# [$^{11}$C]Starch Analysis in *Nicotiana tabacum*: Using a Combination of Spectrophotometry, Thin Layer Chromatography and Autoradiography

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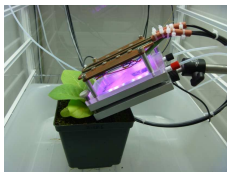
**Introduction:** Starch is one of the most important carbohydrates in the human diet. It is a polysaccharide with the chemical formula $(C_6H_{10}O_5)_n$ which consists of a large number of α-D-glucose-units. This macromolecule is produced in all green plants as an energy store. Starch synthesis starts with $CO_2$ which is converted into glucose in the photosynthesis process, using the energy from sunlight. The glucose is stored mainly in form of starch granules in chloroplasts. The goal of this experiment was to develop new and rapid bioassays for quantifying plant uptake of radioactive carbon-11 as $^{11}CO_2$. To investigate how much of the $^{11}CO_2$ was fixed into glucose and how much [$^{11}$C]glucose was converted into starch in *Nicotiana tabacum*, a combination of spectrophotometry, TLC (thin layer chromatography) and autoradiography was used.

**Experimental:** $^{11}CO_2$ ($^{11}$C $t_{1/2}$=20.38 min.) was produced *via* the $^{14}N(p,\alpha)^{11}C$ nuclear transformation from a 50 ml volume high-purity nitrogen gas target using 17 MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at BNL, and captured on a molecular sieve (4A) [1]. Two types of *Nicotiana tabacum* were supplied with $^{11}CO_2$ gas, a wild type (WT) and an asparaginase overexpressor type (AsNase) which produces more starch at the expense of protein. These two plant samples were prepared by using 10 mg frozen plant tissue for each sample, adding 80% ethanol, heating for 5 min at 85°C and centrifuging for 2 min at 21130 rcf to get rid of free sugars and amino acids and to get a starch pellet. The *Starch Assay Kit* (Sigma Aldrich, Product Code STA20) was used for the starch digestion: [$^{11}$C]Starch was broken down to [$^{11}$C]glucose by adding the digestion enzymes *α-Amylase* and *Amyloglucosidase*:



$$\text{Starch} + (n\text{-}1)\,H_2O \xrightarrow{\text{Amylase, Amyloglucosidase}} n\ \text{(D-glucose)}$$

At this point, two methods can be used for quantitative glucose analysis: (a) spectrophotometry and (b) TLC/autoradiography:

**(a)** Glucose is oxidized to gluconic acid and hydrogen peroxide by adding *Glucose Oxidase* (all enzymes from *Starch Assay Kit)* to both samples:

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{D-gluconic acid} + H_2O_2$$

Hydrogen peroxide reacts with *o-dianisidine* in the present of *Peroxidase* to form a colored product:

$$H_2O_2 + \underset{\text{(colorless)}}{\text{reduced o-Dianisidine}} \xrightarrow{\text{Peroxidase}} \underset{\text{(brown)}}{\text{oxidized o-Dianisidine}}$$

Reaction stops by adding sulfuric acid and a more stable colored product is formed:

$$\underset{\text{(brown)}}{\text{oxidized o-Dianisidine}} \xrightarrow{H_2SO_4} \underset{\text{(pink)}}{\text{oxidized o-Dianisidine}}$$

The intensity of the pink color, measured at 540 nm, is proportional to the glucose concentration. A glucose standard curve was created by measuring the intensity of known glucose concentrations (Figure 1).

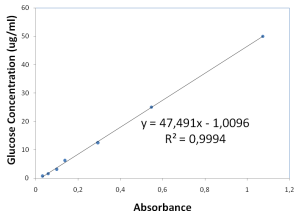



*Figure 1: Glucose Standard Curve (Spectrophotometry)*

**(b)** A glucose standard curve for TLC analysis (Figure 2) was made by spotting known glucose concentrations on Silica gel TLC (60 F254, EMD) and measuring the spot density. The TLC was developed in a solvent of 73.5% acetonitrile and 26.5% water. The glucose spots were visualized by dipping into a mixture of 1-naphtol, ethanol, water and sulfuric acid and heating with a hot air gun.

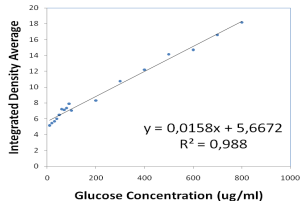



*Figure 2: Glucose Standard Curve (TLC)*

**Results**: Both analytical methods showed a higher glucose concentration in the transgenic plant than in the wild type *Nicotiana tabacum* (Table 1).

| sample | Spot Density | Absorbance | Calculated Glucose Concentration (µg/ml) |
|---|---|---|---|
| WT | 10.657 | 0.404 | 302.23 |
| AsNase | 11.657 | 0.411 | 329.12 |

*Table 1: Glucose Concentration (µg/ml)*

5.5% of $^{11}CO_2$ was fixed in starch in the wild type *Nicotiana tabacum*. The starch specific activity is 0.913 µCi/mg. As expected, the asparaginase overexpressor type (AsNase) fixed more $^{11}CO_2$ than the wild type: 7.7% of $^{11}CO_2$ with the starch specific activity 1.040 µCi/mg. Figure 3 shows (a) the TLC plate with 6 spots and (b) the same TLC plate as an autoradiography image. The four left spots on (a) TLC are standard glucose concentrations (600, 500, 400 and 300 µg/ml). The 5th spot is glucose of the wild type *Nicotiana tabacum* and the 6th spot is glucose of AsNase *Nicotiana tabacum*. [$^{11}$C]Glucose spots of both *Nicotiana tabacum* types are visible in (b) autoradiography image.

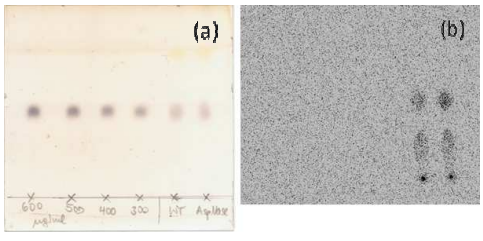



*Figure 3: (a) TLC, (b) Autoradiography Image of TLC*

**References**
[1] N. Hanik, S. Gomez, M. Best, M. Schueller, C.M. Orians, R.A. Ferrieri, *J Chem Ecol* **36**,1061 (2010).

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