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ARTICLE

Development and validation of an LC-MS/MS method for the analysis of L-DOPA in oat

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ABSTRACT L-DOPA (L-3,4-dihydroxyphenylalanine), currently the most effective known therapeutic agent for the symptomatic relief of Parkinson's disease, is one of the highly active allelochemicals that inhibit the growth and development of certain plant species. A tropical legume, *Mucuna pruriens*, has been shown to contain from 2 to 7% L-DOPA. However, little is known about the L-DOPA contents of other plants. The aim of our work was to develop and validate a simple method for the LC-MS/MS determination of L-DOPA in different oat varieties. During the development of the method, various LC and MS parameters were optimized from the aspect of sensitivity. In the final method, the calibration curve was linear over the concentration range 10-10,000 pg/µl. L-DOPA could be detected at 18 µg/kg level through use of the $[(M+H)-NH_3]^+$ fragment of the $[M+H]^+$ molecular ion. The intraday precision and accuracy were all within acceptable ranges.
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L-DOPA (L-3,4-dihydroxyphenylalanine) is currently widely accepted as the most effective therapeutic agent for the symptomatic relief of Parkinson's disease. This compound is produced by certain plants, and especially a tropical legume, *Mucuna pruriens* or velvet bean, which has been reported to contain up to 7% L-DOPA (Daxenbichler et al. 1971). *M. pruriens* is used in the traditional Indian therapeutics and a number of attempts have been made to utilize it as a modern medicine (Nagashayana et al. 2000). On the other hand, the legume demonstrates a significant allelopathic effect, which means that other plants can hardly live in its vicinity. This phenomenon has been ascribed to its high L-DOPA content (Nishihara et al. 2004). Allelopathic plants and allelochemicals are of considerable potential in agriculture as their application has the result that the amounts of artificial fertilizers can then be significantly decreased with them. If this is to be achieved, extended experiments are needed as regards the allelochemical contents of different plants. The aim of the present study was the determination of L-DOPA in oat, certain species of which exhibit notable allelopathy (Schumacher et al. 1983; Bertoldi et al. 2009)

Various methods are available for the quantitative analysis of L-DOPA. Modern capillary electrophoresis and high performance liquid chromatography (HPLC) methods are proper (Tsunoda 2006; Shalini et al. 2011), but the selectivity can be further increased by mass spectrometric (MS) detection, especially if the MS/MS mode is at hand (Manini et al. 2000; Bourcier et al. 2006).

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We therefore set out to develop and validate a simple method for the LC-MS/MS determination of L-DOPA in different oat varieties.

Materials and Methods

Materials

L-DOPA and L-tyrosine standards were purchased from SERVA (Hungary), while α-methyldopa, ascorbic acid standards and formic acid, acetic acid and ammonium acetate were from Sigma-Aldrich (Hungary). Membrane-filtered, deionized water for HPLC runs was produced with Millipore (Merck, Hungary) water purification equipment. HPLC-grade acetonitrile (ACN), methanol (MeOH) were purchased from Molar Chemicals (Hungary).

Stock solutions of L-DOPA, L-tyrosine and α-methyldopa were prepared by dissolution of the solid standards in an aqueous solution of 0.1% (v/v) formic acid and 0.1% (m/v) ascorbic acid to obtain a concentration of 1 mg/ml. Stock solutions were diluted to the necessary concentrations, typically to 10 ng/µl for parameter optimizations.

HPLC parameters

HPLC investigations were performed on an Agilent 1100 (Agilent, Palo Alto, USA) modular HPLC system, equipped with a degasser (G1379A), a binary pump (G1376A) and a micro-well plate autosampler (G1229A).

Separation was carried out on a Synergi Hydro-RP 250 x 2 mm, 4 µm analytical column (Gen-Lab, Hungary) in front of which a Hydro-RP 4 x 2.0 mm guard column (Gen-Lab,

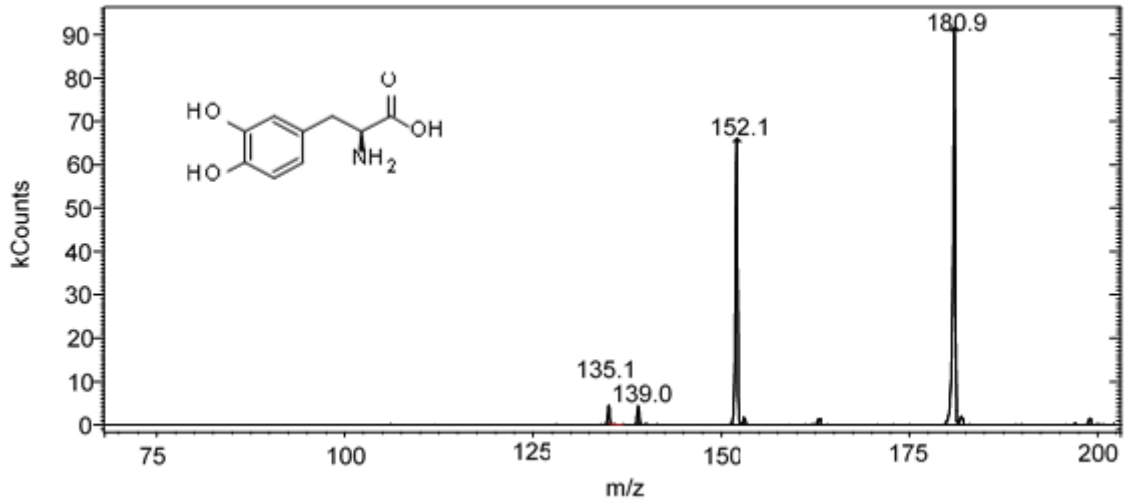

Figure 1. MS/MS spectrum of the L-DOPA after fragmentation of the molecular ion $[M+H]^+$ (*m/z* 198.2).

Hungary) was situated. The column temperature was maintained at 30 °C with a Jones Model 7990 Space column heater (Jones Chromatography, UK). Eluent A consisted of 0.1% (v/v) formic acid and 3% (v/v) eluent B. Eluent B contained ACN/MeOH at a volume ratio of 75/25 containing 0.1% (v/v) formic acid. The gradient elution started with 0% B, which was increased linearly to 100% B within 5 min, held there for 2 min, then decreased to 0% in 3 min, and kept there for 5 min. Before the next injection, a 5 min equilibration period was held. The flow rate was set to 200 µl/min. The injection volume was 5 µl. The retention times of L-DOPA, L-tyrosine and α-methyldopa were 5, 6.5 and 7.8 min, respectively.

The HPLC was controlled by Chemstation B.02.01 software.

Ion trap MS conditions

The MS measurements were performed with a Varian 500MS Ion Trap mass spectrometer equipped with an electrospray ionization (ESI) source. The ion trap parameters were tuned in positive ion mode by continuous infusion (5 µl/min) of the standard solutions (10 ng/µl) with the built-in syringe pump of the instrument. The parameters were set as follows: capillary voltage, 45V, RF loading, 67%; needle voltage, 4350 V; spray shield voltage, 600 V; fragmentation voltages, 0.5, 0.71 and 1.86 V for L-DOPA, L-tyrosine and α-methyldopa, respectively. The ion source parameters were optimized in flow injection analysis without a column. In the final experiments the ion source parameters were as follows: spray chamber temperature, 50 ºC; drying gas (N_2) pressure and temperature, 25 psi and 350 ºC, respectively; nebulizer gas (N_2) pressure, 60 psi.

The MS was controlled by MS Workstation 6.6 software.

Sample preparation

GK Iringo, *GK Kormorán* and *GK Zalán* were chosen as a probe oat species. They were grown in an experimental field in Kiszombor and Újszeged (Csongrád County, Hungary). The *M. pruriens* seeds were grown in greenhouse. The seeds were finely ground with a laboratory grinder and 1 g of the powder was soaked in 6 ml of an aqueous solution of 0.1% (m/v) ascorbic acid and 1% (v/v) MeOH. The mixture was shaken vertically with a Stuart STR4 vertical shaker for 5 h, then centrifuged at 4500 rpm for 5 min. The supernatant was filtered through a GF/B glass microfiber filter. Twenty µl of 0.6 µg/ml internal standard α-methyldopa solution was added to 1 ml of the filtered extract.

Results and Discussion

The development of this method related not only to L-DOPA, but also to its biological precursor, L-tyrosine. α-Methyldopa was selected as internal standard and added to the samples immediately before the analysis in order to correct for the fluctuations in the MS performance.

Because of the H^+-accepting property of the amino group (El Aribi et al. 2004) positive ion mode was applied for the detection of the analytes. Under ESI conditions, L-DOPA (L-tyrosine and α-methyldopa too) can be effectively transformed into a protonated ion. The fragmentation of $[(M+H)]^+$ yields different fragment ions (Fig. 1), the most abundant in the case of L-DOPA being $[(M+H)\text{-}NH_3]^+$ at m/z 181 and $[(M+H)\text{-}H_2O\text{-}CO]^+$ at m/z 152. To obtain the highest $[(M+H)\text{-}NH_3]^+$ fragment peak intensity (m/z 181, 165 and 195 for L-DOPA, L-tyrosine and α-methyldopa, respectively), optimization of the ESI parameters was carried out.

The effects of the nebulizer gas pressure and temperature

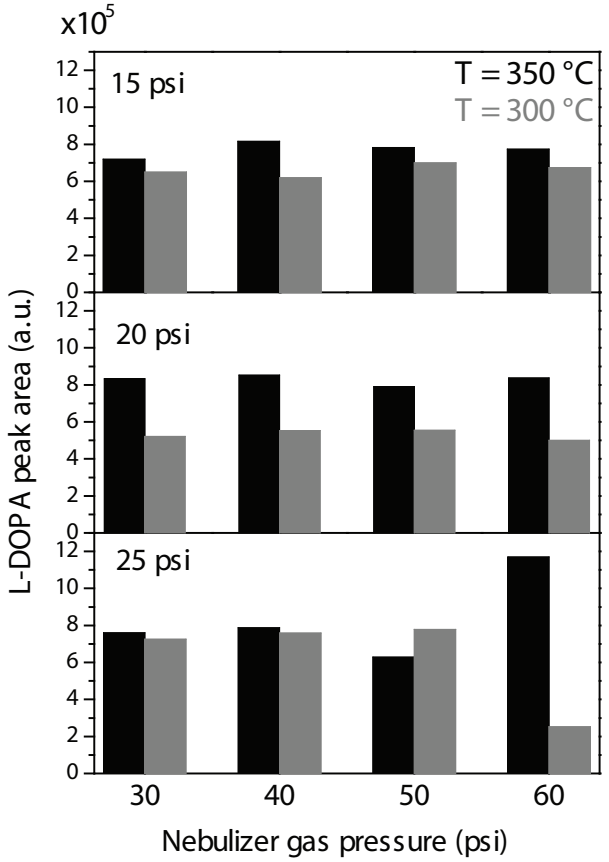

Figure 2. The effect of the nebulizer gas (x axis) and drying gas pressures (upper left hand corners) on the L-DOPA signal. The experiments were carried out at two different nebulizer gas temperatures (350 and 300 °C, black and grey bars).

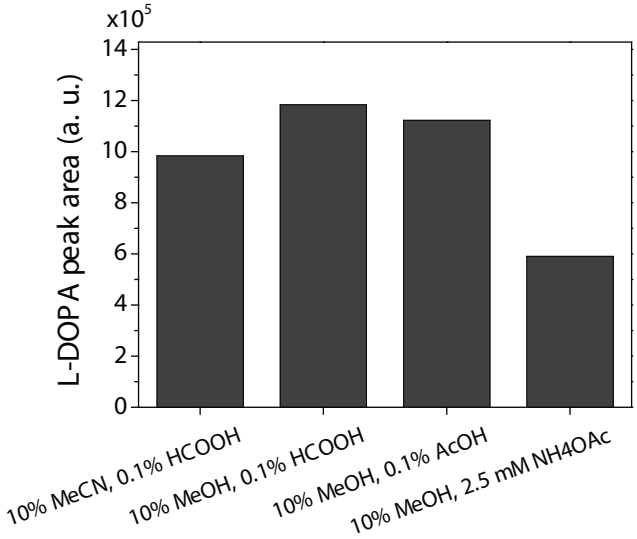

Figure 3. The effect of different mobile phase composition on the L-DOPA signal.

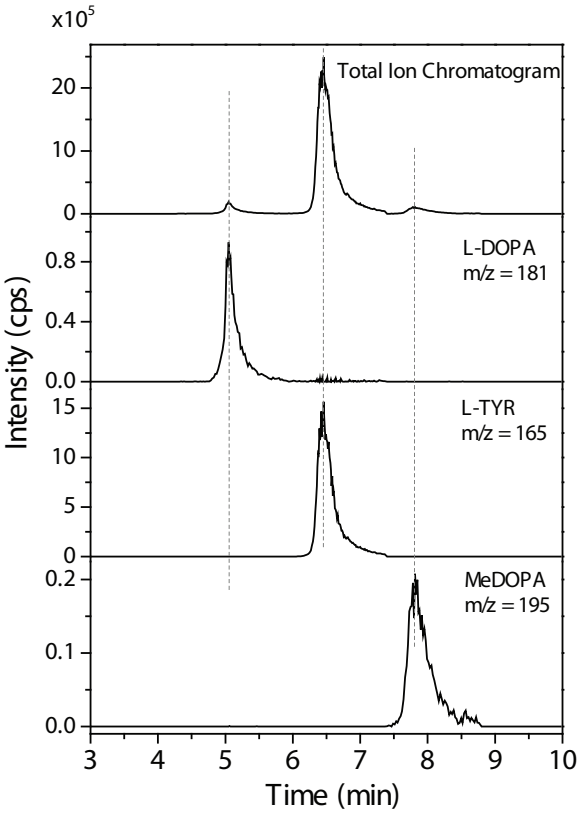

Figure 4. Total ion chromatogram and selected ion chromatograms for L-DOPA, L-tyrosine and α-methyldopa of the *GK Kormorán* variety.

and the drying gas pressure were studied. The results revealed that the investigated parameters did not have significant effects on the ionization efficiency of L-DOPA (Fig. 2). However, an outstanding intensity was observed at a nebulizer gas pressure of 60 psi and a drying gas pressure of 25 psi at a nebulizing gas temperature of 350 °C.

After the establishment of the MS parameters, the influence of the mobile phase composition on the efficiency of L-DOPA ionization was investigated via flow injection analysis by using acetonitrile, methanol, formic acid, acetic acid and ammonium acetate. It was found that formic acid and acetic acid were more suitable than ammonium acetate as mobile phase additives (Fig. 3). No matter which individual solvent was used, there was no marked effect on the signal intensity. On the other hand, in the course of the HPLC separation, methanol provides higher selectivity, while acetonitrile has lower viscosity. In order to take advantage of both of these beneficial features, ACN/MeOH mixture was chosen as eluent B at 75/25 (v/v), and eluent A also contained 3% (v/v) of eluent B. Gradient elution proved suitable for the total separation of the L-DOPA, L-tyrosine and α-methyldopa signals (Fig. 4).

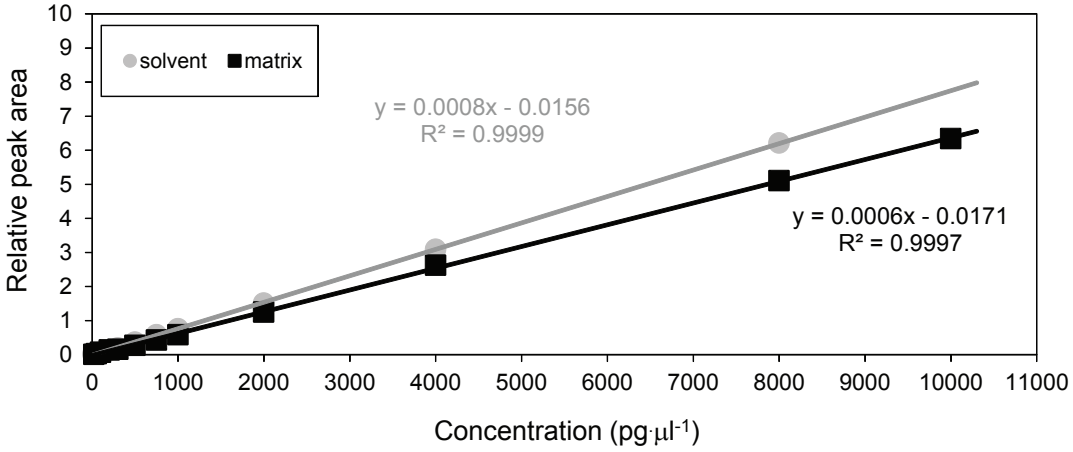

Figure 5. Calibration curves created by dilution with the extraction solvent (0.1% (m/v) ascorbic acid and 1% (v/v) MeOH) and corn extract (blank matrix).

In order to test the linearity of the response versus the amount of L-DOPA injected, various concentrations of standard solutions in the range from 10 to 10 000 pg/µl were analyzed. Figure 5 presents two calibration curves. The stock solution of the analyte was diluted with the extraction solvent, or with corn extract (blank matrix). Both plots exhibited good linearity throughout the whole concentration range. Observations in plant analytical chemistry (Dams et al. 2003; Lattanzio et al. 2007) indicate that the slopes of the calibration curves differed due to the matrix effect. The ionization of L-DOPA is suppressed by the matrix components: the response is 1.35 times higher in the absence of the matrix.

During validation of the method the mean recoveries of four replicates at three different spiking levels (0.6, 3 and 6 mg/kg) were 95.2%, 99.6% and 95.2%, respectively. The repeatability of the measurements was calculated by performing four injections of one of the spiked sample extracts. RSD % was found to be better than 4%, as were the intraday precision and accuracy. The limit of detection was 15 pg or 18 µg/kg (*S/N*=3).

The validated method was applied for the determination of the L-DOPA contents of different oat varieties. Some *M. pruriens* seeds were also analyzed, for comparison (Table 1.). It was concluded that the concentration of L-DOPA in oat varies within the interval 2-20 mg/kg, which is three orders of magnitude lower than that in *M. pruriens* samples. A further important observation was made that the L-DOPA content depends not only on the variety, but also on the time of harvesting and the production site.

Table 1. L-DOPA content of some selected oat species and *Mucuna pruriens* samples.

Sample	L-DOPA content (mg/kg)
GK Iringó	10.02
GK Zalán	2.17
GK Kormorán (Kiszombor, 2007.)	7.83
GK Kormorán (Kiszombor, 2008.)	2.27
GK Kormorán (Kiszombor, 2009.)	3.88
GK Kormorán (Újszeged, 2009.)	11.89
M. pruriens 1.	37423
M. pruriens 2.	64019

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