

Intrinsic water use efficiency estimate: an isotopic method

Protocol from sampling to analysis



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Protocol summary and key steps

This protocol describes in detail how to calculate the intrinsic water use efficiency (WUE_i) from ^{13}C measurements in phloem samples collected at breast height in trees. Basically, the isotopic signature of photosynthesized carbohydrates ($^{13}\text{C}/^{12}\text{C}$) in the phloem sap inform on the assimilation condition: a higher ($^{13}\text{C}/^{12}\text{C}$) is associated with a lower transpiration rate, increasing heavy isotope (^{13}C) use by the plant (Dawson et al. 2002). A higher $^{13}\text{C}/^{12}\text{C}$ in the phloem content reflect a higher proportion of assimilated C compared to transpiration rate, in other words, a higher WUE_i . The method described here is based on the isotopic discrimination of $\delta^{13}\text{C}$ in trees and considers ecophysiological processes of stomatal and mesophyll conductance (g_s and g_m , respectively). The protocol aims to provide the operator with a step-by-step method from the collection of phloem samples in the field to the final calculation. The protocol is already described in the scientific literature (Klein et al. 2016; Seibt et al. 2008; Vernay et al. 2020) but this document addresses more practical and technical details that a practitioner might expect. WUE (water use efficiency) corresponds to plant productivity or the productivity of an organ of the plant regarding the amount of water used to produce this fresh matter (Thérour Rancourt 2014). At an individual scale, WUE considers a ratio of the biomass produced compared to the amount of water used to produce this biomass. At the leaf level, WUE_i (intrinsic WUE) determines the ratio of CO_2 assimilation per amount of water transpired and can take into account the micro-environment of the leaf, by considering g_s/g_m to be more precise (Violet-Chabrand 2013).

Introduction

Water use efficiency is defined as the amount of CO_2 taken up by plant photosynthesis relative to the amount of water lost through transpiration or evapotranspiration (Cernusak 2018). It can be determined using several methods according to the targeted scale for the study and the experimental design of the experiment (Zhang et al. 2023). Briefly, intrinsic water use efficiency (WUE_i) considers ecophysiological processes driving CO_2 influx and water efflux without being sensitive to vapour pressure deficit (but see de Almeida Lobo et al. 2023). Therefore, this method, applicable on C_3 plants, is particularly suitable for estimating WUE at the tree scale. Calculations of WUE_i have recently been improved to embed the impact of mesophyll conductance on CO_2 diffusion in leaf cells (Stangl et al. 2019; Vernay et al. 2020).

WUE_i is an interesting integrative variable to characterize plant stress at scales from individual to ecosystem level. Compared to remote methods such as eddy-covariance measurements, WUE_i brings information on tree ecophysiology and may give a functional ecology perspective to results. For instance, associated to sap-flux measurements you can estimate the gross primary production at stand scale (Vernay et al. 2020) and even quantify the contribution of the different species in mixed-forest (Vernay et al., under review). WUE_i is a critical variable helping to understand how forest may help to mitigate carbon accumulation (Zhang et al. 2023). A recent global study showed that WUE_i increased as the atmospheric concentration increased but this tree functioning accommodation seems to slow down now, which could be interpreted as a limit for tree accommodation to global change (Adams, Buckley, et Turnbull 2020). There are still some limitations in the calculations of WUE_i , mainly due to some lack of knowledge about mesophyll conductance in the leaves (Stangl et al. 2019, 2022) or in the

fractionation processes occurring from the carbon assimilation to its transfer in the phloem (Busch et al. 2020; Tcherkez et al. 2017; Vernay et al. 2020). Despite these challenges that will be hopefully solved in the future, actual WUE_i calculations are strongly reliable.

The cited literature provides more details on WUE_i , and we encourage readers to consult the references to delve deeper into the different concepts. This document is addressed to practitioners or field experimenter and give them an easy-to-read and turnkey protocol to carry out sample collection. It covers sample collection, sample preparation prior to spectrometer analysis and WUE_i calculation (spectrometer analysis is not described as it was contracted out to a platform). The different steps necessary to calculate WUE_i described in this protocol are summarized in Figure 1.

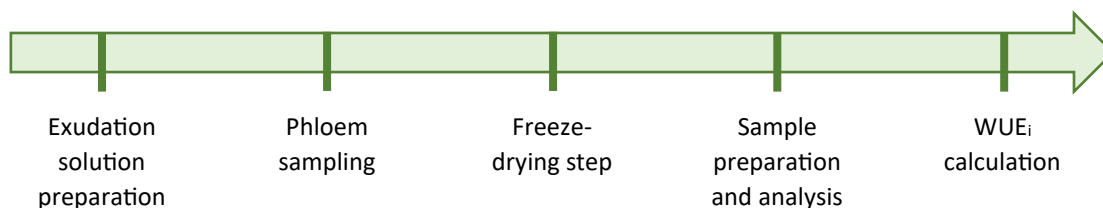


Figure 1: Time-scale summarizing the different steps of the protocol to estimate WUE_i of tree

Materials

- Exudation solution preparation
 - Eppendorf tubes (2mL)
 - Deionized water (volume depends on the number of Eppendorf tubes you will use)
 - Sodium hexametaphosphate (65 – 70% P_2O_5 basis from sigma-aldrich)
 - Becher (size depends on the total volume you need for all your Eppendorf tubes)
 - Magnetic mixer
 - Magnet
 - Pipet
- Phloem sampling:
 - Hammer
 - Cork-corer (8-12mm in diameter)
 - Knife or scalpel
 - Grafting sealant
 - Eppendorf (1.5-2mL) filled with exudation solution.
- Freezer (-80°C)
- Freeze-dryer and pump
 - Device functioning described in this protocol: CHRIST ALPHA 1-4 LD_{plus} → has to be adapted according to your equipment
 - Pump
- Sample preparation for spectroscopy analysis
 - Thin capsules
 - Laboratory clamp

- 96 wells microplates
- Oven
- Deionized water (150µL per sample)

Before field work

Tree labelling: If you plan to sample different tree, prepare a handy labelling protocol. You will use this labelling for the Eppendorf tubes (that are relatively small!) and spectroscopy analysis.

Preparation of exudation solution

The exudation solution is a polyphosphate buffer (Gessler, Rennenberg, et Keitel 2004) which facilitates the solubilization of the phloem content. Therefore, phloem samples collected in the field must be immersed into this solution to collect C content. It must be prepared before going out into the field and can be stored in the fridge for several days before going on the field.

To prepare this solution, you need Sodium hexametaphosphate (65 – 70% P₂O₅ basis from sigma-aldrich) and deionized water.

Preparation for 1 Eppendorf tube filled with 2 mL of the solution:

- Molar concentration (sodium hexametaphosphate) = $C = 15\text{mM} = 0.015 \text{ mol/L}$
- Amount of product = $n = C \times V = 0.015 \times 0.002 = 0.00003 \text{ moles}$
- Molar mass (sodium hexametaphosphate) = $M = 611.77 \text{ g/mol}$
- Mass of product to use = $m = n \times M = 611.77 \times 0.00003 = 0.0183531 \text{ g}$

So, for an Eppendorf tube of 2 mL, you need to add 0.02 g of sodium hexametaphosphate in 2mL of deionized water.

Samples collection

Position the cork-corer at breast height (~1.3m above the forest floor) perpendicular to the trunk and hit with the hammer until the wood layer is reached. This operation do not have detrimental consequences for the tree functioning and should not influence next sampling campaign, if you repeat the sampling (Portier et al. 2023).

Remove the cork-corer from the trunk.

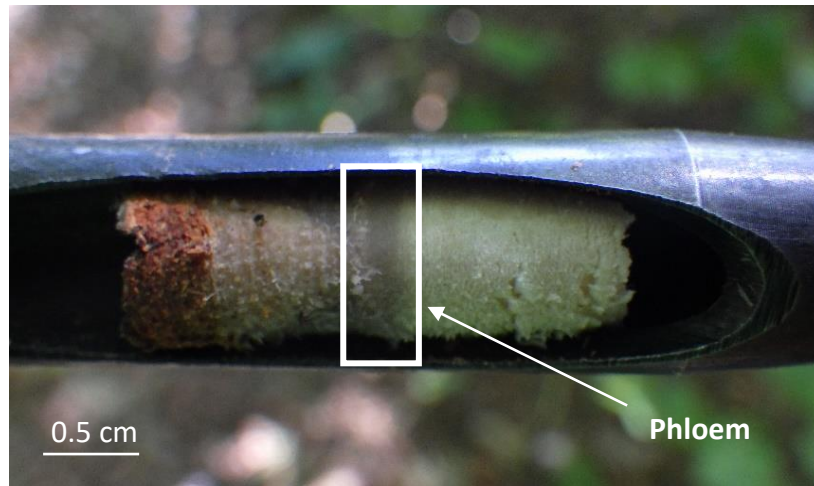


Figure 2: Phloem sample in the core extracted from a tree trunk at breast height.

Once you have extracted the core as shown in Figure 2, carefully collect it by removing the phloem tissue, the thin wet parts located between the cork and the wood with the scalpel. Field collectors report that the core may break when you remove it from the cork-corer. The phloem disc could be on one of the sides of the two parts of the core, you recognize it as it is a wet tissue, soft and easy to cut compared to cork or wood.

Then place the phloem disc in an Eppendorf tube filled with an exudation solution, correctly labelled according to the tree sampled and lay it down a couple of hours, until you return to the lab.

Fill the hole in the tree by putting back the woody part and the cork part previously removed from the core. Then, seal the hole with grafting sealant.

Back to the lab

Remove phloem discs from the Eppendorf tube and store the tubes in a freezer at -80°C before freeze-drying step.

Freeze dryer

Before analysis, samples must be freeze-dried to remove water from the samples, turning it directly from ice to vapour (sublimation). This step avoids modification of the ^{13}C content by microorganisms which can occur when samples return to the liquid phase. Samples must therefore remain frozen.

Tubes' lids must be perforated to allow solid water to evaporate during lyophilization.

The following steps are valid for the freeze-drier we use in our lab and the procedure should be adjusted to your own equipment.

Freeze-dryer functioning:

- Pump (EDWARDS® vacuum, mod. 5)
 - Switch it on approximatively 20 min before using it to pre-heat the device.
 - Verify that there is enough oil in the pump.

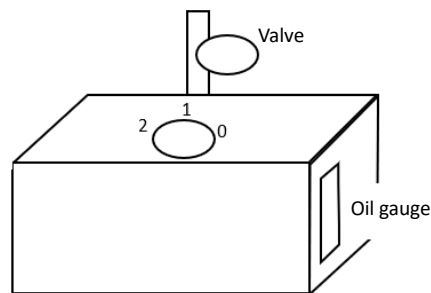


Figure 4: Scheme of the freeze-dryer's pump

- Freeze-dryer
 - Switch on using the button on the right of the machine.
 - Place your Eppendorf tubes with a holed cap to remove water from the sample.
 - Put the cover back on top.
- To begin the manipulation:
 - Turn clockwise the valve on the left of the freeze-dryer to close it
 - Choose the mode "primary desiccation".
 - If asked, choose "NO" for the pre-heating of the pump (choose "YES" if you didn't switch it on 20 min beforehand)
 - Vacuum the freeze-dryer chamber by raising the pump valve slightly, the value of pressure indicated on the screen needs to decrease.
 - Check that there are no air leaks. If so, lower the valve to close and look for the leak. Otherwise, keep raising the valve.
 - When pressure on the screen indicates a value < 0.1 mbar, close the air ballast on 0 on the pump.
- At the end of the manipulation:
 - The samples must be dry: leave them drying for 2 to 3 days, then take them back.
 - Close the valve of the pump by lowering it and put the air ballast on 2.
 - Turn anticlockwise the valve at the left of the freeze-dryer to open it.
 - Turn off the freeze-dryer with the right button.
- Turn off the pump.

Samples preparation for spectrometry

- After removing Eppendorf tubes from the freeze-drier, add 150 μ L of distilled water in each Eppendorf tube.
- Rock the solution by pipetting several times in the Eppendorf tubes and transfer 50 μ L of this solution in a tin capsule located in 96 wells microplate.
- Repeat this for each sample.
- Place the microplate at least 12h in an oven at 60°C.
- Close the thin capsule with laboratory clamps by folding the capsule.
- Give your microplate to spectroscopy platform for analysis. Be careful, they may ask you to add or leave some wells empty in order to add standards. **Check with the colleagues carrying this step out before filling your microplate (Figure 5).**

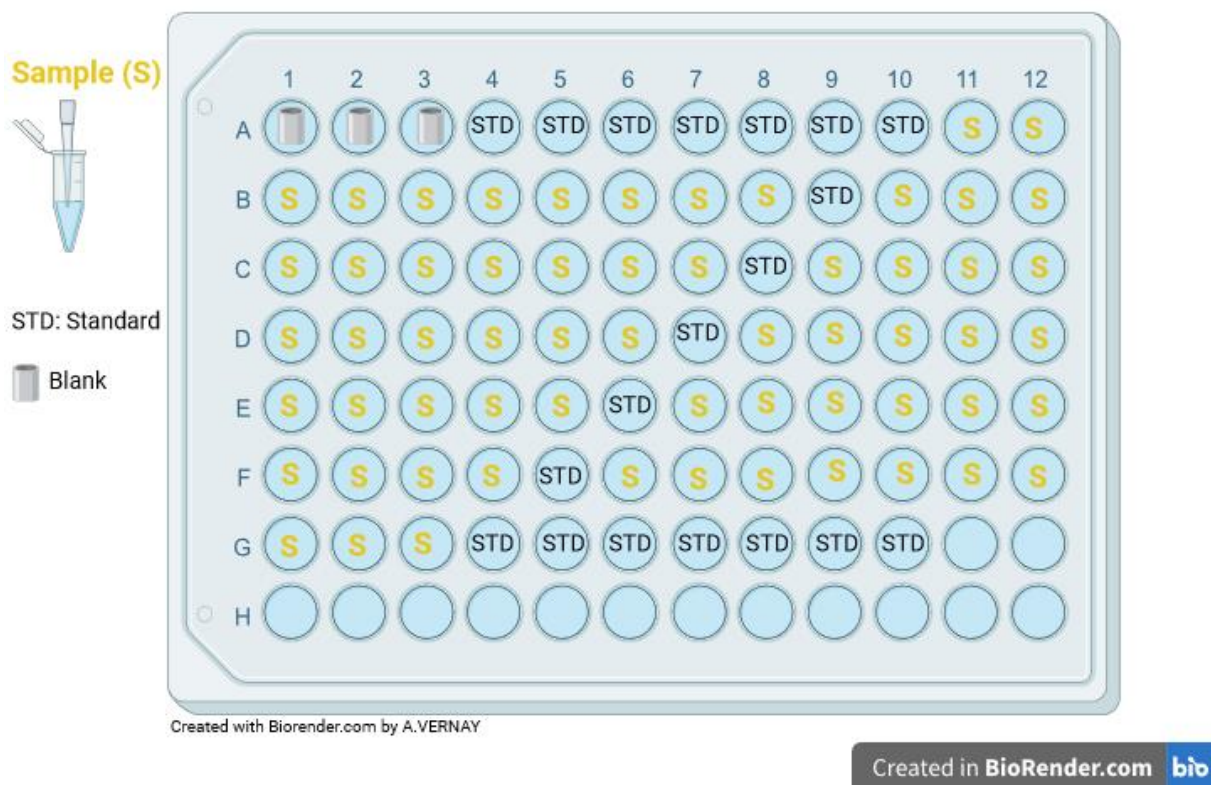


Figure 5: Example of a 96-wells microplate filled with tin capsule containing samples (S) and some standards (STD) according to spectrometry platform instructions.

Intrinsic water use efficiency calculation

$\delta^{13}\text{C}$ (per mille - ‰) value represents the ratio between ^{13}C and ^{12}C , two stable isotopes of carbon. It can vary in function of kinetic fractionation (Cernusak 2018). Plants preferentially assimilate ^{12}C because this isotope is lighter than ^{13}C . A more negative $^{13}\text{C} / ^{12}\text{C}$ ratio indicates that the organism captured more ^{12}C .

From $\delta^{13}\text{C}$ values, Δ (i.e. isotopic carbon discrimination) can be calculated as followed (‰ – (Farquhar, Ehleringer, et Hubick 1989)):

$$\Delta = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 + \frac{\delta^{13}\text{C}_p}{1000}}$$

where $\delta^{13}\text{C}_a$, the ratio of heavy to light ^{13}C isotope in the air, admitted constant at -8‰ (except for the highest and lowest latitudes) and $\delta^{13}\text{C}_p$, the ratio of heavy to light ^{13}C isotope in phloem content (your values of $\delta^{13}\text{C}$ obtained previously by spectrometer analysis).

Intrinsic water use efficiency (WUE_i) can be estimated as followed (Klein et al. 2016; Vernay et al. 2020):

$$\text{WUE}_i = \frac{C_a}{r} \times \frac{b - \Delta - f \times \frac{\Gamma^*}{C_a}}{b - a_a + (b - a_i) \times \frac{g_s}{g_m}}$$

with C_a , CO_2 atmospheric concentration (ppm), r the ratio of the diffusivity of water vapor to CO_2 in the air (1.6), a_a the fractionation of diffusion through air (4.4 ‰) and a_i the fractionation and dissolution in water (1.8 ‰), f the fractionation during photorespiration (16.2 ‰) and b the fractionation during carboxylation (29 ‰). g_m and g_s are the conductance of the mesophyll and the stomatal conductance respectively (in $\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{day}^{-1}$). The g_m/g_s ratio was estimated at $0.5 \text{ mol CO}_2 \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (Klein et al. 2016; Th  roux-Rancourt,   thier, et Pepin 2014) in our case for white poplar, but it varies with the species studied (Xiong, Douthe, et Flexas 2018). The value of g_s/g_m is still under debate: even if a recent study showed that a constant g_s/g_m gives a better estimate of WUE_i than considering g_m as a constant with a variable g_s , or worst, infinite (Vernay et al. 2020), more recent studies showed that this ratio has a seasonal pattern (Stangl et al. 2022). To keep modelling at the simplest, we recommend to use a constant g_s/g_m ratio, with species specific values or a value of 0.5 if there is no information in the literature (Klein et al. 2016; Seibt et al. 2008).

Γ^* ($\mu\text{mol} \cdot \text{mol}^{-1}$) correspond to the CO_2 compensation point:

$$\Gamma^* = 42.75 \times e^{\frac{37830 \times (T_K - 298)}{298 \times T_K \times R}}$$

with T_K the ambient temperature in Kelvin and R the universal gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$).

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