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A straightforward method for glucosinolate extraction and analysis with High Pressure Liquid Chromatography (HPLC) --Manuscript Draft--

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Abstract:	<p>Glucosinolates are a well-studied and highly diverse class of natural plant compounds. They play important roles in plant resistance, rape seed oil quality, food flavoring and human health. The biological activity of glucosinolates is released upon tissue damage, when they are mixed with the enzyme myrosinase. This results in the formation of pungent and toxic breakdown products, such as isothiocyanates and nitriles. Currently, more than 130 structurally different glucosinolates have been identified. The chemical structure of the glucosinolate is an important determinant of the product that is formed, which in turn determines its biological activity. The latter may range from detrimental (e.g. for progoitrin) to beneficial (e.g. for glucoraphanin). Each glucosinolate-containing plant species has its own specific glucosinolate profile. For this reason, it is important to correctly identify and reliably quantify the different glucosinolates present in brassicaceous leaf, seed and root crops or, for ecological studies, in their wild relatives. Here we present a well validated, targeted and robust method to analyze glucosinolate profiles in a wide range of plant species and plant organs. Intact glucosinolates are extracted from ground plant materials with a methanol-water mixture at high temperature to disable myrosinase activity. Thereafter, the resulting extract is brought on an ion-exchange column for purification. After sulfatase treatment, the desulfoglucosinolates are eluted with water and the eluate is freeze-dried. The residue is taken up in an exact volume of water, which is analyzed by High Pressure Liquid Chromatography (HPLC) with a photodiode array (PDA) or Ultra Violet (UV) detector. Detection and quantification is achieved by comparisons of retention time and UV spectra of commercial reference standards. The concentrations are calculated based on a sinigrin reference curve and well established response factors. The advantages and disadvantages of this straightforward method in comparison to faster and more technologically advanced methods, are discussed.</p>
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TITLE:

A straightforward method for glucosinolate extraction and analysis with high-pressure liquid chromatography (HPLC)

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KEYWORDS:

Arabidopsis, *Brassica*, cabbage, European community official method, mustard, high-pressure liquid chromatography-photodiode array (HPLC-PDA), indole glucosinolates, oilseed rape, response factors

SHORT ABSTRACT:

Here, we describe in great detail an established and robust protocol for the extraction of glucosinolates from ground plant materials. After an on-column sulfatase treatment of the extracts, the desulfoglucosinolates are eluted and analyzed by high-pressure liquid chromatography.

LONG ABSTRACT:

Glucosinolates are a well-studied and highly diverse class of natural plant compounds. They play important roles in plant resistance, rapeseed oil quality, food flavoring, and human health. The biological activity of glucosinolates is released upon tissue damage, when they are mixed with the enzyme myrosinase. This results in the formation of pungent and toxic breakdown products, such as isothiocyanates and nitriles. Currently, more than 130 structurally different glucosinolates have been identified. The chemical structure of the glucosinolate is an important determinant of the product that is formed, which in turn determines its biological activity. The latter may range from detrimental (*e.g.*, progoitrin) to beneficial (*e.g.*, glucoraphanin). Each

glucosinolate-containing plant species has its own specific glucosinolate profile. For this reason, it is important to correctly identify and reliably quantify the different glucosinolates present in brassicaceous leaf, seed, and root crops or, for ecological studies, in their wild relatives. Here, we present a well-validated, targeted, and robust method to analyze glucosinolate profiles in a wide range of plant species and plant organs. Intact glucosinolates are extracted from ground plant materials with a methanol-water mixture at high temperatures to disable myrosinase activity. Thereafter, the resulting extract is brought onto an ion-exchange column for purification. After sulfatase treatment, the desulfoglucosinolates are eluted with water and the eluate is freeze-dried. The residue is taken up in an exact volume of water, which is analyzed by high-pressure liquid chromatography (HPLC) with a photodiode array (PDA) or ultraviolet (UV) detector. Detection and quantification are achieved by conducting comparisons of the retention times and UV spectra of commercial reference standards. The concentrations are calculated based on a sinigrin reference curve and well-established response factors. The advantages and disadvantages of this straightforward method, when compared to faster and more technologically advanced methods, are discussed here.

INTRODUCTION:

It is estimated that plants produce over 200,000 different types of chemical compounds¹. Only the minority of these compounds seems to play a role in the plants' primary metabolism, which fuels growth and reproduction; the majority are so-called secondary metabolites. Despite their name, secondary metabolites are often critical for plant survival and reproduction, as they serve to attract pollinators or to defend the plant against pathogens and herbivores¹.

Glucosinolates are a very diverse class of secondary metabolites that have been studied for over 150 years². To date, more than 130 structurally different glucosinolates have been identified³. Broadly, glucosinolates can be subdivided into different classes based on the amino acid from which they are synthesized. Indole glucosinolates, for example, are synthesized from the amino acid tryptophan, whereas phenylalanine provides the base skeleton for the synthesis of aromatic glucosinolates⁴. Within classes, there is a high level of structural diversity, which is brought about by sequential chain elongation steps in the biosynthetic pathways, such as in the class of aliphatic glucosinolates, or by side chain modifications (*e.g.*, hydroxylation)^{4,5}. One glucosinolate plant species may contain up to 37 different glucosinolates belonging to different structural classes⁶. Even though plant species have typical glucosinolate profiles, intraspecific variation for the types of glucosinolates is frequently found among individuals and populations^{6,7}. Intact glucosinolates are stored in the vacuoles of plant cells and can be found in any aboveground or belowground organ⁷⁻⁹. Upon cell rupture (*e.g.*, by herbivory), glucosinolates are released and are mixed with the enzyme myrosinase, setting off a mustard oil bomb¹⁰. Due to the activity of myrosinase, and depending on the structure of the glucosinolate, the reaction conditions, and the presence of modifying enzymes, different pungent, toxic, or noxious compounds are formed, such as nitriles and (iso)thiocyanates¹¹. The reaction products have high biological activities; for example, they serve as repellents of generalist insect herbivores¹². The heritability and biosynthetic pathways of glucosinolates are well studied, mainly because of their importance for herbivore and pathogen resistance, their value as flavor components in mustards and cabbages, and their negative (pro)growth and

positive (glucoraphanin) effects on human health^{5,13,14}.

Because of the great interest in glucosinolates as biologically active compounds, extraction and detection methods based on reversed-phase high-pressure liquid chromatography (HPLC) equipped with ultraviolet (UV) or photodiode array (PDA) detectors have been commonly used since the 1980s¹⁵. Based on this method, the European Communities issued a standard protocol that was tested and validated in several labs for the analysis of glucosinolates in oilseeds (*Brassica napus*, colza, Canola¹⁶). Others added to this method (*e.g.*, by determining additional response factors for glucosinolates that are not present in oilseed rape)¹⁷. Despite the increasing availability of liquid chromatography-mass spectrometry (LC-MS) platforms and high-throughput protocols for glucosinolate analysis^{18,19}, the original HPLC-UV/PDA method is still widely used by scientists. The main reasons are that this method is straightforward, cost-effective, and relatively accessible to labs without an extensive chemical-analytical knowledge infrastructure. To serve this community, we here detail the protocol for the extraction of glucosinolates from plant materials and the analysis of their desulfo-forms with HPLC-PDA.

PROTOCOL:

1. Preparation of solutions needed for the glucosinolate extraction

1.1. Prepare 500 mL of 70% methanol (MeOH) in water (ultrapure) in a glass bottle. For 100 samples and 5 standards, 420 mL of 70% MeOH is needed.

1.2. Prepare 20 mM sodium acetate (NaOAc) (pH = 5.5) by adding 0.82 g of NaOAc or 1.36 g of NaOAc x 3 H₂O in 500 mL of water; adjust the pH with hydrogen chloride (HCl). Store the NaOAc in the refrigerator. For 100 samples and 5 standards, 370 mL of 20 mM NaOAc (pH = 5.5) in water is needed.

1.3. To prepare the column material, mix 10 g of cross-linked dextran gel (type G-25) with 125 mL of ultrapure water. Store the resulting mixture in a refrigerator (4 °C).

1.4. Preparation of the sulfatase solution

1.4.1 Dissolve 10,000 units of aryl sulfatase (type H-1 from *Helix pomatia*) in 30 mL of ultrapure water and add 30 mL of absolute ethanol (EtOH). Mix the solution well and transfer it to one 250-mL centrifuge tube or multiple smaller tubes, depending on availability.

1.4.2 Centrifuge the mixture at 2,650 x g for 20 min at room temperature (RT). Transfer the supernatant to a centrifuge tube or tubes; add 90 mL of EtOH (or aliquots when using smaller tubes) and centrifuge at 1,030 x g for 15 min at RT.

1.4.3 Discard the supernatant and dissolve the remaining pellet in 25 mL of ultrapure water (or aliquots when using smaller tubes). Vortex well, dispense in 1-mL tubes, and store in a freezer at -20 °C; they will keep for at least one year.

1.5 Preparation of the sinigrin reference solution

1.5.1 Weigh around 9 mg of sinigrin monohydrate with 1- μ g accuracy (*e.g.*, 8.769 mg) into a 10-mL volumetric flask. Dissolve the sinigrin monohydrate in 10.0 mL of ultrapure water.

1.5.2 Calculate the molarity of the stock solution and prepare five sinigrin references between 50-750 μ M by diluting of the stock solution. For a detailed example, see Supplementary File S1.

1.5.3 Dispense the different sinigrin references in 1.5-mL reaction tubes and freeze them at -20 °C. Include a series of five sinigrin references in each extraction batch. Always use the correct molarity values for the currently used sinigrin references when calculating the concentrations.

2. Preparation of the extraction setup

2.1. Prepare a column rack for the Pasteur pipette columns (for the preparation of columns, see step 3.1). Label the rack or the columns to keep track of the sample numbers (see Figure 1).

2.2. Prepare a block holding the same number of labelled 2-mL microcentrifuge tubes (see step 3.3 and Figure 1)

[Place Figure 1 here]

3. Preparation of the columns and microcentrifuge tubes

3.1. Prepare one column per sample and five extra columns for the five sinigrin reference samples.

3.1.1. In each glass pipette, place a piece of glass wool approximately 1 cm x 1 cm. Use a wooden or glass stick to push the glass wool down to the point where the pipette narrows. The glass wool should prevent the column material from running out, but do not use too much, as it will slow the elution of the columns. Wear gloves when working with the glass wool.

3.2. Place the columns on the column rack (see Figure 1). Place the column rack in a lab tray (waste tray) to catch the eluents used for the consecutive column washes.

3.3. Cut the first 5 mm off the tip of a plastic 1-mL pipette tip and pipette 0.5 mL of the prepared column material (G-25 gel in water, see step 1.3) into each column. Shake the bottle containing the column material well before pipetting. Discard leaking columns (G-25 material running out through the glass wool layer) and replace with new ones.

3.3.1. After pipetting the column material into the columns, add 1 mL of ultrapure water to flush down the material.

3.4. Prepare one 2-mL reaction tube per sample and five tubes for the five sinigrin reference samples; label them. Use a dissecting needle to make holes in the caps for later freeze-drying and place the prepared tubes into the tube block in the exact same pattern as the columns (step 2.2, see Figure 1).

4. Extraction of glucosinolates

4.1. Weigh freeze-dried and finely-ground plant material (usually 50.0-100.0 mg of dry weight; the final glucosinolate concentrations in the extract should be in the range of the reference curve) to the nearest 0.1 mg in 2-mL, labeled, round-bottom reaction tubes. Add two small metal balls (3 mm in diameter) as boiling retardants to each tube.

Note: The protocol can also be applied to fresh, flash-frozen materials, that have been ground under liquid nitrogen and kept frozen until extraction. Increase the amount of material weighed for extraction and the percentage of MeOH in the extraction liquid to 85% to compensate for dilution by the water in the materials¹⁹.

4.2. Pipette 1 mL of 70% MeOH into each tube and vortex briefly. Close the tubes and seal them with safety caps before placing them as quickly as possible into a hot water bath (90-92 °C) for a few minutes (~5 min), until the 70% MeOH just boils. Caution: Wear safety goggles during this step!

4.3. Place the sample tubes in an ultrasonic bath for 15 min. Meanwhile, take the sulfatase and the five sinigrin reference samples out of the freezer to thaw them at RT.

4.4. After ultra-sonication, centrifuge the sample tubes at 2,700 x g in a benchtop centrifuge for 10 min at RT; a pellet should form in each tube. Add the supernatants to the labelled columns and pipette the five reference samples onto separate columns.

4.4.1. While pipetting the supernatants, keep the tip well over the pellet to avoid pipetting plant materials. Note that when dried samples are used, the supernatant volume will be less than 1.0 mL.

4.5. Add 1 mL of 70% MeOH to the remaining pellets in the sample tubes and vortex the tubes before placing them in an ultrasonic bath for 15 min. Centrifuge the tubes again, as in step 4.4, and add the supernatants to the respective columns; due to the properties of the column material, the negatively charged sulfate group of the glucosinolates will be specifically retained on the column.

4.6. Wash the columns with the extracts in three sequential steps.

4.6.1. Pipette 2 x 1 mL of 70% MeOH onto each column. Wait for the column to run dry before adding the next 1 mL; this will remove more apolar compounds from the extracts (*e.g.*, chlorophyll).

4.6.2. Flush out the MeOH by adding 1 mL of ultrapure water to each column.

4.6.3. Pipette 2 x 1 mL of 20 mM NaOAc buffer to each column to create the optimal conditions for the sulfatase reaction.

4.7. Take the rack with the columns out of the waste tray and dry the feet of the rack with a tissue. Place the rack over the block with vials and labeled tubes. Make sure that each column tip is in the corresponding, labeled, 2-mL tube (see Figure 1).

4.8. Add 20 μ L of sulfatase solution to the columns. Ensure that the sulfatase reaches the surface of the column material. Pipette 50 μ L of NaOAc buffer onto each column to flush down the sulfatase. Cover the columns with aluminum foil and let stand overnight.

Note: Due to the activities of the sulfatase, the sulfate group will be removed, releasing the desulfoglucosinolates from the column so that they can elute with the water. For a detailed description, see Crocoll et al.¹⁹.

4.9. The next day, elute the desulfoglucosinolates by pipetting 2 x 0.75 mL of ultrapure water onto each column. When all columns have run dry, lift the column rack and remove it from the reaction tubes.

4.9.1. Cap the tubes (make sure that there are holes in the caps) and freeze them in liquid nitrogen or in a -80 °C freezer for 30 min. Freeze-dry the samples for 12-24 h (depending on the number of samples and the capacity of the freeze-drier) to remove all water.

4.10. After freeze-drying, re-dissolve the residue in an exact volume (usually 1.0 mL) of ultrapure water. Transfer the samples and the five sinigrin references to labeled HPLC vials. Keep the samples in a refrigerator (4 °C) for up to two weeks or a freezer (-20 °C) for up to one year before analyzing them with HPLC.

4.11. Let the glass columns dry under the hood overnight and dispose 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.

5. HPLC measurements of extracted samples

5.1. Separate the glucosinolates on a reversed-phase C₁₈ column (4.6 x 150 mm, 3 μ m, 300 Å) with an acetonitrile-water gradient (see Table 1) and a flow of 0.75 mL/min and a column temperature of 40 °C. Perform detection and quantification at 229 nm.

5.2. Measure the sinigrin references on the same column but with an adapted gradient method to reduce solvent use (see Table 2). Start with injecting the five sinigrin references, beginning with the reference with the lowest concentration. Then inject the samples, including a methanol injection (blank) after every tenth sample to clean the column and to prevent the carryover of peaks.

[Place Table 1 here]

[Place Table 2 here]

6. Glucosinolate identification and quantification

6.1. Identification

6.1.1. Compare the UV spectra and retention times of the peaks in the samples with commercially available, pure glucosinolate references. See Figure 2 for examples of UV spectra from the various glucosinolate classes.

[Place Figure 2 here]

6.1.2. Identify unknown glucosinolates with reference standards or on LC-MS^{18,19}, if possible.

6.1.3. Identify unknown glucosinolates with nuclear magnetic resonance (NMR), if possible.

6.1.4. Compare the UV spectra and retention times of the peaks with those in Figure 2 and Table 3, databases, or literature references.

6.2. Quantification

6.2.1. Integrate the area under the peaks of the five sinigrin references and the samples.

6.2.2. Calculate a calibration curve of the five measured sinigrin references based on the integrated area. Use the equation of the regression line (see Equation 1) to calculate the interpolated amount of glucosinolates (see Equation 2).

6.2.3. To calculate the concentration of the different glucosinolates, multiply the interpolated amount (x) by the response factor (M) for detection at 229 nm from EC (1990)¹⁶, Buchner (1987)¹⁵, or Brown *et al.* (1993)¹⁷; the consensus response factors of the most commonly detected desulphoglucosinolates are given in Table 3.

Note: Response factors may vary between systems—this may be the reason that there are different values given by different references (see References 15-17). Nevertheless, the values are mostly quite close and in a similar range with structural classes. Following the convention, set the response factor of unidentified glucosinolates to 1, although for unknown indole glucosinolates with a UV spectrum similar to that of glucobrassicin and other indole glucosinolates (Figure 2), it would be advisable to use 0.25 as a response factor to obtain a realistic estimate of the concentration (Table 3).

6.2.4. To calculate the final amount of glucosinolates (x_t) in the sample, take the dilution factor (D) and the mass of sample (w) used for the analysis into account (see Equation 3).

$$y = mx + c \quad (1)$$

$$x = \frac{y-c}{m} \quad (2)$$

$$x_t = \frac{x \cdot M \cdot D}{w} \quad (3)$$

y = Peak area

m = slope of the reference 5-point regression curve

c = the intercept of the regression line with the y-axis

x = amount of glucosinolates in the extract

x_t = concentration of glucosinolates in the plant sample

D = dilution factor

M = response factor for the detection at 229 nm from Buchner (1987) or Brown *et al.* (1993)

w = mass of sample used for extraction

REPRESENTATIVE RESULTS:

This method enables the detection and separation of commonly found desulfoglucosinolates in all structural classes (Figure 3). The detrimental 2-hydroxyglucosinolate progoitrin comes relatively early in the chromatogram and is clearly separated from the beneficial glucosinolate glucoraphanin, the only methylsulfinylglucosinolate in this sample. Sinigrin, gluconapin, and glucobrassicinapin form an eluotropic series of alkenyl glucosinolates with increasing side-chain lengths (C3, C4, and C5, respectively). A similar logical series is visible for the two methylthioalkenyl glucosinolates, glucoiberin (C3) and glucoerucin (C4). The peaks of the three indole glucosinolates, glucobrassicin and its derivatives 4-hydroxy and 1-methoxyglucobrassicin (neoglucobrassicin), are also clearly separated. It should be noted that the peaks of neoglucobrassicin and glucoerucin, as well as gluconasturtiin, the only aromatic glucosinolate in this sample, are particularly high in this *Brassica* extract due to the addition of root extracts to the mix⁹.

[Place Figure 3 here]

Longer chain methylsulfinyl glucosinolates, which are commonly found in the model plant *Arabidopsis*, also show an eluotropic series, as seen in a root extract of *Rorippa austriaca*. Glucohesperalin (C6), glucosiberin (C7), glucohirsutin (C8), and glucoarabin (C9) appear at regular intervals on the chromatogram (Figure 4). Together with the UV spectra of the peaks, such eluotropic logical series may be used to classify, and provisionally identify, unknown glucosinolates.

[Place Figure 4 here]

Figure 1: Rack for glucosinolate extraction.

Front view (left) and top view (right) of a self-made column rack and block for the glucosinolate extraction. Height: 100 mm, distance between shifted holes (to hold the Pasteur pipette columns): 30 mm (x-axis) x 15 mm (y-axis).

Figure 2. UV spectra of the most common glucosinolate classes.

UV absorption spectra (200-350 nm) of six desulfoglucosinolates (GSL), based on solutions made of commercially available reference compounds extracted as described here, representing the most common structural classes. The common name, structural name (between brackets), and structural class are given. (A) Sinigrin (2-propenyl GSL), alkenyl; (B) glucoerucin (4-methylthiobutyl GSL), thioalkenyl; (C) Glucoraphanin (4-methylsulfinylbutyl GSL), sulfinyl; (D) Glucobrassicin (indol-3-ylmethyl GSL), indole; (E) gluconasturtiin (2-phenylethyl GSL), aromatic; (F) Sinalbin (4-hydroxybenzyl GSL), aromatic.

Figure 3: Chromatogram of a glucosinolate extract.

Detail (1-32 min) of an HPLC chromatogram resulting from the analysis of combined root and shoot samples from *Brassica nigra*, *B. rapa*, and *B. oleracea*. The peak labels indicate the retention time and the abbreviations for identified desulfoglucosinolates (GSL). PRO = progoitrin (2-OH-3-butenyl GSL); RAPH = glucoraphanin (4-methylsulfinylbutyl GSL); SIN = sinigrin (2-propenyl GSL), GNA = gluconapin (3-butenyl GSL); 4OH = 4 hydroxyglucobrassicin; IBV = glucoiberberin (3-methylthiopropyl GSL); GBN = glucobrassicinapin (4-pentenyl GSL); ERU = glucoerucin (4-methylthiobutyl GSL); GBC = glucobrassicin (indol-3-ylmethyl GSL); NAS = gluconasturtiin (2-phenylethyl GSL); NEO = neoglucobrassicin (1-MeOH-glucobrassicin).

Figure 4: Chromatogram (frame 9-30 min) of the desulfoglucosinolates in a *Rorippa austriaca* root extract.

The peak labels indicate the retention time and the abbreviations for identified desulfoglucosinolates (GSL). HES = glucohesperin (6-methylsulfinylhexyl GSL); SBE = glucosiberin (7-methylsulfinylheptyl GSL); GBC = glucobrassicin (indol-3-ylmethyl GSL); HIR = glucohirsutin (8-methylsulfinyloctyl GSL); ARA = glucoarabin (9-methylsulfinylnonylglucosinolate); NEO = neoglucobrassicin (1-MeOH-glucobrassicin).

Table 1: Acetonitrile-water gradient for glucosinolate separation and analysis on reversed-phase HPLC

Table 2: Shortened acetonitrile-water gradient for quantification of the five sinigrin references used for the quantification of glucosinolates

Table 3: Response factors of the most commonly found desulpho-glucosinolates in plant extracts and their approximate retention times on C18 columns. Eluents, gradient, column temperature, and flow rate as in Table 1.

DISCUSSION:

The greatest advantages of this established and widely used method are that it is robust, rather

straightforward, and relatively low-cost per sample. Most of the equipment needed for the extraction and analysis should be available in a standard laboratory or can be self-built, with the exception of the HPLC-PDA. Another advantage is that desulfoglucosinolates dissolved in water are chemically quite stable when kept cool and in air-tight (HPLC) vials, so the extracts could easily be shipped for HPLC analysis elsewhere. In contrast to LC-MS platforms, which require specialized training and extensive hands-on experience for managing the software and analyzing the data, HPLC-UV/PDAs can be easily run after a short training period. This not only reduces the costs of the procedure, but also makes this method more accessible to a broad range of scientists, including students.

Generally, when the procedures described above are followed carefully, few problems should occur. In general, the glucosinolate peaks are very well separated in the chromatogram. If this is not the case, the gradient program could be adapted by decreasing the rate of increase of acetonitrile in the eluent. Alternatively, building in a new pre-column (200-500 injections) or column (1,500 -2,000 injections) may solve the issue. Occasionally, chromatograms of single samples in a batch may show very small or no peaks. This is usually due to pipetting errors when adding the sulfatase (*e.g.*, a column has been skipped or the sulfatase was not properly washed down into the column). Alternatively, the glucosinolate concentration in the experimental materials may have been lower than expected and too little material was used for the extraction. If the latter is the case, the injection volume may be increased to 100 μ L, or an exact aliquot (*e.g.*, 800 μ L) of the extract could be concentrated. The latter could be achieved by freeze-drying the extract, dissolving the residue in a smaller volume (*e.g.*, 100 μ L) of water, and reinjecting using the same reference curve. In the calculations for the original concentration of the extract, the numbers should be multiplied by the dilution factor. If this does not solve the issue, the materials should be extracted again using more starting material. If this is more than 100 mg, the volume of the extraction solvents and the size of the tubes should be adjusted proportionally to maintain the extraction efficiency.

An additional advantage is that this method has been well-validated . This is because it has been described as a standard method for the quantification of glucosinolates in rapeseed, for which the procedures and accuracy were confirmed in several laboratories¹⁶. In addition, the genetic background, biosynthesis, and biological functions of glucosinolates are subject to intense research efforts, in the model plant species *Arabidopsis thaliana* among others^{4,6,12}. Therefore, many response factors for the exact quantification of desulfoglucosinolates in relation to sinigrin are well defined and publicly available^{15,17}. Even though LS-MS-based protocols are more high-throughput, more sensitive, and are able to (tentatively) identify glucosinolates for which no standards are available¹⁸⁻²⁰, the lack of universal response factors for LC-MS limits the exact quantification of glucosinolate concentrations¹⁸. Moreover, these methods usually do not include a freeze-drying step, and the amount of water in the fresh plant material is unaccounted for in the calculations, making exact quantification difficult. Lastly, because our extraction method involves a column-based purification and concentration step, it can also be applied to “dirty” samples with low concentrations of glucosinolates, such as soils²¹.

Compared to LC-MS-based methods that usually extract freshly frozen materials, use 96-well

plates for extraction, and do not include a sulfatase step^{18,19}, our method is relatively time-consuming and labor intense. With the column racks described in this paper, a single person can extract about 100-150 samples in one day. Elution (next day), freeze drying (overnight), and re-dissolving can take place within the following two days. With an automated HPLC injector, a run and equilibration time of 40-45 min per injection, and no unforeseen events, it would take 3-4 days to acquire the data for this sample set. When the HPLC software allows automatic quantification based on the sinigrin curve, a manual check of the chromatograms and peak assignments for 100 samples may only take another 1 or 2 h before the data can be used for statistical analyses.

Despite the increasing availability of glucosinolate standards, only a small fraction of the more than 130 candidates can currently be commercially bought. However, with a few references for each of the biosynthetic classes; access to literature databases specifying the compounds previously found in the plant species (*e.g.*, Fahey *et al.*²²); basic knowledge of chromatographic principles, such as the logic of elutropic series (*e.g.*, for increasing numbers of Cs on the side chain in aliphatic compounds, Figures 3 and 4); and the validation of single samples on LC-MS¹⁹ or isolated glucosinolates on NMR²³, one may easily overcome this limitation. Most protocols for glucosinolate analyses use internal reference curves (*i.e.*, a certain concentration for the extraction of sinigrin or sinalbin to the extraction solvent^{16,17,19}). Principally, internal reference curves are more appropriate to correct for individual sample processing errors and thus theoretically yield a higher precision. Despite this advantage, we prefer to use a five-point external reference curve, as we often analyze different wild species, some of which contain high levels of sinigrin (*e.g.*, *Brassica nigra*²⁴) or sinalbin (*e.g.*, *Sinapis alba*²⁵), the two glucosinolate references for which response factors are available. Moreover, adding internal standards to each sample increases the cost of the analyses, as high-grade glucosinolate reference standards are usually quite expensive.

In conclusion, despite the time-consuming steps, this protocol provides a straightforward and accessible method to extract and quantify glucosinolates in plant samples. However, it is important to consider that the glucosinolate levels themselves are only an indication of the potential biological activity, seen as the necessity to react with myrosinase, and variation in the reaction products may arise from a single glucosinolate¹¹. Validation assays must be performed to confirm the biological relevance.

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DISCLOSURES:

The authors have nothing to disclose.

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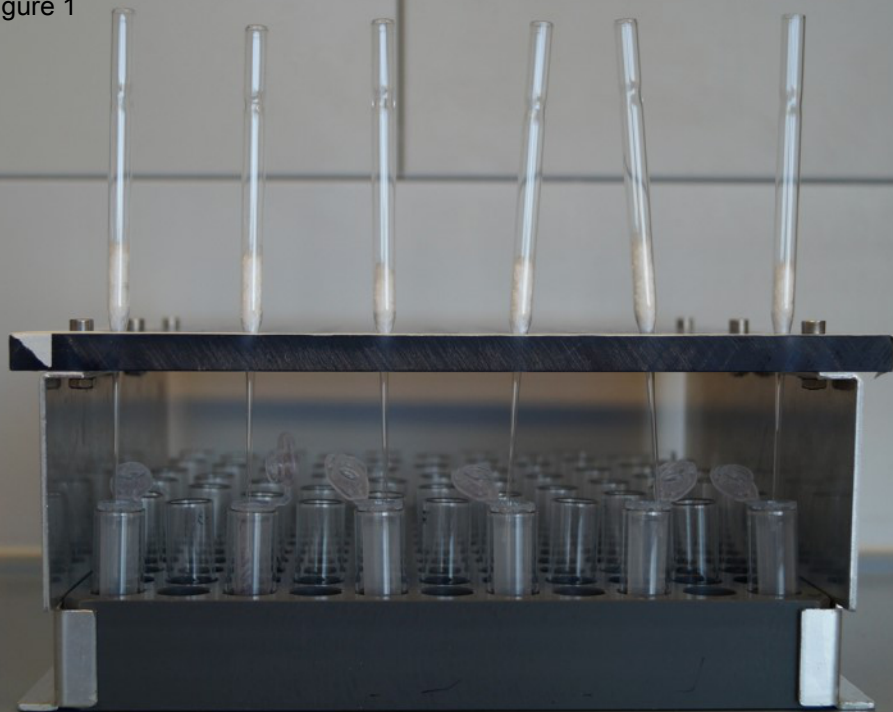
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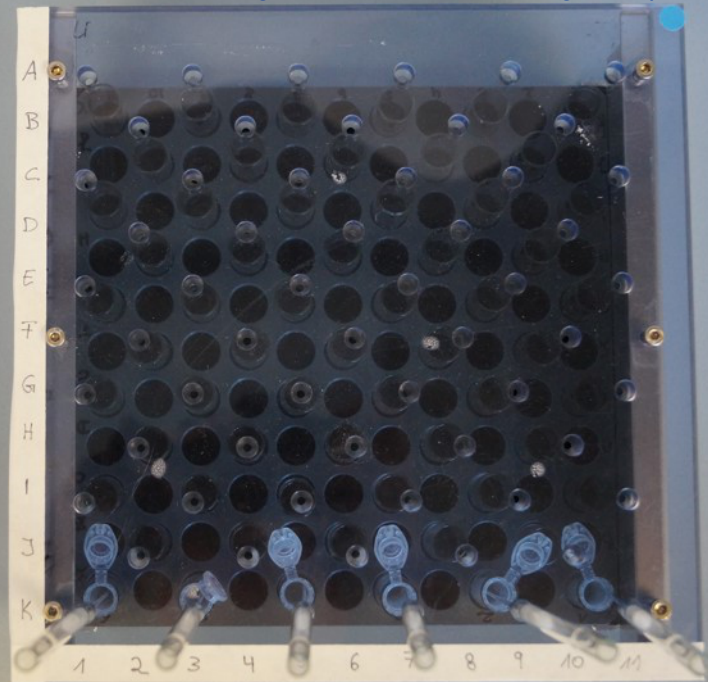
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Figure 1



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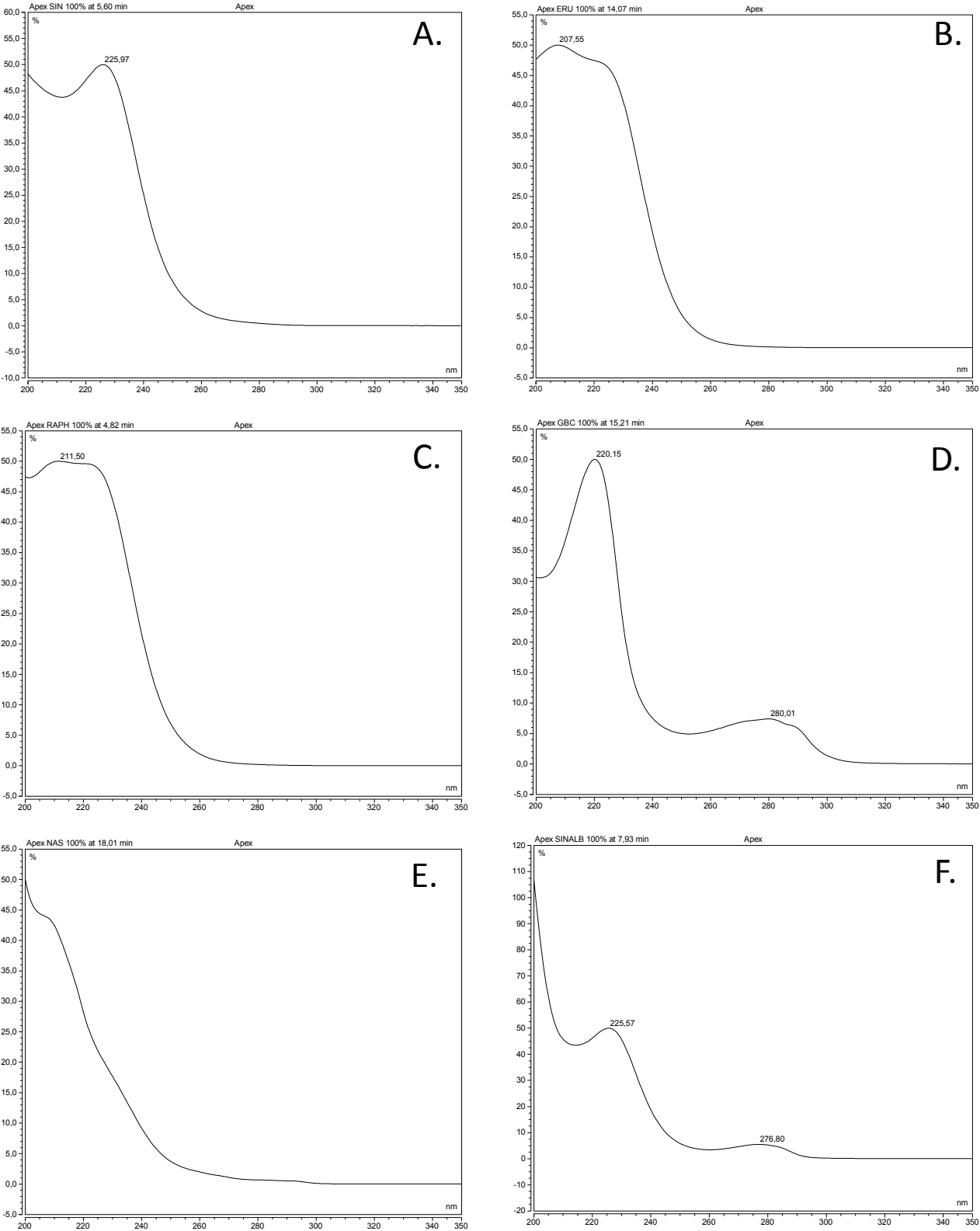


Figure 2

Figure 3

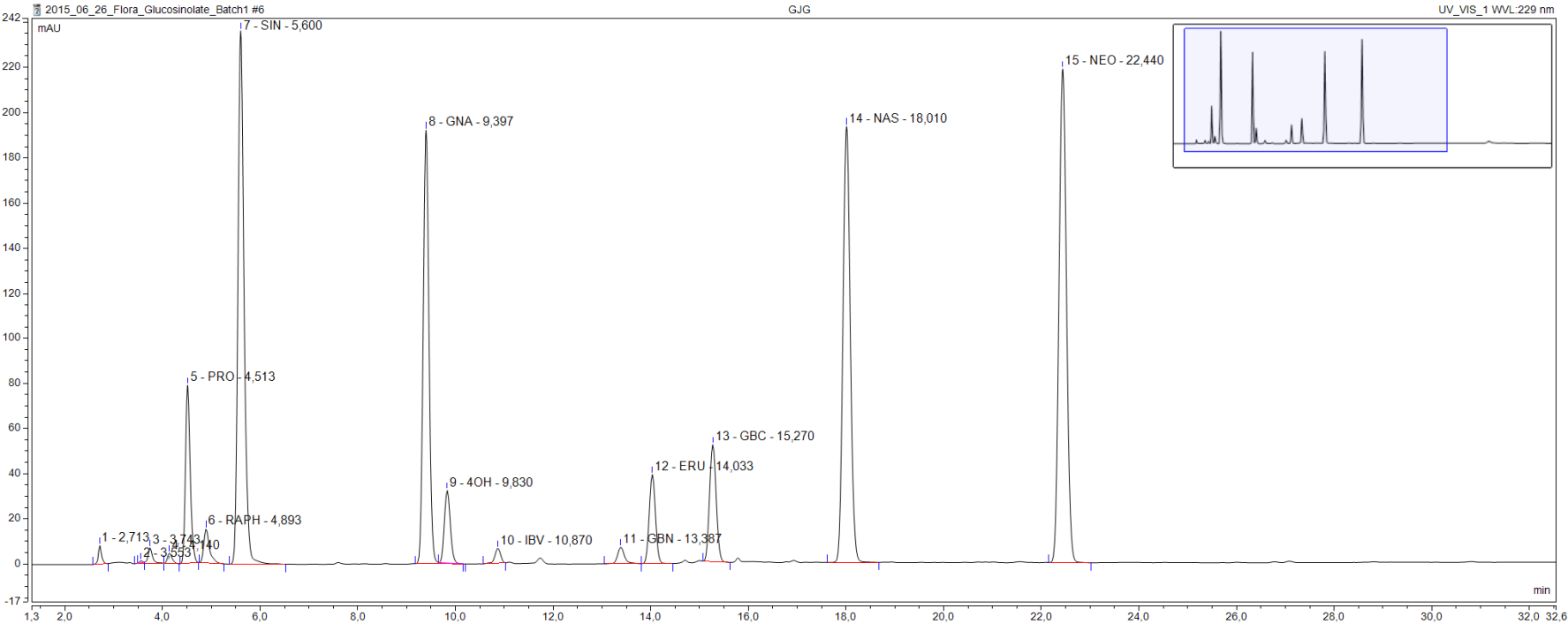
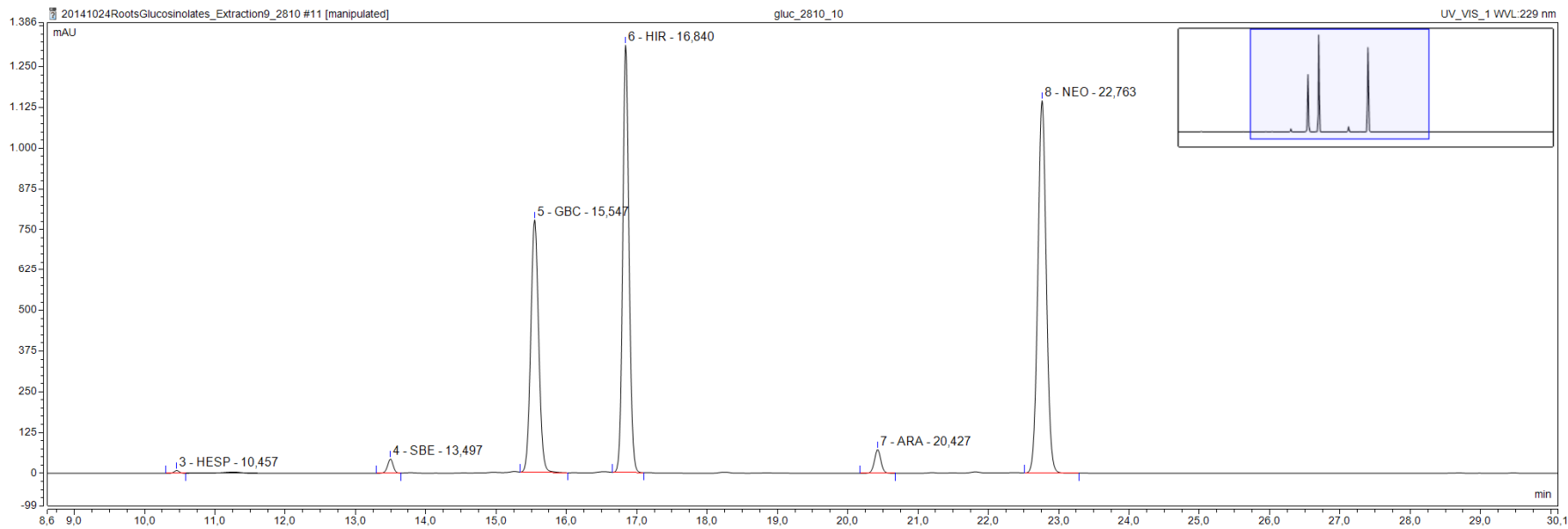


Figure 4



Time [min]	Flow [ml\min]	% A Water	% B ACN
1	750	98	2
35	750	65	35
40	750	98	2

Column Temperature 40°C.

Time [min]	Flow [ml\min]	% A Water	% B ACN
1	750	98	2
10	750	65	10.7
11	750	98	2

Column Temperature 40°C.

Common name	Side chain structure	Rt (min)*	229nm
<i>aliphatic glucosinolates</i>			
Glucocapparin	methyl	3.5	1
Sinigrin	2-propenyl	5.5	1
Gluconapin	3-butenyl	9.5	1.11
Glucobrassicinapin	4-pentenyl	13.5	1.15
Glucoiberin	3-methylthiopropyl	10.9	0.8
Glucoerucin	4-methylthiobutyl	14.0	0.9
Glucoiberin	3-methylsulfinylpropyl	3.7	1.2
Glucoraphanin	4-methylsulfinylbutyl	4.9	0.9
Glucoalyssin	5-methylsulfinylpentyl	7.6	0.9
Glucohesperin	6-methylsulfinylhexyl	10.5	1
Glucosiberin	7-methylsulfinylheptyl	13.5	1
Glucohirsutin	8-methylsulfinyloctyl	16.8	1.1
Glucoarabin	9-methylsulfinylnonyl	20.5	1
Glucocheirolin	3-methylsulfonylpropyl	4.2	0.9
Progoitrin	2(R)-OH-3-butenyl	4.5	1.09
Gluconapoleiferin	2-OH-5-pentenyl	8.3	1
<i>indole glucosinolates</i>			
4-hydroxyglucobrassicin	4-hydroxyindol-3-ylmethyl	11.2	0.28
Glucobrassicin	indol-3-ylmethyl	15.3	0.29
4-Methoxyglucobrassicin	4-methoxyindol-3-ylmethyl	18.2	0.25
Neoglucobrassicin	1-methoxyindol-3-ylmethyl	22.5	0.2
<i>aromatic glucosinolates</i>			
Sinalbin	4-hydroxybenzyl	8.1	0.5
Glucosibarin	2(R)-OH-2-phenylethyl	12.1	0.95
Glucobarbarin	2(S)-OH-2-phenylethyl	12.7	0.95
Glucotropaeolin	benzyl	13.8	0.95
Gluconasturtiin	2-phenylethyl	18.0	0.95
<i>unknown - aliphatic/aromatic like UV spectrum</i>			1

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Methanol HiPerSolv	VWR	20,864,320	
Sodium acetate (NaOAc)	Sigma-Aldrich	W302406-1KG-K	
HCL	VWR	1,090,571,000	
Sephadex	Sigma-Aldrich	A25120-10G	
(-)-Sinigrin hydrate from horseradish	Sigma-Aldrich	S1647-500MG	
Aryl Sulfatase	Sigma-Aldrich	S9626-10KU	
Ethanol	VWR	20,816,298	
Pasteur Pipette	Carl Roth	4518.1	
Glass Wool	Carl Roth	7377.1	
Glass /wooden stick	VWR	HERE1080766	
2 mL reaction tubes	VWR	211-2606	
Dissecting needle	Carl Roth	KX93.1	
Rotilabo-lab dishes	Carl Roth	0772.1	Waste tray
Freeze Dryer Freezone 12 L	Labconco	7960030	
Acetonitril super gradient grade	VWR	83,639,320	
Water bath	VWR	462-5112	
Ultrasonic bath	Fisher Scientific	FB 15061	
PH Electrode	Thermo Fisher Scientific	STARA2115	
Centrifuge Heraeus Multifuge X1	Thermo Fisher Scientific	75004210	
Pre-Column	Thermo Fisher Scientific	69697	C ₁₈ column (4.6 x 10 mm, 5 µm, 300 Å)
Column Acclaim 300	Thermo Fisher Scientific	60266	C ₁₈ column (4.6 x 150 mm, 3 µm, 300 Å)
HPLC Ultimate 3000	Thermo Fisher Scientific		with column oven and UV or PDA detector
Flask 1000 mL	VWR	215-1595	
Glucosinolate reference compounds	Phytoplan	various	http://www.phytoplan.de/



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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 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:

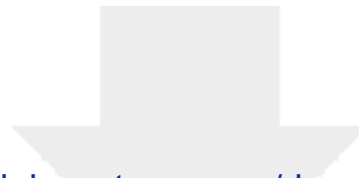
N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A



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Supplemental File (as requested by JoVE)

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