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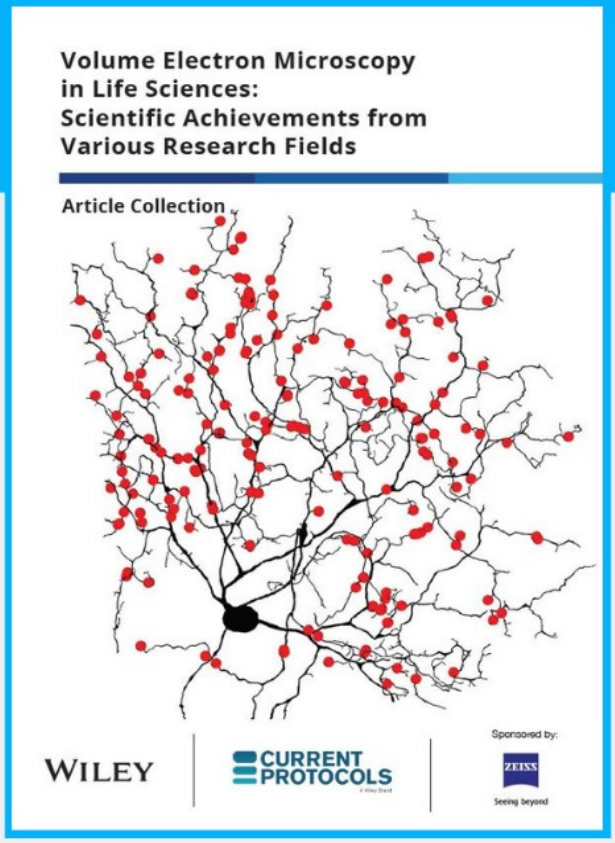

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A High-Throughput Absolute Quantification of Protein-Bound Tryptophan from Model and Crop Seeds

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This protocol describes a high-throughput absolute quantification protocol for the aromatic essential amino acid, tryptophan (Trp). This procedure consists of a milligram-scale alkaline hydrolysis followed by an absolute quantification step using a multiple reaction monitoring tandem mass spectrometric (LC-MS/MS) detection method. The approach facilitates the analysis of a few hundred samples per week by using a 96-well plate extraction setup. Importantly, the method uses only ~4 mg of tissue per sample and uses the common alkaline hydrolysis protocol, followed by water extraction that includes L-Trp-d5 as an internal standard to enable the quantification of the absolute level of the bound Trp with high precision, accuracy, and reproducibility. The protocol described herein has been optimized for seed samples for *Arabidopsis thaliana*, *Glycine max*, and *Zea mays* but could be applied to other plant tissues. © 2023 Wiley Periodicals LLC.

Basic Protocol: Analysis of protein-bound tryptophan from seeds

Keywords: alkaline hydrolysis • amino acids • high-throughput method • LC-MS/MS • seeds • tryptophan

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INTRODUCTION

Tryptophan (Trp) is an aromatic amino acid that is synthesized via the shikimate/chorismate pathway and is transformed into bioactive metabolites post-consumption (Friedman, 2018; Palego et al., 2016). While Trp residues are generally low in most proteins, they are necessary for protein stability and recognition (Santiveri & Jimenez, 2010). Due to the hydrophobic nature of Trp and its bulkiness, Trp can participate in large-scale van der Waals interactions within the hydrophobic core of proteins (Palego et al., 2016). Trp is also one of the amino acid residues most susceptible to oxidation and has been reported to undergo chemical alteration that leads to deleterious alterations in protein function (Corpas et al., 2021; Erland et al., 2015; Vitalini et al., 2020). Moreover, tryptophan is one of the most deficient amino acids in corn, rice, barley, rye, sorghum, and wheat (Boyer et al., 1992; Galili et al., 2005; Shewry, 2007; Shewry & Halford, 2002; Ufaz & Galili, 2008) despite being an essential amino acid (EAA) that cannot be made in the body and must be directly obtained from the diet (Vitalini et al., 2020; Wu, 2009).

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Many studies have attempted to improve the balance of EAAs in seed proteins and there is a need for improving Trp in our protein diets, among other EAAs. However, most studies targeting protein-bound amino acids (PBAAs) in plant materials exclude this amino acid from their measurements because the gold standard acid hydrolysis used for amino acid analysis (Moore et al., 1958) degrades its indole ring rendering it unquantifiable (Friedman, 2018). Moreover, the current alkaline method (Hugli, 1972) used for Trp analysis is lengthy and tedious and only adds information of one additional amino acid out of the full amino acid profile (Dahl-Lassen et al., 2018; Gorissen et al., 2018). To address this issue, we developed a high-throughput method for the quantification of Trp based on alkaline hydrolysis using a 96-plate setup, followed by a 6-min targeted amino acid analysis using a tandem mass spectrometric (UPLC-MS/MS) method as described (Yobi & Angelovici, 2018).

The protocol has 4 major steps: (a) alkaline hydrolysis, (b) extraction from the hydrolysate, (c) quantification using UPLC-MS/MS, and (d) collecting and processing data. Using the same 6-min method as described previously (Yobi & Angelovici, 2018) makes it easy to analyze this amino acid alongside the remaining amino acids without having to change LC or MS conditions.

BASIC PROTOCOL

ANALYSIS OF PROTEIN-BOUND TRYPTOPHAN FROM SEEDS

In this Basic Protocol, we will detail all the instruments and materials needed for bound Trp analyses from plant seeds. We will start by describing: first, the alkaline hydrolysis procedure for the release of bound Trp from proteins; second, Trp extraction; third, Trp analysis with UPLC-MS/MS; and fourth, data collection and analysis as depicted in Figure 1.

Materials

Arabidopsis seeds, or ground seed from other species
4 N sodium hydroxide (NaOH) solution (Fisher Scientific, cat. no. SS411-4)
Standard, serially diluted (see recipe)
Extraction buffer, EB2 (see recipe)
Perfluoroheptanoic acid (PFHA) (Sigma-Aldrich, cat. no. 342041).
Acetonitrile, HPLC-grade
H_2O, Milli-Q

Disposable, anti-static microspatula (e.g., LevGo, cat. no. 17231B)
Sub-milligram analytical balance (e.g., Mettler Toledo XS 105; Fisher Scientific, cat. no. 01-913-892)
1.1-ml non-coded screw cap tubes V-bottom bulk (VWR, cat. no. 101975-256)
96-well racks with lids (NOVA Biostorage, part no. MPW51BCPK)

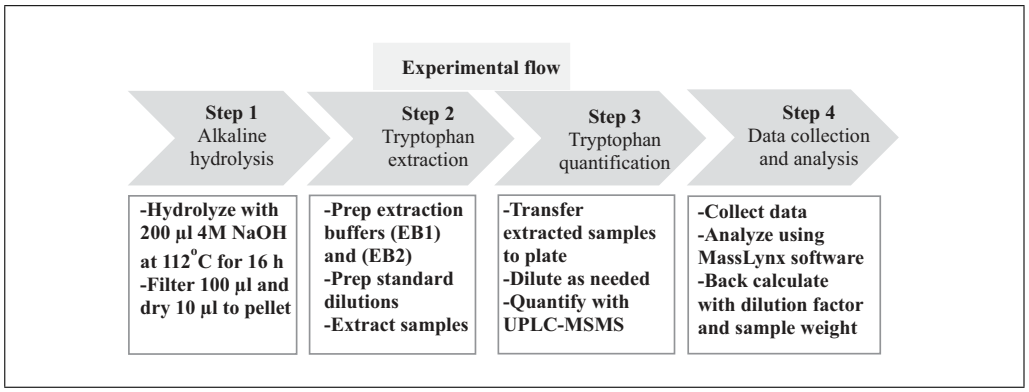

Figure 1 An overview of the tryptophan analysis experimental flow.

3-mm solid glass beads (e.g., Sigma-Aldrich, CLS72683)
3-mm bead dispenser, 96-well (e.g., Qiagen, cat. no. 69973)
Chemical hood
8- and 12-multichannel pipettes (200- and 10-µl volumes) and pipette tips
50-ml reagent reservoir (e.g., Fisher Scientific, cat. no. 07200127)
Pierceable capband-8 in a capmat format (e.g., Micronic, cat. no. MP53002)
High-energy, high-throughput cell disrupter (e.g., Mini-Beadbeater-96 with 1400 to 2400 rpm speed; BioSpec Products, cat. no. 1001)
Cooler centrifuge equipped with 96-well plate rotors (e.g., Sorvall Legend XTR; Fisher Scientific, cat. no. 75-004-523)
Oven that can maintain 112°C (e.g., Heratherm Oven OGS60; Fisher Scientific, item no. T9FB2187511)
Heat-resistant gloves
Manual decapper (e.g., Micronic, MP54001)
0.45-µm filter plate, hydrophilic, PTFE, clear, non-sterile (e.g., EMD Millipore, cat. no. MSRLN0450)
Filter plate vacuum manifold, optional (e.g., VWR, cat. no. 16003-836)
SpeedVac evaporator equipped with 96-well plate rotors (e.g., Savant SC250 EXP; Fisher Scientific, cat. no. SC250EXP-115) coupled with a refrigerated vapor trap (e.g., Savant RVT5105 Refrigerated Vapor Traps; Fisher Scientific, cat. no. RTV5105-115) and vacuum pump (e.g., VLP120 series; Fisher Scientific, cat. no. 50-870-639)
96-well V-bottom microplate, 520 µl (Dot Scientific, cat. no. PC63241-NS6)
96-round well, pre-slit, silicone sealing mat (MidSci, cat. no. MID-RD-9-PRT)
Kinetex 2.6 µm C18 100 Å LC column, 100 × 2.1 mm (Phenomenex, part no. 00D-4462-AN)
MassLynx (version 4.0)

Tryptophan alkaline hydrolysis

1. Weigh out 4 mg of Arabidopsis seeds or ground seed from other species and place in 1.1-ml tubes using a disposable antistatic microspatula and a sub-milligram balance.

   Arabidopsis seeds are small and will be crushed using the mini bead-beater. Crop seeds are larger in size and require crushing prior to this step.

2. Place tubes in a 96-well rack following the order: A1 to A12, B1 to B12, etc.
3. Add three 3-mm glass beads to each tube using the bead dispenser.
4. In a chemical hood and using a multichannel pipette and a 50-ml reagent reservoir, add 200 µl 4 N NaOH to each sample tube and to 3 empty tubes as negative controls.

   Always include 3 negative controls in which no sample is added to the 4 N NaOH to ensure impurities are not contaminating samples.

5. Close lids firmly with a capmat to preserve sample during hydrolysis.
6. Shake rack for 4 min using a mini-bead beater.
7. Centrifuge rack 5 min at 1000 × *g*, room temperature, to bring samples down to bottom of tubes.
8. Place rack into a heat-resistant container and close.

   Although capping the 1.1 ml tubes with capmat prevents any leak, samples are placed in a sealed container as an additional safety measure.

9. Place container in oven preheated to 112°C.
10. Incubate at 112°C for 16 hr.

11. Remove container from oven using heat-resistant gloves and cool to room temperature.
12. Remove rack from the container.
13. Centrifuge 15 min at 3700 × *g*, room temperature, to precipitate debris.
14. Remove the capmat with a manual decapper and transfer 100 µl of the hydrolyzed sample onto a filter plate using multichannel pipette.

    Make sure to maintain the same order of samples.

15. Place filter plate on a vacuum manifold, apply vacuum, and recover the hydrolysate with a collection plate.

    This filtering step is important because particles can damage the LC-MS/MS or interfere with its proper operation or detection. It is not sufficient to pellet plant tissues and other contaminants via centrifugation prior to injection. If a vacuum manifold is not available, sample filtering can be done by centrifugation for 15 min at 3000 × g, room temperature. Higher speeds can damage the filter membrane.

16. Transfer 10 µl of filtered samples to new 1.1-ml tubes and place in a new rack.

    Store remaining filtered samples at −20°C for up to a week, if you wish to repeat the experiment.

17. Dry completely using a SpeedVac.

    It is important to dry completely as any NaOH remnants can interfere with detection and quantification.

18. Cover tubes with a capmat and store at 4°C until ready for analysis.

Tryptophan extraction

19. Re-suspend each pellet (from alkaline hydrolysis, step 18) with 400 µl of EB2 using multichannel pipette and a 50-ml reservoir.
20. Seal firmly with capmat.
21. Shake for 4 min using a mini bead beater.
22. Centrifuge 15 min at 3700 × *g*, 4°C.
23. Transfer 70 µl of each sample to its corresponding well in a 96-well V-bottom microplate plate using a multichannel pipette.
24. Transfer 70 µl from the standard serial dilution to the plate containing the samples using a multichannel pipette.
25. Dilute all samples and standard by half by adding 70 µl EB2 using a multichannel pipette.

    The final concentration of the standards after dilution are 0, 0.1, 0.5, 1, 5, 10, 50, and 100 µM. This step is necessary to diminish ion suppression and reduce concentrations of interfering contaminants and, thus, improve sensitivity and accuracy of detection.

26. Seal plate with a 96-round well, pre-slit, silicone sealing mat
27. Analyze immediately or store at −80°C until ready for analysis.

    For stored samples only, remove from the −80°C freezer, let thaw, and centrifuge 5 min at 3000 × g, 4°C, to remove bubbles. We recommend analyzing within 48 hr.

Table 1 Mobile Phase Gradient Used for UPLC

Time (min)	PFHA (A; 1 mM in H_2O)	Acetonitrile (B)	Flow (ml/min)
0	98%	2%	0.3
0.1	80%	20%	0.3
2.3	60%	40%	0.3
3.6	60%	40%	0.3
4.0	98%	2%	0.3
6.0	98%	2%	0.3

Table 2 MS/MS-MRM Transitions and Conditions Used to Target Individual Amino Acids

AA	Parent-ion (m/z)	Daughter-ion (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (eV)
Trp	205	188	0.02	18	15
Trp[a]	210	150	0.02	18	15

[a] Represents the deuterated amino acid (internal standard).

Tryptophan detection and quantification using LC-MS/MS

Several analytical instruments can be used for amino acid detection after hydrolysis. In this method, we used Xevo TQ-Absolute UPLC-MS/MS (Water's Corporation) with the settings outlined below.

28. Thaw plate containing samples, prior to injection.
29. Spin 5 min at 3000 × *g*, 4°C, to remove air bubbles.
30. Adjust LC settings as follows:
    a. Use a Phenomenex Kinetex 2.6 µm C18 100 Å LC column 100 × 2.1 mm (part no. 00D-4462-AN).

       Any C18 column could be used instead, but LC conditions should be modified accordingly.

    b. Set column oven temperature to 30°C.
    c. Set autosampler temperature to 10°C.
    d. Set injection volume to 10 µl.
    e. Set mobile phase to 1 mM PFHA for A and acetonitrile for B and the flow rate to 0.3 ml/min.

       The flow gradient of the mobile phase is shown in Table 1.

31. Adjust MS/MS settings as follows:
    a. Acquire mass spectra using electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM).
    b. Set voltage of capillary, cone, and source offset to 3.17, 18, and 50 V, respectively.
    c. Set flow of cone gas and desolvation to 150 L/hr and 500 L/hr, respectively.
    d. Set nebulizer to 7 bars.
    e. Set desolvation temperature to 350°C.
    f. Set collusion gas flow to 0.15 ml/min.

       The detection method is composed of one ESI+ function (0 to 6.0 min). Collision energies and source cone potentials were optimized for each transition using Water's IntelliStart (Table 2).

32. Inject samples.
33. Acquire data using MassLynx.

34. Process calibration and quantification of the analytes using TargetLynx software (MassLynx V4.1 SCN919; Water's Corporation).

35. Export data to a spreadsheet.

36. Back calculate using the total volume and sample weight to obtain the final amount in nmol/mg tissue.

REAGENTS AND SOLUTIONS

Use sterile Milli-Q H_2O in all recipes and protocol steps.

Extraction buffer, EB2

To prepare the extraction buffer EB, first calculate the number of samples, standard curve dilutions, and extra sample volume that will be included.

We recommend including a duplicate series of 8 dilutions in the proper range and an extra buffer to account for pipetting errors. We also recommend including an amount that is enough to extract 12 samples as shown in the formula: $N + 16 + 12 = N + 28$.

Prepare extraction buffer in a clean 50-ml conical tube or bottle where N = number of samples as follows:

H_2O (ml) = (N + 28) × 0.456
10 mM DTT (µl) = (N + 28) × 0.9
Heavy L-Trp-d5 standard (ml) = (N + 28) × 0.024

Stock solution of DTT is 10 mM and the desired concentration is 19 µM.

Place 1 ml of the EB into a clean 1.5-ml Eppendorf tube and label "EB1".

EB1 is used only to make the 200 µM dilution of the initial concentration of the serial dilution for plotting the standard curve.

Dilute remaining EB by 20% with HPLC-grade H_2O to make EB2.

Store both EB1 and EB2 on ice for the duration of the experiment.

Heavy L-Trp-d5 stock solution

Prepare a 20 mM L-Trp-d5 (CDN Isotopes, Inc., CAS No. 62595-11-3) stock solution using 19 µM DTT (Fisher, cat. no. R0861) for Trp internal standard. Dilute with 19 µM DTT to bring the concentration of the working L-Trp-d5 standard to 100 µM. Aliquot solution according to usage (e.g., 1200 µl; two aliquots of 1200 µl are enough for the extraction of a 96-well plate). Label with name and date and store up to 6 months at −80°C.

The procedure to make the internal standard stock and working solutions is shown in Table 3.

Proteinogenic L-Trp stock solution

Prepare a 20 mM Trp (Sigma-Aldrich, CAS no. 73-22-3) stock solution standard. Dilute with 19 µM DTT (Fisher, cat. no. R0861) to bring the concentration of the working Trp standard to 1 mM. Aliquot into small volumes (e.g., 100 µl). Label with name and date and store up to 6 months at −80°C.

The procedure to make the external standard stock and working solutions is shown in Table 4.

Standard, serially diluted

Prepare heavy L-Trp-d5 and proteinogenic L-Trp stock solutions (see recipes). Prepare extraction buffers EB1 and EB2 (see recipe). Transfer 40 µl of L-Trp

Table 3 Internal Standard Used for Protein-Bound Tryptophan Analysis[a]

Deuterated	Vol.	Manufacturer	Item no.	MW (g/mol)	[Stock] (mM)	[Stock mix] (mM)	Vol. (µl) to add to mix	Used to
L-Trp-d5		CDN Isotopes	D-1522	209.26	20	100	90	Trp
Preparing 19 µM DTT solution								
Water	20 ml							
DTT (10 mM stock)	38 µl							
Final Std stock solution								
19 µM DTT solution	17,910 µl							
Internal Std	90 µl							
Total	18,000 µl							
Aliquot[b]	1200 µl							

[a] The table also shows stocks and needed volumes to make 18 ml of standards, which is enough for 10 plates.
[b] Store at −80°C.

Table 4 L-Tryptophan Standard[a]

C^{12} std.	Manufacturer	Item no.	MW (g/mol)	[Stock] (mM)	[Stock mix] (mM)	Vol (µl) to add for the mix[b]
L-Trp	Sigma-Aldrich	T0254	204.23	20	1	90

[a] The table also shows the stock and volume needed to make 17 runs.
[b] Bring the final volume to 1800 µl with 19 µM DTT and make 100 µl aliquots and store at −80°C.

proteinogenic amino acid solution standard mix and 160 µl of EB1 to a tube labeled "200 µM Standard" and mix well by pipetting up and down.

In this step, we dilute the internal standard with EB1 by 20%; therefore, all further dilutions are made with EB2, which is already diluted by 20%.

Use EB2 and the initial 200 µM standard to prepare an 8-concentration serial dilution for a standard curve.

We recommend a standard curve with the following concentrations: 0, 0.2, 1, 2, 10, 20, 100, and 200 µM. Each point including 0 contains a fixed amount of the internal standard since it is diluted with EB2 (i.e., 4 µM final concentration). These concentrations can be adjusted as needed.

Place the standard curve dilutions on ice until ready to transfer to the designated plate.

COMMENTARY

Background Information

Previously, we developed a high-throughput analysis method for absolute quantification of protein-bound amino acids from plant tissues (see Current Protocols article, Yobi & Angelovici, 2018), a method that can quantify 16 amino acids, representing 18 amino acids. This method could not, however, quantify Trp that was destroyed during acid hydrolysis. As Trp is an essential amino acid that is scarce in many crop plants (Boyer et al., 1992; Galili et al., 2005; Shewry, 2007; Shewry & Halford, 2002; Ufaz & Galili, 2008), many methods have been developed to quantify Trp from plants tissues, which is a prerequisite for any biofortification effort. As this amino acid is destroyed during the gold standard acid hydrolysis, many alternative methods have been explored. One is to use acid hydrolysis, but with additives such as

phenol, 2-mercaptoethanol, and tryptamine (Fountoulakis & Lahm, 1998). For example, when 1% 2-mercaptoethanol and 3% phenol were added to 6 M HCl during hydrolysis, 79% Trp was recovered (Adebiyi et al., 2005). 3 M mercaptoethanesulfonic acid at 166°C for 25 min also resulted in 92% Trp recovery in a protein sample (Maeda et al., 1984). The acid ninhydrin method, which utilizes ninhydrin in a mixture of formic and hydrochloric acid for 10 min at 100°C, causes Trp to transform into a yellow product that can be quantified spectrophotometrically (Zahnley & Davis, 1973). However, for carbohydrate-containing foods, the thioglycolic and organic acid methods appear ineffective (Friedman, 2018). Also, spectrophotometric methods are inherently less reliable and lower in resolution when compared to the high-resolution LC-MS/MS detection methods. Other detection alternatives such as GC-MS and GC-MS/MS require time-sensitive sample chemical derivatization, or they have lower resolution and sensitivity compared to the LC-MS/MS method. However, the most used method for Trp quantification is alkaline hydrolysis (Hugli & Moore, 1972). This method uses sodium hydroxide for hydrolysis (la Cour et al., 2019; van Wickern et al., 1997; Yust et al., 2004), although lithium hydroxide and barium hydroxide have also been used (Hugli & Moore, 1972; Lucas & Sotelo, 1980). Although our method is based on alkaline hydrolysis, our low sample size, low reagents use, short run time, and exclusion of additives make it more convenient for the analysis of large set of samples. This method will serve as one of three complementary high-throughput methods that can be utilized to analyze all the 20 amino acids from large populations, which will greatly benefit the scientific community, especially those working in the field of quantitative genetics (Yobi et al., 2023; Yobi & Angelovici, 2018).

Critical Parameters and Troubleshooting

The following practices can help ensure a successful analysis and quantification of Trp:

a. All materials used for hydrolysis (e.g., plastic tubes containing samples, mat covering the tubes, rack holding the tubes, SpeedVac) must be able to handle a combination of a strong base and high temperature.

b. The SpeedVac evaporator should be equipped with a cold trap to collect any residues, which must be disposed as regulated by your institution.

c. Handle 4 M NaOH in the hood and avoid touching it with bare hands or spilling it in on surfaces.

d. Do not use 4 M NaOH past its expiration date, as this can result in a partial hydrolysis of the sample.

e. It is essential to have an analytical instrument (e.g., HPLC/UPLC-MS/MS) equipped with a 96-well format autosampler. Other instruments, such as the cooled centrifuge and SpeedVac, also should also be equipped with 96-well plate and rack rotors.

f. Use multichannel pipettes during all stages of the protocol to save time and prevent pipetting errors.

g. Prepare two serial dilutions and use to plot the standard curve. We recommend injecting a complete series before the first sample and the second series after the last sample to help ascertain that any changes in sensitivity and retention times during the run were considered.

h. Renew the mobile phase A (PFHA) every 2 weeks to avoid a shift in retention time and contaminants.

i. Wash after every 24 samples, before and after each standard, and between treatments to reduce the risk of cross contamination. Run a 20-min wash of 97% methanol at the end of the cycle to clean the column and the UPLC instrument.

j. Use a clean column. A dirty column can cause a long retention time and poor detection.

Understanding Results

To validate this method, we quantified Trp from ∼11 mg bovine serum albumin (BSA, Gold Biotechnology, cat. no. A-420-10). We choose a high amount of BSA because Trp is very low in this protein compared to other amino acids. Our results show that the quantification of Trp from three replicates (n = 3) was comparable with the expected values deduced from the sequence. BSA has 2 Trp residues that in theory corresponds to 30.3 nmol/mg Trp. Our results show that the average of the 3 BSA samples measured is 25.97 nmol/mg, which corresponds to 1.7 residues.

To apply this method to model and crop plants, we analyzed ∼4 mg seed tissue from *Arabidopsis*, maize, and soybean (n = 12) using our method. Our results show very highly reproducible results as the highest percentage of the relative standard deviation (RSD) was 10.8% for the 12 maize technical replicates (Table 5). We then used three different *Arabidopsis* seed weights (2, 4, and 6 mg) to test if using different weights affects the accuracy

Table 5 Protein-Bound Tryptophan Analysis from *Arabidopsis,* Maize, and Soybean Seeds[a]

	nmol/mg seed		
Replicate	*Arabidopsis*	Maize	Soybean
1	8.18	4.18	21.06
2	7.88	3.55	20.94
3	8.36	3.57	20.15
4	9.62	4.80	19.81
5	8.51	4.18	19.44
6	8.85	3.96	20.77
7	8.98	3.98	19.59
8	9.46	3.35	20.20
9	9.35	3.77	20.20
10	8.82	4.60	20.28
11	8.32	3.78	17.86
12	8.65	3.76	20.23
Average	8.75	3.96	20.04
StDev	0.54	0.43	0.85
%RSD	6.15	10.80	4.22

[a] The results show the levels of amino acids in three replicates (nmol/mg seed) as well as the average and relative standard deviation (RSD%).

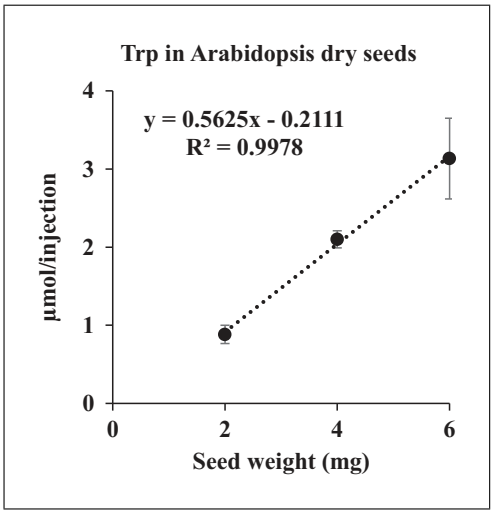

Figure 2 Tryptophan analysis from *Arabidopsis* seeds at different sample weights. The figure shows a linear response from 2-6 mg seed.

of the measurements. Our results show that in Arabidopsis, sample weights from 2 to 6 mg produce a linear response (Fig. 2), which is another indication of the reliability of our method.

To further validate this method, we sent soybean samples to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories (ESCL; *https://aescl.missouri.edu*) from the same batch as the one used for Table 5. ESCL utilizes the gold standard method developed by the Association of Official Analytical Collaboration (AOAC) International that employs alkaline hydrolysis and ion-exchange [AOAC 982.30 E(b), chp 45.3.05, 2006] and therefore its findings are considered reliable. Results from the ESCL analysis showed an average of 18.93 nmol/mg. The average of tryptophan measured from our analysis was 20.04 nmol/mg, which is not significantly different ($p < 0.05$) from the one measured by ESCL (Table 6).

Table 6 Comparing Protein-Bound Tryptophan Analysis Averages from Soybean Seeds Between Our Analysis and ESCl Analysis[a]

	Trp (nmol/mg)			
Species	Our analysis	ESCL[b]	Ratio	*t*-test
Soybean	20.04	18.933	0.94	0.0534

[a] n = 12 in our analysis and n = 3 in ESCL analysis 3.
[b] University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories.

Time Considerations

From start to finish, the method takes 3 days. To take full advantage of the method, analyze two plates at a time, as the time required to analyze two plates at once does not double the time of analyzing one plate. The following is a breakdown of the approximate time needed for each step:

a. 2 hr to weigh a plate and add glass beads to each tube using a bead dispenser.
b. 30 min to add 4 M NaOH, shake, spin down, and place in oven.
c. 16 hr for hydrolysis.
d. 2 hr to cool, filter, and dry samples.
e. 2 hr to make extraction buffer, standard preparation, sample extraction, and final dilution.
f. For the LC-MS/MS, 1 hr to equilibrate the column, prep the instrument, and prepare sequence table.
g. 10 to 11 hr to run a full plate.
h. 30 min for data analysis.

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Author Contributions

Huda Ansaf: Formal analysis, validation, writing original draft, **Abou Yobi:** Formal

analysis, validation, writing original draft, **Ruthie Angelovici:** Conceptualization, supervision, writing review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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