SUPPLEMENTARY MATERIAL

Metabolism, transformation and dynamic changes of alkaloids in silkworm during feeding mulberry leaves

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Abstract: Metabolism, transformation and dynamic changes of five alkaloids, DNJ, 2-O-\textalpha-D-Gal-DNJ, fagomine, isofagomine and 4-O-\textbeta-D-Glc-fagomine from mulberry leaves in silkworm at different instars was observed. UPLC-Q/TOF-MS and UPLC-TQ/MS methods were adopted for qualitative analysis and quantitative analysis respectively. Mulberry leaves of three species were used to feed the silkworm as controls. By analyzing and comparing the content changes of DNJ, fagomine and their derivatives in different instars’ silkworms and silkworm excrement, we revealed the dynamic changes, confirmed the enrichment effect on the polyhydroxy alkaloids by silkworm, and inferred the conversion process behind this effect. The experimental result indicated that DNJ and its derivatives turned into some intermediate substances in the metabolic process, and finally they converted back and the
content increased. Fagomine and its derivatives interconverted into each other in the process, 4-O-β-D-Glc-fagomine transformed into fagomine, while fagomine transformed into isofagomine.

**Keywords:** mulberry leaves; silkworm; alkaloids; dynamic changes; metabolism; transformation
1. Experimental

1.1. Instruments and equipment

Waters ACQUITY UPLC system (Waters, USA). Xevo Mass Spectrometer Detector (Waters). MassLynx™ Mass Spectrometer Workstation Software (Waters). EPED-E2-30TF ultra-pure water machine (Nanjing Yeap Esselte Technology Development Co., Ltd.). KQ-250E ultrasonic cleaner (Kunshan Wo Chong Ultrasonic Instrument Co., Ltd.). Anke GL-16GII centrifuge (Shanghai Anting Scientific Instrument Factory). BT125 electronic balance (Sartorius Scientific Instrument Co., Ltd.).

1.2. Materials and reagents

DNJ (T-021-120611) standard substance was purchased from National Institutes for Food and Drug Control, fagomine (3-MSC-25-6) standard substance was purchased from Toronto Research Chemicals Inc.

Mulberry leaves, silkworm and silkworm excrement were collected in June, 2013 in the Institute of Sericulture, Chinese Academy of Agricultural Sciences, Jiangsu University of Science and Technology. Baiyu × Qiufeng silkworm samples were collected from the National Silkworm Germplasm Resource Bank of Jiangsu University of Science and Technology. We fed them the mulberry leaves of three varieties of 9703, 711 and Jihu4, respectively as controls.

1.3. Experimental method

1.3.1. Preparation of samples

We selected Baiyu×Qiufeng silkworm and fed them the three species mulberry leaves of 9703, 711 and Jihu4, respectively. We divided groups according to the silkworm instars and each group took three parallel experiments, recorded the weight of mulberry leaves eat by silkworm. The different instars’ molting silkworm and each day’s silkworm in fifth instar, starvation treatment 24 h, after being freeze-dried, crushed and sieved, preserved at -80 ºC.
The mulberry leaves of 9703, 711 and Jihu4, and the different instars’ silkworm excrement were selected, after being freeze-dried, crushed and sieved, preserved at -80 ºC.

1.3.2. Standard curve

We chose a series of reference solutions of DNJ and fagomine with different concentration, determined them under the chromatography conditions. The concentration of reference solutions (X) is used as abscissa and the integrations of the chromatographic peak area (Y) used as ordinate. The linear regression equation, Linear Range, LOD and LOQ of the five alkaloids are shown in Table S2. The content of 2-O-α-D-Gal-DNJ was calculated by using DNJ as reference, while the content of isofagomine and 4-O-β-D-Glc-fagomine was calculated by using fagomine as reference.

1.3.3. Methodological study

1.3.3.1. Precision. The mother liquor of reference substance was injected 6 times under the chromatography conditions. The RSDs of the contents of DNJ and fagomine were 1.0% and 1.1% respectively.

1.3.3.2. Reproducibility. The same sample solution was injected 6 times under the conditions. The RSDs of the content of DNJ and fagomine were 1.2% and 1.1%.

1.3.3.3. Stability. The same sample solution was injected at 0, 4, 8, 12, 24, 48 h. The RSDs of the content of DNJ and fagomine were 0.8% and 1.0% respectively.

1.3.3.4. Recovery. We disposed nine samples which the contents of alkaloids were known, added the reference of DNJ and fagomine according to the known mass fraction of 80%, 100% and 120% to the nine samples. The recoveries were 94.2% and 97.6%, and the RSDs were 2.6% and 2.5%, respectively.

2. Tables and Figures

Table S1 The MRM parameters of the five alkaloids
| Analyte               | [M+H] - (m/z) | MRM transitions       | Cone voltage (eV) | Collision energy (eV) |
|----------------------|--------------|-----------------------|-------------------|-----------------------|
| Isofagomine          | 147.90       | 147.90→111.98         | 22                | 14                    |
| 2-O-α- D-Gal-DNJ     | 325.95       | 325.95→163.98         | 20                | 18                    |
|                      |              | 325.95→82.25          |                   |                       |
| 4-O-β-D-Glc-Fagomine | 310.30       | 310.30→147.99         | 22                | 14                    |
|                      |              | 310.30→111.98         |                   |                       |
| DNJ                  | 163.33       | 163.33→82.25          | 20                | 18                    |
| Fagomine             | 147.90       | 147.90→111.98         | 22                | 14                    |

Table S2 The standard curve, Linear Range, LOD and LOQ of the five alkaloids

| alkaloids              | standard curve       | $r^2$  | Linear Range (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|------------------------|----------------------|-------|----------------------|-------------|-------------|
| DNJ                    | Y=2867x+1341         | 0.9997| 1.45~14.5            | 0.035       | 0.138       |
| 2-O-α- D-Gal-DNJ       | Y=2867x+1341         | 0.9997| 1.12~32.5            | 0.037       | 0.140       |
| Fagomine               | Y=54400x+10534       | 0.9998| 1.26~17.8            | 0.0098      | 0.028       |
| Isofagomine            | Y=54400x+10534       | 0.9998| 1.2~12               | 0.0078      | 0.024       |
| 4-O-β-D-Glc-Fagomine   | Y=54400x+10534       | 0.9998| 0.42~22.6            | 0.010       | 0.032       |

Figure S1 UPLC-TQ/MS of five alkaloids
Figure S2 The possible metabolic transformation process of DNJ in silkworm excrement

Figure S3 The possible degradation reaction of glycosides in silkworm excrement

Figure S4 The possible Isomerization Reaction in silkworm excrement