

## Determination of Nectar Nicotine Concentration in *N. attenuata*

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**[Abstract]** In this protocol, the determination of the nicotine concentration in nectar of *Nicotiana attenuata* is described. This method is applicable for the investigation of small amounts of nectar (above 1  $\mu$ l). It is a high-throughput protocol optimized and streamlined for one skilled person to process approximately 100 nectar samples per day.

### **Materials and Reagents**

1. *Nicotiana attenuata*
2. Nicotine-D3 (Cambridge Isotope laboratories, catalog number: DLM-1818-0.5)
3. Ammonium hydroxide 25% (Fluka, catalog number: 44273-100 ML-F)
4. Methanol (Merck KGaA, catalog number: 1.06007.2500)
5. Solvent A (see Recipes)
6. Solvent B (see Recipes)

### **Equipment**

1. Varian 1,200 triple quad LC-MS system
2. Phenomenex Gemini NX 50 x 2 mm column, 3  $\mu$ m C18 (Phenomenex, catalog number: 593381-9)
3. 1.5 ml Eppendorf tubes
4. 1.5 ml GC vials ([www.wicom.de](http://www.wicom.de))
5. 25  $\mu$ l glass capillary (BLAUBRAND<sup>®</sup>, intraMark) (BRAND GMBH + CO KG, Wertheim, catalog number: 708722)

### **Procedure**

1. The best time to collect nectar from *N. attenuata* is in the morning between 4:00 and 7:00 am, as the maximum nectar accumulation is reached around 4:00 am and remains stable until the sun rises or the lights in the glasshouse are switched on. As flowers of *N. attenuata* remain open for two nights, one must make sure that the same floral stage is

used for collections. Usually newly opened flowers are used, which requires removal of all open flowers 24 h before the actual sampling in order to be sure to collect floral nectar from the same floral stage-- one- and two-day-old flowers are hard to tell apart from each other.

2. Nectar from flowers is collected by inserting a clean 25  $\mu$ l glass capillary into the corolla tube until it reaches the base of the nectaries (Figure 1). With practice, a complete nectar sample can be obtained by holding the capillary with one hand and the corolla tube with the other and removing the tube against the counter-pressure of the inserted capillary (Video 1). This technique requires some training in order to avoid damage to the ovary and contamination of the nectar. Alternatively, the complete corolla can be detached from the rest of the flower by simply pulling on the corolla tube. The nectar remains at the base of the tube from which it can be collected with a 25  $\mu$ l glass capillary (Video 2).



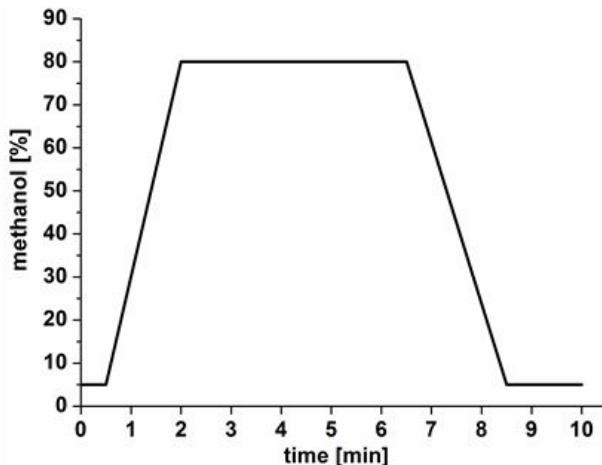
**Figure 1. Floral nectar collection with a glass capillary**

**Video 1. Nectar collection from *N. attenuata* flowers - method 1**



**Video 2. Nectar collection from *N. attenuata* flowers - method 2**

3. Nectar of single flowers is collected separately in 1.5 ml Eppendorf tubes. 2  $\mu$ l or 1.5  $\mu$ l (for flowers with low nectar volume) of nectar are transferred into a new tube containing 400  $\mu$ l water and 20 ng of the internal standard nicotine-D3.
4. Particles are removed by centrifugation (10 min at 12,000  $\times$  g, at 4 °C) and the supernatant is transferred into 1.5 ml GC vials.
5. 10  $\mu$ l of the solution are analyzed using a Varian 1,200 triple quad LC-MS system (<http://www.varianinc.com>) connected to an ESI source with a capillary voltage of 35 V, solvent A ; Solvent B. The gradient (min/% B): 0/5; 0.5/5; 2/80; 6.5/80; 8.5/5; 10/5 (Figure 2) is used with a Phenomenex Gemini NX 5  $\times$  2 mm column, particle size 3  $\mu$ m. The pH of the mobile phase is the most important parameter which determines nicotine's retention on the column. The starting conditions focus nicotine on the column, while sugars and salts are eluted. The steep gradient guarantees sharp nicotine peaks. The time for reconditioning the column is strongly instrument dependent. The short reconditioning time in our program is adapted to accommodate our slow auto sampler. The transition of the precursor ion nicotine  $[M + H]^+$  = 163 and nicotine-D3  $[M + H]^+$  = 166 to the fragment ( $m/z$ ) = 130 at a collision energy of 14.5 V is recorded for quantification. Quantification was achieved by isotope dilution and can be calculated by the following formula: amount nicotine (ng/ $\mu$ l nectar) = area of targeted compound/area ISD (internal standard) = nicotine - D3)  $\times$  amount ISD (ng/ $\mu$ l nectar).



**Figure 2. Methanol gradient**

## Recipes

1. Solvent A
2. Add 1 ml of 25% ammonium hydroxide solution to 1 L Milipore H<sub>2</sub>O  
Mix carefully, adjust pH to 10 [according to the pka of nicotine, which is 8.05 (Fujita *et al.*, 1971) the pH should be 10 or higher, to maintain nicotine in its neutral form. The upper pH is limited by the column chemistry and may be adjusted with concentrated ammonia or a few drops of 1:10 diluted formic acid].
3. Solvent B  
Methanol

## Acknowledgments

This work was supported by the Max Planck Gesellschaft. The protocol was adapted from the publication: Kessler *et al.* (2012).

## References

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2. Kessler, D., Bhattacharya, S., Diezel, C., Rothe, E., Gase, K., Schottner, M. and Baldwin, I. T. (2012). [Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in Nicotiana attenuata](#). *Plant J* 71(4): 529-538.