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A rapid laser probing method facilitates the non-invasive and contact-free determination of leaf thermal properties

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Abstract:	Plants can produce valuable substances such as secondary metabolites and recombinant proteins. The purification of the latter from plant biomass can be streamlined by heat treatment (blanching). A blanching apparatus can be designed more precisely if the thermal properties of the leaves are known in detail, i.e. the specific heat capacity and thermal conductivity. The measurement of these properties is time consuming and labor intensive, and usually requires invasive methods that contact the sample directly. This can reduce the product yield and may be incompatible with containment requirements, e.g. in the context of good manufacturing practice. To address these issues, a non-invasive, contact-free method was developed that determines the specific heat capacity and thermal conductivity of an intact plant leaf in about one minute. The method involves the application of a short laser pulse of defined length and intensity to a small area of the leaf sample, causing a temperature increase that is measured using a near infrared sensor. The temperature increase is combined with known leaf properties (thickness and density) to determine the specific heat capacity. The thermal conductivity is then calculated based on the profile of the subsequent temperature decline, taking thermal radiation and convective heat transfer into account. The associated calculations and critical aspects of sample handling are discussed.
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Aachen, April 7th 2016

Cover letter for invited manuscript

Dear Alison Hamlin,
We would like you to consider the enclosed manuscript, entitled "A rapid laser probing method facilitates the non-invasive and contact-free determination of leaf thermal properties" by Johannes Buyel *et al.*, for publication in *Journal of Visualized Experiments*. We have written this manuscript upon your personal request.
The manuscript describes a contact-free non-destructive near infrared (NIR) laser probing method to determine the specific heat capacity and thermal conductivity of leaves and other solid biological samples. The two thermal parameters are important for the design of equipment used for heat treatment of biological materials as in food and feed applications as well as in molecular pharming. Additionally, the method will be of interest to environmental biologists. We used an approach that requires minimal laboratory equipment, that can be set up quickly and facilitates a high sample throughput with measurement times of three minutes per specimen. The method comprises a two-staged measurement phase. First, the sample is heated up locally by a short laser pulse after which the specific heat capacity of the sample is calculated based on the maximum temperature difference between sample and environment as well as its mass. Next, the temperature profile of the sample is recorded with an infrared sensor for up to one minute. The observed decrease in temperature over time is combined with an energy balance for conduction, convection and thermal radiation to calculate the thermal conductivity of the sample. Hence, both thermal properties, specific heat capacity and thermal conductivity, can be determined in a single rapid measurement which is contact-free and non-destructive. These are important advantages that, to our knowledge, have not been reported for previously described methods. In the protocol section of the manuscript we provide all the required details to prepare the samples, set up the apparatus for measurement, collect the temperature profiles and conduct the calculations for the specific heat capacity and thermal

conductivity. In the results section we provide data acquired for two plant species, *Nicotiana tabacum* and *N. benthamiana*. We discuss the parameters that have the strongest impact on the measurement and calculations as well as approaches how both can be improved. If solely based on a written description, the protocol may be difficult to reproduce, especially the measurement apparatus. Therefore, visual instructions will greatly facilitate the implementation and success rate of the method when transferred to other laboratories, which is why we believe the manuscript will be of great interest to your readers.
Johannes Buyel contributed the computational work, data, text, figures and scientific knowledge as well as experiment planning and data interpretation in this manuscript. Hannah Gruchow contributed the laboratory work, data interpretation, manuscript writing and figure design. Martin Wehner provided expert knowledge in optics as well as engineering expertise to effectively set up the apparatus. During the submission process, we received assistance from you, Alison Hamlin, Associate Editor - Environment of the *Journal of Visualized Experiments*.
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We look forward to hearing from you in due course.
Yours sincerely,

(Dr. Johannes F. Buyel, Head of Department)

TITLE:
A rapid laser probing method facilitates the non-invasive and contact-free determination of leaf thermal properties

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KEYWORDS:
Heat capacity; Near infrared laser; Plant-derived biopharmaceuticals; Plant growth monitoring; Process optimization; Thermal conductivity

SHORT ABSTRACT:
A method was developed to determine the specific heat capacity and thermal conductivity of leaf tissue by non-invasive, contact-free near infrared laser probing, which requires less than 1 min per sample.

LONG ABSTRACT:
Plants can produce valuable substances such as secondary metabolites and recombinant proteins. The purification of the latter from plant biomass can be streamlined by heat treatment (blanching). A blanching apparatus can be designed more precisely if the thermal properties of the leaves are known in detail, i.e. the specific heat capacity and thermal conductivity. The measurement of these properties is time consuming and labor intensive, and usually requires invasive methods that contact

the sample directly. This can reduce the product yield and may be incompatible with containment requirements, *e.g.* in the context of good manufacturing practice. To address these issues, a non-invasive, contact-free method was developed that determines the specific heat capacity and thermal conductivity of an intact plant leaf in about one minute. The method involves the application of a short laser pulse of defined length and intensity to a small area of the leaf sample, causing a temperature increase that is measured using a near infrared sensor. The temperature increase is combined with known leaf properties (thickness and density) to determine the specific heat capacity. The thermal conductivity is then calculated based on the profile of the subsequent temperature decline, taking thermal radiation and convective heat transfer into account. The associated calculations and critical aspects of sample handling are discussed.

INTRODUCTION:

The large-scale processing of biological materials often requires heat-treatment steps such as pasteurization. The equipment for such processes can be designed more precisely if the thermal properties of the biological materials are well characterized, including the specific heat capacity ($c_{p,s}$) and thermal conductivity (λ). These parameters can be determined easily for liquids, suspensions and homogenates by calorimetry [1]. However, measuring such parameters in solid samples can be labor intensive, and often requires direct contact with the sample or even its destruction [2]. For example, photothermal techniques require direct contact between the sample and detector [3]. Such limitations are acceptable during food processing, but are incompatible with highly regulated processes such as the production of biopharmaceutical proteins in plants in the context of good manufacturing practice [4]. In such a context, repeated (e.g. weekly) monitoring of thermal properties may be required during a seven-week growth period for individual plants as a quality control tool. If such a monitoring would require and consume a leaf for each measurement, there would be no biomass left to process at the time of harvest.

Additionally, using only leaf parts instead would cause wounding to the plant and increase the risk of necrosis or pathogen infection, again diminishing the process yield. The likelihood of pathogen infection may also increase if a method with direct contact to the sample would be used, inducing the risk that an entire batch of plants can be infected through contact with a contaminated sensor device. Similar aspects have to be considered for the monitoring of plant stresses like drought, e.g. in an ecophysiological context. For example, water loss is often monitored by a change in the fresh biomass, which requires an invasive treatment of the plants under investigation [5], e.g. dissecting a leaf. Instead, determining the specific heat capacity, which depends on the water content of a sample, in a non-invasive manner as describe here, can be used as a surrogate parameter for the hydration status of plants. In both scenarios (pharmaceutical production and ecophysiology), artificial stresses induced by destructive or invasive measurement techniques would be deleterious as they can distort the experimental data. Therefore, previously reported flash methods [6] or the placement of samples between silver plates [7] are unsuitable for such processes and experiments because they either require direct contact to the sample or are destructive.

The parameters $c_{p,s}$ and λ must be determined in order to design the process equipment for a blanching step that can simplify product purification and thus reduce manufacturing costs [8-10]. Both $c_{p,s}$ and λ can now be rapidly determined by contact-free non-destructive near infrared (NIR) laser probing in a consistent and reproducible manner [11] and this new method will be explained in detail below. The results obtained with this method were successfully used to simulate heat transfer in tobacco leaves [12], allowing the design of appropriate processing equipment and the selection of corresponding parameters such as the blanching temperature.

The method is easy to set up (**Figure 1**) and has two phases, measurement and analysis, each of which comprises two major steps. In the measurement phase, a leaf sample is first locally heated by a short laser pulse and the maximum sample temperature is recorded. The temperature profile of the sample is then recorded for a duration of 50 s. In the analysis phase, leaf properties such as density (easily and accurately determined by pycnometric measurement) are combined with the maximum sample temperature to calculate $c_{p,s}$. In the second step, the leaf temperature profile is used as the input for an energy balance equation, taking conduction, convection and radiation into account, to calculate λ.

Detailed step-by-step instructions are provided in the protocol section, expanding on the contents of the accompanying video. Typical measurements are then shown in the results section. Finally, the benefits and limitations of the method are highlighted in the discussion section along with potential improvements and further applications.

[Place Figure 1 here]

PROTOCOL:

1. Plant cultivation and sample preparation

1.1) Flush each mineral wool block with 1–2 L of deionized water and subsequently with 1 L of 0.1% [m/v] fertilizer solution. Place one tobacco (*Nicotiana tabacum* or *N. benthamiana*) seed in each block and gently flush with 0.25 L of fertilizer solution without washing away the seed.

1.2) Cultivate the plants for 7 weeks in a greenhouse or phytotron with 70% relative humidity, a 16-h photoperiod (180 µmol s^{-1} m^{-2}; λ = 400–700 nm) and a 25/22°C light/dark temperature regime.

1.3) Move the plants to the measurement apparatus. If the plants are immobile, harvest single leaves for the measurement of thermal properties.

2. Determine leaf thickness and density

2.1) Determine the leaf thickness

2.1.1) Prepare a 2% [m/v] agarose solution in phosphate-buffered saline (PBS) and autoclave it. Let the solution cool down to 40°C and embed a leaf sample placed in a

Petri dish. Solidify the agarose by placing the Petri dish in a refrigerator at 4°C for 30 min.

2.1.2) Cut the agarose block into 200-µm slices using a vibratome with a razor blade cutting angle of 15°. Use a cutting velocity of 1.0 mm s^{-1} and an amplitude of 0.5 mm.

2.1.3) Mount five transversal leaf sections on a glass slide using cyanoacrylate as a fixative. Determine the leaf thickness under a microscope with a 20× objective and an eyepiece with 10× magnification, using the measurement tools built into the microscope software according to the manufacturer's instructions.

2.1.4) Determine the leaf thicknesses in sample areas without veins.

2.1.5) Alternatively, determine the leaf thickness with a dial-gauge at a vein-free area of the leaf blade. Make sure the dial-gauge is held perpendicular to the plane of the leaf blade.

CAUTION: Cyanacrylate is a skin irritant and may also glue fingers together if not handled with care.

2.2) Determine the leaf density
2.2.1) Determine the empty mass (m_0) of a dry pycnometer, then fill it with water and determine the mass again (m_1). Dry the pycnometer completely, place a leaf inside and determine the mass (m_2) once more. With the leaf inside, carefully fill up the pycnometer with water and determine the mass (m_3).

2.2.2) Calculate the leaf density (ρ_S) using Equation 1:

Equation 1: $\left(\frac{m_2-m_0}{(m_2-m_0)-(m_3-m_1)}\right) \times \rho_W = \rho_S$

3. Determine the spectral transmission and reflection of leaves

3.1) Place a leaf in the sample chamber of a UV/VIS spectrophotometer by fixing it between sample-holding clamps. For transmission measurements, place the leaf in front of the detector. For reflection measurements place the leaf at the rear of the detection chamber.

3.2) Launch the spectrophotometer control software. Select a spectrum from 900 nm to 1600 nm. Start a new scan and record the values for transmission (μ_T) and reflection (μ_R) displayed by the UV/VIS spectrophotometer software, based on the spectral curve.

3.3) Perform all measurements with at least three biological replicates. Increase the number of biological replicates to five or more if a heterogeneous sample quality can be expected, i.e. variation in leaf surface morphology and thickness.

3.4) Calculate the power for transmission (P_T) and reflection (P_R) by multiplying the measured μ_T or μ_R values by the measured laser power P_{Laser} according to Equations 2

and 3.

Equation 2: $P_T = \mu_T \times P_{Laser}$

Equation 3: $P_R = \mu_R \times P_{Laser}$

NOTE: The transmission can be also determined with a photodiode sensor during the measurement (see **6.3**).

4. Set up the measurement apparatus

4.1) Mount a fiber-coupled single-bar NIR diode laser (wavelength = 1550 nm) into a 25.4-mm diameter cone on a stainless-steel holder. Connect a controller to set the output power (P_{Laser}) of the NIR laser to 4–6 W.

4.2) Place a bi-convex lens with a focal length of 25.4 mm at the end of the cone to adjust the beam width to 13 mm.

4.3) Place a photodiode power sensor 354 mm below the bottom of the lens. Then attenuate the photodiode by placing a neutral density filter with an optical density of 1.0 and a 22-mm ceramic layer above the sensor.

4.4) Connect the photodiode power sensor to an oscilloscope using a coaxial cable.

4.5) Connect a 10 × 10 cm frame which has a 6 × 6 cm sample exposure area with the scaffold of the measurement setup at a height of 308 mm below the lens (**Figure 1**). Fix the leaf position in space by mounting it into the 10 × 10 cm frame.

4.6) Connect a NIR detector to a personal computer using a universal serial bus (USB) cable and install the interface software for the detector.

4.7) Place the detector at a 45° angle to the laser beam 135 mm above the ceramic layer. Align the measurement area of the detector to the laser spot on the sample by varying the sensor position and angle until the maximum temperature signal is observed.

4.8) Use the laser control interface software to adjust the output laser power to 5 W and the duration of the laser pulse to 0.5 s. Select the “Current control” command in the control options window below the graphical representation of the laser power and adjust the laser power by typing “5” into the “Power [W]” field. Adjust the laser pulse duration by typing “0.5” into the “Time [s]” field.

4.9) To determine the absolute laser power for each set of experiments, replace the photodiode power sensor with a thermal surface absorber power sensor at the end of each set of experiments and measure the laser output power for 20 s without a sample.

5. Prepare the leaf samples

5.1) Use intact and undamaged leaves for the measurements.

5.2) If relevant for the investigation, mimic typical leaf damage types by piercing the leaf with a scalpel, rubbing the leaf between latex gloves, exposing the leaf to an open flame or a laser beam for 2–3 s, or use other techniques to simulate other types of damage.

5.3) Carefully but quickly mount the leaf sample between sample-holding clamps.

6. Take the temperature measurements

6.1) Avoid direct contact between the leaf and the ceramic attenuator placed above the photodiode sensor to prevent artificial heat transfer that interferes with the calculation of $c_{p,s}$ and λ (see section 9).

6.2) Use the temperature measurement software to collect the temperature profile of the leaf sample for a total of 60 s via the NIR detector. First, record the temperature baseline for 10 s, then activate the laser for 0.5 s and continue data collection for 49.5 s.

6.2.1) Start a measurement by clicking “Measurement” and then “New Measurement”. Afterwards click the green arrow above the graphical representation of the thermal profile. Save the temperature profile by clicking on the “Save” icon (a stylized disk) above the graphical representation of the profile.

6.3) Confirm the transmitted laser power using the photodiode power sensor by calculating the difference in signal for measurements with and without a leaf sample using an oscilloscope connected to the photodiode power sensor via a coaxial cable (**Figure 2**).

6.3.1) Determine the height of the two flanks ($f_{1,S}$ and $f_{2,S}$) in the voltage profile acquired with the oscilloscope.

6.3.2) Repeat the measurement without a leaf sample as a reference ($f_{1,0}$ and $f_{2,0}$). Calculate transmission μ_T as the ratio of these measurements according to Equation 4 (see also **Figure 2**).

Equation 4: $\mu_T = \frac{f_{1,0}}{f_{1,s}} = \frac{f_{2,0}}{f_{2,s}}$

[Place Figure 2 here]

7. Calculate the specific heat capacity of the leaf sample

7.1) Calculate the maximum temperature difference ΔT [K] during the laser pulse by subtracting the room temperature T_0 [K] from the maximum leaf temperature T_{max} [K] (Equation 5).

Equation 5: $\Delta T = T_{max} - T_0$

7.2) Calculate the energy absorbed by a leaf (E_S [J]) based on the effective laser power and laser pulse duration (Equation 6), where P_R [W] is the reflected laser power and P_T [W] is the transmitted laser power.

Equation 6: $E_S = (P_{Laser} - P_R - P_T) \times t_{Laser}$

7.3) Calculate the mass of the heated leaf area (m_S [kg]) using Equation 7, where d_S [m] is the leaf thickness according to 2.1), r_{Laser} [m] is the radius of the laser spot, V_S [m^3] is the heated leaf volume, and ρ_S [kg m^{-3}] is the leaf density according to 2.2).

Equation 7: $m_S = V_S \times \rho_S = d_S \times {r_{Laser}}^2 \times \pi \times \rho_S$

7.4) Calculate $c_{p,s}$ [J kg^{-1} K^{-1}] according to Equation 8 by dividing the absorbed energy E_S by the product of the heated leaf area mass m_S and maximum temperature difference ΔT.

Equation 8: $c_{p,s} = \frac{E_S}{m_S \times \Delta T} = \frac{(P_{Laser} - P_R - P_T) \times t_{Laser}}{V_S \times \rho_S \times (T_{max} - T_0)} = \frac{(P_{Laser} - P_R - P_T) \times t_{Laser}}{d_S \times {r_{Laser}}^2 \times \pi \times \rho_S (T_{max} - T_0)}$

8. Prepare the temperature profile data for thermal conductivity calculations

8.1) Use the "Export" command of the NIR sensor control software to export the time and temperature raw data as a *.dat file and open the file in a spreadsheet processor.

8.2) Apply 1:100 data reduction, *e.g.* using an "IF(MOD(Value;100)=0;"x";"0")" command, resulting in a data density of one data point per 0.1 s.

8.3) Calculate the average baseline temperature T_B [°C] for each temperature profile over the initial 10 s of a measurement, during which the laser was still off. Then, calculate the difference between T_B and the actual ambient temperature T_0 [°C].

8.4) Use this difference to individually normalize each profile by shifting it towards T_0 (y-normalization), *e.g.* if $T_B - T_0$ = 2.0 K, then subtract 2.0 K from each temperature value in the temperature profile (**Figure 3A**).

8.5) Normalize the time coordinate of each temperature profile (x-normalization) by deleting every data point before the maximum sample temperature (T_{max}) and assign new time values starting with t = 0 for T_{max} (**Figure 3B**).

8.6) Screen each profile for sudden temperature shifts, *i.e.* temperature differences that are more than three times the baseline noise level, which is typically 3 × 0.31 K ≈ 1.0 K. Remove these regions from the data set because they correspond to measurement artifacts (**Figure 3C**).

8.7) Fit an exponential decay function (Equation 9) to the data using a spreadsheet processor, where T_t [K] is the fitted leaf sample temperature at time t [s], T_0 is the ambient temperature, A [K] is the amplitude and t_1 [s] the decay constant (**Figure 3D**).

Equation 9: $T_t = T_0 + A \times e^{-\frac{t}{t_1}}$

8.8) Use the fitted function to calculate the temperature decline in the leaf sample from 0–80 s after the laser pulse.

8.9) Transform the temperature data measured in [°C] to the [K] scale by adding a value of 273.15 to each temperature data point (**Figure 3E**).

[Place Figure 3 here]

9. Calculation of the thermal conductivity of the leaf sample

9.1) Calculate the temperature difference between the leaf sample and the environment for each 0.1-s interval according to Equation 10, where ΔT_x [K] is the temperature difference, T_t [°C] is the fitted leaf sample temperature and T_0 [°C] the ambient temperature (**Figure 3E**).

Equation 10: $\Delta T_x = T_t - T_0$

9.2) Assume that the decline in temperature is due to the combined effect of convective heat transfer, thermal radiation and thermal conduction. Use the corresponding energy balance (Equation 11) as a basis for the calculation of λ, where ΔE_{Temp} [J] is the difference in the thermal energy of the sample at two consecutive time points, ΔE_{rad} [J] is the energy difference due to thermal radiation, ΔE_{conv} [J] is the energy difference due to convective heat transfer, and ΔE_{cond} [J] is the energy difference due to thermal conduction.

Equation 11: $\Delta E_{temp} = E_{rad} + \Delta E_{conv} + \Delta E_{cond}$

9.3) Substitute the general terms in the energy balance with the actual physical properties yielding Equation 12, where ΔT_t [K] is the difference in the fitted leaf sample temperature, ε the unitless emissivity, σ [kg s^{-3} K^{-4}] the Stefan-Boltzmann constant, A_{rad} [m^2] the area of thermal radiation, h [J s^{-1} m^{-2} K^{-1}] the convective heat transfer coefficient, A_{conv} [m^2] the area of convective heat transfer, A_{cond} [m^2] the area of thermal conduction and l [m] the characteristic length.

Equation 12: $\Delta T_t \times V_s \times \rho_s \times c_{p,s} =$

$$[(\varepsilon \times \sigma \times ({T^4}_t - {T^4}_0) \times A_{rad}) + (h \times \Delta T_x \times A_{conv}) + \left(\frac{\lambda \times \Delta T_x \times A_{cond}}{l}\right)]dx$$

9.4) Calculate the characteristic length l based on the correlation: $l = V/A$.

9.5) Use the heated sample volume V_S and the cross-sectional area of the leaf sample to calculate A [m^2]. The cross-sectional leaf area corresponds to A_{cond} according to Equation 13, where A_{cond} is the area where conduction occurs, r_{Laser} is the radius of the laser spot and d_s is the leaf thickness.

Equation 13: $A = A_{cond} = 2 \times r_{Laser} \times \pi \times d_s$

9.6) Calculate A_{rad} and A_{conv} according to Equation 14, where A_{Laser} is the area of the laser spot.

Equation 14: $A_{rad} = A_{conv} = 2 \times A_{Laser} = 2 \times {r_{Laser}}^2 \times \pi$

9.7) Substitute Equations 9, 12 and 13 into Equation 11 and resolve the latter for λ, yielding Equation 15 where t_{Laser} is the laser pulse duration [s].

Equation 15: $\lambda = \left[\left(\frac{(T_t - T_{t+1}) \times t_{Laser} \times (P_{Laser} - P_R - P_T)}{dt \cdot (T_{max} - T_0)}\right) - \left(\varepsilon \times \sigma \times \left({T_t}^4 - {T_0}^4\right) \times {r_{Laser}}^2 \times \pi \times 2\right) - \left(h \times (T_t - T_0) \times {r_{Laser}}^2 \times \pi \times 2\right)\right] \times \frac{l}{(T_t - T_0) \times 2 \times (r_{Laser} + l) \times \pi \times d_S}$

9.8) Assume a value of 0.94 for ε and calculate *λ* for each 0.1-s time interval over the first 20 s of the temperature profile. Average the 200 values for *λ* obtained in this way and calculate the standard deviation (**Figure 3F−H**).

REPRESENTATIVE RESULTS:

Measurement of leaf properties

Using the above microscopic method, a leaf thickness of 0.22–0.29 × 10^{-3} m was determined for both *N. tabacum* (0.25±0.04 × 10^{-3} m, n=33) and *N. benthamiana* (0.26±0.02 × 10^{-3} m, n=24), which is well within the 0.20–0.33 × 10^{-3} m range previously reported for the leaves of various plant species [3]. Determining the thickness with a dial-gauge yielded values of ~0.28 × 10^{-3} m (n=10), which was within one standard deviation of the results from the microscopic measurement. Thus, the dial-gauge measurement may be preferred over the microscopic method for thickness determination in routine applications as it was easier to apply and the results for $c_{p,s}$ and *λ* deviated less than 10% from the more labor intensive technique. The density of *N. tabacum* and *N. benthamiana* leaves was 750±10 kg m^{-3} (n=20), which matches the 631–918 kg m^{-3} range previously reported for leaves in other species [3].

Calculation of the specific heat capacity

Temperature profiles collected for *Nicotiana* species showed a rapid increase over the time of the laser pulse until the maximum temperature (T_{max}) was reached within less than 1 s. After the pulse, the temperature decreased exponentially until it reached ambient temperature (T_0) (**Figure 3A−E**). The specific heat capacity ($c_{p,s}$) was calculated according to Equation 8 yielding values of 3661 ± 323 J kg^{-1} K^{-1} for *N. tabacum* and 2252 ± 285 J kg^{-1} K^{-1} for *N. benthamiana*. Two cultivation settings and durations were used for each species (see section 1.2) but this did not affect $c_{p,s}$ (**Figure 4**). However, the $c_{p,s}$ values decreased linearly from the old (bottom) to young (top) leaves (R^2 = 0.85) in the case of *N. tabacum* (**Figure 4A**), which correlated to the water content [g g^{-1} biomass] that had been determined as the difference of wet biomass at the time of harvest and the mass after 72 h incubation at 60°C [11]. This correlation between water content and specific heat capacity was in agreement with previous observations by other authors [13]. An inverse correlation was observed for *N.*

benthamiana (R^2 = 0.79), where the difference between the specific heat capacities of leaves of different degrees of maturity (bottom = old; top = young) were only 13% compared to 21% for *N. tabacum*. This difference may originate in the fact that the water content in leaves of *N. benthamiana* is almost constant over the different degrees of leaf maturation [11]. A sensitivity analysis revealed that differences in $c_{p,s}$ were proportional to fluctuations in the measurement parameters in Equation 8. The effect of the reflected and transmitted laser power was sub-proportional, because these parameters were not individual factors in Equation 7. Accordingly, the effect of errors in these two parameters was smaller than those caused by fluctuations in the laser power or ambient temperature. In general, the measurement was considered to be robust because all parameters involved in the calculation of $c_{p,s}$ had a coefficient variation of less than 10% (**Figure 4C** and **D**).

[Place Figure 4 here]

Calculation of the thermal conductivity

The thermal conductivity (ʎ) was calculated from the temperature profiles by exponential fitting (**Figure 3**) combined with equations for conductive and convective heat transfer as well as thermal radiation. Equation 15 yielded average values of 0.49 ± 0.13 J m^{-1} s^{-1} K^{-1} (n = 19) for *N. tabacum* and 0.41 ± 0.20 J m^{-1} s^{-1} K^{-1} (n = 25) for *N. benthamiana*. There was no correlation between ʎ and plant age or cultivation setting, although a correlation between the leaf age and ʎ was observed for *N. benthamiana* (**Figure 4B**), agreeing with previously reported age-dependent differences in other plant species [14]. As discussed above, the water content was an unlikely reason for this difference as it was found to be homogenous across leaves of varying maturity for *N. benthamiana*. Instead, we speculate that changes in the leaf tissue, e.g. the cell wall composition, were responsible for this observation by altering the heat transfer properties of the leaves and thus affecting the value of ʎ. The determination of ʎ was sensitive to changes in the ambient temperature. A sensitivity analysis revealed that fluctuations of ±2.3 K altered the value of ʎ by 64–125%. According to Equation 15, the ambient temperature has an effect by the power of four on the thermal radiation and thus directly affects the value of ʎ.

Evaluation of the measurement apparatus

It was possible to set up the measurement assembly within 3 h. Once this was complete, the start-up time of the system was approximately 15 min per measurement series. Single measurements took less than 3 min, including sample preparation and the entire measurement cycle. Analysis of the laser exposure time revealed that a heating time of 0.5 s resulted in a temperature increase of 19.9±4.3°C (n=55) was the best compromise between the high ΔT (achieved by long laser pulses) required for a good signal-to-noise ratio (SNR) and the low ΔT (achieved by short laser pulses) required to avoid tissue damage. Pulse durations longer than 0.5 s resulted in the loss of mass from the sample, probably reflecting the evaporation of water and/or damage to the leaf tissue as the sample temperature reached up to 70°C, whereas only 42.9±4.2°C (n=55) were observed for 0.5 s laser pulses. For durations of less than 0.5 s, the temperature noise of ±0.31 K (standard deviation, n = 25) accounted for more than 5%

of ΔT and was thus a significant part of ΔT. In contrast, at 0.5 s the noise accounted only for 2.5% of the signal and was thus regarded as insignificant. Additionally, the samples did not heat up to more than ~45°C, which is a temperature that tobacco plants can also be exposed to in the natural tropic to sub-tropic habitat and which is only detrimental to plant species found in tundra habitats [15]. The power density of the laser was 170 kW m^{-2}, whereas natural solar radiation is typically in the range of 1.0–1.4 kW m^{-2} [16,17]. However, due to the very short time of the pulse, this higher energy dose did probably not damage the leaf tissue as indicated by a recently published microscopic analysis [11]. The temperature data used to calculate ʎ were restricted to the initial 20 s after the laser pulse because only during this period did the noise (±0.31 K) account for less than 5% of the sample's temperature signal and was thus regarded as insignificant. When temperature data from beyond the 20 s time frame were used, the values calculated for ʎ declined (**Figure 3F**). A possible explanation was that some of the assumptions made for the calculation of ʎ did not apply for low values of ΔT. Especially, the term describing thermal radiation in Equation 15 might have been affected as it is affected by the forth power of temperature. Also, the leaf area surrounding the sample spot exposed to the laser might have heated up slightly and thus might not have been the ideal heat sink assumed in the model reducing the effective ΔT_x and ultimately the calculated ʎ.

FIGURE LEGENDS:

Figure 1: Apparatus used to determine leaf thermal properties. A. Photograph of the measurement apparatus used to determine the specific heat capacity and thermal conductivity of leaves. The peripheral devices (computers, oscilloscope) are not shown. B. Schematic representation of the measurement apparatus. The laser and connected equipment are highlighted in red, the NIR detector for temperature measurement is shown in purple, the leaf sample is green and the photodiode power sensor is blue. C. Drawing of the elements of the measurement setup with the same color code as in B. The size bar indicates 0.1 m. D. Screenshot illustrating the typical elements of the laser control software.

Figure 2: Measuring leaf transmission using a photodiode power sensor. A. Typical voltage profile for a reference experiment without a leaf sample visualized using an oscilloscope. B. Voltage profile with a leaf sample mounted in the apparatus. In both cases, the transmitted laser power is proportional to each of the two flanks.

Figure 3: Data processing scheme for the calculation of λ. A. After data reduction, the temperature profiles are normalized to the ambient temperature. B. Next, all data points before the maximum sample temperature (T_{max}) are removed. C. Measurement artifacts (shown in the "inconsistent" data set) are identified based on temperature shifts larger than three times the baseline noise and removed from the dataset prior to fitting to an exponential function. D. The Celsius temperature scale is converted into the Kelvin scale. E. For each time interval, λ is calculated based on the temperature profile. F. A window of 20 s is defined in which a relevant temperature change can be observed. G. Based on the selected time window, the average and standard deviation are calculated for λ. H. Representative results for two different *N. tabacum* leaf

samples. Orange arrows and lines indicate the effect of the corresponding processing step on the presented data.

Figure 4: Specific heat capacity and thermal conductivity values determined for *N. tabacum* and *N. benthamiana*. A. Specific heat capacity and thermal conductivity of *N. tabacum* leaves according to the leaf position on the plant (bottom = old leaves; middle = mature leaves; top = young leaves). Stars and triangles indicate plants that were 49 and 56 days old, respectively. B. Specific heat capacity and thermal conductivity of *N. benthamiana* leaves according to the leaf position on the plant. Stars and triangles indicate plants that were cultivated in a phytotron or greenhouse, respectively. C. Sensitivity of specific heat capacity values to changes in the input parameters. Triangles show specific heat capacity values resulting from a 10% increase (red, upward) or decrease (blue, downward) in single model parameters. D. Sensitivity of thermal conductivity values to changes in the input parameters. Triangles mark shoe thermal conductivity values resulting from a 10% increase (red, upward) or decrease (blue, downward) in single model parameters. Error bars in A and B indicate the standard deviation (n≥3), while in C and D they represent the complete range of values obtained during 10% variation sensitivity analysis.

DISCUSSION:

The contact-free, non-destructive measurement method described above can be used to determine $c_{p,s}$ and ʎ in a simultaneous and reproducible manner. The calculation of ʎ in particular depends on several parameters that are sensitive to errors. Nevertheless, the impact of these errors was either linear or sub-proportional, and the coefficient of variation for all parameters was found to be less than 10%. Even though the method can thus be regarded as robust, some technical improvements can be made to reduce the remaining sources of error.

Mounting the sample into the assembly was technically challenging because a flat leaf surface is preferable for measurement but the sample naturally has an undulating surface. This problem could be overcome by designing a dedicated sample holder with geometries precisely adjusted to the leaf sample, *e.g.* leaf thickness and width, clamping the sample in the preferable orientation. This approach would make the measurements more reproducible, but would compromise the contact-free nature of the measurement because firm contact between the sample and holder would be required to pull the leaf surface flat. The benefits of using this kind of holder would therefore depend on the context of the measurement, *i.e.* whether the precision or contact-free nature of the measurement is most important. In contrast, such considerations may not be necessary at all for leaves with an inherently flat surface, *e.g.* rice and related species.

Convective heat transfer due to air movement in the environment of the sample should be kept to a minimum during measurements because this strongly affects the calculation of both $c_{p,s}$ and ʎ [18]. The apparatus should therefore be located away from air streams generated by air conditioning systems, radiators or other equipment, such as computer with integral cooling fans. This is also important because changes in the

relative water content of the leaves [19] that might occur before or during the measurement due to evaporation, which can be increased by air movements [20], were not accounted for in the model. Thus, measurements, especially with detached leaves, should be carried out rapidly as described in the protocol section to avoid errors during data acquisition. In the future, the effects of evaporation on the measurement may be reduced or avoided if the measurement is conducted in an at least partially enclosed measurement chamber with an implemented humidity control.

The accuracy of $c_{p,s}$ and ʎ values can be increased by measuring the parameters used in the corresponding equations more precisely. In the case of $c_{p,s}$ these parameters are the laser power, maximum and ambient temperature and sample volume, *i.e.* the product of laser spot area and thickness, and sample density (Equation 8). The latter two parameters must be determined in experiments accompanying the actual measurement and their reliability can be improved if several representative biological replicates are tested. However, even when a simple dial-gauge measurement was used, the difference in leaf thickness compared to a microscopic analysis was only 11%, which affected the values calculated for $c_{p,s}$ and ʎ by the same degree. In contrast, temperatures and laser power can be monitored throughout the measurement. The accuracy of $c_{p,s}$ can be improved if these online data are used instead of fixed values for laser power and ambient temperature, and the data are collected using well-calibrated sensors. These considerations also apply to ʎ, but the ambient and sample temperatures are the most important parameters because both affect the calculated value by the power of four.

The current calculation of ʎ was based on several assumptions regarding convective heat transfer and thermal radiation. For example, the emissivity (ε) and convective heat transfer coefficient (h) were not measured or calculated explicitly in the method presented above, but were derived from previous publications [18,21]. The accuracy of ʎ could therefore be improved by determining these two parameters under the actual measurement conditions. However, using the literature data for calculations nevertheless yielded ʎ values that were within the range experimentally determined for other plant species for which similar properties can be expected due to their phylogeny to *Nicotiana* species and their physiology, *i.e.* herbaceous plants [3]. Even if the values for ε and h were varied over the entire range previously reported for these values in plants, e.g. 0.93–0.98 for ε [21], their effect on the final value of ʎ was <10% and thus within the natural variation observed here.

The method presented above was not only able to determine the thermal properties of intact unharmed leaves and detached leaves, but it also correctly identified different types of more severe damage introduced intentionally before measurement. Therefore, different types of leaf samples can be readily distinguished, providing a tool to remove, prior to analysis, any poor samples that would yield low-quality data. This feature could be used for quality control when monitoring biological materials, *e.g.* samples failing to meet specifications in terms of $c_{p,s}$ and ʎ could be excluded from further processing. This would be an asset in the context of a highly regulated processes such as molecular farming [4].

The advantages of this new method compared to others in the literature include the rapid sample handling, minimal preparation, contact-free and non-destructive simultaneous measurement of $c_{p,s}$ and λ, and the use of common equipment that can be found in many optical laboratories. This will facilitate broader applications of the method compared to those requiring specialized and expensive devices such as differential scanning calorimeters. Furthermore, calorimetry requires direct contact with the sample[22] so there is a risk of damage, and the method is usually limited to the measurement of specific heat capacity [22]. In contrast, whereas thermal imaging can detect necrosis or physical changes in leaves or entire plants in a contact-free manner [23], it also requires complex image analysis and dedicated specialized devices [24] which might be overcome in the future by more cheaper and more powerful IR cameras and accompanying peripheral devices. Spectral analysis is another contact-free method for the analysis of water content and chlorophyll levels [25], but it has not yet been used to determine specific heat capacity and/or thermal conductivity.

The measurement approach reported herein is a robust method to determine the thermal properties of plant leaves with low investment costs and short measurement times. It was successfully used to determine $c_{p,s}$ and λ in *N. tabacum* and *N. benthamiana*, two species that are relevant in the area of molecular farming [4]. The values calculated for both parameters based on leaf temperature profiles were in good agreement with those previously reported for other plant species [3]. The method is non-destructive, contact-free, and does not require complex sample preparation, providing advantages over all current alternative methods for the analysis of thermal properties. The simple design may also facilitate the development of hand-held devices to increase flexibility.

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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure1

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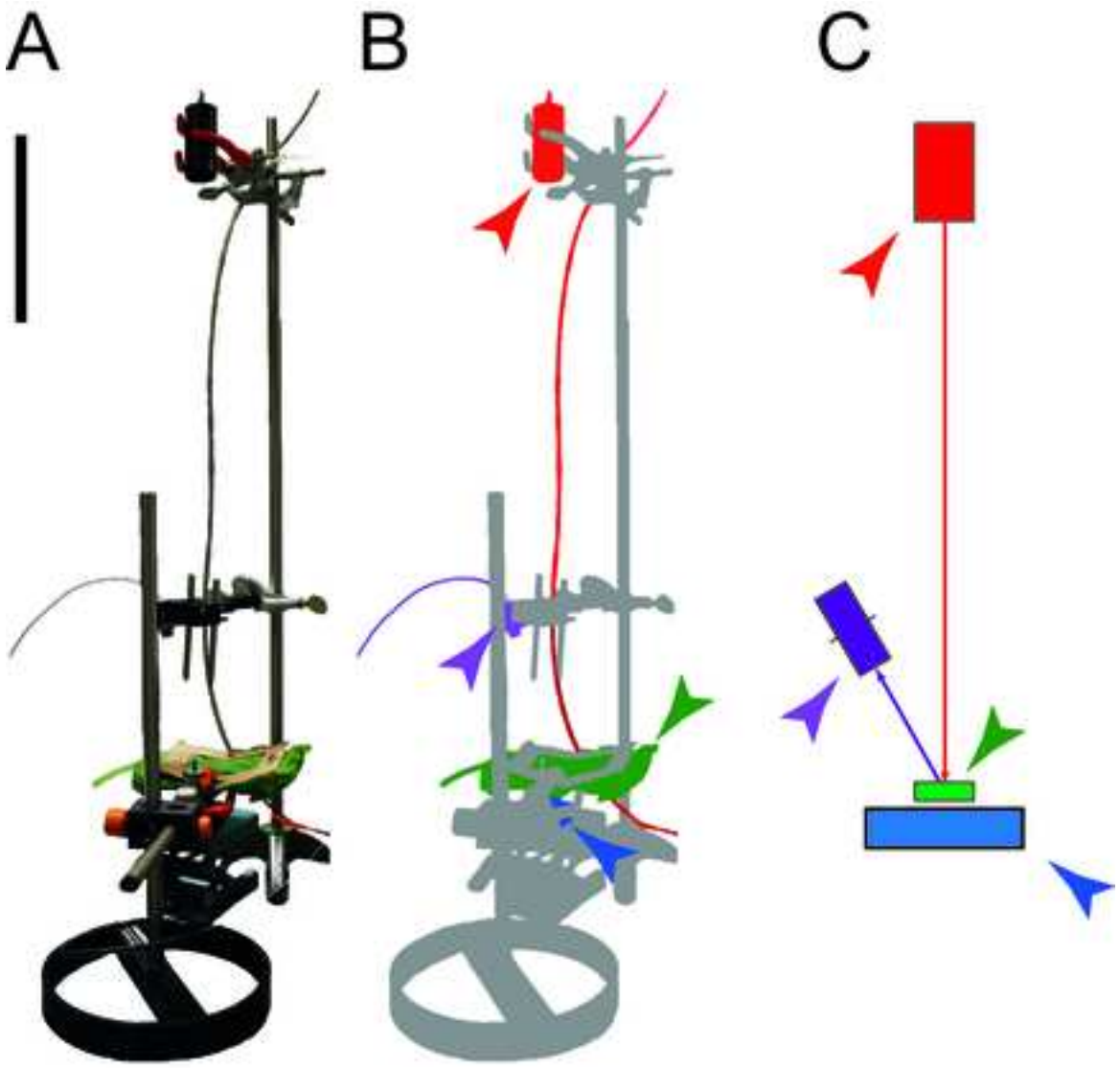

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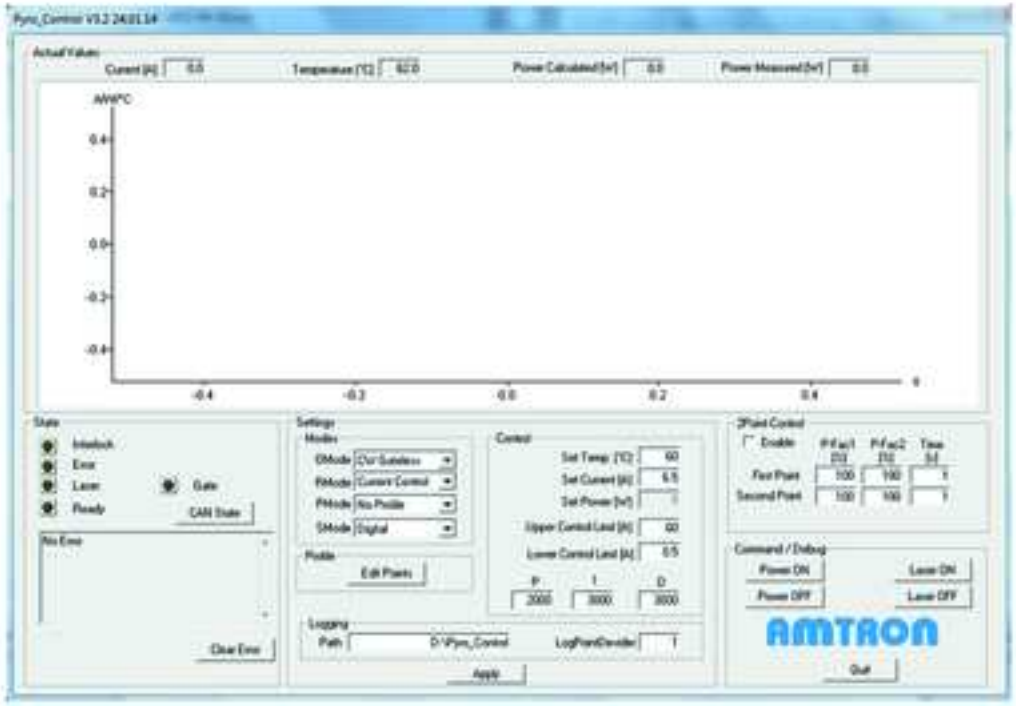

Figure2

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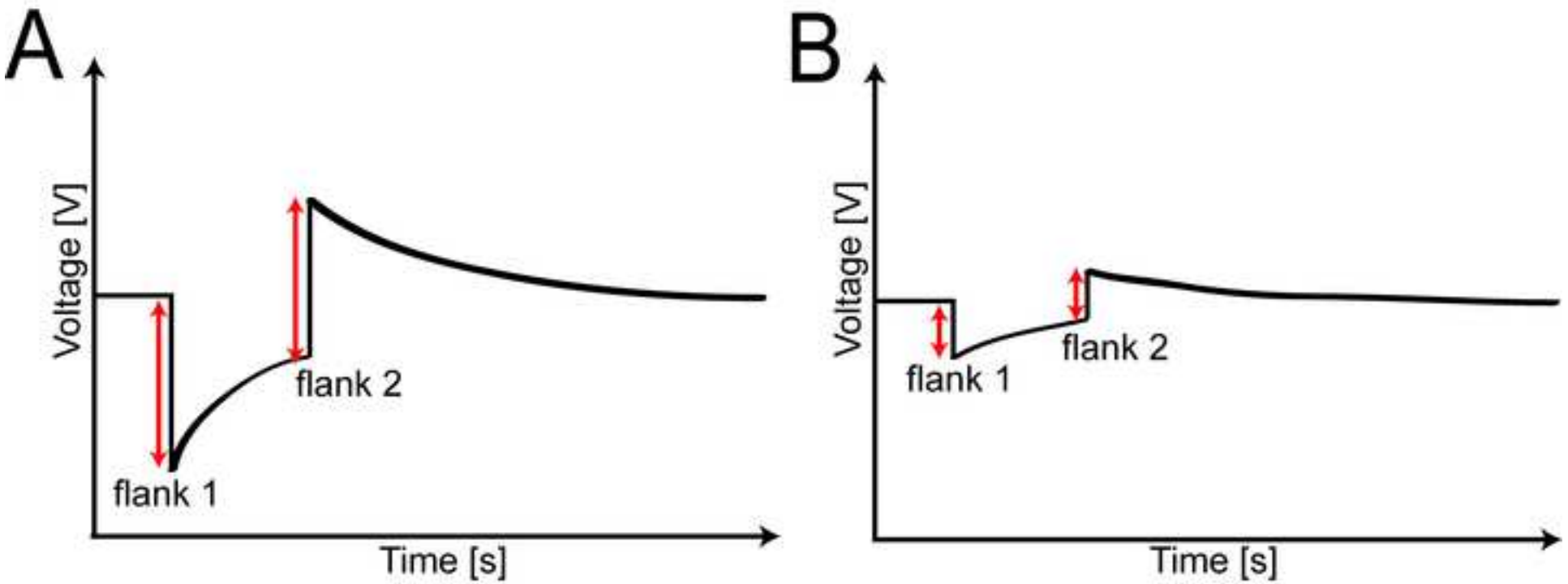

Figure3

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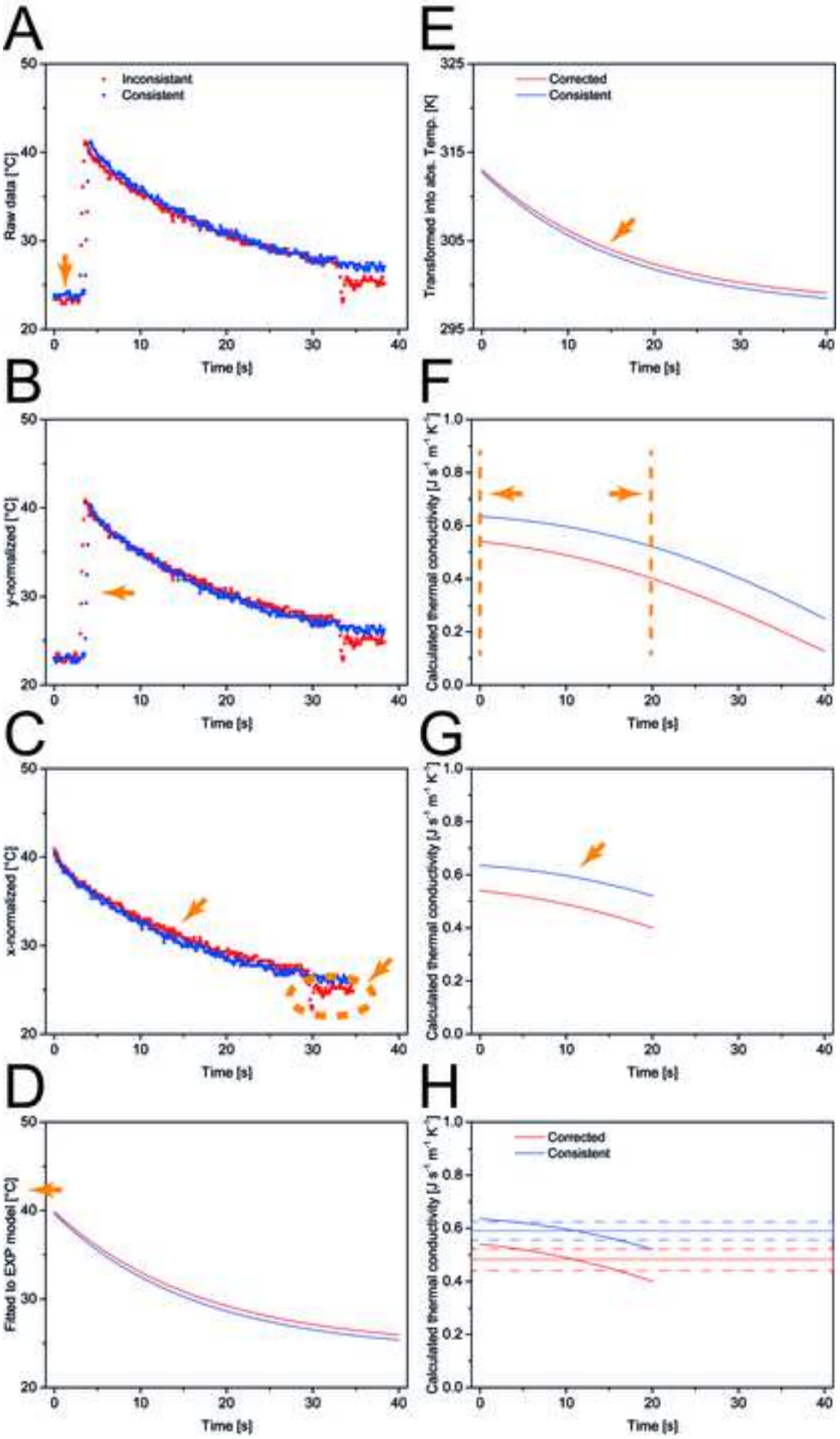

Figure4

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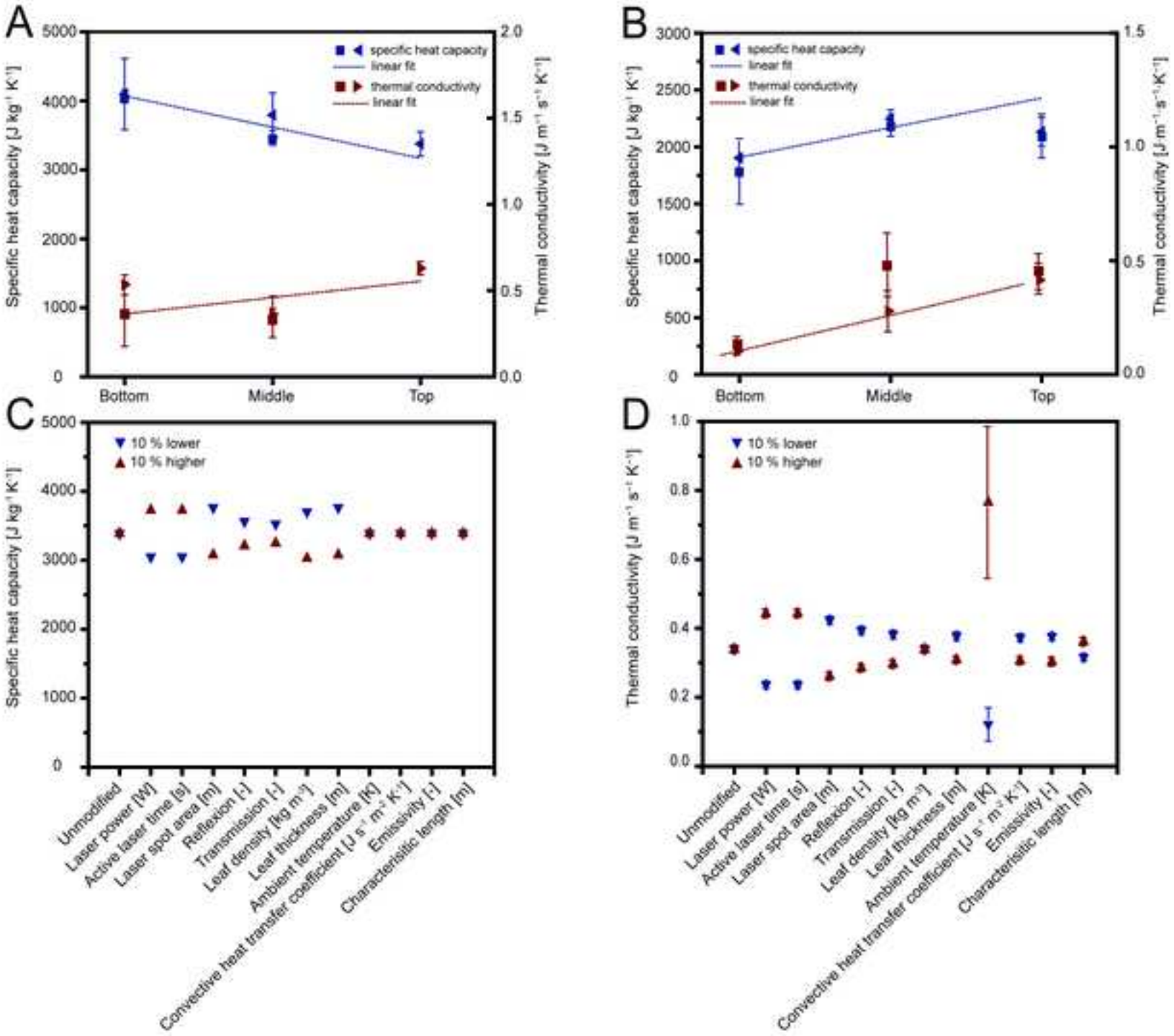

Name of Material/Equipment	Company	Catalog Number
1" tube	Thorlabs	SM1L10E
Agarose	Sigma Aldrich	A0701
Bi-Convex lense f=25.4	Thorlabs	LB1761
Digital Handheld Optical Power and Energy Meter Console	Thorlabs	PM100D
Digital Phosphor Oscilloscope	Tektronix	DPO7104
DMR light microscope	Leica	n.a.
Falcon 50mL Conical Centrifuge Tubes	Fisher Scientific	14-432-2
Ferty 2 Mega	Kammlott	5.220072
Fiber holder	Thorlabs	
Forma -86C ULT freezer	ThermoFisher	88400
Greenhouse	n.a.	n.a.
Grodan Rockwool Cubes 10x10cm	Grodan	102446
Infrared Detector Optris CT	Optris	OPTCTLT15
Infrared Detector Software Compact Connect	Optris	n.a.
Lambda 1050 UV/Vis spectrophotometer	PerkinElmer	L1050
Laser 400µm, 1550nm Conduction Cooled Single Bar Fiber Coupled Module	DILAS	M1F-SS2.1
Laser cover	Amtron	LM200
Laser Driver	Amtron	CS 408
Osram cool white 36 W	Osram	4930440
Photodiode sensor	Thorlabs	PDA20H-EC
Precision weight Ohaus Analytical Plus	Ohaus	80251552
Sample frame	Fraunhofer ILT	n.a.
Software Pyro Control	Amtron	n.a.
Stainless-steel-holder	n.a.	n.a.
Teflon plates 2cm	Fraunhofer ILT	n.a.
Thermal surface absorber Power sensor	Thorlabs	S314C
Vibratome	Leica	1491200S001
Zoc/Pro 6.51	EmTec Innovative Software	n.a.

Comments/Description
Tube for fiber holder
Agarose
Lense
Console for thermal surface absorber sensor
Oscilloscope
Light microscope
Pycnometer
Fertilizer
Fiber holder
Freezer
For plant cultivation
Rockwool block
Infrared detector
Control software for infrared detector
UV/VIS Spectrophotometer
Laser
Laser Cover
Laser Driver
Light source
Power sensor for transmission measurements
Precision weight
Fixation of the leaf sample
Laser Power Control Software
Holder for measurement set-up
Teflon attenuation
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Editor comments

•Formatting:

E1: Please be consistent with units of time. Use “s” for second(s).

Author response E1: We are very sorry to say but this editor comment is highly inconsistent with previous feedback from the JoVE editors. For our manuscript JoVE54343 "Comparison of tobacco host cell protein removal methods by blanching intact plants or by heat treatment of extracts" we have received this Editorial comment: "Please use “hr” and “sec” for hours and seconds respectively.". Therefore, we would kindly ask the JoVE Editorial office to provide a consistent guideline in terms of units and stick to it. We have now changed the units back to standard SI terms but will not convert them back anymore.

E2: Caution statement for cyanacrylate should be moved below 2.1.3.

Author response E2: We have shifted the statement as requested.

E3: Please explicitly define the terms in all equations.

Author response E3: We have added the missing explicit definitions for equations 6, 10 and 15.

•Additional detail is required:

E4: 3.2 – How is the measurement obtained? Is a particular scan run?

Author response E4: We have expanded this section to contain the missing information.

E5: 3.4 note – What does the “ff” in 6.3 ff mean?

Author response E5: The “ff” was an editing artifact which we have removed.

E6: 6.3 – How does one confirm this with the photodiode power sensor?

Author response E6: We have modified the section to answer the Editors question.

•Results:

E7: Please describe what the orange arrows/circles/lines indicate in Figure 3.

Author response E7: The orange arrows and lines indicate the effect of the corresponding processing step on the presented data. We have added this information in the figure legend.

E8: Please define the error bars in Figure 4 (SD, SEM, etc.).

Author response E8: We have added the missing definition of the error bars in the figure legend.

Reviewers' comments:

Reviewer #1:

Comment R1.1: However, the context justifying the development of this method should be more clearly stated, and in particular the field of application as well as why it is important to develop a non-invasive and contact-free method.

Author response R1.1: We have added an additional text section in the introduction justifying the development of a contact-free and non-invasive method.

Comment R1.2: In a general viewpoint, the method is described in a clear and detailed way, and an effort of discussion of the application is made. However, there is a major issue that remains unclear for me: the temperature attained by the sample is not mentioned in an absolute way (only normalized), which

prevents from evaluating the risk of degradation of the sample during tests. If the temperature is too high, then the method loses its validity as it does not measure leaf heat capacity but an addition of several contributions…The author mentions this issue but does not give any convincing argument about it.
Author response R1.2: We disagree with the reviewer, the absolute sample temperatures are shown in Figure 3 (e.g. 3A) with the maximum being ~40°C which is a temperature that tobacco plants can also be exposed to in their natural habitat. We have added this information in the text as well.

Comment R1.3: Moreover, the issue of sample moisture content is also problematic for two reasons. Firstly, the way it is measured is not mentioned. Then, the influence of moisture content is found to follow the opposite trend as that commonly observed and no explanation is searched for this unexpected trend. Based on this, to my opinion, this paper cannot be published in this form and require major revisions.
Author response R1.3: A detailed description of the determination of the water content of the leaves is given in the original publication that our current methods manuscript is based on and which we reference in the text, e.g. in the results section under "Calculation of the specific heat capacity" (reference 11). We have now also included a brief description of how the moisture content was determined in the text in the same section and mention which mechanism may be behind the different correlations observed for *N. tabacum* and *N. benthamiana*.

Comment R1.4: In particular, the issue of temperature attained by sample must be clarified with the supply of data in absolute mode.
Author response R1.4: We have addressed this comment as described in response 1.2.

Comment R1.5: To help the authors in such revision, I have also enclosed a series of more specific comments/ proposals/issues directly in the text (please see attached PDF).
Author response R1.5: The comments provided in the pdf file were in part redundant with the above comments. We have clarified what the state of the art was; we have altered the spelling accordingly; the density is easily and accurately determined as described in the protocol and shown in the results section; the species mentioned are only examples, but JoVE requires to state the precise method and not "options"; the reference mentioning the moisture data refers to the same experiments presented here and the temperature is not shown in normalized but in absolute values; an explanation for the inverse observation made for the specific heat capacity of *N. benthamiana* compared to *N. tabacum* is provided; an explanation for the leaf age dependence of lambda is provided; we changed the reference to the correct equation; we clarified that the fluctuations were part of an in silico sensitivity analysis; as stated before Tt is provided in the manuscript but we have now also added this information in the results section highlighted by the reviewer; we have expanded our explanation why we think using literature values of h and ε is justified at this point; we have changed the misleading position of the reference.

Reviewer #2:
Comment 2.1: The abstract is too ambitious. A knowledge of specific heat capacity and thermal conductivity are not really needed to design a blanching apparatus.
Author response R2.1: We agree with the reviewer and have modified the abstract and introduction accordingly.

Comment 2.2: Abstract says a set of measurements can be completed in one minute. Upon going through the paper one will realize that this is not true.

Author response R2.2: It would be helpful if the reviewer could be a bit more specific on why he/she thinks that a measurement cannot be conducted in one minute. We agree that the plural used in the abstract ("intact leaves") may allow a misinterpretation that several leaves are measured in this time. This is not the case, we have used the plural to imply that the method is not limited to a specific type of leaf but we have now modified this to avoid any misinterpretation. As can be seen in the methods section 6 (especially 6.2) a single measurement takes indeed 1 min. Of course this measurement requires preparation (setting up the devices etc.), but these are one-time tasks and independent of the number of samples. The data processing we have described in the following sections (7-9) can be automatized using software (e.g. MatLab or even Excel) and will take only several milliseconds on typical desktop computers. Therefore, we believe that our statement is valid with the modifications we described above.

Comment 2.3: The paper is not written in a style generally followed by international journals.
Author response R2.3: We agree that the style of the manuscript deviates from that of other journals, but this applies to all publications in JoVE and is a requirement of the journal. Therefore we suggest the reviewer may best discuss this issue with the Editor after consulting the journals guidelines for authors.

Comment 2.4: Upon going through the paper one gets the feeling that these experiments can be performed only with the specific equipment (model) and software available with the authors. A scientific paper should provide sufficient details for anybody to replicate the experiment or calculation, but should not be equipment model specific.
[Editorial recommendation: Please keep JoVE's protocol requirements in mind as you address the above comment - the protocol must contain sufficient details in order to enable users to accurately replicate your technique. In addition these details are required for our scriptwriters to most accurately plan and write for your video. We recommend NOT removing any details from the protocol text.]
Author response R2.4: We agree with the reviewer that the methods should enable colleagues to repeat the experiments with other equipment. Focusing on the actual equipment we used is a requirement of JoVE and we have been told to avoid general descriptions (e.g. "use a spectrophotometer" instead of "use spectrophotometer xy") in the course of our previous submissions to JoVE, which is why we have avoided them here. Given the editorial recommendation above, we think that a consolidated statement from the Editor and the reviewer is required before we can address this issue properly. As a matter of fact, the method can be conducted with any type of laser etc. and is not limited to the specific equipment we used.

Comment 2.5: The quality of figures 3 and 4 are poor. These are not legible.
Author response R2.5: We have provided all figures in 600 dpi resolution which is double of what is required by the journal. Has the reviewer downloaded the original high resolution figures or only looked at the low resolution images included in the pdf? Or does the Reviewer think that the font size is too small? We require further input before we can address this point properly.

Comment 2.6: The authors state that the power of the IR laser used was 5 W or so. What was the power density? A laser power of 5 W will easily damage soft plant tissues even if the exposure time is less than a second. This aspect is not clearly discussed in the paper.
Author response R2.6: We have added the power density and compared it with the typical solar radiation as well as microscopic tissue analysis presented in our previous work.

Comment 2.7: From figure 3 it is obvious that thermal conductivity changes significantly with time. This may be due to changes in tissue structures with heat from the laser. This aspect is not clearly discussed in the paper.

Author response R2.7: The reviewer raises a valid point and we have added more details in the results section at the end of paragraph “Evaluation of the measurement apparatus”. We do not believe that changes in the tissue are responsible for decline in calculated values for lambda because the sample is already close to ambient temperature.

Comment 2.8: From figure 4 it follows that thermal conductivity and specific heat are very different at different locations of a leaf. This result is not acceptable. Again, this result is not convincingly explained or discussed in the paper.
Author response R2.8: We believe that our initial description might have been misleading. We did not claim that the thermal conductivity and specific heat capacity vary for different positions on a single leaf. Instead we meant to convey that leaves of different degrees of maturation, i.e. leaves at the bottom of a stem (= mature) have different thermal properties than those at the top (= young). We have added additional content discussing potential reasons for this difference as well as a more detailed explanation as to what “position” means in the results section.

Comment 2.9: All claims about the advantages of this method over others are not true and cannot be accepted completely. After all the methodology followed has been well known to the photothermal community for a long time.
Author response R2.9: We agree with the reviewer that the measurement principle is well known for a long time, but our focus is its application for the determination of leaf thermal properties in a non-invasive manner. Apart from our initial publication (reference 11 in the current manuscript) we have not found any other paper using the technique in this context. Additionally, the JoVE format is intended to provide a visual demonstration of current methods used in various scientific fields and again we have not found that the technique we present has been describe in a video format before. Moreover, we have been specifically invited by the JoVE Editors to report this method. As for the advantages of the method, we kindly ask the reviewer to provide some more details as to which claims may be illegitimate in his/her opinion and why so we can either remove such unjustified claims or provide more evidence supporting our statements.

Reviewer #3:
Comment 3.1: In the introduction I suggest to indicate other uses of the methodology besides molecular farming purposes. I suggest to emphasize the potential relevance of the method to other relevant commercial applications or studies (e.g. plants stress ecophysiology)
Author response R3.1: This is a good suggestions and we have added additional text in the introduction.

Comment 3.2: -Line 113. The methodology used to assess leaf thickness seems complex. It is not clear from the text how much time is required to do such measurement. I wonder if other simpler methods could be proposed (e.g. dial-gauge or a digital micrometer) and how these simpler approaches would affect robustness of results. Moreover, authors should specify which part of leaves is being effectively assessed for thickness. This because this parameter varies over the leaf surface (generally being thickest at the midrib, primary veins, margins and leaf base). I suggest an extra figure showing determination of leaf thickness and density
Author response R3.2: The reviewer raises valid points here. We have determined the thickness in vein-free areas of the leaves and have now stated this in the methods section (2.1.4). We had initially determined the leaf thickness with a dial-gauge as proposed by the reviewer but then switched to microscopy for higher accuracy. We have now added this alternative method in the methods section (2.1.5) and provide a comparison of the according calculation for the thermal properties in the first paragraph of the results section.

Comment 3.3: -Line 111. Authors suggest that the method can be used both attached and detached leaves? Is it possible that leaf severance affects Relative Water Content and leaf thermal properties? It is not absolutely clear how the authors take in account the influence of evaporative cooling due to transpiration and this can be affected in detached leaves
Author response R3.3: This is a valid point. Yes, we suggest that attached and detached leaves can be measured. We have now pointed out in the discussion section that especially for detached leaves the measurements should be conducted quickly in order to prevent water evaporation from affecting the results. We have added an according section in the protocol too (5.3).

Comment 3.4: -Line 153. Authors suggest a minimum of 3 biological replicates. However this is too few if there is a high variability (e.g. variation within leaf surface morphology and thickness);
Author response R3.4: We agree with the reviewer that 3 biological replicates can be too few for variable samples. Therefore, we had already stated in our initial version that at least 3 replicates be used. We have now taken up the reviewers suggestions and recommend a minimum of 5 replicates for samples that exhibit a variable morphology.

Comment 3.5: -Line 193. An extra figure to visualize the laser control interface software is advisable
Author response R3.5: We have added an according figure as panel D of figure 1.

Comment 3.6: -Line 355 . Why considering emissivity of 0.94? It is slightly lower than the values described in literature for leaf material (0.96-0.97, see e.g. Jones 2004 Adv,Bot Sci.)
Author response R3.6: The same author cited by the reviewer also provides a range of 0.93-0.98 for the emissivity of leaves (Jones et al. in Journal of Experimental Botany, Vol. 54, No. 384, pp. 879-889, March 2003) which includes the value we selected. Therefore we believe that our selection is justified.

Comment 3.7: -Line 457, authors do not discuss anything about limitations and potential errors posed by leaf thickness (see above comments)
Author response R3.7: We disagree in part with the reviewer, in former lines 485-490 we had pointed out that the calculation of thermal conductivity and specific heat capacity would benefit from a more accurate determination of the leaf thickness. However, in the previous version we did not mention the quantitative effect of the thickness measurement, which we have now included at the according point in the manuscript.

Comment 3.8: -Line 479, I believe that it is not solely convective heat transfer. If leaves are alive and stomata are still functional, we will have evaporative cooling as well due to leaf transpiration; This suggests that the method and set up has still limitations to be used under unstable environmental conditions, e.g. in the greenhouse or climate chamber and that improvements in the set up are needed to minimize such type of disturbances. Maybe the authors could propose solutions
Author response R3.8: We agree with the reviewer and have proposed a possible solution in the according discussion section.

Comment 3.9: -Line 507. Can we say that a detached leaf is a "unharmed leaf"? Severance from the mother plants is a major stress. Leaf hydraulics/hormonal regulation is particularly affected influencing stomatal regulation and related transpiration water loss
Author response R3.9: We agree with the reviewer that detaching is a form of harm to a leaf. In the context of former line 507 we had in mind to discriminate between a damage that we thought of as severe at the site of measurement (e.g. burning) but we have now listed detached leaves as a separate group of samples in the according discussion section.

Comment 3.10: -Line 522. The disadvantage of thermal imaging may be partly solved in future by cheaper high resolution IR cameras
Author response R3.10: We agree with the reviewer and have amended the text accordingly.

Comment 3.11: -Line 529 , what do you mean by low costs. Please provide info on the range of costs related to the set up and its maintenance in order to support your statement
Author response R3.11: The devices we used were 10+ years old, which is why we unfortunately cannot provide any documentation about the costs.

Comment 3.12: -Figure 1, authors could use a "a scale" to easily evaluate the size of the equipment used. In addition an extra plate (Fig. 1B) could be added to show the overall set up including the perifericals; The artwork presented by the same authors in a previous article (Buyel et al., 2016, J. Biotechn. 217) has much higher quality and is more clear that the one presented here. Maybe the authors could use an adapted version of that plate
Author response R3.12: We have added a scale bar as requested. Showing the peripherals would not add relevant information in our opinion, because that mean showing black/grey boxes (for the lasers) and a computer screen. However, we agree with the reviewer that the last version of the figure was not so clear and thus we have now added an additional schematic view of the measurement setup. The quality of the figure is according to the journals standards (600 dpi), but the pdf for review only contains low-res images with links on the upper right corner to download the high-res counter parts. Has the reviewer tried to download these?

Comment 3.13: -Figure 3. The order of the different plates composing Figure 4 is not correct. The second column of figures should be from top to the bottom E, F, G, H; Be consistent along the paper
Author response R3.13: We have modified the figure accordingly.

Comment 3.14: -Figure 4. Improve quality of the figure for a matter of clarity. Use other symbols than "stars" and "triangles". At the present, in Fig. 4 symbols are used to represent different ages, whereas in figure 4b they represent other variables (different growing conditions, greenhouse vs climate chamber). Moreover, plates 4c and 4d from figure 4 are quite similar to the data/figures published in a previous paper of the authors (see Buyel et al., 2016, J. Biotechn. 217).
Author response R3.14: We have changed the “stars” into “squares” so they can be differentiated easily from the triangles. As described before, the low quality of the figures is due to the pdf format. We agree with the reviewer that panels C and D are similar to those in our previous publication, however, they are useful to illustrate the sensitivity analysis which we believe provides important information on the robustness of the method. Hence, we would like to include them in this manuscript.

Comment 3.15: -Line 127. Write "Mount five leaf slices" (more correct would be transversal sections)
Author response R3.15: We have changed the text accordingly.

Comment 3.16: -Line 193. An extra figure to better visualize the laser control interface software is advisable
Author response R3.16: We addressed this point as part of comment 3.5.

Comment 3.17: Line 204-211 . I believe that the points 5.1. and 5.2. could be combined
Author response R3.17: We agree that these points may be combined, but as they describe two distinct sets of samples we would like to keep them separate.

Comment 3.18: -Line 425, Instead of "is" use "are"
Author response R3.18: We have changed the text accordingly.