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 ** 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.