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Quantification of allantoin in various *Zea mays* L. hybrids by RP–HPLC with UV detection

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A RP-HPLC method for quantification of allantoin in silk of fifteen maize hybrids (*Zea mays* L., Poaceae) was described. Following extraction of the plant material with an acetone-water (7 : 3, V/V) mixture, filtration and dilution, the extracts were analyzed without previous chemical derivatization. Separation and quantification were achieved using an Alltech Econosil C18 column under isocratic conditions at 40 °C. The mobile phase flow (20% methanol - 80% water with 5 mM sodium laurylsulfate added at pH 2.5, adjusted with 85% orthophosphoric acid; pH of water phase was finally adjusted at 6.0 by addition of triethylamine) was maintained at 1.0 mL/min. Column effluent was monitored at 235 nm. This simple procedure afforded efficient separation and quantification of allantoin in plant material, without interference of polyphenols or other plant constituents of medium to high polarity, or similar UV absorption. Our study revealed that the silk of all investigated maize hybrids could be considered relatively rich in allantoin, covering the concentration range between 215 and 289 mg per 100 g of dry plant material.

1. Introduction

In traditional medicine, the herbal drug Maydis stigma (dried cut stigmata of maize, *Zea mays* L., Poaceae female flowers) is recognized and used as a mild diuretic, to pass stones and gravel from kidneys and urinary bladder, against benign prostatic hyperplasia, cystitis, gout, chronical nephritis and similar ailments (Willuhn 2002). Similar findings were published in several extensive ethnopharmacological studies (Bastien 1983; Cáceres et al. 1987; Yeşilada et al. 1995).

It is generally accepted that maize can neither be found in the nature as a wild-growing plant, nor is capable to survive without human influence and care (Jevtić 1986). Instead of indigenous types, one can find a number of maize hybrids on fields all over the world, which represent the plant material M. stigma exclusively comes from today. Only in Serbia and Montenegro, there are more than 100 internationally recognized domestic maize hybrids, bred from the genetic base of 16 autochthonous populations, formed and differentiated by spontaneous cross-fertilization that lasted from the 16th century (estimated date of the first maize introduction to Serbia) to the middle of the 20th century (when indigenous maize populations were replaced by hybrids) (Radović et al. 1995). Similar considerations apply to almost every place where maize is cultivated today. Hence, it is reasonable to bear in mind that plant material available for the production of the herbal drug M. stigma is presumably heterogeneous.

Available phytochemical data about M. stigma are scarce and frequently inconsistent. According to Willuhn (2002), M. stigma contains essential oil, fats (waxes), flavonoids, saponins, tannins, gums, bitter principles, mucilage and nitrogen-containing constituents. Previous communications confirmed only the presence of volatile compounds (Flath et al. 1978; Zeringue 2000) and flavonoids (Styles et al. 1975; Elliger et al. 1980; Snook et al. 1993; Widstrom et al. 1998) in silk of several maize hybrids. However, the presence and/or distribution of allantoin in maize silk has not been found reported yet.

Pursuing our research on the phytochemical and pharmacological profile of M. stigma, we designed and performed an extensive set of various phytochemical and pharmacological studies on the silk of fifteen commercially important domestic maize hybrids. In those experiments, certain hybrids were included strictly according to their economic importance: all the selected are prevalent on the fields in Serbia. Taking into account that the herbal drug M. stigma in Serbia is produced completely from the silk collected after harvesting the grain, selected hybrids could be considered the best representatives of available plant material. In this communication, we present the results of the study on identification, distribution and quantification of allantoin in silk of selected maize hybrids.

2. Investigations, results and discussion

2.1. Chromatographic separation

Allantoin [(2,5-dioxo-imidazolidinyl)-urea] is a highly polar, amphoteric substance. Achieving a satisfactory separation of this compound in biological samples using HPLC based methods is frequently considered a major analytical problem, due to possible interference of the other constituents with similar polarity and UV absorption (Czauderna

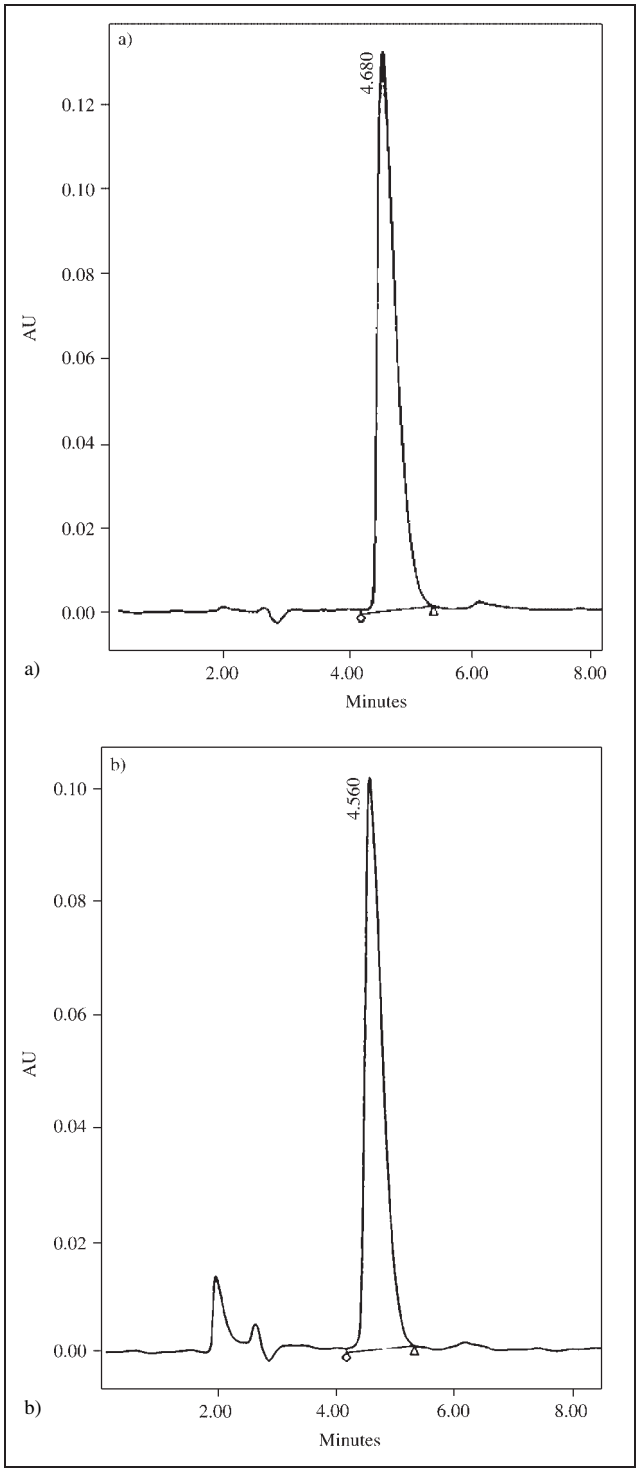

Fig.: (a) Chromatogram of allantoin standard. (b) Representative chromatogram of analyzed extracts

et al. 1997; Shingfield et al. 1999; Czauderna et al. 2000). In addition, highly polar substances, especially those capable for ionisation in water, usually have peak tailing and poor peak symmetry when separated with RP-HPLC methods. Those events could be prevented by addition of certain modifiers to the mobile phase, such as acidic or basic substances, or ions of opposite charge as well. By our oppinion, the most acceptable approach was to adjust the pH value of the mobile phase, as it is well known that ionisation degree of solutes, stationary phase and mobile phase additives are affected by the pH and may lead to better selectivity.

Table 1: Basic attributes of the calibration curve

Data points (N)	5
Concentration range (μg/ml)	1.0–10.0
Calibration equation ($y = a + bx$)	$y = 28.1910 + 562.6594x$
Correlation coefficient (r)	0.9997
Residual standard deviation ($S_{y,x}$)	57.8
Standard error of intercept (S_a)	50.2
Standard error of slope (S_b)	8.3
Significance of intercept (t_a)	0.6

In our case, the pH value of the initial mobile phase (methanol-water, 20 : 80 V/V) has varied from 3.0 to 6.0. Severe peak tailing was observed when a separation was performed at pH values lower than 4.5. With addition of triethylamine, major improvements were noticed. Triethylamine improved the peak shape by interacting strongly with acidic silanol groups on the silica surface. A slight decrease in allantoin retention time was achieved, but the resolution of separation was not disturbed.

Further improvement in peak symmetry was gained with addition of sodium laurylsulfate to the water phase. In a concentration of 5 mM, the addition of sodium laurylsulfate resulted in a significant improvement of allantoin peak shape, without resolution changes. Sodium laurylsulfate acts as a surfactant, which improves wetting and lowers the influence of the stationary phase. Hence, the retention time of allantoin was even shortened, but no interference with other medium to high polar constituents, or compounds with similar UV absorption occured, as shown at representative chromatograms of standard substance and one extract analyzed (Fig.).

2.2. Linearity of the method

A calibration curve was constructed and the linearity of the method was estimated by regression analysis of allantoin peak area against the concentration. After quantification, Student's t-test for unpaired data was applied to achieve a comparison between the groups. Mean values were considered significantly different if $P < 0.05$.

Allantoin peak identification was performed by comparing its retention time with that of authentic standard. The regression equation of peak area (y) against compound concentration (x, μg/mL) and the other important parameters of the calibration curve are given in Table 1. Significance of the intercept was estimated by the statistical method, and found insignificantly different from zero, at the probability level of 0.05. Our results indicate that a very good linearity of the method was achieved under the chromatographic conditions described.

2.3. Results of the quantification of allantoin in silk of selected maize hybrids

Using the described method, the presence of allantoin was confirmed in all investigated extracts. Our study revealed that, although a greater variability of results was expected, the concentration of allantoin in silk of the maize hybrids investigated covered a narrow range between 215 and 289 mg per 100 g of dry plant material, with insignificant difference of means between two series (Table 2). In other terms, the quantity of allantoin appeared to be independent or, at least, just slightly influenced by the biological source: the silk of all the investigated maize hybrids contained similar levels of allantoin.

Table 2: Results of allantoin quantification in selected maize hybrids

N°	Hybrid	Allantoin concentration (mg/100 g dry plant material)
1	ZP 360	271.2
2	ZP 434	258.5
3	ZP 539	288.8
4	ZP 599	272.8
5	ZP 677	233.8
6	ZP 680	271.2
7	ZP 704	244.2
ZP average[a]		262.9 ± 18.8
8	NS 444	249.2
9	NS 501	261.8
10	NS 606	249.7
11	NS 607	215.0
12	NS 640	267.3
13	NS 663	277.2
14	NS 6666	262.3
15	Balkan	222.2
NS average[a]		250.6 ± 21.8
Total average[a]		256.3 ± 20.7

[a] Results are presented as mean ± standard deviation

The low variability of allantoin levels might be in accordance with the physiological role of this secondary metabolite in higher plant tissues: among the other ureides, amino acids, nitrates or amides, allantoin is considered one of the forms in which nitrogen is transported through the plant organism (Mazzafera et al. 1999). This type of nitrogen transport is frequent in the plant kingdom: so far, allantoin is detected in various organs of plants belonging to 23 families; in some of them (Boraginaceae, Fabaceae), allantoin is a typical constituent (Dikhtyarev et al. 1983; Schliemann 1984; Mazzafera et al. 1999). These facts could partially explain the low variability observed in our study: plant tissues either contain allantoin at genetically determined levels, or could be considered allantoin-free.

In pharmacy, dermatology and cosmetology, allantoin is frequently applied both topically and orally, mainly as astringent and keratolytic, in numerous preparations for skin care, or for regeneration of damaged tissues in cases of hemorrhoids, ulcers and cold sores (Parfitt 1999; Gennaro 2000). Necessary quantities of allantoin are mainly produced synthetically, by oxidation of uric acid with alkaline potassium permanganate, or by heating urea with dichloroacetic acid (Budavari 1989). Because of economic advantages of synthesis over isolation from plant material, allantoin-containing herbal drugs have lost their importance as sources for the industrial production of allantoin. However, those drugs still have an importance in dermatology and cosmetology, because adequate extracts are frequently used.

The use of herbal drug Maydis stigma in dermatology and cosmetology does not have an ethnopharmacological background. However, a novel type of application of this herbal drug could be evaluated now. Allantoin level in maize silk could be considered intermediate, if compared to one of the most frequently used allantoin-containing herbal drugs – Symphyti radix (dried roots of *Symphytum officinale* L., Boraginaceae), which contains approximately 0.6–0.8% of allantoin (Willuhn 2002). But, the use of Maydis stigma instead of Symphyti radix could have several clear advantages. First, being considered a by-product in the grain production, maize silk is inexpensive and readily available at a large scale. Further, due to the presence of significant quantities of pyrrolizidine-type alkaloids, prolonged peroral use of Symphyti radix and adequate extracts can induce the development of hepatotoxic, carcinogenic and mutagenic effects (Willuhn 2002). Finally, excessive collection of maize female flowers after harvesting the maize grain can never threaten its biological source with extinction.

3. Experimental

3.1. Reagents

Acetone (Zorka-Pharma, Šabac, Serbia and Montenegro), used for the extraction of plant material, was of an analytical grade. Triethylamine and sodium laurylsulfate (Merck, Darmstadt, Germany), 85% orthophosphoric acid and methanol (Carlo Erba, Milano, Italy) were all of HPLC grade. Distilled, deionized water was obtained from a Simplicity 185 purification system (Millipore S. A., Molsheim, France). The standard substance – allantoin (Sigma, St. Louis, MO, USA) was used without further purification.

3.2. Plant material

Fully developed, mature silk of fifteen maize hybrids (Table 2) were gathered from the field collections of the Maize Research Institute "Zemun Polje" in Belgrade (ZP series) and the Maize Department of the Institute of Field and Vegetable Crops, Novi Sad, Serbia and Montenegro (NS series). Collected plant material was dried *in toto* in a shaded and well-ventilated place and kept refridgerated in dark all-glass containers until extracted.

3.3. Sample preparation

Plant material (1 g per sample) was reduced to a fine powder and extracted with 25 mL of an acetone-water mixture (7 : 3, V/V) under 20 min sonication in an ultrasonic bath at the ambient temperature. The extracts were rapidly vacuum-filtered through a sintered glass funnel and kept refridgerated before assay. Prior to analysis, the extracts were diluted with water phase (1 : 20, V/V).

The stock solution was prepared by dissolving the working standard substance (allantoin) in acetone-water mixture (7 : 3, V/V), to obtain the concentration of 0.1 mg/mL. Five standard solutions, covering the concentration range between 1.0 and 10.0 μg/mL, were prepared by diluting the aliquots of stock solution with water phase and used for the calibration curve construction.

3.4. Chromatographic conditions

RP-HPLC analysis of investigated extracts was performed on the Breeze Waters chromatographic system, consisted of Waters 1500 HPLC binary pump and Waters 2487 UV–VIS detector (Waters Corporation, Milford, MA, USA). Chromatographic separations were performed on the Alltech Econosil C18 column (Alltech Associates, Inc., Deerfield, IL, USA; 4.6 × 250 mm, 5 μm particle size), without the use of a precolumn. The samples were introduced through a Rheodyne injector valve, with a 20 μL sample loop. Data acquisition and analysis were performed using Breeze Software.

Determination of allantion was performed using the mobile phase: methanol – water (20 : 80, V/V). The water phase was prepared by dissolving 1.442 g of sodium laurylsulfate in 1000 mL of water (pH of water was adjusted to 2.5 with 85% orthophosphoric acid). Finally, the pH of the water phase was adjusted to 6.0 with triethylamine. Both solvents were filtered through a 0.45 μm Alltech pre-cut nylon filter and degassed in an ultrasonic bath for 15 min.

Chromatography of standard solutions and investigated extracts was performed under isocratic conditions, at a flow rate of 1.0 mL/min. Separation was achieved at 40 °C, with a total run time of 20 min. Column effluent was monitored at 235 nm. Allantoin peaks in the investigated extracts were identified by comparing their retention times with those of authentic standards.

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