*Responses to editors and reviewers are given in italics after each comment.*

Editorial comments:  
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55425\_R1\_092016.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.  
  
1. Formatting:  
-4.4 – Please use “x g” rather than rpm for centrifuge speeds.

*All values are recalculated from rpm to “x g”*

-Please define all abbreviations (RT, etc.).

*All abbreviations are defined. For example: room temperature (RT).*

2. Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance and should particularly address usage of prepositions and articles (a, an, the).

*We apologize, this is partly due to using ‘protocol language’ which is short rather than grammatically correct. In addition to the changes below, we made several other minor changes to improve the text.*

Some example errors are listed below:  
-Title should read “with High Pressure…” rather than “on”.

*Changed*-Please use “refrigerator” rather than the informal “fridge”.

*Fridge was changed to refrigerator*

-Line 159 – “to run out” should be “from running out”.

*Changed*-4.11 – “in glass bin”, “disposing the tubes”

*Sentence was changed to:*

*”Let the glass columns dry under the hood overnight and dispose of them when dry. Recover the metal balls from the sample tubes used in step 4.5 for reuse and put the tubes in the waste disposal bin.”*

-6.1.1 – “glucosinolates samples”

*Changed to glucosinolate samples*-Line 369 – “In contrasts”

*Changed to “In contrast””*-Line 372 – “of procedure”  
*This was changed*

3. Additional detail is required:  
-3.1.1 – What constitutes a small piece?

*This is now specified as follows:*

*“In each glass pipette, place approximately 1 x 1 cm piece of glass wool . Use a wooden or glass stick to push the glass wool down until the point where the pipette narrows.”*

-4.11 – Please include a citation for quantification.

*We do not get this remark, 4.11 is about the disposal of the glass columns and tubes.*

-6.1.2 – Please provide a citation.

*We now refer to the reference by Glauser et al (2012) and Crosell et al (2016)*

4. Discussion: Please discuss the critical steps and any modifications/troubleshooting that can be performed. Please also discuss the future applications of the protocol.

*The method in itself is rather robust, nevertheless we discussed several aspects of troubleshooting, such as ‘no peaks in the chromatogram, co-eluting peaks ’ in the discussion. This method is and can be used for extracting glucosinolates from all types of samples, including root, shoot, seed and soil samples, liquids (references added), and food stuffs.*

Reviewers' comments:  
**Reviewer #1:**  
*Manuscript Summary:*  
The manuscript titled "A straightforward method for glucosinolate extraction and analysis on High Pressure Liquid Chromatography (HPLC)" by Nicole van Dam et al., provided a robust method to analyze glucosinolates by HPLC. The extraction of glucosinolates method in this manuscript provides a practical and easy protocol for rapid detection of glucosinolates.  
  
1. For glucosinolate extraction, different solvents such as deionized water aqueous methanol solution have been used and published. For the quality of glucosinolate, the deionized water seems to be the best solvent for extraction. Methanol provides, however, higher extraction efficiency. The authors of this manuscript are invited to compare different solvents for the quality of the extracted glucosinolate and for its extraction efficiency.

*The method described here has been optimized for extraction efficiency by previous authors in the 1980-ies (Buchner 1987). The method we describe here is a well validated method that is widely used by many laboratories around the world and is described at by the European Committee as the standard for extracting and determining glucosinolates in oil seed rape materials (see EC 1990). We would not dare to pretend that we can improve on this method. Moreover, seen the polarity of the glucosinolates, 70%MeOH seems to be a logical choice.*

2. In the 70 % of aqueous methanol solution, what would be the deactivation rate of the endogenous myrosinase? The deactivated endogenous myrosinase could in turn degrade glucosinolates.

*It is not the 70%MeOH that kills the enzyme, but the boiling of the extract in the first step. Instead of adding boiling 70% MeOH – as some other protocols describe, but which harbors various (health) risks during the extraction - we chose to add the extraction solution at room temperature and quickly transfer the locked tubes to a boiling water bath to reduce the time in which the myrosinase can be reactivated– if at all in 70% MeOH. Some colleagues argue that the boiling may not be needed at all. Indeed, in LCMS protocols which use fresh materials, extract intact glucosinolates including a more comprehensive metabolomics profile, this step is often omitted. In addition, the boiling increases extraction efficiency.*

3. Some articles showed completely extract glucosinolates need more than once extraction because the plant samples could not dissolve completely. Since the to-be-extracted glucosinolates were "wrapped" by insoluble material, is a solution of 70% aqueous methanol solution capable of extracting the glucosinolates in only one extraction?

*We do not quite understand this remark; we describe in our method that the materials are to be extracted twice with 70% MeOH. The last author has once -in 2006- determined the extraction efficiency by extracting triplicate samples of 3 different root and shoot samples. After the first extraction the solvent was brought on a Sephadex column as usual and the plant residue was extracted one more time with 70% MeOH, vortexing and 15 minutes ultrasonic bath (second extraction). The latter extract was brought on a separate Sephadex column. Both extracts were processed as if they were independent samples, and injected on HPLC. The results showed that less than 2% of the total amount of glucosinolates present in the first extraction was found in the second extract. This suggests the extraction as we describe, with 2 extraction steps, is exhaustive.*

*\*\* NM van Dam : Question to the editors: should we mention this experiment somewhere? If so, where and to which detail? I have the data, but never worked out the details as it was just a test for the procedure. I would need some time to write a paragraph and work out the data in more detail.\*\**

Have the authors compared the extraction efficiency using 70% aqueous methanol solution for extraction to that using other solvent systems.

*No we did not, see answer to question 1*

4. Authors used plant material dry weight to control the material amount. The authors should provide the fresh plants drying method because the tubes may be filled with the fresh plant, such as broccoli floret which contains almost 95% water.

*The reason for using dried plant materials is exactly this: when using fresh materials the glucosinolate concentration can be determined much less exactly. It greatly depends on the condition and the treatment of the fresh materials (e.g. how were they stored, were plants watered just before the harvest, were roots flushed with water to remove soil just before they were frozen?) how much water will be in the sample. Moreover, it is easier to store and process freeze-dried materials during the extraction. Fresh materials need to be flash frozen and ground under liquid nitrogen, and then weighed into a tube while keeping them frozen. Not only is this less precise (liquid nitrogen will evaporate during weighing), but the sample may thaw before extraction and myrosinase will convert the glucosinolates. In our experience, dried materials will be ground to a much finer powder much easier, which enhances extraction efficiency. It will be always possible to calculate the concentration on a per fresh mass basis by drying pre-weighed aliquots of the materials to constant weight and weigh them again after drying to determine the percentage dry mass. This is now mentioned in a shorter form as a note between point 4.4 and 4.5.*

5. Authors should choose glucosinolates content not plant material dry weight to control the material amount

*We do not understand this remark. See our answer to question 4 for the reasons to use dried materials. What is meant exactly by ‘content’? As specified above, this value can be calculated (e.g. 100 g of fresh broccoli contains x micromoles of glucosinolates)*

…and provide the detection limit of the glucosinolates content.

*The detection limit depends greatly on the concentration of the glucosinolates in the materials, the individual glucosinolates and the analytical platform used. Preferably the concentrations of the samples are within the range of the reference curve. We now mention this specifically at point 4.1 and refer to Crocoll and all for the detection limit (0.5-2 nmol)*

*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
The authors should to provide accurate description of the experimental details throughout the manuscript, such as the flow rate in table 1 and 2, and the ratio of ACN at 10 min in table 2.

*We do not understand this remark. As far as we know, we gave these details in Table 2.*  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**  
*Manuscript Summary:*  
This manuscript describes a well-known protocol for glucosinolate quantification based on desulfation followed by HPLC-DAD analysis. I think it will constitute a nice video paper which will be useful to plant scientists. The manuscript is well written and describes all steps of the procedure in details. The advantages and limitations of the technique are well described and the authors do not try to "oversell" the method.  
  
*Major Concerns:*  
No major concern.  
  
*Minor Concerns:*  
I only have a few comments which may help improve the manuscript:  
-I believe the readers would benefit from two additional tables for both retention times on the specific type of chromatographic column that was used, and for response factors at 229 nm. In such way, one would not necessarily need to consult several papers from the literature. This comment is valid provided there is no copyright issue, naturally.

*This information is now given in a Table (Table 3).*

-Line 243: either give the exact brand of the column, or a range of possible columns which may be used.

*We were asked by the editors to ignore this remark*-Line 103: from, not form.

*Changed*-Line 317: show, not shown.

*Changed*  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**  
*Manuscript Summary:*  
The manuscript describes in a clear way a method for analysis of glucosinolates by HPLC, and as such the manuscript will be of value for many people wanting to measure these compounds.  
  
General comments:  
L. 55, the sentence 'identification of the glucosinolates takes place…' . This may mislead people to think that novel structures can be identified this way, which is not correct. Please rephrase.

*Changed to “the detection and quantification”*L.119, sulfatase, please add purchaser.

*We were asked by the editors to ignore this remark*

L.265, this is not quite specific enough. Identification of novel GLS is only possible with reference standard, otherwise only with NMR.

*We added this to this to line and mentioned it again in the discussion, using De Graaf et al. 2015 as an example.*

L. 289, M (response factor) is only available for 40 glucosinolates in the literature (Brown et al 2003), so not for all. Should be stated explicitly.

*We added the response factors for the most commonly found glucosinolates in plant materials in Table 3.*

L.348, should be stated that for HPLC-UV/DAD one needs to learn to interpret the UV spectra.

*For these reasons we added the example UV spectra.*

*Question to the editors: should we really add that this needs to be learned? This seems obvious to us.*

L.382, response factors for LC-MS is not lacking, but they are instrument-specific. Please add the following ref: Crocoll C, Halkier, BA, Burow, M (2016) Analysis and quantification of glucosinolates. Curr Protoc Plant Biol, 1, 385-409.

*Sorry to have missed this recent paper, we included it now in the references and cite at various places in the text.*

L.386-389, LC-MS can easily be used for 'dirty' samples as one can also do desulfo-GLS analysis on LC-MS, not only intact GLS. LC-MS can be used for both freeze-dried or fresh samples, - it will only determine if values are given pr. g f.w. or d.w. Please add the following ref: Zhang, J., et al., Metabolite profiling of Arabidopsis seedlings in response to exogenous sinalbin and sulfur deficiency. Phytochemistry, 2011.

*Indeed, LC-MS can be used for desulphoglucosinolates as well, which is why we state it could be used to identify ‘unknown’ glucosinolates. We chose to refer to the more recent reference Crocoll et al. who also mention this aspect.*

L.410, other external references can be used, as they can be calculated relatively to other GLS.

*This might indeed be possible, but that would mean the response factors for each glucosinolates (class) have to be determined again. As stated in the answers above, our aim is to present this well-validated standard method for the community of glucosinolate researchers without access to high grade research infrastructures.*   
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A