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Quantitative determination of 1-deoxynojirimycin in mulberry leaves using liquid chromatography–tandem mass spectrometry

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Abstract

A novel HPLC–MS/MS method was developed for the quantitative determination of 1-deoxynojirimycin (DNJ), a potent glucosidase inhibitor present in mulberry leaves (*Morus alba* L.). DNJ was isolated from the mulberry leave extract on a TSKgel Amide-80 column using a mixture of 0.1% formic acid and acetonitrile as a mobile phase at a flow rate of 0.6 ml/min. A triple quadrupole mass spectrometry using electrospray ionization source in a positive ion mode under multiple reaction monitoring with the $[M+H]^+$ ions, *m/z* 164.4/109.9 was used. The detection limit (S/N = 3) was 75 pg and quantitation limit (S/N = 10) was 100 pg. The comparison of mulberry leaves of different ages showed that the DNJ level was higher in mulberry shoots than young and mature leaves.

Keywords: 1-Deoxynojirimycin; Mulberry leaves; Mulberry shoots; LC–MS/MS; MRM

1. Introduction

Mulberry (*Morus alba* L.; Moraceae) has been cultivated in many Asian countries such as China, Korea, Japan and Thailand. The infusion of its leaves is consumed as antihyperglycemic nutraceutical foods for patients with diabetes mellitus [1]. The leaves are rich in alkaloid components including 1-deoxynojirimycin (DNJ) which is known as one of the most potent α-glycosidase inhibitors [1–5]. It inhibits the enzyme from decomposing starch and sugar and preventing the glucose absorption, resulting in a decrease of blood sugar level.

DNJ (Fig. 1) is a polyhydroxylated piperidine alkaloid, which lack of chromophore in its molecule and therefore difficult to be detected by HPLC-UV analysis. The sample pretreatment such as derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) was necessary for HPLC-fluorescence detection [1,6]. Evaporative light scattering detection (ELSD) [7] as well as mass spectrometry [8,9] was an alternative detection method for compounds without a chromophore like DNJ. Another difficulty of DNJ analysis is the separation method as DNJ is highly polar. The interaction between the stationary phase of reversed phase columns and DNJ is so weak that DNJ does not retain in the column. Kimura et al. [7] successfully separated DNJ from an extract of mulberry leaves on a TSKgel Amide-80 column, a hydrophilic interaction chromatography (HILIC) column, using HPLC-ELSD. In addition, cation exchange chromatography with pulsed amperometric detection was also used for quantitation of an *n*-alkylated imino sugar [10].

Liquid chromatography coupled with mass spectrometry (LC–MS/MS) has been proven to be a promising technique for quantitation in biological samples. A triple quadrupole LC–MS system that acquires a mass spectrum of the product ions produced from the fragmentation of the selected precursor ion has been extensively used for quantitation with multiple reaction monitoring (MRM). This technique allows the simultaneous

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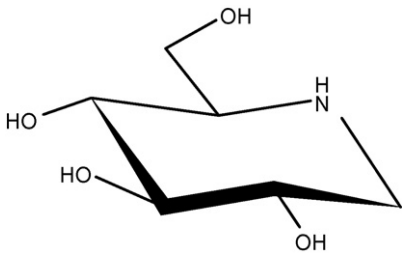

Fig. 1. Chemical structure of 1-deoxynojirimycin.

monitoring of more than one pair of parent/daughter fragment ions with its high selectivity, specificity and sensitivity.

Because of the increasing interest in mulberry leaves as a possible nutraceutical product for diabetic patients, there is a demand for efficient quality control measurement to ensure the authenticity and content of DNJ in these products and to verify the labeled claims. Here, we propose a fast, sensitive and selective method for the quantitation of DNJ in mulberry leaves by using LC–MS/MS. This is the first time that the use of LC–MS/MS in MRM mode of DNJ is reported.

2. Experimental

2.1. Chemicals and solvents

Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol (HPLC grade) were obtained from Labscan (Labscan Asia, Bangkok, Thailand). The reference substance, 1-deoxynojirimycin hydrochloride, was purchased from Sigma–Aldrich (St. Louis, MO, USA) purity 99%. Deionized water was purified using Elga USF system (Bucks, England).

2.2. Standard and sample solutions

Stock solution of DNJ standard was made in methanol. A working solution was prepared in methanol-water (70:30, v/v) and diluted to provide a series of analytical standards ranging from 0.1 μg/ml to 10 μg/ml for constructing calibration curves. The stock solution was stable up to 3 months when kept at 4 °C before used.

Leaves from seven mulberry cultivars, i.e., Burirum 60, Chiang Mai, Khun Phai, Nakhonratchasima 60, Noi, PMN 9 and PMN 14 were taken from different positions of the tree and classified into three groups, i.e., the shoots (leaf bud and two youngest leaves from the top of the tree), the young leaves (the third to the tenth leaves from the top), and the mature leaves (the leaves below the 10th leave). The leaves were gathered from the mulberry plantation of the Department of Queen Sirikit Institute of Sericulture (Udonthani Center, Thailand) in July 2005. The leaves were harvested, cleaned, air dried and ground into powder and sieved. The material that passed through an 80 mesh sieve was collected and stored in a glass bottle at 4 °C until used. Fifty milligrams of the powder was added to 10 ml of 70% aqueous methanol, and then sonicated in an ultrasonic bath for 15 min. The extracts were filtered through filter paper no. 1 (Whatman), and the dried pellets were re-extracted twice and pooled together. The filtrate was adjusted to a volume of 25 ml with 70% methanol. The filtrate was filtered through a 0.2 μm nylon syringe filter (Chromtech, USA) before injected into the LC–MS system.

2.3. LC–MS/MS instrument and conditions

A series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) was equipped with a binary pump, a degasser, an autosampler, a thermostated column compartment and a control module connect with a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with a electrospray (ESI) interface. The Analyst 1.3.2 software was used for data acquisition and processing. The chromatographic separation was on a TSKgel Amide-80 column (5 μm, 250 × 4.6 mm i.d.) (Tosoh, Tokyo, Japan) operated at 40 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B). The elution program was employed from isocratic elution A:B (80:20, v/v) in 6 min, followed by linear gradient elution from A:B (80:20, v/v) to A:B (10:90, v/v) in 0.5 min. A:B ration was constant at 10:90 (v/v) for 1.5 min then changed to A:B (80:20, v/v) in 0.5 min and the column was equilibrated with A:B (80:20, v/v) for 3.5 min before a new sample was injected. The flow rate was set at 600 μl/min. The injection volume was 5 μl. The mass spectroscopy was performed in a positive mode using MRM. Optimal operating parameter of ESI with maximum signal intensity of molecular ions and fragment ions were obtained by direct infusion of the standard solution of DNJ (100 ng/ml) in methanol, using a Harvard syringe pump (Syringe Pump 11 plus, Harvard apparatus Inc., Holliston, USA) at a flow rate of 5 μl/ml. The optimum conditions of the interface were as follows: ion spray voltage of 5500 V, curtain gas of 12 psi, ion source gas 1 of 65 psi, ion source gas 2 of 55 psi. The interface temperature was set at 450 °C. The entrance and declustering potential were 10 V and 45 V, respectively. The fragmentation was induced with collision gas (CAD) of 5 psi, collision energy (CE) of 22 V and collision cell exit potential (CXP) of 5 V. MRM of MS/MS was used for specific detection of DNJ by measuring the characteristic ion transitions of *m/z* 164.4 (parent ion) to *m/z* 146.1, 128.2 and 109.9 (product ions). The dwell time per transition was set at 200 ms. The mass parameters for each pair of parent and product ions were shown in Table 1.

2.4. Calibration and validation

The standard curve was prepared over a concentration range of 0.1–10 μg/ml with five different concentration levels. Stan-

Table 1
MS/MS transition, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) used for DNJ analysis

Transition (*m/z*)	Retention time (min)	DP (V)	CE (V)	CXP (V)
164.35 → 146.15	8.12	45	17	7
164.35 → 128.20	8.12	45	20	6
164.35 → 109.95	8.12	45	22	5

dard curves were made on each analysis day. The coefficient of correlation (r) was used to judge linearity. The calibration was performed using an external standard method and calibration curves were obtained by plotting the exact ion chromatogram (XIC) peak area versus concentration using linear regression $1/x^2$ weighting. The calibration range was set according to its expected levels in leaves.

Mulberry leaf cultivar Burirum 60 was used for validation testing. Intra- and inter-day precision and accuracy were accessed through triplicate experiments and triplicate injections in 3 days. The limit of detection (LOD) and quantitation (LOQ) were determined as the analyte signal having a peak area equal to three times (S/N = 3) and 10 times (S/N = 10) of that of noise using Analyst Software.

2.5. Statistics

The amounts of DNJ in samples were presented as means ± standard deviations (SD). They were statistically evaluated using the Kruskal–Wallis test. Individual differences were then assessed using a post hoc test. In all cases, $p < 0.05$ denoted significance. Each experiment was done in triplicate.

3. Results and discussion

3.1. Column selection

Several columns such as Atlantis dC18 (Waters, USA), Inertsil NH_2 (GL Sciences, Japan), Zorbax SB-Aq and Eclipse SDB C8 (Agilent Technologies, USA), Pinacle II amino (Restek, USA), Develosil RP-Aqueous (Nomura Chemical, Japan) and Luna phenyl-hexyl (Phenomenex, USA), were used for the separation and determination of DNJ in mulberry leaves. Due to its highly hydrophilic nature, DNJ did not retain in all columns used and some slight peak tailings were observed. The mobile phase containing various buffers such as ammonium formate, ammonium acetate, acetic acid and formic acid were tested in combination with the organic modifiers, methanol and acetonitrile. Finally, a TSKgel 80-amide column which is a more polar column was tested. It gave excellent peak shape and a suitable retention time of DNJ (Table 2). This was in agreement with the previous report [7]. Acetonitrile was chosen as the organic modifier in combination with formic acid for preventing peak tailing and enhancing the positive ionization of DNJ.

3.2. MS condition

The first step in the development of the LC–MS/MS procedure was to determine the type of ionization. Standard DNJ in methanol was infused directly to mass detector and tested on electrospray ionization (ESI) in positive ion and negative ion modes. ESI in positive ion mode was found to be superior in comparison to the negative ionization mode (Fig. 2). Due to adequate sensitivity of the ESI mode, other ionization techniques like atmospheric pressure chemical ionization (APCI) were not tested. Full scan positive ion ESI mass spectra were observed at m/z 164.2 (Fig. 2b). The $[M+H]^+$ ion was selected by the first quadrupole and fragmented in the collision cell (Q2) to yield a diagnostic product ion mass spectrum which was characteristic of the structural moieties present in the analytes. The fragmentation of DNJ gave intense peaks at m/z 146.3, 128.1 and 110.1 (Fig. 2c). The molecular ion was used as the precursor and three product ions were selected as daughter ion candidates for MRM analysis (Fig. 3). The mass transition with the best linearity and the lowest LOD was used. Although the mass transition at 146.3 gave a high intensity compared with the other two m/z values but it showed tailing at the low concentration. Therefore, the MRM analysis was performed using m/z 164.4/109.9 for quantitation (Fig. 3c).

3.3. Validation of the assay

Some analytical parameters such as linear range and a limit of quantitation of the developed method were evaluated. The data clearly showed a linear behaviour of the calibration curve over the range tested. A weighting factor of $1/x^2$ was applied to

Table 2
Tailing factor calculated on different LC columns and mobile phases used for DNJ analysis

Column type	Mobile[a] phase (solvent A:solvent B)	t_R (min)	T_f[b]
Atlantis dC18 3.9 mm × 150 mm, 5 μm	0.01% Formic acid:MeOH	1.35	1.30
Inertsil NH_2 4.6 mm × 250 mm, 5 μm	0.01% Formic acid:MeOH	5.99	1.90
Zorbax SB-Aq 4.6 mm × 150 mm, 5 μm	0.1% Formic acid:MeOH	2.62	1.60
Zorbax Eclipse SDB C8 4.6 mm × 150 mm, 5 μm	0.1% Formic acid:MeOH	1.96	1.30
Pinacle II amino 4.6 mm × 150 mm, 3 μm	0.1% Formic acid:MeOH	3.08	1.30
TSK gel 80-amide 4.6 mm × 150 mm, 3 μm	0.1% Formic acid:ACN	8.11	1.30
Develosil RP-Aqueous 4.6 mm × 150 mm, 5 μm	0.1% Formic acid:MeOH	2.31	1.20
	10 mM ammonium acetate:ACN	2.83	1.70
	10 mM ammonium formate:ACN	2.65	1.50
Luna Phenyl-Hexyl 4.6 mm × 150 mm, 5 μm	0.1% Formic acid:MeOH	1.86	1.38
	0.1% Formic acid:ACN	1.99	1.31
	0.01% Formic acid:ACN	1.98	1.33
	0.01 mM acetic acid:0.2%Formic acid in MeOH	2.13	1.11

[a] Mobile phase used in gradient elution.
[b] T_f (tailing factor) = $2B/(A+B)_{5\%}$.

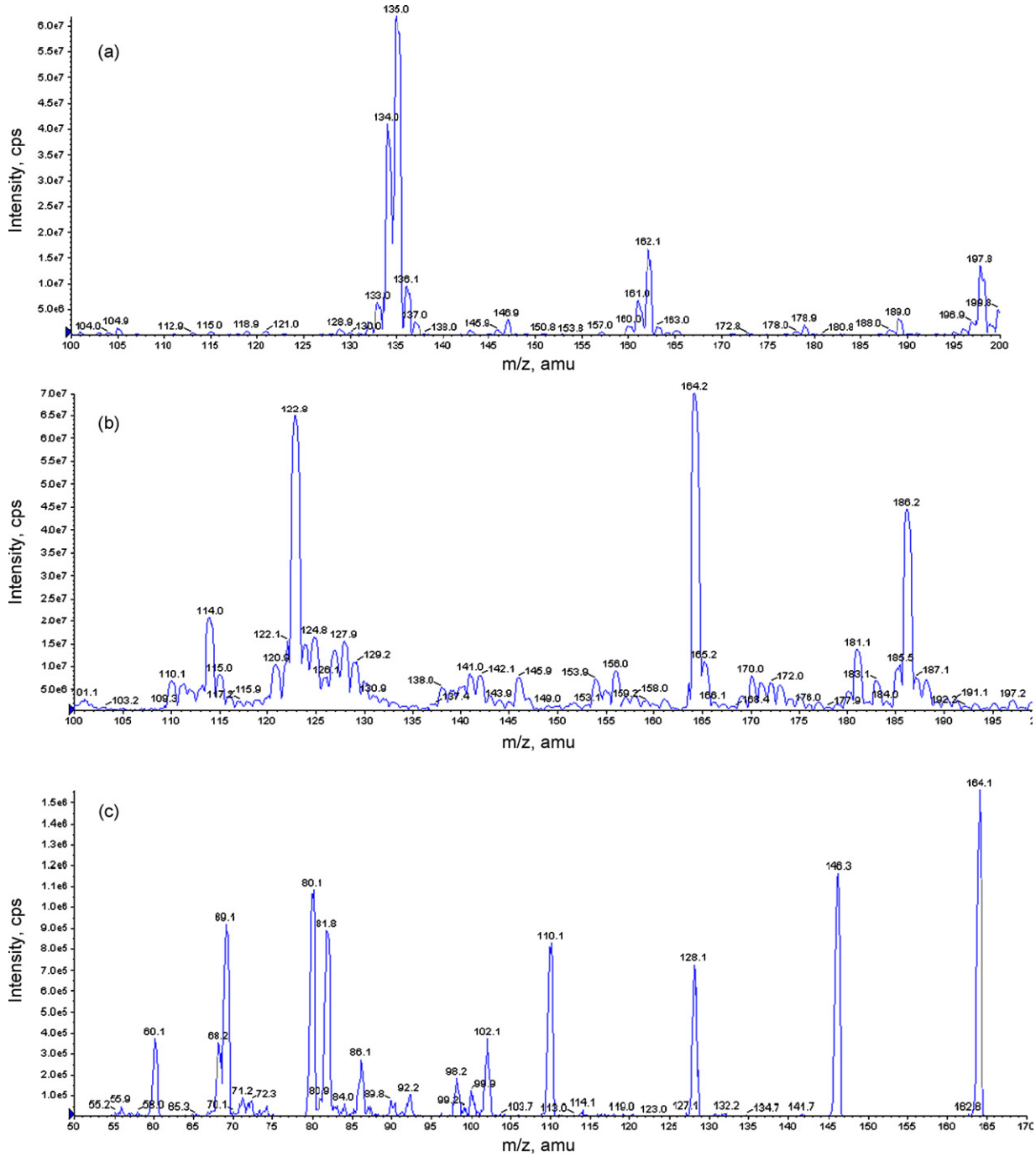

Fig. 2. Full scan mass spectra of DNJ in (a) negative ionization mode, (b) positive ionization mode and (c) MS2 product ion mass spectra of DNJ.

reduce the influence of the lowest concentration tested on the slope of the calibration curve. The calibration curve was linear in the range studied (0.1–10 μg/ml), showing a correlation coefficients (r) of 0.9995. A mean linear regression equation for the calibration curve is $y = 128x + 6.98$, where y is the peak area and x is the concentration of the analyte. The working calibration curve was generated in the range of 0.1–10 μg/ml which was the expected concentration of DNJ in the extracted samples. The recovery rates were greater than 85%. Results of intra- and inter-day precision and accuracy are shown in Table 3. CV% values were less than 6% for both intra- and inter-day analyses. The triplicate injection precision was less than 1%. The limit of detection (LOD) and limit of quantitation (LOQ) were 75 and 100 pg, respectively indicating excellent sensitivity of this method comparing to that of the previous methods [1,7].

3.4. Application

The shoots, young leaves and mature leaves from seven cultivars of mulberry trees were analyzed using LC–MS/MS developed method. The highest content of DNJ was found

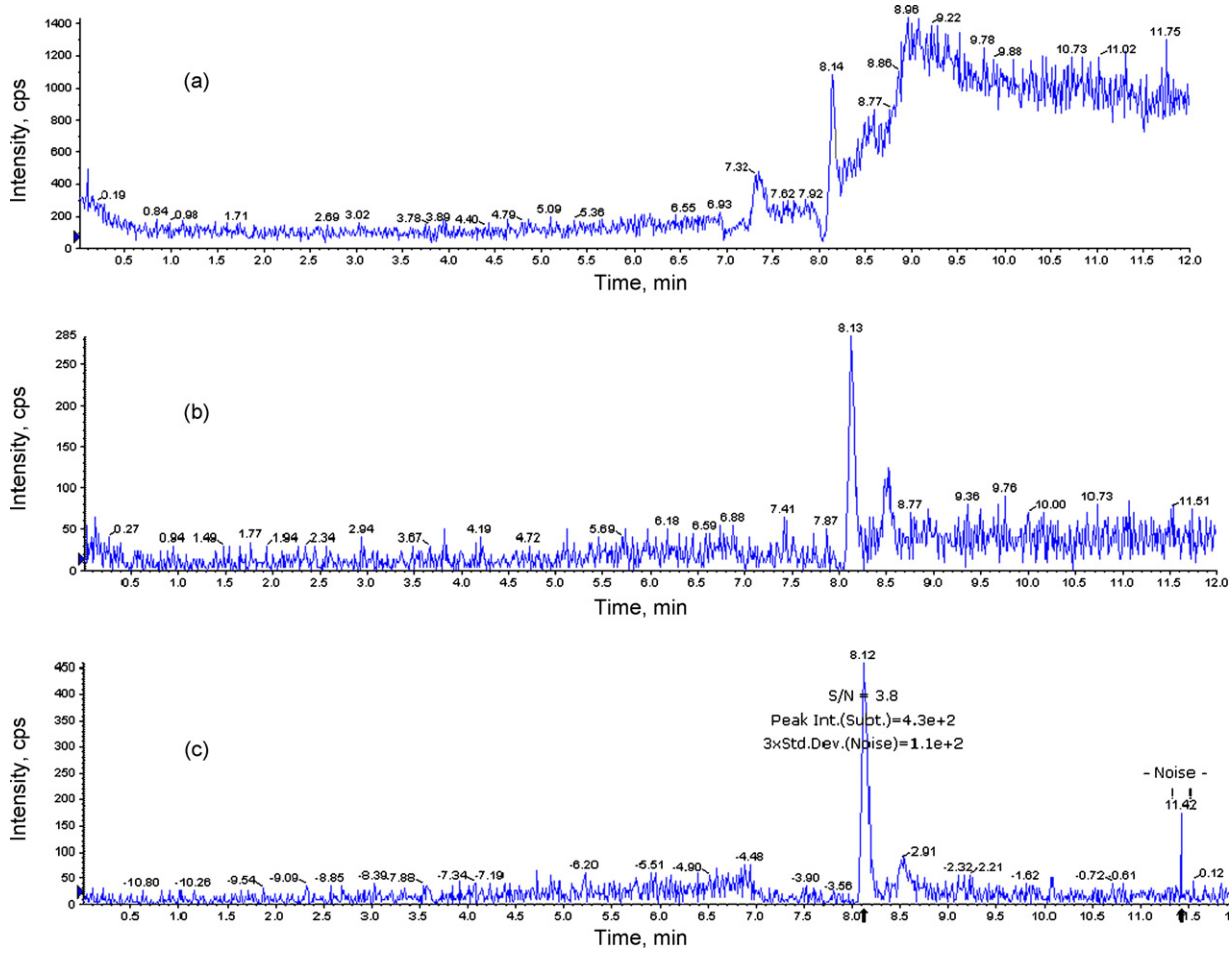

Fig. 3. Comparison of sensitivity for the three MRM spectrum of DNJ at concentration of 0.015 μg/ml (a) *m/z* 164.4/146.1, (b) *m/z* 164.4/128.2, and (c) *m/z* 164.4/109.9; (S/N = 3S.D., LOD = 75 pg on column).

in the shoots. The amounts of DNJ were in the range of 2.24–3.08 mg/g in the shoots, 0.62–1.61 mg/g in the young leaves and 0.47–0.96 mg/g in the mature leaves (Table 4). It was noted that the younger the leave were, the more they contained DNJ. Among seven cultivars, the shoots of the native cultivars, Khun Phai and Noi gave the highest amount of DNJ. It is in the same range in comparison to the values found in mulberry leaves from Japan and Korea [1,7,8].

Table 3
Intra- and inter-day accuracy and precision for DNJ in mulberry leaves (Burirum 60)

Spiked amount (μg/sample)	Mean concentration (μg/sample)	Standard deviation	Coefficient of variation (%)	Recovery (%)
Intra-day precision ($n = 3$)				
Non-spiked	1.08	0.02	2.03	
500	1.53	0.02	1.59	90.44
5000	6.10	0.14	2.30	100.39
Inter-day precision ($n = 3$)				
Non-spiked	1.13	0.06	5.64	
500	1.56	0.04	2.77	86.23
5000	6.09	0.23	3.79	99.14

Table 4
The amount of DNJ found in. Mulberry leaves in seven cultivars and in three portions of the plants harvested in July 2005

Cultivar	DNJ concentration (mg/g)[a]		
	Shoots	Young leaves	Mature leaves
Burirum 60	2.24 ± 0.05	0.62 ± 0.02	0.47 ± 0.02
Chiang Mai	2.62 ± 0.09	1.59 ± 0.06	0.96 ± 0.03
Khun Phai	3.08 ± 0.16	1.61 ± 0.02	0.72 ± 0.04
Nakhonratchasima 60	2.42 ± 0.13	0.93 ± 0.02	0.67 ± 0.02
Noi	2.97 ± 0.16	0.83 ± 0.09	0.48 ± 0.02
PMN9	2.83 ± 0.13	1.27 ± 0.09	0.70 ± 0.01
PMN14	2.43 ± 0.11	1.30 ± 0.06	0.63 ± 0.02

[a] Values are means ± standard deviations, $n = 3$.

4. Conclusions

An HPLC–MS/MS method for the quantitative determination of DNJ in mulberry leaves was developed and validated. Only simple sample preparation step was needed. The method gave high sensitivity and selectivity. It can be applied as routine quality control procedure for the determination of DNJ both in raw materials used for manufacturing and the herbal products in the market.

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