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Untargeted liquid chromatography-mass spectrometry-based metabolomics analysis of wheat grain

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November 26th, 2019

Dear Benjamin,

Revised protocol manuscript submission: Untargeted liquid chromatography-mass spectrometry-based metabolomics analysis of wheat grain

On behalf of my co-authors I am submitting the enclosed revised protocol manuscript which we trust will be suitable for publication in Journal of Visualized Experiments. We would like to thank the editors and reviewers for their careful consideration of this work and constructive comments and suggestions. We have provided responses to each comment and have made all of the suggested corrections which we feel have improved the manuscript.

We would be grateful if this manuscript was now seen as meeting the requirements for a Journal of Visualized Experiments publication.

Kind regards,

Hayley Abbiss

TITLE:

Untargeted Liquid Chromatography-Mass Spectrometry-Based Metabolomics Analysis of Wheat Grain

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KEYWORDS:

Wheat, field-grown wheat, wheat variety, wheat grain, agriculture, metabolomics, untargeted metabolomics, liquid chromatography, mass spectrometry

SUMMARY:

A method for the untargeted analysis of wheat grain metabolites and lipids is presented. The protocol includes an acetonitrile metabolite extraction method and reversed phase liquid chromatography-mass spectrometry methodology, with acquisition in positive and negative electrospray ionization modes.

ABSTRACT:

Understanding the interactions between genes, the environment and management in agricultural practice could allow more accurate prediction and management of product yield and quality. Metabolomics data provides a read-out of these interactions at a given moment in time and is informative of an organism's biochemical status. Further, individual metabolites or panels of metabolites can be used as precise biomarkers for yield and quality prediction and management. The plant metabolome is predicted to contain thousands of small molecules with varied physicochemical properties that provide an opportunity for a biochemical insight into physiological traits and biomarker discovery. To exploit this, a key aim for metabolomics researchers is to capture as much of the physicochemical diversity as possible within a single analysis. Here we present a liquid chromatography-mass spectrometry-based untargeted metabolomics method for the analysis of field-grown wheat grain. The method uses the liquid chromatograph quaternary solvent manager to introduce a third mobile phase and combines a traditional reversed-phase gradient with a lipid-amenable gradient. Grain preparation,

metabolite extraction, instrumental analysis and data processing workflows are described in detail. Good mass accuracy and signal reproducibility were observed, and the method yielded approximately 500 biologically relevant features per ionization mode. Further, significantly different metabolite and lipid feature signals between wheat varieties were determined.

INTRODUCTION:

Understanding the interactions between genes, environment and management practices in agriculture could allow more accurate prediction and management of product yield and quality. Plant metabolites are influenced by factors such as the genome, environment (climate, rainfall etc.), and in an agriculture setting, the way crops are managed (i.e., application of fertilizer, fungicide etc.). Unlike the genome, the metabolome is influenced by all of these factors and hence metabolomics data provides a biochemical fingerprint of these interactions at a particular time. There are usually one of two goals for a metabolomics-based study: firstly, to achieve a deeper understanding of the organism's biochemistry and help explain the mechanism of response to perturbation (abiotic or biotic stress) in relation to the physiology; and secondly, to associate biomarkers with the perturbation under study. In both cases, the outcome of having this knowledge is a more precise management strategy to achieve the goal of improved yield size and quality.

The plant metabolome is predicted to contain thousands[1] of small molecules with varied physicochemical properties. Currently, no metabolomics platforms (predominantly mass spectrometry and nuclear magnetic resonance spectroscopy) can capture the entire metabolome in a single analysis. Developing such techniques (sample preparation, metabolite extraction and analysis), which provide as great a coverage of the metabolome as possible within a single analytical run, is a key aim for metabolomics researchers. Previous untargeted metabolomics analyses of wheat grain have combined data from multiple chromatographic separations and acquisition polarities and/or instrumentation for greater metabolome coverage. However, this has required samples to be prepared and acquired separately for each modality. For example, Beleggia et al.[2] prepared a derivatized sample for the GC-MS analysis of polar analytes in addition to the GC-MS analysis of the nonpolar analytes. Das et al.[3] used both GC- and LC-MS methods to improve coverage in their analyses; however, this approach would generally require separate sample preparations as described above as well as two independent analytical platforms. Previous analyses of wheat grain using GC-MS[2-4] and LC-MS[3,5] platforms have yielded 50 to 412 (55 identified) features for GC-MS, 409 for combined GC-MS and LC-MS and several thousand for an LC-MS lipidomics analysis[5]. By combining at least two modes into a single analysis, extended metabolome coverage can be maintained, increasing the richness of biological interpretation while also offering savings in both time and cost.

To permit the efficient separation of a wide range of lipid species by reversed-phase chromatography, modern lipidomics methodologies commonly use a high proportion of isopropanol in the elution solvent[6], providing amenability to lipid classes that might otherwise be unresolved by the chromatography. For an efficient lipid separation, the starting mobile phase is also much higher in organic composition[7] than the typical reversed phase chromatographic methods, which consider other classes of molecules. The high organic

composition at the start of the gradient makes these methods less suitable to many other classes of molecules. Most notably, reversed phase liquid chromatography employs a binary solvent gradient, starting with a mostly aqueous composition and increasing in organic content as the elution strength of the chromatography is increased. To this end, we sought to combine the two approaches to achieve separation of both lipid and non-lipid classes of metabolites within a single analysis.

Here, we present a chromatographic method that uses a third mobile phase and enables a combined traditional reversed phase and lipidomics-appropriate chromatography method using a single sample preparation and one analytical column. We have adopted many of the quality control measures and data filtering steps that have previously been implemented in predominantly clinical metabolomics studies. These approaches are useful in determining robust features with high technical reproducibility and biological relevance and excludes those which do not meet these criteria. For example, we describe repeat analysis of the pooled QC sample[8], QC correction[9], data filtering[9,10] and imputation of missing features[11].

PROTOCOL:

This method is appropriate for 30 samples (approximately 150 seeds per sample). Three biological replicates of ten different field-grown wheat varieties were used here.

1. Preparation of grains

1.1. Retrieve samples (whole grains) from -80 °C storage.

NOTE: Freeze-drying of seeds is recommended shortly after harvest if samples are being collected from multiple seasons. This minimizes any changes in metabolite concentration that may occur after varying periods of storage. To do this, transfer seeds to a 15 mL plastic centrifuge tube (approximately 300 seeds will fill the tube) and cover with aluminum foil. Pierce the foil two-three times using a pin and freeze dry the whole grains overnight (approximately 24 h). Samples can either be returned to the -80 °C freezer at this stage or the next step can be carried out.

1.2. Grind the seeds using a laboratory blender for two runs on high mode for 20 s.

NOTE: The blender used for this protocol requires a minimum of approximately 150 seeds to fill the blender to blade height and give a relatively homogenously ground grain sample.

1.3. Remove the blender from the base and tap the side of the blender to bring any coarsely ground grain to the surface of the sample. Coarse material can be discarded or stored.

1.4. Transfer powder-like finely ground material from the blender to a 2 mL plastic microcentrifuge tube.

NOTE: Wash the blender with deionized water and rinse with LC-MS-grade MeOH between samples. Ensure the blender is completely dry before proceeding to the next sample.

1.5. Return finely ground grain samples to the freezer or proceed to the next step (metabolite extraction).

2. Preparation of extraction solvent

NOTE: Prepare extraction solvent on the same day as performing the extractions.

2.1. Prepare at least 2 mL of 1 mg/mL of each standard. Use acetonitrile (ACN) to prepare 2-aminoanthracene, miconazole and d_6-transcinnamic acid. Use water to prepare $^{13}C_6$-sorbitol.

2.2. Take 2 mL of each 1 mg/mL standard and add to a 100 mL volumetric flask.

2.3. Fill the volumetric flask to the line with acetonitrile. Ensure that 100 mL of acetonitrile contains 20 µg/mL of each of the internal standards: 2-aminoanthracene, miconazole, $^{13}C_6$-sorbitol, d_6-transcinnamic acid.

3. Metabolite extraction

3.1. Weigh 200 mg of finely ground grain into a 2 mL microcentrifuge tube.

3.2. Add 500 µL of extraction solvent to 200 mg of finely ground grain sample.

3.3. Mix using a homogenizer for 2 runs of 20 s at 6,500 rpm.

3.4. Centrifuge at 4 °C for 5 min at 16,100 x *g*.

3.5. Transfer the supernatant to a 2 mL plastic tube.

3.6. Repeat this procedure from steps 3.1 to 3.5 twice more to give a total supernatant volume of approximately 1.5 mL.

3.7. Vortex to mix the supernatant.

3.8. Transfer an equal volume (55 µL) of each extract to a separate 2 mL tube to make a pooled grain extract sample.

3.9. Transfer a 50 µL aliquot of the extract to a glass vial.

NOTE: Extracts can be frozen (-80 °C) at this point or proceed to the next step and follow through to the LC-MS analysis.

4. Preparation of solutions for LC-MS analysis

CAUTION: For concentrated acid, always add acid to water/solvent.

4.1. Prepare 50 mL of 1 M ammonium formate stock solution. Weigh 3.153 g of ammonium formate and transfer to a 50 mL volumetric flask. Fill volumetric flask to the line with LC-MS grade H_2O.

4.2. Prepare 1 L of the mobile phase A consisting of 10 mM ammonium formate, 0.1% formic acid. To do so, add approximately 500 mL of LC-MS grade water to a 1 L volumetric flask. Add 10 mL of 1 M ammonium formate stock and 1 mL of formic acid. Fill volumetric flask to the line with LC-MS grade H_2O. Transfer to a 1 L bottle and sonicate for 15 min to degas.

4.3. Prepare 1 L of mobile phase B consisting of 10 mM ammonium formate in 79:20:1 acetonitrile:isopropyl alcohol:water, 0.1% formic acid ratio. Add 200 mL of isopropanol to a 1 L volumetric flask. Add 10 mL of 1 M ammonium formate stock and 1 mL of formic acid. Fill volumetric flask to the line with acetonitrile. Transfer to a 1 L bottle and sonicate for 15 min to degas.

NOTE: Dilute the 1 M ammonium formate into the isopropyl alcohol (IPA) before adding the ACN. Ammonium formate is insoluble in CAN.

4.4. Prepare 1 L of mobile phase C consisting of 10 mM ammonium formate in the ratio of 89:10:1 isopropyl alcohol:acetonitrile:water. To do so, add approximately 500 mL of isopropanol, 10 mL of 1 M ammonium formate stock and 100 mL of acetonitrile to a 1 L volumetric flask. Fill to the line with isopropanol. Transfer to a 1 L bottle and sonicate for 15 min to degas.

4.5. Preparation of LC-MS system wash solvents

4.5.1. Replace the pump-head wash solution with fresh solution. Use 50% methanol, 10% isopropyl alcohol or other as recommended by the manufacturer.

4.5.2. Prepare strong and weak needle wash solutions for washing the injection fluidics prior to and following sample injection. For the strong wash, add equal volumes of ACN and IPA. For the weak wash, prepare a solution of 10% ACN (requiring approximately 500 mL and 1 L of each of the strong and weak washes respectively for this protocol and number of samples) in separate bottles.

4.5.3. Set the needle wash volumes to 600 µL and 1800 µL for the strong and weak washes, respectively.

5. Preparation of samples for LC-MS analysis

5.1. As per the manufacturers standard operating procedure for the preparation of 400 ng/µL leucine enkephalin, pipette 7.5 mL of water into the 12 mL leucine-enkephalin vial containing 3 mg of leucine-enkephalin. Freeze at -80 °C in 50 µL aliquots.

5.2. Prepare 100 mL of 5% ACN containing 200 ng/mL leucine-enkephalin (50 µL of 400 ng/µL leucine enkephalin). Prepare on the same day as LC-MS analysis.

5.3. Add 950 µL of 5% acetonitrile containing the injection standard leucine-enkephalin to the 50 µL sample aliquot prepared from step 3.

5.4. Vortex to mix the prepared sample.

6. LC-MS setup

NOTE: A detailed description of instrument and acquisition method setup is described in the manufacturer's user guide. A general guide and the details specific to this protocol are outlined below. The following steps can be completed at any time prior to acquiring the data.

6.1. Open an LC-MS hardware profile.

6.2. Set up the chromatographic method as outlined in **Table 1**. Ensure that the LC system is equipped with a quaternary solvent manager to set up this gradient.

NOTE: IPA is a viscous solvent. It should be introduced at a low flow rate and a sufficient equilibration time should be used before increasing the composition to 98.0%. These steps will prevent the LC system from overpressuring and stopping.

6.3. Set up the mass spectrometer acquisition methods for each of the positive and negative ToF-MS modes over the *m/z* range 50-1,300.

NOTE: The instrument used for the work presented here requires positive and negative methods to be calibrated and run individually (i.e., polarity switching within a method is not possible).

6.4. If the LC column is new, condition the column according to the manufacturer's recommendation.

NOTE: The following steps should be completed directly before data acquisition.

6.5. In an 'MS only' hardware profile, calibrate the mass spectrometer according to the manufacturer's recommendations. Complete this step prior to each mode of acquisition, ensuring that the system has stabilized in each given modality before calibration.

6.6. Purge and flush the LC fluidics system using LC-MS grade solvents, including mobile phase

and wash solvents.

6.7. Equilibrate the LC system using the LC method starting conditions, ensuring that column pressure has stabilized.

6.8. Inject sodium formate (0.5 mM in 90% IPA) at the beginning of the sample sequence (described below) to check the instrument calibration.

6.9. Set up the instrument sequence table so that solvent and preparative (extraction) blanks are analyzed first; followed by pooled QC samples (6-10) for system conditioning; then the randomized sample list with QC samples run at regular intervals (e.g., every fifth injection) as technical replicates. Run two QC samples at the end of the sequence.

NOTE: It is helpful to include the date and injection/acquisition order in the sample filename as well as the sample ID. For example: YYYY MMDD_Injection number_Variety_Biological replicate. Before pressing start, ensure the LC column pressure is stable and that the LC is connected to the MS.

7. Data processing

NOTE: A general data processing workflow is presented in **Figure 1.**

7.1. Check the data quality (internal standard mass accuracy (calculation below) and signal reproducibility) while the sequence is running. To check signal reproducibility, visual inspection of overlaid spectra should suffice.

NOTE: Mass error (ppm) = ((Theoretical mass – measured mass) / theoretical mass) x 10^6

7.2. Generate an aligned peak intensity matrix containing samples x internal standards (aligned by retention time and *m/z* values).

7.2.1. Open the data processing software (see **Table of Materials**). Under **Home > Open**, click **Data**. Navigate to the appropriate file location and open all data files.

7.2.2. Under **Home > Sequence > Processing Type**, select **Quantitation** from the drop-down menu.

7.2.3. Under **Home > Method > Quan**, click **Calibration Components**. Fill in each field using the details provided in **Table 2**. Click **OK**.

7.2.4. On the left panel in the columns next to the data files, fill in the sample type by right clicking on the cell and selecting **Unknown**. Fill in the level as **n/a**.

7.2.5. Under **Home > Processing**, select **Sequence**. Choose a location to save the sequence and

then click **Process**.

7.2.6. Under **Home > Results**, select **Quan**. From the **Quan** viewer, select **Export runs to matrix analyzer**.

7.2.7. From matrix analyzer results viewer, click **Export to csv**. Save the file as a spreadsheet file.

7.2.8. In spreadsheet software, calculate the average, standard deviation and relative standard deviation of the intensity (peak area) of each internal standard.

7.3. Generate an aligned peak intensity matrix containing samples x untargeted features (aligned by retention time and *m/z* values).

7.3.1. Open the small molecule discovery analysis software (see **Table of Materials**) and select **File** > **New** to create a new experiment. Name the experiment and choose the location to save and store experiment files. Click **Next**.

7.3.2. Select the type of instrument used (high resolution mass spectrometer), data format (profile), and the polarity (positive or negative). Click **Next**. Select all adducts available in the library and edit adduct library as required. Click **Create Experiment**. A new page will load where the rest of the data processing will continue/occur.

7.3.3. Import data files. Select the file format and then select import. Browse to data location and select the data files to be imported. The progress will be shown for each file on the left panel of the page.

7.3.4. Once data files are imported, select **Start Automatic Processing**. Choose a method for selecting an alignment reference. Either let the software assess all runs for suitability, give a list of suitable samples (i.e., QC samples) or choose the reference most suitable i.e. a mid-sequence QC sample. Select **Yes, automatically align my runs** > **Next**.

7.3.5. Select **Next** on the experiment design page (this can be set up later).

7.3.6. Select **Perform Peak Picking** and then **Set Parameters**. Under the **Peak Picking Limits** tab, select **Absolute Ion Intensity** and enter **100**. Select apply a minimum peak width and enter 0.01 min. Select **OK > Finish**. When processing is complete, select **Close**.

NOTE: Peak picking limits can be optimized for other data file types as necessary.

7.3.7. On the bottom right of the screen, select **Section Complete**. Review aligned runs and make sure each sample is aligned to the reference. The alignment scores were >90% for the data presented here. Select **Section Complete**.

7.3.8. On the next page, select between subject design. Name the design. Select **Group the runs manually** and **Create design**. Add condition, click on **Condition 1** and name the group appropriately. Click **Section Complete**.

NOTE: Continue to add conditions as appropriate to use statistics within the software. Since we only used the software to generate an untargeted matrix, we used a single condition labelled 'all'.

7.3.9. On the next page, select **Section Complete**. Do not re-do peak picking.

7.3.10. Review the deconvolution and then click **Section Complete**.

7.3.11. Go to **File** > **Export Compound Measurements**. Deselect any properties not wanted in the output. Click **OK**. Choose a location to save the .csv file. Click **Save > Open File > Open Folder** or **Close**.

7.4. Filter the data using the extraction blanks to remove artefacts (spreadsheet software).

7.4.1. For each RT x *m/z* feature, in a new column, calculate the average response in extraction blanks.

7.4.2. For each RT x *m/z* feature, calculate the average response in all other samples (including QC samples).

7.4.3. Calculate the % peak intensity of blanks in samples (average response in blank/average response in samples x 100).

7.4.4. Sort the percent contribution column from lowest to highest values.

7.4.5. Remove features which have >5% intensity contribution from blanks.

7.5. Filter missing values and correct the feature signals to signals in pooled QC samples.

7.5.1. Open the data processing software (**Table of Materials**). Click the **View MatrixAnalyzer** button. Click the **Open Data File** button.

7.5.2. Navigate to the .csv file location containing the peak intensity of RT x m/z features for each sample (untargeted matrix). Select **Open**.

7.5.3. Under the QC samples tab, fill out each parameter as required. For the data set described here, use QC category=QC, ignore categories=empty, impute type=none, coverage threshold=80, scale using=no scaling, uncheck log transform, correct using=smoothing spline, smoothing=0.25.

7.5.4. On the top right of the matrix panel, click the play button **QC correction**.

7.5.5. After the correction has been performed on the top right of the matrix panel, click **Save Results**. Navigate to an appropriate location and save the .csv results file.

7.6. Filter the data to remove features which have >20% RSD in QC samples.

7.6.1. Arrange the data so that samples are in rows and features are in columns.

7.6.2. In a new column, calculate the average peak intensity for QC samples.

7.6.3. In a new column, calculate the standard deviation of the peak intensity for QC samples.

7.6.4. Calculate the relative standard deviation of the peak intensity for QC samples: (QC standard deviation/QC average) x 100.

7.6.5. Sort the features from lowest to highest %RSD and remove features which have a QC RSD >20%.

7.7. Filter the data to remove features with low RSD_{sample}/RSD_{QC} ratios (e.g., <1).

7.7.1. In a new column, calculate the average peak intensity for samples.

7.7.2. In a new column, calculate the standard deviation of the peak intensity for samples.

7.7.3. Calculate the relative standard deviation of the peak intensity of the samples: (sample standard deviation/sample average) x 100.

7.7.4. In a new column, divide the samples RSD by the QC RSD.

7.7.5. Sort the values (RSD_{sample}/RSD_{QC} ratios) from highest to lowest and remove features with a ratio <1.

7.8. Impute missing values (several methods available online).

7.8.1. Format the spreadsheet so that samples are in rows and features are in columns. The first column should be the samples filename. Create an additional column next to the filenames and enter the sample groupings. In this case, the samples were grouped by variety.

7.8.2. Save the spreadsheet as a .csv file.

7.8.3. Go to the homepage for the web-based analytical pipeline for high-throughput metabolomics (see **Table of Materials**) and click **Click Here to Start**.

7.8.4. Click **Statistical Analysis**. Under **Upload Your Data**, select Data Type: peak intensity table, Format: Samples in rows (unpaired) and then **Choose File**.

7.8.5. Navigate to .csv file, select **Open** and then select **Submit**.

7.8.6. On the next page, select **Missing Value Estimation**. Uncheck step 1 (this was performed in AnalyzerPro XD). In step 2, choose a method to estimate missing values.

NOTE: For the data presented here - 'estimate missing values' with 'KNN' was selected.

7.8.7. At this stage, the data matrix can be downloaded (select **Download** from the left panel of the web page) or proceed to perform further statistical analyses.

REPRESENTATIVE RESULTS:

The plant metabolome is influenced by a combination of its genome and environment, and additionally in an agricultural setting, the crop management regime. We demonstrate that genetic differences between wheat varieties can be observed at the metabolite level, here, with over 500 measured compounds showing significantly different concentrations between varieties in the grain alone. Good mass accuracy (<10 ppm error) and signal reproducibility (<20% RSD) of internal standards (**Figure 2**) were observed for both negative and positive ionization modes (**Table 3**). The described sample preparation and liquid chromatography-mass spectrometry-based analysis yielded >900 deconvoluted features in negative ionization mode and >1300 deconvoluted features in positive ionization mode. Preparative blanks (**Figure 3**) were included to determine whether the sample preparation and analysis methods introduced artefact features, and thus all non-biological influences eliminated from the data matrix. It was found that 421 signals in the negative mode and 835 signals in the positive mode had signal intensities equal to or greater than 5% of the average signal intensity in grain samples. These features were removed and after further data filtering steps (step 7 and **Figure 1**), the negative mode returned 483 features and the positive mode returned 523 features, forming the metabolic snapshot. The method was successful in detecting features, which had significantly different intensities between wheat varieties (**Figure 4**) with >500 significant features across both ionization modes. In negative ionization mode, the majority of significant features were in the reversed phase gradient and in positive ionization mode, the majority of significant features were in the lipid gradient (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1. The workflow used in this analysis for data checking, processing and filtering. Step 1 is conducted using the data acquisition/viewing software on the instrument so that 'on-the-fly' assessments can be conducted. This includes calculating the mass error (ppm) of internal standards and overlaying internal standard peaks for visual assessment of data reproducibility. Steps 2-7 describe the data processing procedure outlined in the protocol, step 7.

Figure 2. Extracted ion chromatograms. Extracted ion chromatograms of $^{13}C_6$-sorbitol (dark blue), leucine-enkephalin (pink), d_6-trans-cinnamic acid (orange), 2-aminoanthracene (green)

and miconazole (light blue) internal standards in positive (top) and negative (bottom) electrospray ionization (ESI) modes. The internal standard retention times and intensities are shown. ESI + and ESI -

Figure 3. Total ion chromatogram (TIC) overlay of preparative blanks showing negative mode (pink) and positive mode (blue) acquisitions. One internal standard, miconazole, is shown.

Figure 4. Total ion chromatogram (TIC) overlay, showing negative mode (pink) and positive mode (blue) acquisitions and number of features significantly different between wheat variety across the chromatographic gradient. In negative mode, the greatest number of significant features was found when mobile phase B composition was high. In positive mode, the greatest number of significant features was found when mobile phase C composition was high. One internal standard, miconazole, is shown.

Table 1. Liquid chromatography timed program of mobile phase compositions.

Table 2. Peak detection parameters for internal standards in positive (and negative) acquisition modes.

Table 3. Sample (*n*=30) internal standard mass accuracy (ppm) and signal reproducibility before and after QC-correction expressed as relative standard deviation (%).

DISCUSSION:

Here, we present an LC-MS-based untargeted metabolomics method for the analysis of wheat grain. The method combines four acquisition modes (reversed phase and lipid-amenable reversed phase with positive and negative ionization) into two modes by introducing a third mobile phase into the reversed phase gradient. The combined approach yielded approximately 500 biologically relevant features per ion polarity with roughly half of these significantly different in intensity between wheat varieties. Significant changes in metabolite concentration in the grain of different wheat varieties indicates altered biochemistry, which may be linked to disease resistance, stress tolerance and other phenotypic traits that are important for grain quality and yield. For example, metabolomics approaches have been used to describe novel defense mechanisms[12] and propose the role of metabolites in drought tolerance[13]. Future applications of this protocol may be able to further link biochemical profiles of particular varieties to genetic traits that are desirable for certain environments and management practices. In turn, this would allow production of optimal grain quality and yield for selected genotypes.

The inclusion of internal standards is critical to this protocol to allow the user to determine changes in signal, retention time shifts and as indicators of mass accuracy. Changes in signal may indicate, for example, sub optimal extraction, injection (including fluidic system blockages), or detector performance. Retention time shifts may indicate poor pump performance, inappropriate mobile phase gradient equilibration or that the LC column stationary phase has deteriorated. Poor mass accuracy can be indicative of a drifted calibration and that the system

requires re-calibration. In all of the above cases, the system should be stopped, and the appropriate maintenance/replacement of parts performed. We included four standards in the extraction solution used to prepare grain and a standard in the final sample added prior to injection. Care was taken to ensure that standards were amenable to each ionization mode and covered a range of retention times; however, we acknowledge that this array of standards could be improved with the inclusion of a labeled lipid standard. It has been shown that wheat grain contains hundreds of triacylglycerols (TAGs)[5], any of which would be a suitable addition to this protocol. The inclusion of preparative blanks and pooled QC samples[8] are also critical steps in this protocol. Thousands of ion features are detected in untargeted mass spectrometry methods and it is important to exclude those which are present only in blank samples and also those which are not reproducibly detected (i.e., high %RSD) throughout the analysis.

Although the current method saves considerable time and resources, if a quaternary solvent manager is not available, standard reversed phase and lipid methods can be used to achieve the same results. The extraction volume used in this protocol would suffice for the analysis of additional acquisition modes. This protocol describes an acetonitrile extraction. Whilst successful, an alternative extraction solvent, or combination of solvents, will provide a different metabolite coverage, which may in turn deliver more features and/or give better (or a lesser) extraction efficiency of some compounds. We have not attempted to establish the metabolite identity of the statistically significant measurements resolved in this protocol; however, mass spectral databases for plant metabolites and lipids are available and developing[5,14,15]. To identify the metabolites, tandem mass spectra (MS/MS) would need to be collected in addition to full scan data. These can be collected during the initial run using pooled samples and an appropriate MS/MS method or on reserved extract (stored at -80 °C) once metabolites of interest have been determined. We observed large fold changes of compounds between varieties so we would recommend doing both and in the second instance, using a variety known to contain a high concentration of the compound of interest to obtain the highest quality MS/MS spectrum.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

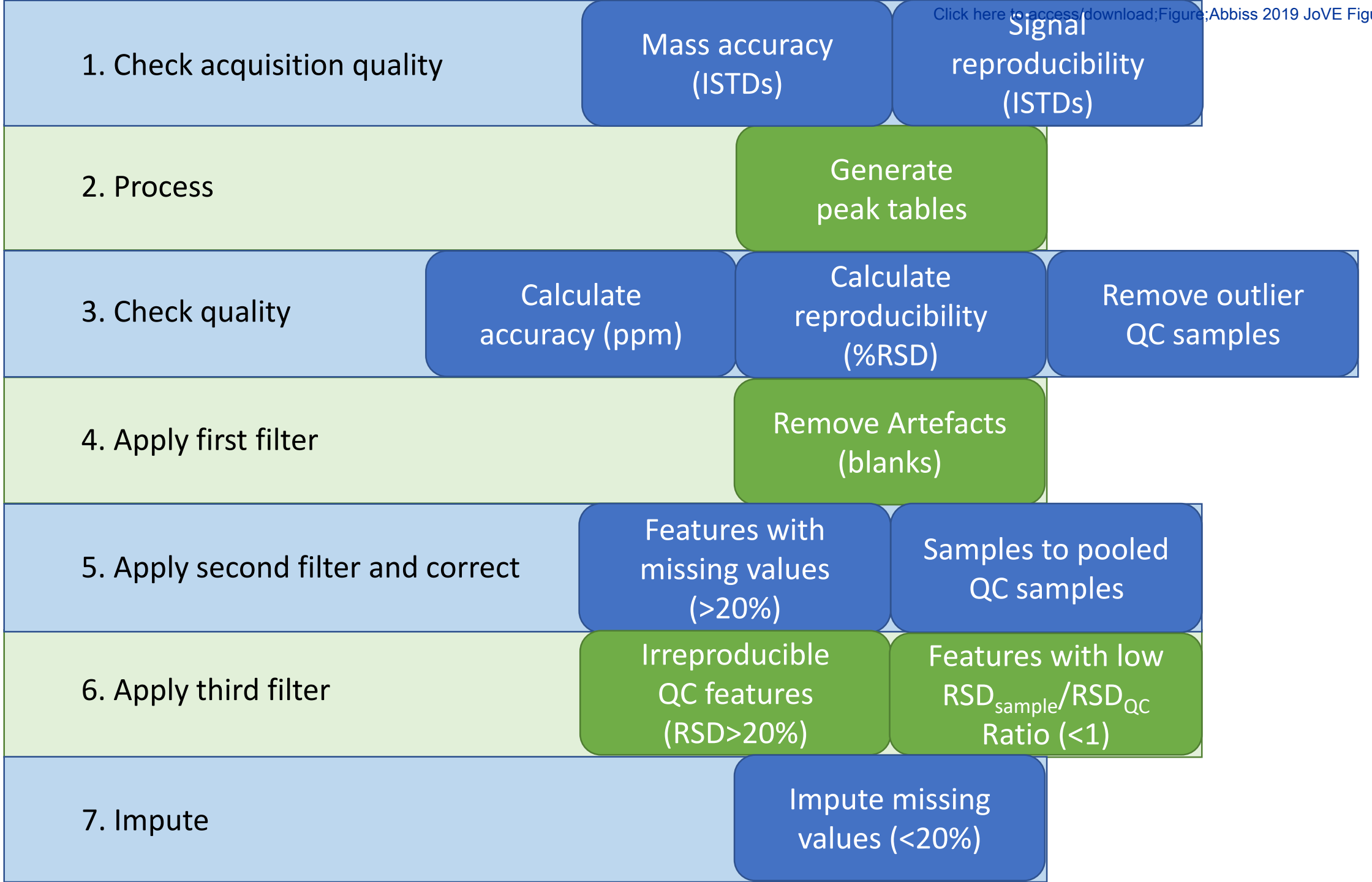

Figure 2

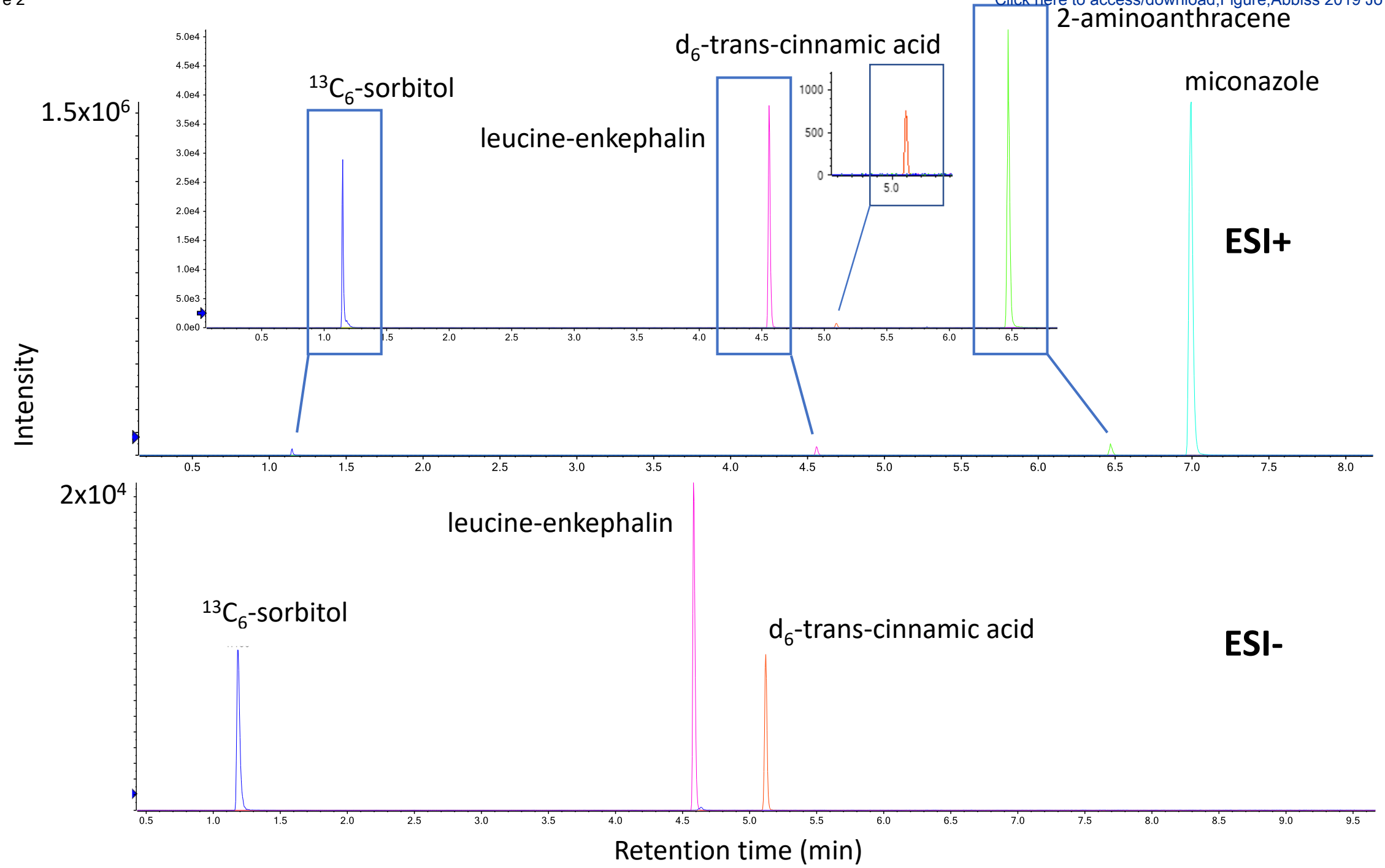

Figure 3

Click here to access/download;Figure;Abbiss 2019 JoVE Figure 3.pdf

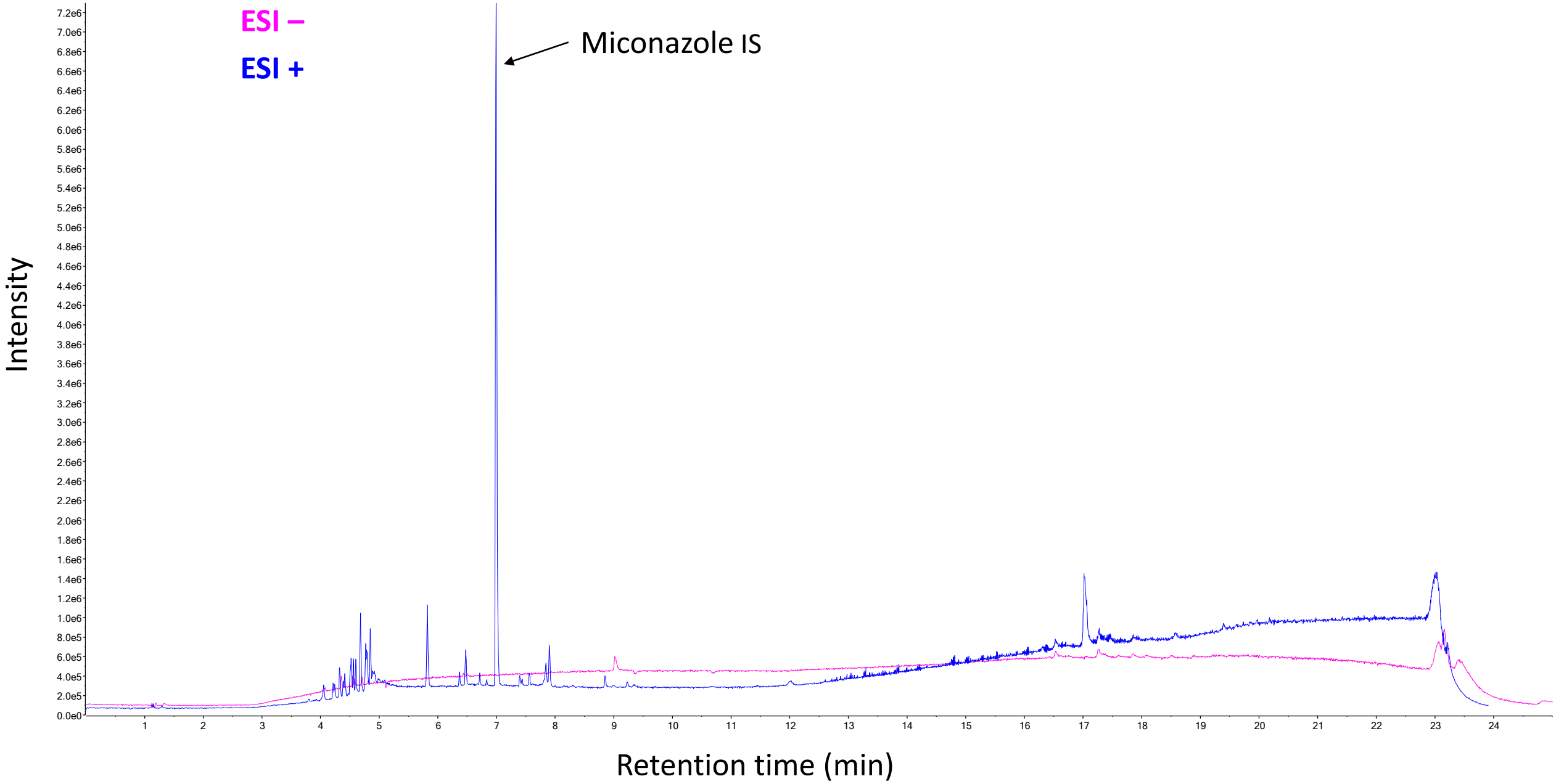

Figure 4

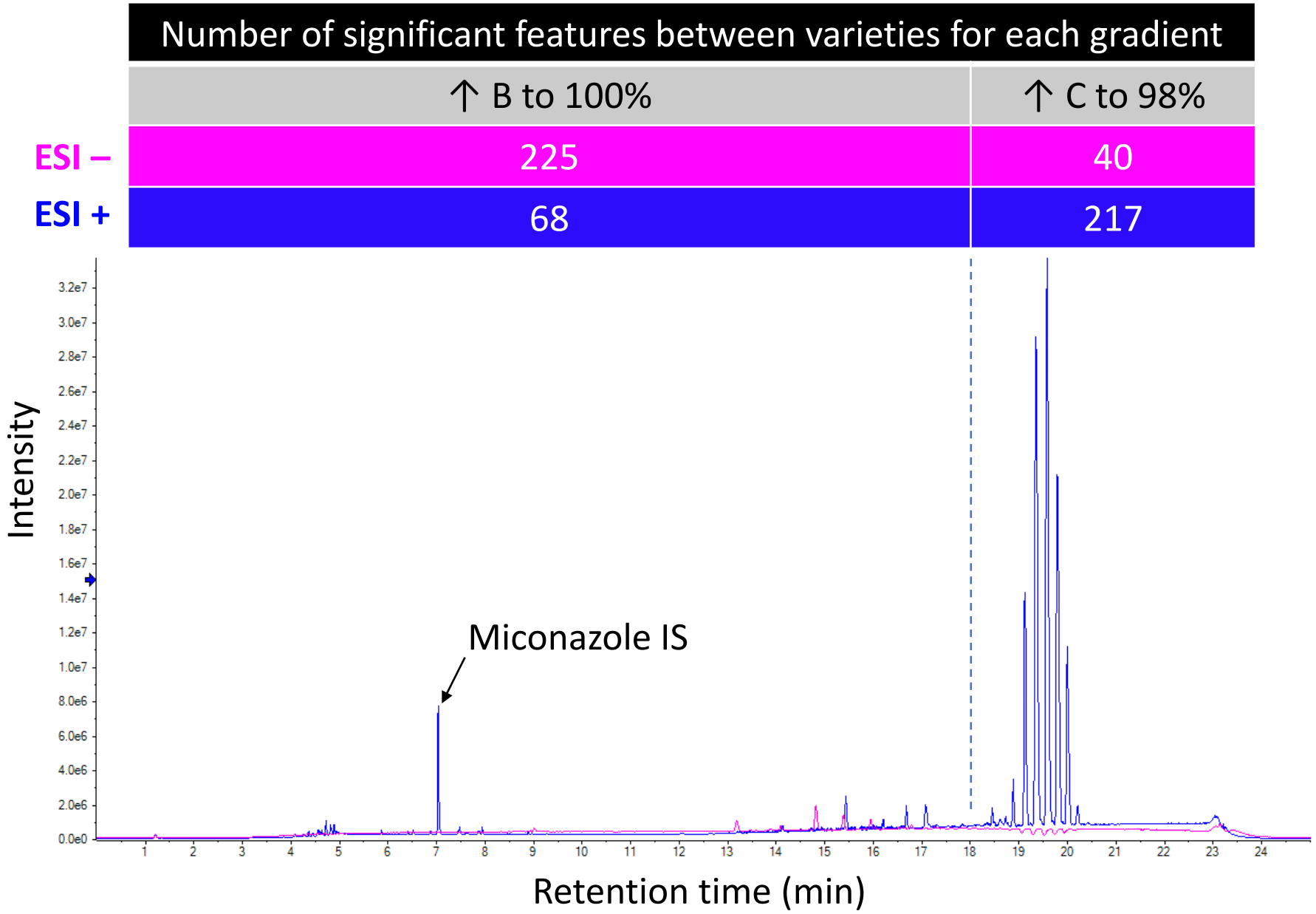

Table 1

Click here to access/download;Table;Abbiss 2019 JoVE Table 1.xlsx

Segment	Time (min)	Flow rate (mL/min)	%A	%B	%C	Curve
1	Initial	0.6	98	2	0	6
2	1	0.6	98	2	0	6
3	7	0.8	2	98	0	6
4	7.1	0.8	0	100	0	6
5	10	0.8	0	100	0	6
6	18	0.4	0	10	90	6
7	21	0.4	0	2	98	6
8	21.1	0.4	98	2	0	6
9	24	0.4	98	2	0	6
10	24.1	0.6	98	2	0	6
11	25	0.6	98	2	0	6

Table 2

Click here to access/download;Table;Abbiss 2019 JoVE Table 2.xlsx

Parameter	Internal standard			
	$^{13}C_6$-sorbitol	Leucine-enkephalin	d_6-transcinnamic acid	2-amino-anthracene
Quan *m/z*	211.09 (187.09)	556.28 (554.26)	155.097 (153.08)	194.1
Mass tolerance (amu)	0.01 (0.05)	0.01 (0.05)	0.01 (0.05)	0.01
Retention time	1.2	4.6	5.1	6.5
Retention time window	0.1 (0.5)	0.1 (0.5)	0.1 (0.5)	0.1
Detection type	Highest	Highest	Highest	Highest
Response type	Area	Area	Area	Area
Area threshold	10	10 (50)	10 (50)	10
Width threshold	0.01	0.01	0.01	0.01
Height threshold	0	0	0	0
Signal-to-noise ratio	5	5	3 (5)	5
Smoothing	5	5 (3)	5 (3)	5

Miconazole
414.99
0.01
7
0.1
Highest
Area
10
0.01
0
5
5

Table 3

Click here to access/download;Table;Abbiss 2019 JoVE Table 3.xlsx

		Mass accuracy (ppm)	%RSD Before QC correction
Negative mode	$^{13}C_6$-sorbitol	4.59	6.12
	D_6-transcinnamic acid	7.94	3.93
	Leucine-enkephalin	0.91	1.8
Positive mode	$^{13}C_6$-sorbitol	5.65	14.1
	Leucine-enkephalin	3	3.24
	D_6-transcinnamic acid	8.03	5.41
	2-aminoanthracene	3.99	7.97
	Miconazole	1.8	3.01

%RSD After QC correction
7.08
5.99
1.96
15.3
5
9.81
5.45
5.72

Name of Material/Equipment

13C6-sorbitol
2-aminoanthracene
Acetonitrile
Ammonium formate
Analyst TF
AnalyzerPro software
AnalyzerPro XD sortware
Balance
d6-transcinnamic acid
Formic acid
Freeze dryer (Freezone 2.5 Plus)
Glass Schott bottles (100 mL, 500 mL, 1 L)
Glass vials (2 mL) and screw cap lids (pre-slit)
Installation kit for Sciex TripleToF
Isopropanol
Laboratory blender
Leucine-enkephalin
Metaboanalyst
Methanol
Miconazole
Microcentrifuge (Eppendorf 5415R)
Microcentrifuge tubes (2 mL)
Microsoft Office Excel
Peak View software
Pipette tips (200 uL, 100 uL)
Pipettes (200 uL, 1000 uL)
Plastic centrifuge tubes (15 mL)
Progenesis QI
Sciex 5600 triple ToF mass spectrometer
Screw-cap lysis tubes (2 mL) with ceramic beads
Sodium formate
Tissue lyser/homogeniser

Volumetric flasks (10 mL, 50 mL, 100 mL, 200 mL, 1 L)
Vortex mixer
Water
Water’s Acquity LC system equipped with quaternary pumps
Water’s Aquity UPLC 100mm HSST3 C18 column

Company	Catalog Number
Merck Sigma-Aldrich	605514
Merck Sigma-Aldrich	A38800-1 g
ThermoFisher Scientific	FSBA955-4
Merck Sigma-Aldrich	516961-100 mL
Sciex	
SpectralWorks Ltd.	
SpectralWorks Ltd.	
Sartorius. Precision Balances Pty. Ltd.	
Isotec	513962-250 mg
Ajax Finechem Pty. Ltd.	A2471-500 mL
Labconco	7670031
Velocity Scientific Solutions	VSS-913 (vials), VSS-SC91191 (lids)
Sciex	p/n 4456736
ThermoFisher Scientific	FSBA464-4
Waring commercial	
Waters	p/n 700008842
https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml	
ThermoFisher Scientific	FSBA456-4
Merck Sigma-Aldrich	M3512-1 g
Eppendorf (Distributed by Crown Scientific Pty. Ltd.)	
SSIbio	1310-S0
Microsoft	
Sciex	
ThermoFisher Scientific	MBP2069-05-HR (200 uL), MBP2179-05-HR (1000 uL)
ThermoFisher Scientific	
ThermoFisher Scientific	NUN339650
Nonlinear Dynamics	
Sciex	
Bertin Technologies	
Merck Sigma-Aldrich	456020-25 g
Bertin Technologies	

IKA Works Inc. (Distributed by Crown Scientific Pty. Ltd.)	
ThermoFisher Scientific	FSBW6-4
Waters	
Waters	p/n 186005614

Comments/Description

Optima LC-MS grade
>99.995%
Version 1.7
Data processing software used for step 7.2. Version 5.7
Data processing software used for step 7.5. Version 1.4

99%

Optima LC-MS grade
Model HGBTWTS3
Tuning solution
Web-based analytical pipeline for high-throughput metabolomics. Free, web-based tool. Version 4.0.
Optima LC-MS grade

5426 No. 0021716

Version 1.2 (64-bit)

Samll molecule discovery analysis software. Version 2.3 (64-bit)

Serial 0001620

001722
Optima LC-MS grade

Responses to editorial and reviewer comments

The authors would like to thank the editors and reviewers for carefully considering this work and providing constructive feedback and suggestions. We have incorporated all of the suggested changes in the revised version of the manuscript and feel that the overall quality of the manuscript has been lifted.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

The document has been proofread and spell checked and the following corrections have been made: analyse has been changes to analyze (section 6.8), lyser has been changed to lyzer (section 3.2).

2. Please ensure that the Introduction includes all of the following with citations:

a) A clear statement of the overall goal of this method

The overall goal of this method was described in the final sentence of the first paragraph of the introduction: "the outcome of having this knowledge is a more precise management strategy and ultimately improved yield size and quality". However, we acknowledge that this may not be clear and have edited this to: "the outcome of having this knowledge is a more precise management strategy to achieve the goal of improved yield size and quality".

b) The rationale behind the development and/or use of this technique

The rationale behind the development and use of the technique is described in paragraph 2 of the introduction: "Currently no metabolomics platforms (predominantly mass spectrometry and nuclear magnetic resonance spectroscopy) can capture the entire metabolome in a single analysis. Developing such techniques (sample preparation, metabolite extraction and analysis) which provide as great a coverage of the metabolome as possible within a single analytical run is a key aim for metabolomics researchers. Increasing the metabolome coverage will increase the richness of biological interpretation and can offer savings in both time and cost".

c) The advantages over alternative techniques with applicable references to previous studies

We have included the following paragraph with appropriate citations in the introduction to outline advantages over previous works which have utilized multiple platforms for greater metabolome coverage:

"Previous untargeted metabolomics analyses of wheat grain have combined data from multiple modes for greater metabolome coverage however this has required samples to be prepared separately for different modes. For example, Beleggia et al. prepared a derivatized sample for the GC-MS analysis of polar analytes in addition to the GC-MS analysis of the non-polar analytes. Das et al. used both GC- and LC-MS methods to improve coverage for their analysis however this approach would generally require separate sample preparations as described above as well as two independent platforms. Previous analyses of wheat grain using GC-MS and LC-MS platforms have yielded 50 to 412 (55 identified) features for GC-MS, 409 for combined GCMS and LC-MS and several thousand for an LC-MS lipidomics analysis. By combining at least two modes into a single analysis, extended metabolome coverage can be maintained while minimizing analysis time and resources used."

d) A description of the context of the technique in the wider body of literature

We have provided a more detailed description of the technique with appropriate citations:

The plant metabolome is predicted to contain thousands of small molecules with varied physicochemical properties. Currently no metabolomics platforms (predominantly mass spectrometry and nuclear magnetic resonance spectroscopy) can capture the entire metabolome in a single analysis. Developing such techniques (sample preparation, metabolite extraction and analysis) which provide as great a coverage of the metabolome as possible within a single analytical run is a key aim for metabolomics researchers. Previous untargeted metabolomics analyses of wheat grain have combined data from multiple chromatographic separations and acquisition polarities and/or instrumentation for greater metabolome coverage. However, this has required samples to be prepared and acquired separately for each modality. For example, Beleggia et al. prepared a derivatized sample for the GC-MS analysis of polar analytes in addition to the GC-MS analysis of the non-polar analytes. Das et al. used both GC- and LC-MS methods to improve coverage in their analyses however this approach would generally require separate sample preparations as described above as well as two independent analytical platforms. Previous analyses of wheat grain using GC-MS and LC-MS platforms have yielded 50 to 412 (55 identified) features for GC-MS, 409 for combined GC-MS and LC-MS and several thousand for an LC-MS lipidomics analysis. By combining at least two modes into a single analysis, extended metabolome coverage can be maintained, increasing the richness of biological interpretation while also offering savings in both time and cost.

To permit the efficient separation of a wide range of lipid species by reversed-phase chromatography, modern lipidomics methodologies commonly use a high proportion of isopropanol in the elution solvent[6], providing amenability to lipid classes which might otherwise be unresolved by the chromatography. For an efficient lipid separation, the starting mobile phase is also much higher in organic composition than the typical reversed phase chromatographic methods which consider other classes of molecules. The high organic composition at the start of the gradient makes these methods less suitable to many other classes of molecules. Most notably, reversed phase liquid chromatography employs a binary solvent gradient, starting with a mostly aqueous composition, and increasing in organic content as the elution strength of the chromatography is increased. To this end, we sought to combine the two approaches to achieve separation of both lipid and non-lipid classes of metabolites within a single analysis.

e) Information to help readers to determine whether the method is appropriate for their application

The following information will help readers determine whether the method is appropriate for their application:

Plant metabolites are influenced by factors such as the genome, environment (climate, rainfall etc.), and in an agriculture setting, the way crops are managed (i.e. application of fertilizer, fungicide etc.). Unlike the genome, the metabolome is influenced by all of these factors and hence metabolomics data provides a biochemical fingerprint of these interactions at a particular time. There are usually one of two goals for a metabolomics-based study. Firstly, to achieve a deeper understanding of the organism's biochemistry and help explain the mechanism of response to perturbation (abiotic or biotic stress) in relation to the physiology. Secondly, to associate biomarkers with the perturbation under study.

3. Please include more citations to cover a wider body of literature in the introduction.

We have included the following 5 additional references to include a wider body of literature:

Beleggia, R. et al. Effect of Genotype, environment and genotype-by-environment interaction on metabolite profiling in durum wheat (Triticum durum Desf.) grain. Journal of Cereal Science. 57 (2), 183-192, (2013).

Blazenovic, I. et al. Structure Annotation of All Mass Spectra in Untargeted Metabolomics. Anal Chem. 91 (3), 2155-2162, (2019).

Castro-Perez, J. M. et al. Comprehensive LC–MSE Lipidomic Analysis using a Shotgun Approach and Its Application to Biomarker Detection and Identification in Osteoarthritis Patients. Journal of Proteome Research. 9 (5), 2377-2389, (2010).

Das, A. et al. Unraveling key metabolomic alterations in wheat embryos derived from freshly harvested and water imbibed seeds of two wheat cultivars with contrasting dormancy status. Frontiers in Plant Science. 8 (1203), (2017).

Francki, M.G. et al. Metabolomic profiling and genomic analysis of wheat aneuploid lines to identify genes controlling biochemical pathways in mature grain. Plant Biotechnology Journal. 14 (2), 649-660, (2016).

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We have ensured that all text in the protocol is written in the imperative tense in complete sentences. Text which cannot be written in the imperative tense has been included as an asterisked note directly after each protocol step where appropriate.

5. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

We have carefully reviewed the protocol to ensure that each step only contains 2-3 actions.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We have added more detail to protocol steps in section 7 to include software navigation and recommended settings.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section.

The manuscript has been formatted: paragraph indentation: 0 for both left and right and special: none, line spacings: single. A single line space between each step, sub step and note in the protocol section has been included.

8. 1.2: Please include the significance of freeze drying at this stage.

In response to this request and that of Reviewer 1, we have included freeze drying as an optional step as a note rather than a critical step of the protocol:

*“*NOTE: We recommend freeze-drying seeds shortly after harvest if samples are being collected from multiple seasons. This minimizes any changes in metabolite concentration that may occur after varying*

periods of storage. To do this: transfer seeds to a 15 mL plastic centrifuge tube (approximately 300 seeds will fill the tube) and cover with aluminum foil. Pierce the foil two-three times using a pin and freeze dry the whole grains overnight (approximately 24 h). Samples can either be returned to the -80 °C freezer at this stage or the next step can be carried out."

9. 1.3: Please include why 150 seed are required per sample? What is the difference between coarse and fine material here? Do you store it in some solution?

*150 seeds is the minimum amount required to fill the blender to blade height. We have updated the protocol to: "Once dry, grind the seeds using a laboratory blender for two runs on Hi for 20 s. *NOTE: The blender used for this protocol requires a minimum of approximately 150 seeds to fill the blender to blade height and give a relatively homogenously ground grain sample".*

After blending, some coarsely ground grain material is likely to be present as well as the desired powder-like ground sample. We have updated the protocol to: "Remove the blender from the base and tap the side of the blender to bring any coarsely ground grain to the surface of the sample. Coarse material can be discarded or stored." And "Transfer powder-like finely ground material from the blender to a 2 mL plastic microcentrifuge tube". No solution is used to store either coarse or fine material.

10. 2: What solution is used to prepare the stock solution? Fill to which line? Where and how do you store this, temperature of storage?

We have reworded the text in this section of the protocol to:

"Preparation of extraction solvent (100 mL acetonitrile containing 20 µg/mL of each of the internal standards: 2-aminoanthracene, miconazole, $^{13}C_6$-sorbitol, d_6-transcinnamic acid).

**NOTE: Prepare extraction solvent on the same day as performing the extractions. Prepare at least 2 mL of 1 mg/mL of each standard. Use ACN to prepare 2-aminoanthracene, miconazole and d_6-transcinnamic acid and water to prepare $^{13}C_6$-sorbitol.*

Take 2 mL of each 1 mg/mL standard and add to a 100 mL volumetric flask.

Fill volumetric flask to the line with acetonitrile".

11. 3: So, the solvent contains internal standard as well?

Yes, the extraction solvent contains internal standard. As above, for clarity, we have reworded the text in this section of the protocol to:

"Preparation of extraction solvent (100 mL acetonitrile containing 20 µg/mL of each of the internal standards: 2-aminoanthracene, miconazole, $^{13}C_6$-sorbitol, d_6-transcinnamic acid).

12. 5.1: Please explain what Water's SOP is or provide citation.

We have included the following explanation in the protocol for the Water's SOP: "As per the manufacturers (Water's) standard operating procedure for the preparation of 400 ng/µL leucine enkephalin, pipette 7.5 mL of water into the 12 mL leucine-enkephalin vial containing 3 mg leucine-enkephalin. Freeze (-80°C) in 50 µL aliquots."

13. For the LC-MS procedure, please include all the button clicks in the software and equipment, knob turns etc. to show how the procedure is performed.

We have directed the reader to the instrument user guide for specific set-up details:

**NOTE: A detailed description of instrument and acquisition method set-up is described in the 5600-system user guide. A general guide and the details specific to this protocol are outlined below.*

14. For the data processing, please include all the button clicks, etc. performed in the software to show how the process is performed. e.g., Right click on the peak to find the intensity, then click "Analyze".

We have now included considerable detail in the data processing section of the protocol (section 7) to describe each software used and how each process is performed.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have now highlighted text that identifies essential steps of the protocol.

16. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. , e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have included the following sentences at the beginning of the results section to describe the result with respect to the experiment performed:

"The plant metabolome is influenced by a combination of its genome and environment, and additionally in an agricultural setting; the crop management regime. We demonstrate that genetic differences between wheat varieties can be observed at the metabolite level, here, with over 500 measured compounds showing significantly different concentrations between varieties in the grain alone."

The paragraph text in the results section refers to all figures and tables with the exception of tables which are referred to in the protocol (i.e. Tables 1 and 2).

17. What are the negative and positive control samples in your experiment? How do you check for negative and positive acquisitions? Where are the peaks for internal standard present in the figures?

We aimed to demonstrate metabolite changes in grain between different wheat varieties rather than changes from a wild type or reference variety. However, the following technical controls were used:

Negative controls were preparative (extraction) blanks and solvent blanks. Including these samples allowed the removal of peaks which were introduced during the sample preparation steps from the final sample data.

Positive controls were extraction blanks and samples containing metabolite internal standards. The detection of metabolite internal standards ensured that the method was suitable for the reproducible detection of metabolite compounds.

With the exception of miconazole, the internal standards are not clearly visible in the total ion chromatogram. We have indicated the peaks for miconazole in figures 3 and 4 and included an additional figure (Figure 2) showing extracted ion chromatograms of each internal standard in positive and negative mode. Internal standard mass and retention time information are presented in Table 2 and mass accuracy and peak area reproducibility data are presented in Table 3.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

This work does not contain figures which have been reused or reproduced from previous publications.

19. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

Each figure legend has been modified to include a brief summary of the data presented:

Figure 1. The workflow used in this analysis for data checking, processing and filtering. Step 1 is conducted using the data acquisition/viewing software on the instrument so that 'on-the-fly' assessments can be conducted. This includes calculating the mass error (ppm) of internal standards and overlaying internal standard peaks for visual assessment of data reproducibility. Steps 2-7 describe the data processing procedure outlined in the protocol, section 7.

Figure 2. Extracted ion chromatograms of $^{13}C_6$-sorbitol (dark blue), leucine-enkephalin (pink), d6-trans-cinnamic acid (orange), 2-aminoanthracene (green) and miconazole (light blue) internal standards in positive (top) and negative (bottom) electrospray ionization modes. The internal standard retention times and intensities are shown.

Figure 3. Figure 3. Total ion chromatogram (TIC) overlay of preparative blanks showing negative mode (pink) and positive mode (blue) acquisitions. One internal standard, miconazole, is shown.

Figure 4. Total ion chromatogram (TIC) overlay, showing negative mode (pink) and positive mode (blue) acquisitions and number of features significantly different between wheat variety across the chromatographic gradient. In negative mode, the greatest number of significant features was found when mobile phase B composition was high. In positive mode, the greatest number of significant features was found when mobile phase C composition was high.

20. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Embedded tables have been removed and replaced with separate .xlsx files. Table titles and descriptions are given after the representative results section.

21. Please sort the materials table in alphabetical order.

The materials table has been sorted in alphabetical order

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper describes a method for analysis of wheat seed by LCMS. The authors need to compare their method with other methods and explain its benefits more in the discussion. Although the authors have included considerable detail a few points need clarification.

Minor Concerns:

* Why are so many seed required (about 150 ground)? Have the authors verified that this number is needed to represent the diversity in a single wheat variety?

*This number of seeds is required to fill the laboratory blender so that grain can be ground relatively homogenously. We have edited the protocol to make this clear: "Once dry, grind the seeds using a laboratory blender for two runs on Hi for 20 s. *NOTE: The blender used for this protocol requires a minimum of approximately 150 seeds to fill the blender to blade height and give a relatively homogenously ground grain sample".*

* Why are the seeds freeze dried? If stored correctly the seed should have little moisture - how much do they lose on freeze drying. i.e. is this step necessary?

We have included freeze-drying to ensure that metabolite changes during storage are minimized since we intend to analyze samples from multiple seasons. Rather than include freeze-drying as a step in the protocol we have amended this section to include freeze-drying as an optional step by adding a note:

*"*NOTE: We recommend freeze-drying seeds shortly after harvest if samples are being collected from multiple seasons. This minimizes any changes in metabolite concentration that may occur after varying periods of storage. To do this: transfer seeds to a 15 mL plastic centrifuge tube (approximately 300 seeds will fill the tube) and cover with aluminum foil. Pierce the foil two-three times using a pin and freeze dry the whole grains overnight (approximately 24 h). Samples can either be returned to the -80 °C freezer at this stage or the next step can be carried out."*

* Line 101 d6-transcinnamic acid should be d6-transcinnamic acid

This has been corrected to d_6.

* The LC method described in Table 1 either has redundant segments or the need for segments has not been described. E.g. segment 5 could be removed. The slow flowrate change from 10 to 18 min is unusual. Again, is this the most efficient method?

Thank you for noticing this redundancy. Segment 5 was from an earlier version of the method and was mistakenly included in the current version. It has now been removed from Table 1.

When the IPA is initially introduced at 10 min, the flow rate needs to be lower to allow for the introduction of such a viscous solvent. The low flow rate prevents over-pressuring the LC system. After and 8-minute equilibration, this is then increased to a higher flow for improved peak capacity. We have included the following note in section 6.1: "IPA is a viscous solvent. It should be introduced at a low flow rate and a sufficient equilibration time should be used before increasing the composition to 98.0%. These steps will prevent the LC system from over-pressuring and stopping".

* It is not clear in the LCMS set-up if the (p5 167-187) if the data is acquired in +/- switching mode or in two independent runs. Please clarify.

Each mode was run independently. We have included a note in section 6.2 to clarify this: "The instrument used in the work presented here requires positive and negative methods to be calibrated and run individually i.e. polarity switching within a method is not possible".

* P5 L199 the authors state: "Correct the feature signals to signals in pooled QC samples" this needs more explanation. What correction is applied and how does this affect the results?

We have included additional protocol steps in section 7.5 to further explain the software used, software navigation, recommended settings and the type of correction applied.

* P7 authors note the importance of internal standards - Why are there no internal standards for the lipid portion of the run?

We agree that the protocol would be improved with the addition of a lipid internal standard and have discussed this further in the discussion section and provided a recommendation for a suitable lipid class and supporting literature:

"We included four standards in the extraction solution used to prepare grain and a standard in the final sample added prior to injection. Care was taken to ensure that standards were amenable to each ionization mode and covered a range of retention times however we acknowledge that this array of standards could be improved with the inclusion of a labeled lipid standard. It has been shown that wheat grain contains hundreds of triacylglycerols (TAGs), any of which would be a suitable addition to this protocol.

Reviewer #2:

The authors described a nice workflow for the metabolomic analysis of grain samples containing an analytical approach to analyze both hydrophilic and hydrophobic compounds in one run using a quaternary pump.

To further complete the paper, it is recommended to elaborate further on both the need of replicate analysis, why only biological replicates were chosen and no technical replicates were used, why a number of three biological replicates was chosen. Is this number of 3 sufficient to highlight all significant changes between the different samples.

The number of biological replicates is not likely to be adequate to observe significant changes in intensity for all metabolites however it is adequate for >500 metabolites where significant changes were observed. This is preliminary work from which power analysis can be performed to allow a more accurate estimation of the number of biological replicates needed.

Pooled samples were used as technical replicates and run for the first 8 injections and then every 5th injection thereafter. There were 17 QC samples in total.

We have amended section 6.8 to:

Set up the instrument sequence table so that solvent and preparative (extraction) blanks are analyzed first; followed by pooled QC samples (6-10) for system conditioning; then the randomized sample list with QC samples run at regular intervals, e.g. every fifth injection, as technical replicates. Run two QC samples at the end of the sequence.

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