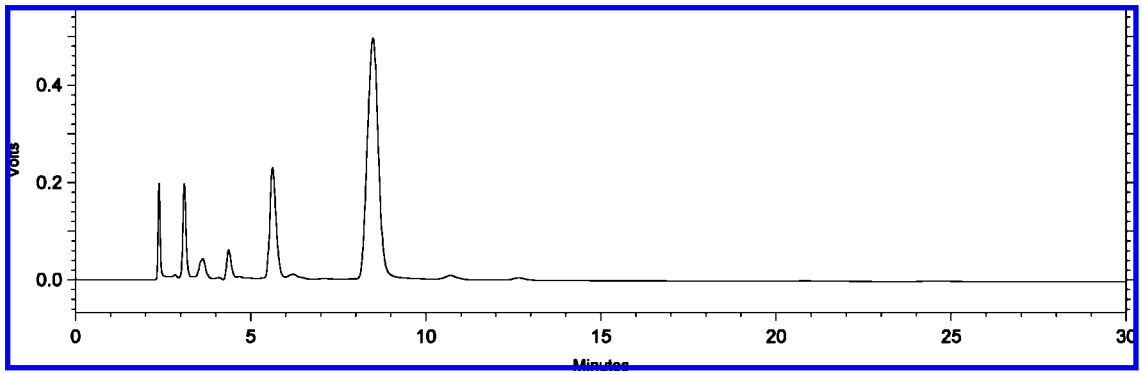

Figure 3. Chromatogram of *Z. mays* silk extract analyzed following the current method on an Econosil column. The retention time of allantoin peak is at 3.1 min.

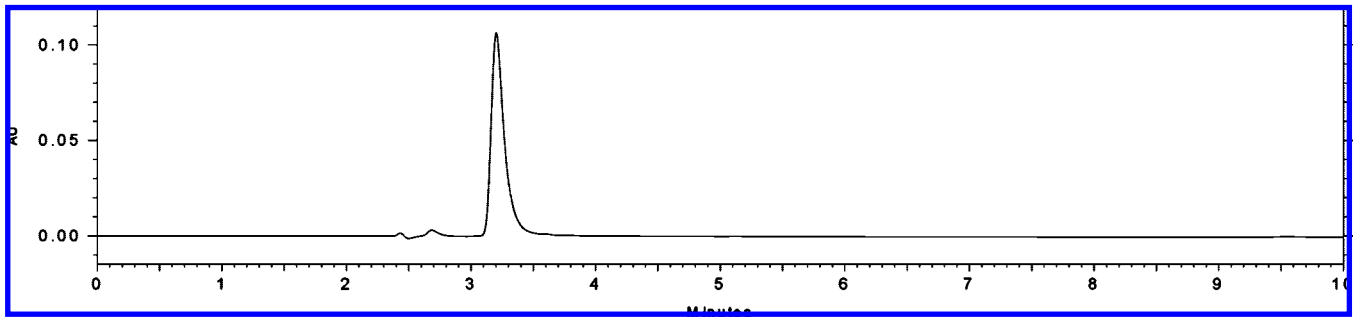

Figure 4. Chromatogram of allantoin standard (50 μg/mL) analyzed following the current method on an Eurospher column at 210 nm. The retention time allantoin peak is at 3.2 min.

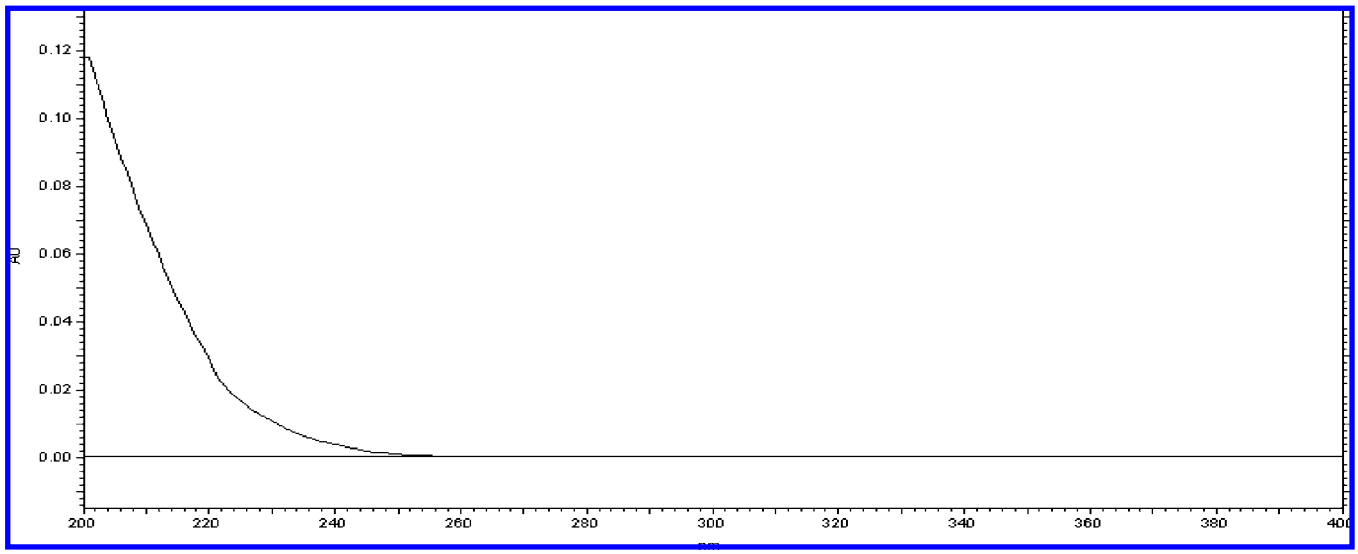

Figure 5. UV spectrum of allantoin standard analyzed following the current method on a fast scanning UV detector k-2600 in the chromatogram of **Figure 4**.

a UV range from 210 to 235 nm, and it was found that 210 nm well represents the profile of the peaks. Analyte sensitivity at this wavelength was 10 times higher than 235 nm. For a satisfactory separation and decreased run time, available stationary phases of C_{18} were examined. The extract was separately analyzed on columns of Eurospher, Nucleosil, and Econosil. HPLC chromatograms of the extract obtained on three columns were compared to each other (**Figures 1**−**3**). The best separation was observed using Econosil column, and its run time was less than 15 min (**Figure 3**). **Figures 4** and **5** represent the HPLC chromatogram and UV spectrum of allantoin standard. The UV spectrum of allantoin in the extract on detector K-2600 is shown in **Figure 6**, which is identical to the UV spectrum of allantoin standard in **Figure 5**.

Finally, efficiency of extraction was determined by extracting the silk with different solvents, such as water, methanol–water mixtures, and methanol, and compared as an extracting solvent. Persistent turbidity was observed in the sample extracted with water that hardly filtered presumably because of the presence of mucilaginous constituents in the extract, while the methanolic extract was clear and stable. Methanol gave a quite acceptable extraction of the analyte. Therefore, methanol was chosen as the solvent for sample preparation. For the determination of the sample/solvent ratio, a 0.25 g sample was extracted with different amounts of methanol and 25 mL was selected as the proper amount (1:100 sample/solvent ratio). Our study revealed that the amount of allantoin in the two kinds of samples in *Z. mays* (seed and silk) covered a wide range from 14 to 271 mg/

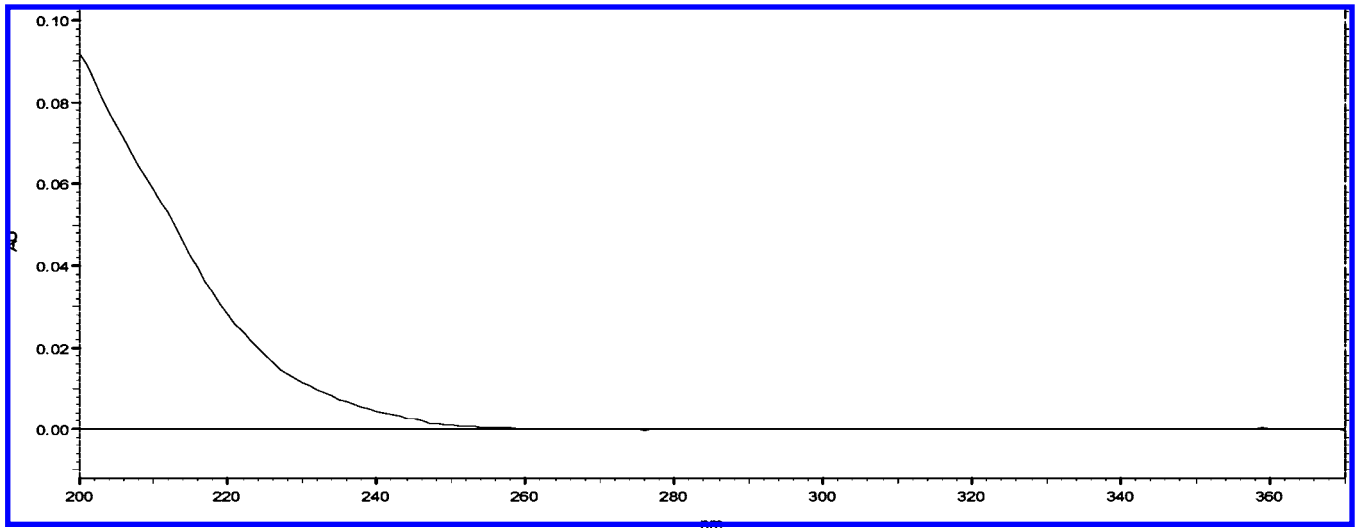

Figure 6. UV spectrum of allantoin in extract analyzed following the current method on a fast scanning UV detector k-2600 in the chromatograms of **Figures 1−3**.

Table 1. Determination of Allantoin/100 g of Dry Material in This Study

sample name		allantoin (mg)
silk	N1	271
silk	N2	123
seed	N1	24
seed	N2	14

Table 2. Repeatability of Method Including Extraction, Evaporation, and Filtration Process of Four Samples of 250 mg of Silk N1 ($n = 3$)

	weight (μg)					standard	relative standard
compound	1	2	3	4	average	deviation (μg)	deviation (%)
allantoin	665	683	677	682	677	8	1.2

100 g of dry plant material and a major difference in allantoin content of silk and seed and even between their silks exists (**Table 1**).

Validation Results. The following validation criteria were used to evaluate the method: sensitivity, linearity, repeatability, and recovery. On the basis of visual evaluation with a signal-to-noise ratio of about 3, the limit of detection (LOD) value of allantoin was found to be 0.05 μg/mL (1.0 ng). Linearity of the detector responses was determined by preparing a calibration graph. A linear relationship of peak area against concentration of standard solution was obtained, and the calculated equation was reported as $Y = 0.017X + 0.008$ [Y is the peak area $\times 10^{-6}$, and X is the concentration (μg/mL)] for allantoin. The linearity of the determination of allantoin was verified by regression analysis (seven-point measurement, correlation coefficient of $r^2 > 0.999$). Limit of quantification (LOQ) value of allantoin was 0.2 μg/mL (4.0 ng).

The repeatability of the current method was evaluated when applied to silk with a higher allantoin content. For this purpose, four samples of 250 mg of silk N1 were extracted, and each of the four extracts were analyzed 3 times by HPLC. The results are given in **Table 2**. The relative standard deviation value is 1.2%.

Recovery experiment was carried out using standard addition method. For assessing recovery of the current method, known quantities of allantoin were added to the samples at four different mass values (250–1000 μg). Triplicate experiments were performed at each level. The recovery method including extraction, evaporation, and filtration process used in sample preparation was determined by HPLC analysis of samples. The results of the recovery studies are presented in **Table 3**. The recovery of allantoin standard added to samples was observed in the range of 98.3–101.6%, with the RSD value of 1.5%.

Table 3. Results of Recovery of Allantoin at Different Amounts Added to 250 mg of Silk N1 ($n = 3$)

compound	initial amount (μg)	average recoveries (%)	relative standard deviation (%)
allantoin	250	98.3	5.3
	500	101.6	5.7
	750	101.1	4.8
	1000	101.4	3.6
pooled data		100.6	1.5

The present paper describes a developed and validated method for the qualitative and quantitative analysis of allantoin in *Z. mays*. The improved HPLC method allows for a satisfactory separation between the peaks of allantoin and other interfering unknown compounds in crude extract (eight peaks) with good specificity. This procedure exhibits high sensitivity with a LOD of 1 ng and good linearity, repeatability, and recovery. The method uses a simple mobile phase for isocratic elution at ambient temperature. The run time of the method using an Econosil C_{18} column is less than 15 min and can be used for routine analysis of allantoin as one of the active components in the quality assessment of *Z. mays* herb.

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