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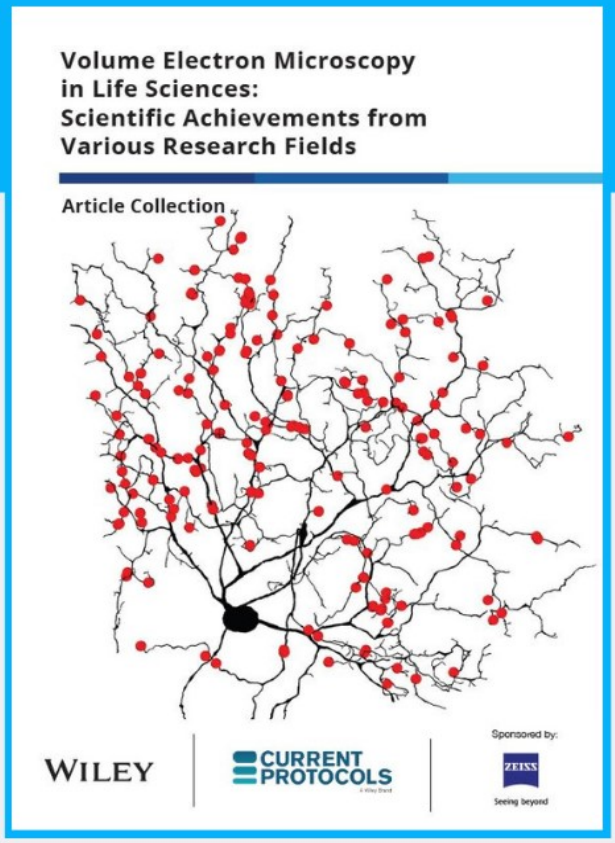

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Through this article collection, we hope to provide researchers with more information on the application of volume electron microscopy techniques, allowing them to further their research in this field.

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A High-Throughput Absolute Quantification of Protein-Bound Sulfur Amino Acids from Model and Crop Plant Seeds

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In this procedure, we describe a high-throughput absolute quantification protocol for the protein-bound sulfur amino acids, cysteine (Cys) and methionine (Met), from plant seeds. This procedure consists of performic acid oxidation that transforms bound Cys into cysteic acid (CysA) and bound Met into methionine sulfone (MetS) followed by acid hydrolysis. The absolute quantification step is performed by multiple reaction monitoring tandem mass spectrometry (LC-MS/MS). 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 acid hydrolysis protocol, followed by water extraction that includes DL-Ser-d3 and L-Met-d3 as internal standards to enable the quantification of the absolute levels of the protein-bound Cys and Met with high precision, accuracy, and reproducibility. The protocol described herein has been optimized for seed samples from *Arabidopsis thaliana*, *Glycine max*, and *Zea mays* but could be applied to other plant tissues.

Basic Protocol: Analysis of protein-bound cysteine and methionine from seeds

Keywords: acid hydrolysis • cysteine • LC-MS/MS • methionine • performic acid oxidation • seeds

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INTRODUCTION

Cysteine (Cys) and methionine (Met) are sulfur-containing amino acids that are indispensable for the survival of all living organisms (Romero et al., 2014). Besides being proteogenic amino acids, they are involved in many biological processes such as protein stability, regulation of catalytic activity, and post-translational modification (Nozaki et al., 2005). For example, Met is the initiating amino acid in eukaryotic protein synthesis (Merrick & Pavitt, 2018) and aside from being an integral part of the hydrophobic core of proteins, its bound form has a role in sequence-independent recognition of protein surfaces, protein stabilization, and can act as an endogenous antioxidant (Aledo, 2019). Unlike Met, which lacks a functional thiol group, Cys has an important role in

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determining protein conformation as it usually exists as cystine in proteins by forming a disulfide bond between two Cys residues inside of the protein (Wiedemann et al., 2020). Cys residues perform vital functions for proteins such as binding various metal ions and serving as a site for post-translational modifications (Fomenko et al., 2008). Furthermore, Cys is a powerful antioxidant and Cys residues in the active sites of proteins have the capability of trapping reactive oxygen species (ROS) due to the redox ability of their thiol groups (Krauth-Siegel & Leroux, 2012; Romero et al., 2014; Ulrich & Jakob, 2019; Wirtz & Droux, 2005). In plants, the inorganic sulfur is a macronutrient that is important for growth and development and Cys biosynthesis plays a critical role in its fixation from the environment (Bonner et al., 2005).

Sulfur amino acids are deficient in some staple crops, which is unfortunate because seeds of staple crops are a major source of proteins for both humans and livestock (Mandal & Mandal, 2000). Legumes such as soybean are deficient in the sulfur-containing amino acids, Cys and Met (Krishnan & Jez, 2018). Met is an essential amino acid and must therefore be obtained from diet, whereas Cys is considered a "conditional" essential amino acid because animals can convert Met to Cys (Krishnan & Jez, 2018). Hence, sulfur-containing amino acids can only be obtained through dietary sources (Krishnan & Jez, 2018). Sulfur amino acid deficiency results in poor disease resistance and can lead to mental and physical abnormalities, especially in countries where diets are primarily based on plants (Krishnan & Jez, 2018).

Although having adequate levels of sulfur-containing amino acids in our diet is critical, most methods used for analyzing protein-bound amino acid profiles exclude Cys because the most commonly used method—acid hydrolysis—destroys it, thus rendering it unquantifiable (Thera et al., 2018; Yobi & Angelovici, 2018). To lessen this problem, several methods have been developed (reviewed in Fountoulakis & Lahm, 1998), but the most commonly used methods are adding 0.2% sodium azide to the acid during hydrolysis or using performic acid prior to hydrolysis. Both methods are, however, not optimized for high-throughput use and mostly require additives (reviewed in Fountoulakis & Lahm, 1998).

To alleviate this problem and conduct high-throughput analysis of Cys from large-scale plant tissues, especially seeds, we developed a 96-plate setup for performic acid oxidation, followed by a 6-min targeted analysis using a tandem mass spectrometric (UPLC-MS/MS) method that was established previously (Yobi & Angelovici, 2018). The protocol has five major steps: (a) PFA oxidation, (b) acid hydrolysis, (c) reagent preparations for AA standards and extraction buffers (EB1, EB2) and serial dilution preparations for the standard curve, (d) extraction of amino acids with extraction buffer EB2, and (e) using UPLC-MS/MS for detection and quantification of Cys. Using the same 6-min method described previously (Yobi & Angelovici, 2018) makes it convenient to analyze these two amino acids alongside the remaining amino acids without having to change LC or MS conditions.

CAUTION: All reactions must be run in a suitable fume hood with efficient ventilation. Many of the reactions in this article are highly exothermic; safety glasses and reagent-impermeable protective gloves should be worn.

BASIC PROTOCOL

ANALYSIS OF PROTEIN-BOUND CYSTEINE AND METHIONINE FROM SEEDS

In the following section, we will detail all the materials and instruments needed for bound Cys and Met analyses from plant seeds. We will also show (1) the steps needed for Cys and Met oxidations to CysA and MetS, respectively; (2) acid hydrolysis and release of

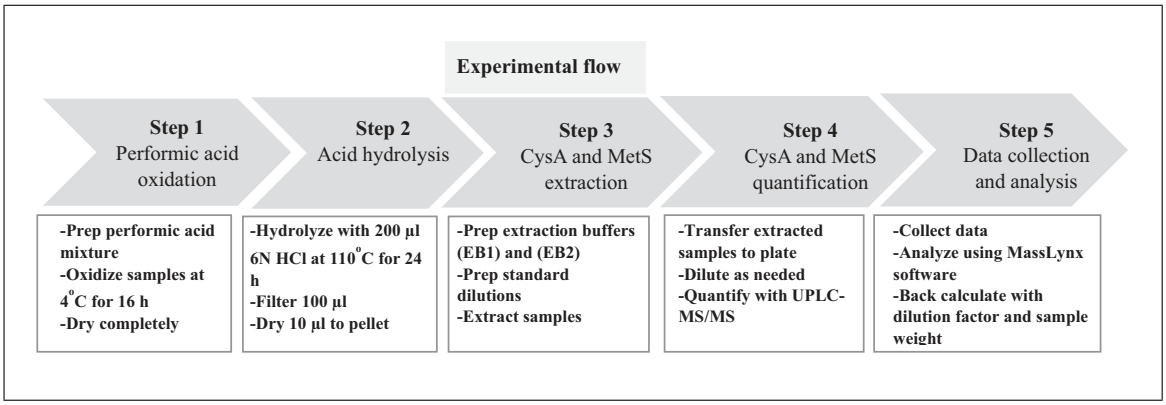

Figure 1 An overview of the sulfur amino acid analysis experimental flow.

CysA and MetS; (3) CysA and MetS extraction; (4) CysA and MetS analysis with UPLC-MSMS; and (5) data collection and analysis, as depicted in Figure 1.

Materials

Arabidopsis seeds or ground seed from other species
Performic acid solution, freshly prepared (see recipe)
HCl, 6 N
Extraction buffer, EB2 (see recipe)
Standard, serially diluted (see recipe)
L-cysteic acid monohydrate (Sigma-Aldrich, CAS no. 23537-25-9)
L-methionine sulfone (Sigma-Aldrich, CAS no. 7314-32-1)
DL-serine 2,3,3-d_3 (CDN, Isotopes, Inc., CAS no. 70094-78-9)
L-methionine-d_3 (S-methyl-d3) (CDN, Isotopes, Inc., CAS no. 13010-53-2)
Dithiothreitol (DTT; Fisher, cat. no. R0861).
Perfluoroheptanoic acid (PFHA; Sigma-Aldrich, 342041).
Acetonitrile (HPLC grade)
Methanol (HPLC grade)
Water (Milli-Q)

Sub-milligram analytical balance (e.g., Mettler Toledo XS 105; Fisher Scientific, cat. no. 01-913-892)
Cooled centrifuge equipped with 96-well plate rotors (e.g., Sorvall Legend XTR; Fisher Scientific, cat. no. 75-004-523)
A 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 racks with lids (NOVA Biostorage, part no. MPW51BCPK)
1.1-ml non-coded screw cap tubes, V-bottom bulk (VWR, cat. no. 101975-256)
Disposable, anti-static microspatula (e.g., LevGo, cat. no. 17231B)
3-mm solid glass beads (e.g., Sigma-Aldrich, CLS72683)
3-mm bead dispenser, 96-well (e.g., Qiagen, cat. no. 69973)
High-energy, high-throughput cell disrupter (e.g., Mini-Beadbeater-96 with 1400 to 2400 rpm speed; BioSpec Products, cat. no. 1001)
Oven that can maintain 110°C (e.g., Heratherm Oven OGS60; Fisher Scientific, item no. T9FB2187511)
Filter plate vacuum manifold, optional (e.g., VWR, cat. no. 16003-836)
Pierceable capband-8 in a capmat format (e.g., Micronic, MP53002)
Manual decapper (e.g., Micronic, MP54001)

Filter plate, hydrophilic, PTFE, 0.45 µm, clear, non-sterile (e.g., EMD Millipore, cat. no. MSRLN0450)
96 round-well V-bottom microplate, 520 µl (Dot Scientific, cat. no. PC63241-NS6)
Sealing mat, silicone, pre-slit, 96-round well (MidSci, MID-RD-9-PRT)
50-ml reagent reservoir (e.g., Fisher Scientific, cat. no. 07200127)
8 and 12 multichannel pipettes (200 and 10 µl volumes) and pipette tips
50-ml Falcon tubes
1.5-ml Eppendorf tubes
Kinetex 2.6 µm C18 100A° LC column 100 × 2.1 mm (Phenomenex, part no. 00D-4462-AN)
Heat-resistant gloves
Permanent marker
Ice
Xevo TQ-Absolute UPLC-MS/MS (Waters Corporation)
MassLynx (ver. 4.0, Waters Corporation)
TargetLynx software (MassLynx ver. 4.1; SCN919; Waters Corporation)

Performic acid oxidation

1. Weigh out 4 mg of *Arabidopsis* seeds or ground seed from other species and place in 1.1-ml tubes.

   Note: Arabidopsis seeds are small and can be easily crushed by using the bead beater. However, for crops, seeds must be crushed prior to this step.

2. Place tubes in a 96-well rack in the following order: A1 to A12, B1 to B12, and so on
3. Add three 3-mm glass beads to each tube using a bead dispenser.
4. Place both the samples and performic acid solution on ice for at least 15 min.
5. In a chemical hood and using a multichannel pipette and a 50-ml reagent reservoir, add 100 µl of performic acid to each sample tube and to 3 empty tubes as negative controls.

   We recommend placing the rack on ice and storing in a refrigerator for 16 hr. This treatment will transform bound Cys to CysA and Met to MetS. We also recommend starting the procedure in the afternoon, so you find the samples ready the next morning. Including the 3 empty negative control tubes will ensure detection of contaminants and systematic errors during the course of the run.

6. The next morning, stop the incubation and dry the entire sample using SpeedVac.
7. Proceed to acid hydrolysis or store the pellet at 4°C or −20°C.

Acid hydrolysis

8. Add 200 µl of 6 N HCl to each sample tube.
9. Close lids firmly with a capmat to preserve sample during hydrolysis.
10. Shake rack for 4 min using the bead beater.
11. Spin rack briefly to bring samples down to the bottom of tubes.
12. Place rack into a heat-resistant container and close.

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

13. Place container in an oven preheated to 110°C.
14. Incubate at 110°C for 24 hr.

15. Remove container from the oven using heat-resistant gloves and cool to room temperature.
16. Remove rack from the container.
17. Centrifuge for 15 min at 3700 × *g* to precipitate debris.
18. Transfer 100 µl of the hydrolyzed sample onto a filter plate using a multichannel pipette.

    Make sure to maintain the same sample order.

19. 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 and room temperature. Higher speeds can damage the filter membrane.

20. 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 1 week, if you wish to repeat the experiment.

21. Dry completely using SpeedVac.

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

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

    If samples are not ready for analysis, dry pellets can be stored at –80°C/–20°C for weeks.

Extract cysteic acid and methionine sulfone

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

    Note: The final concentrations 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, thus improving the sensitivity and accuracy of detection.

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

    Note: For stored samples only, remove from the −80°C freezer, let thaw, and centrifuge briefly to remove bubbles. We recommend analyzing within 48 hr.

Table 1 The Mobile Phase Gradient Used for UPLC

Time (min)	PFHA (A; 1 mM)	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
5.98	98%	2%	0.3

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

AA	Parent ion	Daughter ion	Dwell (s)	Cone voltage	Collusion energy
Met[a]	153	107	0.02	18	15
Ser[a]	109	63	0.02	18	15
MetS	182	56	0.02	18	16
CysA	170	106	0.02	18	16

[a] Deuterated amino acid (internal standard).

Detect and quantify cysteic acid and methionine sulfone using LC-MS/MS

Several analytical instruments could be used for AA detection after hydrolysis. In this method, we used a Xevo TQ-Absolute UPLC-MS/MS with the following settings:

32. Thaw PCR plate containing samples prior to injection.
33. Spin briefly at 4°C to remove air bubbles.
34. Adjust LC settings.
    a. We used a Phenomenex Kinetex 2.6 µm C18 100 Å LC column 100 × 2.1 mm.
       Note: 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.
35. Adjust MS/MS settings.
    a. Acquire mass spectra using electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM).
    b. Set voltages of the 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 bar.
    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 min). Collision energies and source cone potentials were optimized for each transition using Waters' IntelliStart (Table 2).

Table 3 The Internal Standard Used for Protein-Bound Cysteine and Methionine Analyses

Deuterated	Manufacturer	Item no.	MW (g/mol)	[Stock] (mM)	[Stock mix] (μM)	Vol (μl) to add to the mix	Used to quantify
L-Met-d_3	CDN Isotopes	D-1293	152.23	20	100	90	MetS
DL-Ser-d_3	CDN Isotopes	D-1583	108.11	20	100	90	CysA
Preparing 19 μM DTT solution							
Water	20 ml						
DTT (10 mM stock)	38 μl						
Final Std stock solution (μl)							
19 μM DTT solution	17910						
Internal Std	90						
Total	18000						
Aliquot[a]	1200						

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

36. Inject samples.
37. Acquire data using MassLynx.
38. Perform process calibration and quantification of the analytes using TargetLynx software.
39. Export data to a spreadsheet.
40. 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 water in all recipes and protocol steps.

DL-Serine 2,3,3-d_3 and L-Methionine-d_3 stock solutions

DL-serine 2,3,3-d3 (CDN, Isotopes, Inc., CAS no. 70094-78-9)
L-methionine-d3 (S-methyl-d3) (CDN, Isotopes, Inc., CAS no. 13010-53-2)
Dithiothreitol (DTT; Fisher, cat. no. R0861).

Note: We used DL-Ser-d_3 as the internal standard for cysteic acid instead of heavy L-cysteine-d2 (DLM-769; Cambridge Isotope Laboratories, Inc.) because the latter was not producing stable peaks under our experimental conditions. In addition, DL-Ser-d3 has almost the same retention time as CysA.

The procedure to prepare the internal standard stocks and working solutions is shown in Table 3.

Prepare heavy 20 mM DL-Serine 2,3,3-d3 and l-Methionine-d3 stock solutions using 19 μM DTT for CysA and MetS internal standards. Dilute with 19 μM DTT to bring the concentrations of both the working DL-Serine 2,3,3-d3 and l-Methionine-d3 standards 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. Store at −80° C for up to 6 months.

L-Cysteic acid monohydrate and L-Methionine sulfone solutions

L-cysteic acid monohydrate (Sigma-Aldrich, CAS no. 23537-25-9)

Table 4 Cysteic Acid and Methionine Sulfone Standards

C^{12} Std	Manufacturer	Item #	MW (g/mol)	[Stock] (mM)	[Stock mix] (mM)	Vol (µl) to add for the mix[a]
L-Methionine sulfone	Sigma-Aldrich	M0876	181.21	20	1	90
L-Cysteic acid monohydrate	Sigma-Aldrich	30170	187.17	20	1	90

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

L-methionine sulfone (Sigma-Aldrich, CAS no. 7314-32-1)
Dithiothreitol (DTT; Fisher, cat. no. R0861)

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

Prepare 20 mM proteinogenic CysA and MetS stock solution standards. Dilute with 19 µM DTT to bring the concentration of the working standards to 1 mM, each. Aliquot into small volumes (e.g., 100 µl). Label with name and date. Store at −80°C for no longer than 6 months.

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 recommend including an amount that is enough to extract 12 samples as shown in the formula: N+16 +12 = N+28.

1. Prepare extraction buffer in a clean conical tube or bottle as follows, where N = number of samples:

Milli-Q water (ml) = (N+28)*0.456

10 mM DTT (µl) = (N+28)*0.9

Heavy standards: 100 µM DL-Serine 2,3,3-d3 and 100 µM l-Methionine-d3 standards (ml) = (N+28)*0.024

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

2. Place 1 ml of the EB into a clean 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.

3. Dilute the remaining EB solution by 20% with Milli-Q water to make EB2.

Note: Keep both EB1 and EB2 on ice for the duration of the experiment.

Performic acid mixture

30% hydrogen peroxide solution (H2H2; Fisher Scientific, cat. no. H325-500)
88% formic acid (Fisher Scientific, cat. no. A118P-500)

To make the performic acid reagent, mix 30% hydrogen peroxide and 88% formic acid (1:9, v/v) in a Falcon tube, cap firmly, let sit at room temperature for 30 min, and place on ice.

Performic acid reagent should be prepared fresh each time (before starting the oxidation).

This procedure should be done in a chemical hood.

Standards, serially diluted

1. Use the extraction buffers EB1 and EB2 (same used for the samples).
2. Transfer 40 µl of each proteinogenic amino acid solution standard mix (1mM L-cysteic acid monohydrate and 1 mM L-methionine sulfone) and 160 µl of EB1 to a tube labeled "200 µM Standard" and mix well by pipetting up and down.

Note: 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% (see Reagents and Solutions).

3. 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 because it is diluted with EB2 (i.e., 4 µM final concentration). These concentrations can be adjusted as needed.

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

COMMENTARY

Background Information

Although we developed a high-throughput method for the quantification of protein-bound amino acids from plant tissues a few years ago (Yobi & Angelovici, 2018), this method was not capable of quantifying Cys. This method is specifically optimized to quantify protein-bound sulfur amino acids Cys and Met, although Met could be quantified with the previous method as well. Cys and Met quantification has interested scientists for years and many methods have also been proposed to quantify sulfur amino acids. For example, performic acid oxidation followed by hydrochloric acid hydrolysis, derivatization with AccQ-Tag Ultra (Waters, Inc.), and separation by ultra-high performance liquid chromatography with fluorescence detection was used to quantify Cys and Met from aquatic invertebrates (Thera et al., 2018). Derivatization via thiol group with 2-chloro-1-methylpyridinium iodide (CMPI), followed by converting Cys to its reduced forms using 2-mercaptoethanol, and analysis using ion-pair reversed-phase HPLC and ultraviolet detection was also used to quantify Cys from human plasma (Chwatko & Bald, 2000). 4 M methane sulfonic acid hydrolysis with a combination of oxidizing agents (hydrogen peroxide, performic acid) and antioxidants (phenol, tryptamine) combined with LC-MS/MS detection were also used to quantify both Cys and Met from ubiquitin, BSA, and Soy protein samples (Kambhampati et al., 2019). Adding 0.2% sodium azide to 6 N HCl has been claimed to be a superior method for the determination of Cys in samples, but the recovery of Met as MetS is partial (Fountoulakis & Lahm, 1998). In addition, sodium azide is toxic at higher concentrations and could cause explosion at these concentrations when mixed with strong acids (Fountoulakis & Lahm, 1998; Manneberg et al., 1995).

All these methods and many others relied on HPLC-based detection that required either derivatization or additives. Although fluorescence-based detection is very sensitive, the derivatives have short-term stability (Kutlan & Molnar-Perl, 2001; Rutherfurd & Gilani, 2009). Unlike GCMS that requires derivatization, which could reduce stability and produce multiple products (Halket et al., 2005), LC is more stable, and when coupled with MS/MS, it produces highly sensitive, reproducible, and accurate results (Chaimbault et al., 1999). On the other hand, adding derivatives is time consuming and adds to the cost, beside the fact that most derivatives are often toxic. Although our method is based on acid hydrolysis, our low sample size, less reagent usage, short run time, and exclusion of additives make it more convenient for the analysis of large populations. With this method, we have established three complementary high-throughput methods that can be employed to analyze all the 20 amino acids from large populations. These methods will be an asset to the scientific community, and especially those

in the fields of population genetics and quantitative genetics (Yobi & Angelovici, 2018; Ansaf, Yobi, & Angelovici, 2023).

Critical Parameters and Troubleshooting

The following practices can help ensure successful amino acid analysis and quantification:

1. All materials used for hydrolysis (e.g., plastic tubes containing samples, mat covering the tubes, rack holding the tubes, and SpeedVac) must be able to handle combinations of strong acids and high temperatures.
2. The SpeedVac evaporator should be equipped with a cold trap to collect any residues, which must be disposed of as regulated by your institution.
3. Do not use 6 N HCl past its expiration date, as this can result in partial hydrolysis of the sample.
4. Handle 6 N HCl in the hood and avoid touching it with bare hands or spilling it on surfaces.
5. 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 cooler centrifuge and SpeedVac, should also be equipped with 96-well plates and rack rotors.
6. Use multichannel pipettes during all stages of the protocol to save time and prevent pipetting errors.
7. Prepare two serial dilutions and use them 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 taken into account.
8. Renew the mobile phase A (PFHA) every 2 weeks to avoid a shift in retention time and contaminants.
9. 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.
10. Use a clean column. A dirty column can cause long retention times and poor detection.

Understanding Results

To validate this method, we analyzed 0.1 mg/ml of bovine serum albumin (BSA; Gold Biotechnology, cat. no. A-420-10) from a 10-mg/ml stock (dissolved in water). We analyzed 12 replicates for each concentration and quantified CysA and MetS. Our results show that the quantification of Cys, recovered as CysA, was comparable with the expected values deduced from the sequence. BSA has 34 oxidized Cys residues and 1 reduced Cys residue (Siriwardana et al., 2015); therefore, upon oxidation, it should result in 35 CysA molecules, which, in theory, corresponds to 530.30 nmol/mg, which is 93% of the 570 nmol/mg we measured (corresponding to 37.6 molecules). For Met, BSA has 4 molecules corresponding, in theory, to 60.61 nmol/mg. We measured 57.33 nmol/mg, which corresponds to 3.78 molecules on average. These results show that there is no significant difference between measured and expected values for both Cys and Met and therefore reflect the accuracy of this method.

We further validated these results in model and crop plant seeds. We took samples from *Arabidopsis*, maize, and soybean and analyzed one set using our method, while sending the other set to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories (ESCL) for comparison. ESCL utilizes the gold standard method developed by the Association of Official Analytical Collaboration (AOAC) International, which utilizes performic acid oxidation and acid hydrolysis [AOAC 982.30 E(b), chp 45.3.05, 2006] and therefore its findings are considered reliable. In brief, we weighed ~ 4 mg from each tissue (3 replicates per species) and analyzed Cys and Met, as described in our method. We first assessed reproducibility among technical repeats ($n = 3$) by calculating the relative standard deviation (RSD); our results showed less than 2.5% RSD across all seeds (Table 5). We then compared our results with those obtained from ESCL and found that there was no significant difference between our finding and their results for both Cys and Met (Table 6). Combining these findings with our BSA measurements, we can deduce that this high-throughput method is reliable and has the advantage of using less tissue, less reagents, no additives, and less time compared to conventional methods.

Time Considerations

From start to finish, the method takes 4 days. The following is a breakdown of the approximate time needed for each step.

1. 2 hr to weigh a plate and add glass beads to each tube using a bead dispenser
2. 1 hr to make performic acid and add to sample

Table 5 Protein-Bound Cysteine and Methionine Analyses from *Arabidopsis*, Maize, and Soybean Seeds

	Cys nmol/mg seed tissue[a]			Met nmol/mg seed tissue[b]		
	Arabidopsis	Maize	Soybean	*Arabidopsis*	Maize	Soybean
Replicate 1	29.16	30.45	48.28	26.86	28.76	34.82
Replicate 2	34.03	30.05	50.78	28.00	26.87	35.38
Replicate 3	31.52	29.16	50.87	30.23	26.60	35.74
Average	31.57	29.89	49.98	28.36	27.41	35.31
StDev	2.43	0.66	1.47	1.71	1.18	0.46
%RSD	7.70	2.20	2.94	6.04	4.29	1.31

[a] Measured as cysteic acid (CysA).
[b] Measured as methionine sulfone (MetS).
[c] The results show the levels of amino acids in three replicates (nmol/mg tissue) as well as the average and relative standard deviation (RSD%).

Table 6 Comparing Protein-Bound Cysteine and Methionine Analysis Averages from *Arabidopsis*, Maize, and Soybean Seeds Between Our Analysis and ESCL Analysis

	Cys averages (nmol/mg)				Met averages (nmol/mg)			
Species	Our analysis	ESCL [a] analysis	Ratio	*t*-test	Our analysis	ESCL[a] analysis	Ratio	*t*-test
Arabidopsis	31.57	31.78	1.01	0.282029	28.36	25.47	0.90	0.108
Maize	29.89	31.78	1.06	0.3258881	27.41	30.83	1.12	0.14353
Soybean	49.98	51.72	1.03	0.1939165	35.31	35.07	0.99	0.53142

[a] n = 3 in our analysis and n = 2 in ESCL analysis for maize and *Arabidopsis*; n = 3 for soybean in both analyses.
[b] University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories.

3. 20 hr for performic acid oxidation and sample drying thereafter
4. 30 min to add 6 N HCl, flush with nitrogen, shake, spin down, and place in oven
5. 24 hr for hydrolysis
6. 2 hr to cool, filter, and dry samples
7. 2 hr to make extraction buffer, standard preparation, sample extraction, and final dilution
8. For LC-MS/MS, 1 hr to equilibrate the column, prep the instrument, and prepare sequence table
9. 10 to 11 hr to run a full plate
10. 1 hr for data analysis

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.

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Author Contributions

Abou Yobi: formal analysis, validation, writing original draft; **Huda Ansaf:** formal analysis, validation, writing original draft; **Ruthie Angelovici:** conceptualization, resources, supervision, writing review & 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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