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Dual column chromatography combined with high-resolution mass spectrometry improves coverage of non-targeted analysis of plant root exudates

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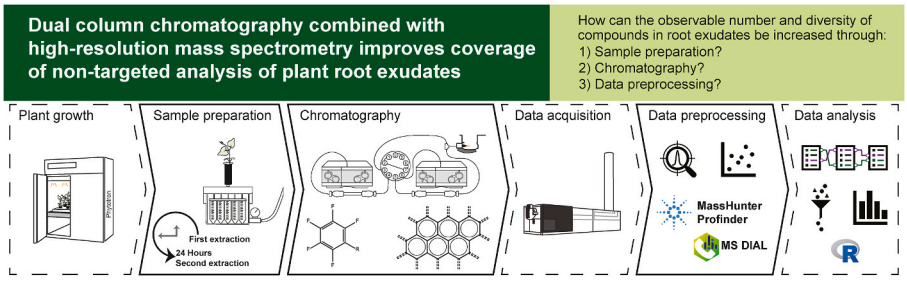
HIGHLIGHTS

- Pre-extraction removal of nutrient solution from rhizosphere samples enhances signal strength and analytical method stability.
- Ultrafiltration of samples leads to the loss of fatty acids and phenolics.
- The developed dual column chromatographic method improves coverage of small polar metabolites.
- MS DIAL proves a viable alternative to MassHunter Profinder for non-targeted analysis workflow.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Within the plant kingdom, there is an exceptional amount of chemical diversity that has yet to be annotated. It is for this reason that non-targeted analysis is of interest for those working in novel natural products. To increase the number and diversity of compounds observable in root exudate extracts, several workflows which differ at three key stages were compared: 1) sample extraction, 2) chromatography, and 3) data preprocessing.

Results: Plants were grown in Hoagland's solution for two weeks, and exudates were initially extracted with water, followed by a 24-h regeneration period with subsequent extraction using methanol. Utilizing the second extraction showed improved results with less ion suppression and reduced retention time shifting compared to the first extraction. A single column method, utilizing a pentafluorophenyl column, paired with high-resolution mass spectrometry ionized and correctly identified 34 mock root exudate compounds, while the dual column method, incorporating a pentafluorophenyl column and a porous graphitic carbon column, retained and identified 43 compounds. In a pooled quality control sample of exudate extracts, the single column method detected 1,444 compounds. While the dual method detected fewer compounds overall (1,050), it revealed a larger number

Abbreviations: (MRE), Mock root exudate; (NTA), non-targeted analysis; (ppmR), mass accuracy in parts per million of reported values; (ppmA), mass accuracy in parts per million of averaged values; (LC), liquid chromatography; (HRMS), high-resolution mass spectrometry; (MS/MS), tandem mass spectrometry.

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of small polar compounds. Three preprocessing methods (targeted, proprietary, and open source) successfully identified 43, 31, and 38 mock root exudate compounds to confidence level 1, respectively.
Significance: Enhancing signal strength and analytical method stability involves removing the high ionic strength nutrient solution before sampling root exudate extracts. Despite signal intensity loss, a dual column method enhances compound coverage, particularly for small polar metabolites. Open-source software proves a viable alternative for non-targeted analysis, even surpassing proprietary software in peak picking.

1. Introduction

The plant kingdom's metabolic profile is highly diverse. There are >374,000 described, accepted, and known plant species [1] and estimates of total plant species are as high as ~422,000 [2]. The chemical space of plants is equally diverse, with estimates of over 1 million metabolites produced by the kingdom. However, with only ~63,700 metabolite entries in the KNApSAcK family as of 2024 (http://www.knapsackfamily.com/KNApSAcK/), this results in fewer than 10 % of plant metabolites being publicly identified. It is for these reasons researchers seek out a method that can both elucidate and quantify as many natural products as possible. Natural products are compounds derived from any living organism and are often of interest as consumer products. These compounds have potential application for many industries ranging from medicinal and pharmaceutical to agricultural and food.

Within the agricultural industry, there is a drive away from the use of synthetic herbicidal chemicals towards the use of more natural products. Some plants, in their competition for the same resources, release compounds which negatively impact other plants into the rhizosphere [3], a zone of intense physical and chemical interaction between plants, microorganisms, and the soil. The prospect of using these compounds as natural and safe herbicidal alternatives makes their assessment highly interesting [4]. However, the rhizosphere is difficult to sample and chemically analyze.

Within the rhizosphere, not only do plant exudates from several interacting species need consideration, but so do interactions with and compounds released by microorganisms [5]. Additionally, the composition and concentration of root exudate compounds in rhizosphere soil is typically changing in a dynamic manner because of degradation [6], adsorption [7], and leaching processes. For these reasons, it becomes pertinent to simplify and normalize the rhizospheric soil solution matrix and utilize a soil substrate with low binding properties which do not leach contaminants to allow for a more controlled experiment with limited interfering factors when assessing root exudates in a non-targeted way.

The need to measure many unknown compounds of diverse chemical space has caused non-targeted liquid chromatography (LC) high-resolution mass spectrometry (HRMS) to become a popular method within metabolomics [8], especially when equipped with a collision cell and second mass analyzer (MS/MS) and able to generate fragmentation patterns [9]. LC-HRMS allows for the measurement and fragment annotation of thousands of ions in a single sample. While HRMS is limited in structural elucidation when compared to the structural resolution of nuclear magnetic resonance or cryo-electron microscopy [10], the use of known standards and recent advances in computational power and machine learning [11] have improved our abilities to annotate molecules based upon the fragmentation patterns in LC-HRMS/MS data.

However, the term non-targeted analysis (NTA) is a misnomer. With the complex chemical diversity that exists within the plant kingdom, it is too time and cost prohibitive to develop a method which is equally as complex. Even then, existing analytical methods available struggle to detect all possible compounds [12] to be considered completely non-targeted. In fact, the Benchmarking and Publications for Non-Targeted Analysis working group asserts that the specific definition of NTA is still debated within the community and that NTA is limited by the chemical space of the analysis and thus defines NTA as focused on chemicals that are either unknown to databases and/or *a priori*. From the beginning of an experiment, decisions and concessions must be made which are favorable to certain types of compounds [13].

Upon compound extraction, there are even more considerations to take which may cause bias in the final data set. The use of different solvents is impactful on the profile of metabolites recovered from samples during extraction (e.g., polar solvents are more likely to recover polar metabolites during extraction) [14]. Filtration or desalting of samples may be required to prevent mechanical or ion suppression issues with LC-MS equipment [15], but filters may lead to overall sample loss or dissimilar binding of non-polar metabolites to filter membrane and/or to filtered particulate when performing ultrafiltration [16,17]. Pre-concentration of samples may increase the signal of less abundant compounds or ones difficult to ionize, but it may also lead to the degradation of less stable ones (e.g., the application of heat during drying may cause thermolysis) [18], the loss of volatile metabolites [19], and, if samples are fully dried, may result in incomplete reconstitution [20]. Stationary and mobile phases of chromatographic separation and ionization mode are critical in determining which compounds are favored during the data acquisition [13,21].

More bias can arise during data acquisition. During chromatographic separation, there is bias from different column chemistries towards different types of compounds, frequently based upon compound polarity [21–23]. State-of-the-art single column methods attempt to overcome this by using stationary phases that utilize multiple types of interactions which can increase their ability to retain more compounds, but this is still quite limited in its capabilities [24]. There has been development of two major two-dimensional liquid chromatography (2D-LC) methods, comprehensive 2D-LC [25] or heart-cutting [26], which also offer some promise in increasing compound coverage and decreasing bias by having multiple column chemistries complimenting each other. However, these have mostly been developed to improve chromatographic separation, especially for isomers. Within the source of the mass spectrometer, different molecules can generate different signals (or lack thereof) of varying intensity based upon the ionization mode, the polarity of the atoms within compounds being measured [27], and different additives within the matrix or mobile phase which may result in adduct formation [28]. Based upon the resulting ionization efficiency, there is bias towards easily ionized and abundant compounds.

There is also data loss when converting data file types (e.g, conversion to .CEF files loses MS2 data information and conversion to .mgf files results in the loss of MS1 isotope and peak shape information) and bias in data manipulation and analysis. Non-targeted mass spectrometry data sets are large and profile data makes file sizes unmanageable for single processor computers. Preprocessing large data files with thousands of signals to focus on a manageable file size and subset of features can have biases as well (e.g., when centroiding [29], converting data [30], setting peak height, blank, and S/N thresholds filters and statistics performed …) [13]. To deal with these issues, there are several working groups which call for the standardization [31–33] of NTA data processing to push for good practices that can minimize these biases and which advocate for the use of proper reporting tools [34,35] to be transparent about inevitable and unavoidable bias.

It is important to be critical of every decision made during the development of an NTA protocol, as this bias will feed into downstream follow up targeted analysis. For this reason, we used a mock root exudate (MRE), a set of diverse physical-chemical standards of known

compounds present in the rhizosphere, to develop and assess methods and ensure awareness of which compound classes are weak within the analytical method. The overarching goal of this study was to cover a high number of diverse compounds when performing non-targeted metabolomics. Two non-targeted data analysis pipelines, one proprietary to the equipment used to acquire the data and another open source, were compared to see which offers results that have the highest mass accuracy and filters out the fewest MRE model compounds to prevent issues with false negatives. These vary between software, as reported mass for a single compound from several aligned samples can be calculated in different ways, e.g., as mean or median values, and because of the different peak picking algorithms. This was assessed first so that all other comparisons could be performed with the optimal data analysis pipeline. The second aspect was to compare two different extractions of a dual extraction method to assess which has better compound coverage. The first extraction contains nutrient solution and is thus closer to the undisturbed biological condition. A second extraction of the same plant's re-exudation is more standardized from the removal of the potentially variable nutrient solution. Lastly, a single reversed phase LC method was compared to a dual column method that was neither a comprehensive 2D-LC nor a heart-cutting method. The purpose of the dual column method is to allow for the retention and robust separation of both small nonpolar and semi-polar metabolites in a single LC-HRMS/MS run in a way that previous single column and 2D-LC methods have not.

2. Materials and methods

2.1. Plant growth conditions and experimental setup

Three plant species were grown for their root exudates, buckwheat (*Fagopyrum esculentum* Moench, variety: Lileja), black oat (*Avena strigosa* Schreb.), and redroot pigweed (*Amaranthus retroflexus* L.), using the protocol published by Ref. [36]. Seeds were sown on 250–400 μm glass beads (Guyson SA) in 60 mL solid phase extraction (SPE) tubes (Bond Elut, Agilent Technologies) covered in black tape to reduce algae growth. Plants were grown in a Phytotron (Aralab) growth chamber with a 16:8-h light/dark photoperiod at 24/18 °C and 70 % relative humidity. Compartments were watered daily with 5 mL of 50 % Hoagland's solution (Sigma-Aldrich, Hoagland's No. 2 Basal Salt Mixture) with pH adjusted to 5.8. Hoagland's solution is comprised of salts and nutrients seen in Table S1.

Five different plant experiments and their resulting data sets were utilized in this paper (Fig. 1) for optimizing sample preparation, the chromatographic separation methods, and data processing. For all experiments, blank samples came from compartments which had no plants, but which received watering with Hoagland's solution. Additionally, all experiments had a pooled QC. This was generated by taking 50 μl from each sample vial generated by an experiment, excluding blanks, and mixing them together. Samples from different experiments were not mixed with one another (i.e., each experiment had its own QC sample). If the dual extraction protocol outline below was performed, there were two QC samples for that experiment for each extraction. The details of each experimental setup can be found in Table S2.

2.2. Sample collection and exudate extraction

Several different sample preparation workflows were followed based upon the experiment and purpose. An overview of the different sample preparation steps and their related experiments can be seen in Fig. S1.

2.2.1. Single extraction

Exudates from buckwheat and pigweed from experiment 0 and experiment 3 were pulled from SPE cartridges using a Macherey-Nagel manifold paired with a vacuum pump (V-300, Buchi) controlled by a Buchi I-300 Pro Interface set at 780 mbar. 30 mL of extraction solvent was distributed over the glass beads over a period of 30 s. Extraction solvent consisted of 5 % Nanopure water (Barnstead, Thermo Fisher Scientific), 0.05 % formic acid (FA) (VWR, HiPerSolv Chromanorm for LC-MS), and 0.5 μmol/L 3,5-di-tert-butyl-4-hydroxybenzoic acid (Sigma-Aldrich) in methanol (Merck Uvasol). The vacuum was maintained for another 30 s to ensure all solution was pulled into 50 mL conical centrifuge tubes (Corning) over a span of 1 min total.

10 mL of sample was dried in in a vacuum concentrator (Genevac EZ-2 Plus) set at 35 °C using HPLC mode for 1 h and then aqueous mode until sample reached approximately 200 μl. Extracts were then reconstituted to 10 % MeOH (Honeywell LC/MS Ultra CHROMASOLV) and 0.1 % FA (Honeywell Fluka) until they reached a 30-fold reduction of the original volume (333 μl).

2.2.2. Ultrafiltration

An aliquot of the pooled QC sample from experiment 3 (Table S2) was used to reconstitute a 10 μmol/L MRE standard mixture (described below). This was then aliquoted into two fractions. One fraction was further processed with a 30 kD centrifugal filter (Amicon, Merck) to remove particulates. The exudate samples were then transferred to amber 1.5 mL HPLC glass vials (Altmann Analytik) and stored at −80 °C until measurement. Storage temperature and time was kept as low as possible to reduce issues with the formation of artifacts over time [37].

2.2.3. Dual extraction

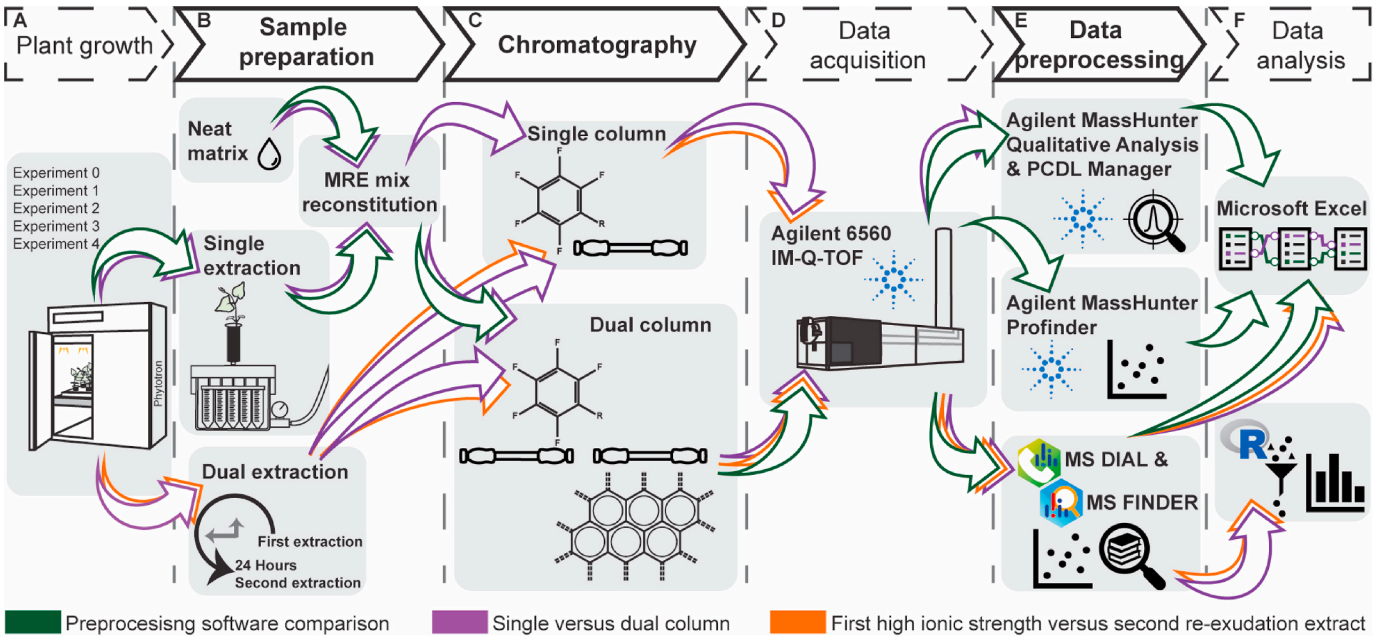
Exudates from experiments 1, 2, & 4 were used to compare the dual extraction method. As with the single extracts, a vacuum was applied to the SPE tube the plants were grown in. For the first extraction of exudates in Hoagland's, 30 mL of Nanopure water was distributed over the glass beads over a period of 30 s. The vacuum was maintained for another 30 s into 50 mL conical centrifuge tubes (Corning) over a span of 1 min total. Plants were then given 15 mL of Nanopure H_2O after the first extraction was completed and allowed 24 h to regenerate and re-release exudates in the Phytotron. Then, the same extraction protocol was performed using 30 mL extraction solvent (5 % H2O and 0.05 % FA in methanol with 0.5 μmol/L 3,5-di-tert-butyl-4-hydroxybenzoic acid).

Ten mL of sample was dried in a Genevac EZ-2 Plus until ~200 μl remained and then reconstituted to a final 30-fold concentration (333 μl) to 10 % MeOH 0.1 % FA. While original extracts were inherently filtered of particulate by the glass beads, the aqueous first extract samples formed particulate during sample pre-concentration and were ultracentrifuged (Sorvall Discovery M150 SE) for 15 min at 125000 x g and 4 °C to remove precipitating salts. Precipitate and particulate were not an issue with the second MeOH extracts. The exudate samples were then transferred to amber vials and stored at −80 °C until measurement. The QC for the second extraction of experiment 4 was spiked with MRE compounds (outlined below) to 10 μmol/L.

2.2.4. Mock root exudate (MRE)

A MRE mixture was made up of a set of 58 analytical standards of compounds known to be exuded by the roots of agricultural plants. The exact standard compounds, the plants they are known to exude from, their sources, and purity are found in Table S3. All standards were made to 250 μmol/L in 10 % MeOH and 0.1 % FA and diluted to 5, 10, or 20 μmol/L (I.e., there were 3 different dilutions of the mixed standards, but all standards within each mix were at the same concentration). The three standard mixes were then individually dried in a Genevac EZ-2 Plus for 1 h in HPLC mode with a max temperature setting of 35 °C until dry. The standard mixes were then stored in the −80 °C until reconstitution and analysis.

These standard mixtures were reconstituted in one of two matrices: 1) a solvent standard of 10 % MeOH with 0.1 % FA or 2) a sample matrix which was comprised of pooled root exudate from experiment 0 and 3. This is to showcase how the analytical and data analysis methods perform in both a best-case (solvent standard) and worst-case (high ionic strength sample matrix) scenario. These samples were not used to assess



Experiment	**Plant species**	**Extraction type**	**Amicon filtered?**	**Used to reconstitute MRE?**	**Final sample description**	**Chromatography method**	**Comparison(s) made**
Solvent standard	none	none	no	yes	MRE at 20, 10, and 5 μmol/L in a solvent standard (n = 9).	single and dual	• Software preprocessing • Single versus dual column
0	buckwheat & black oat	single	no	yes	MRE at 20, 10, and 5 μmol/L in a sample matrix. Pooled with experiment 3. n = 9, except 20 and 5 μmol/L for the single column method were n=3.	single and dual	• Software preprocessing • Single versus dual column
1	buckwheat & pigweed	dual	no	no	A set of samples from different experimental conditions and varying salt concentrations (n=3-5).	dual	• Fist versus second extract
2	black oat & pigweed	dual	no	no	A set of samples from different experimental conditions and varying salt concentrations (n=3-5).	dual	• Fist versus second extract
3	buckwheat & pigweed	single	yes	yes	MRE at 20, 10, and 5 μmol/L in a sample matrix. Pooled with experiment 0 for software and column comparisons. n = 9, except 20 and 5 μmol/L for the single column method were n=3.	single and dual	• Software preprocessing • Amicon filtration • Single versus dual column
4	black oat & pigweed	dual	no	no	A pooled QC sample for each extraction type, including pigweed metabolites. Second extract was spiked with 10 μmol/L MRE (n=1, no statistics performed).	single and dual	• Fist versus second extract • Single versus dual column (only second extract)

Fig. 1. Workflow
The entire workflow was comprised of six stages: A) plant growth, B) sample preparation, C) chromatographic separation, D) data acquisition on a QTOFMS instrument, E) data preprocessing, and F) data analysis. Buckwheat, black oat, and pigweed plants were grown in five different experiments which were used for different comparisons within the workflow. The stages of the workflow where different comparisons were made (sample preparation, chromatography, and data prepressing) are highlighted in bold in the guide above. Additionally, the specific workflow for each comparison is shown in different color arrows. Each extraction of a dual extraction protocol was compared to one another and highlighted by orange arrows. A single versus dual column chromatographic comparison can be followed with the purple arrows. Last, three different data preprocessing software and strategies (suspect screening in MassHunter qualitative analysis with a PCDL manager generated database, proprietary non-targeted analysis in MassHunter Profinder, and open-source MS DIAL non-targeted analysis assisted with a MS FINDER generated database) were compared with the workflow shown in green arrows. Within the table, the five different plant experiments performed, and their root exudate extractions used for different comparisons are described. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the extraction method or extraction efficiency as the compounds were spiked in and not necessarily extracted from sample.

Nine technical replicates were obtained by injecting the 10 μmol/L MRE samples in sets of three on three different days. On the days of analysis, 10 μmol/L MRE sample aliquots were reconstituted in solvent standard or sample matrix, as mentioned above, and the same sample was injected three times. All statistics performed were based on these 10 μmol/L samples (n = 9). Data for both the 5 μmol/L and 20 μmol/L samples were collected with an equal number of replicates in the same way on the same days as the 10 μmol/L samples, except for the MRE samples in sample matrix using the single column method. For these samples, injections occurred only once per day (yielding a total of three replicates spread over three days). Statistics were not performed on these samples with only three replicates.

2.3. LC-HRMS/MS

2.3.1. Instrument design

Agilent MassHunter acquisition software (version 10.1) controlled the Agilent Technologies Inc. (Santa Clara, CA) liquid chromatography quadrupole time-of-flight (LC-QTOF-MS) system comprised of a cooled autosampler unit (1290 Infinity II), two binary pumps (1290 Infinity II), a nano pump (1260 Infinity) for reference mass solution (HP-0921, Purine, $TFANH_4$, Agilent Technologies Inc), a temperature-controlled column compartment (1290 Infinity II), and a 6560 ion mobility QTOFMS with a Dual AJS electrospray ionization (ESI) interface. The whole system was manufactured by Agilent Technologies Inc. (Santa Clara, CA).

For all methods, samples were thawed (and reconstituted for MRE samples), vortexed and then kept in the autosampler at 4 °C. Injection volume was 5 μL with a constant flow rate of 350 μL min^{-1} and a column temperature of 50 °C.

2.3.2. Single column method

The single column chromatography method stationary phase was a Discovery HSF5 (2.1 × 150 mm, 3 μm particle size, Sigma-Aldrich) pentafluorophenyl (PFP) column paired with a Discovery HS F5 Supelguard Cartridge (Sigma-Aldrich) guard column. Mobile phase A was ultrapure H_2O with 0.1 % FA and mobile phase B was MeOH with 0.1 % FA (% v/v). All mobile phase ultrapure H_2O was sourced from a MilliQ system with an LC-Pak attachment (Merck). MeOH was Honeywell LC/MS Ultra CHROMASOLV and Honeywell Fluka FA. The gradient is

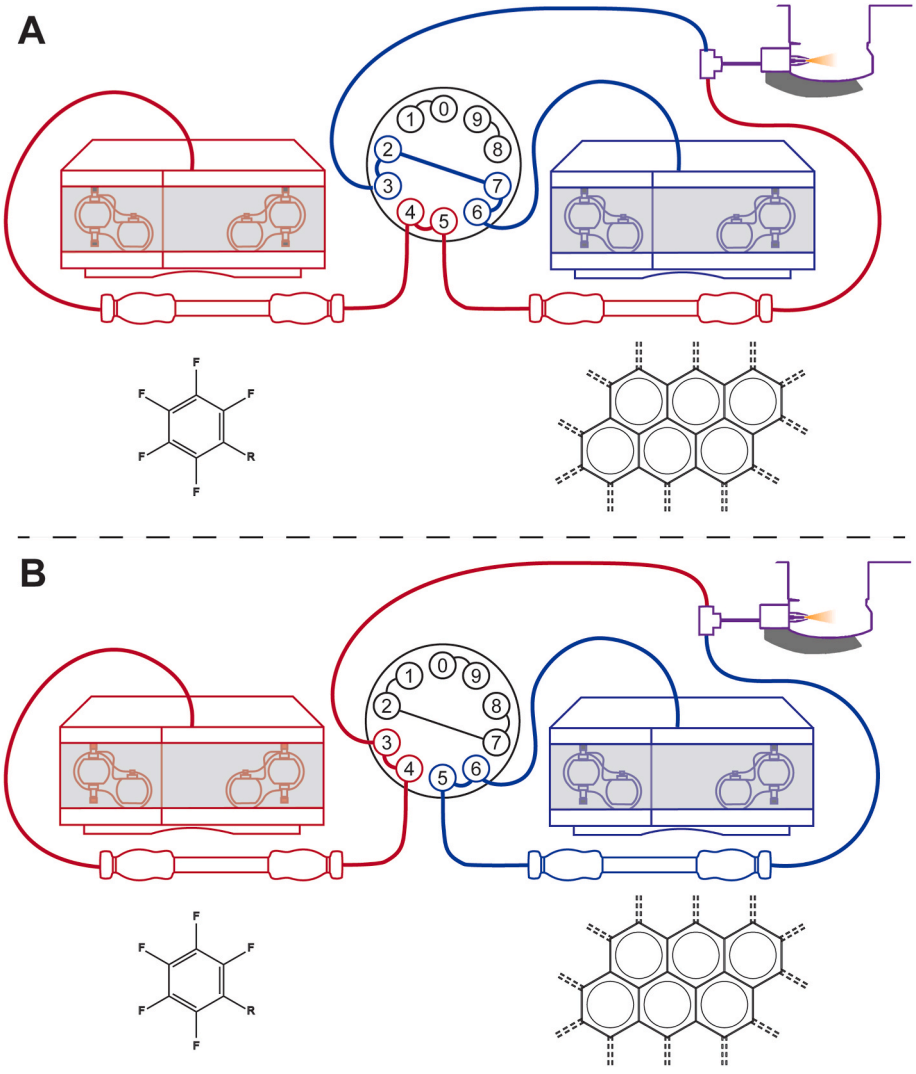


Fig. 2. Dual column setup
The dual column setup was separated by two distinct stages. In stage A of the dual column setup, pump 1 is transferring the injected sample with aqueous mobile phase through both pentafluorophenyl (PFP) and porous graphic carbon (PGC) columns. Metabolites which are not retained by the first column go sequentially to the second column. After metabolites are retained on their respective columns, a valve switches to stage B, where each column has its own pump and LC gradient for analyte separation. The eluents of the two columns are merged by a T-joint before entering the ESI sprayer.

shown in Table S4A with total analysis time of 15.5 min per sample.

2.3.3. Dual column method

A dual column method was adapted [38] and utilized both the Discovery HSF5 PFP column with guard column and a Hypercarb porous graphitic carbon (PGC) column (2.1 × 150 mm, 5 μm particle size, Thermo Scientific) paired with a Thermo Scientific Hypercarb Drop-in Guard Cartridge as the stationary phase. In stage 1 (Fig. 2A), the retention stage, the two columns were connected in tandem with pump 1 running mobile phase A through both columns. After 1.8 min, the column selection valve was switched automatically through the Agilent MassHunter acquisition software program to begin stage 2 (Fig. 2B), the elution stage, and thereafter each column had its own pump and gradient. Pump 1 had the same stationary and mobile phases as the single column method, but the gradient was different in holding an aqueous phase longer at the beginning of the method to account for loading of both columns and at the end to account for equilibration and charge regeneration of the PGC column. Pump 2 mobile phase A remained the same, but mobile phase B was acetonitrile (ACN) (Honeywell LC/MS Ultra CHROMASOLV) with 0.01 % FA (% v/v). The gradient for this method is shown in Tables S4B and C.

2.3.4. Mass spectrometric method

Mass spectrometric data acquisition was the same for both dual column and single column methods using the Agilent 6560 IM-QTOF. Reference mass solution was pumped to the secondary sprayer of the Dual AJS ESI. Data was acquired using DDA (data dependent acquisition) with negative and positive ionization mode performed as separate runs with a scan range of 50–1700 *m/z*. The top five most abundant ions above 2000 counts (with an active exclusion of 3 spectra and 0.3 min enabled) were fragmented. Collision energy information and the HR-MS/MS acquisition method report can be seen in Table S4D. During acquisition, 3,5-di-tert-butyl-4-hydroxybenzoic acid was used as a marker to ensure samples were consistently injecting, retaining, and ionizing.

2.4. Data preprocessing

Before data analysis, all data was centroided and recalibrated with Agilent's reprocessing software (MassHunter Workstation version 12.0).

Data was then preprocessed using three different software (MassHunter Qual version 10.0, MassHunter Profinder version 10.0, and MS DIAL version 4.9). MassHunter Qual and Profinder come from the same line of proprietary software from Agilent, but MassHunter Qual is often utilized for qualitative browsing of the data whereas Profinder is used for non-targeted analysis. However, Qual can be used for targeted feature extraction. MS DIAL is an open-source software capable of NTA and screening.

2.4.1. Targeted/suspect screening

An in-house database was generated with Agilent PCDL manager (version B.08.00) using the molecular formulas of all compounds in the MRE mix. This was used to search for compounds of interest using Agilent MassHunter Qual Browser (version 10.0) find by formula method. From these results, *m/z*, the fragmentation patterns, retention times, and isotope patterns were considered when confirming peak assignment. With this, the in-house database was updated with the retention times of compounds that were detectable and fragmentation patterns if available. No peak height or width filters nor void volume filters were applied for the targeted analysis. Peaks eluting before the dual column void volume of 2.50 min were observed to ensure their presence within the void volume but were not included in statistical analysis. Data was analyzed twice. First it was analyzed so that MS1 values excluded spectra above 0 % of TOF spectra saturation as this is more like how MSDIAL analyzes the data. Then it was analyzed again with a 60 % cutoff to see if this improved the mass accuracy of the final reported *m/z* across aligned samples. More of the method parameters can be found in Table S5.

2.4.2. Proprietary non-targeted analysis

Agilent's proprietary MassHunter Profinder (version 10.0) software was used as a baseline for analysis. Profinder's batch recursive feature extraction for small molecules performed peak picking. Spectra above 60 % of TOF spectra saturation were excluded. Only peaks with a minimum height of 1E3 (counts) and a minimum peak width of 6 data points were picked. Peaks eluting before the dual column void volume of 2.50 min were picked, but they were not considered during further analysis. The Profinder software aggregates both isotopologues and adducts into a single numeric value (ion volume). Peaks were matched across samples with an MS1 tolerance of 15 mDa and a retention time tolerance of 0.2 min and minimum of 60 % group presence filtration was applied. More Profinder preprocessing details can be found in Table S6.

2.4.3. Open-source non-targeted analysis

MS DIAL (version 4.9 [39]) was the open-source software utilized for peak picking and alignment. It was selected as the open-source software as it was shown within the 2022 Critical Assessment of Small Molecule Identification (CASMI) challenge to provide excellent results. Again, only peaks with a minimum height of 1E3 and a minimum peak width of 6 data points were picked. For MRE samples, compounds which eluted before the void volume (1.60 min for the single column method and 2.50 min for the dual column) were picked but not considered during analysis. For analysis of compounds exudated by roots in experimental samples and not of MRE standards, a void volume filter was applied in MS DIAL. Isotopologues were aggregated and adducts annotated. A secondary in-house database was generated using MS FINDER (version 3.6 [40]), and this was applied to the MS DIAL data. Peaks were then aligned across samples with an MS1 tolerance of 15 mDa and a retention time tolerance of 0.2 min. After alignment, gap filling of missing values was performed. Last, a further blank (5-fold sample average/blank average) and minimum of 60 % group presence filtration was applied. More detailed MS DIAL pre-processing parameters can be found in Table S7.

2.5. Data analysis

2.5.1. Open source versus Agilent MassHunter

Comparison of the different preprocessing software was done using the same dual column data. This analysis was done both for MRE solvent standard samples and MRE samples which were reconstituted in root exudate extracts from experiment 0 and 3 (Fig. 1: green pathway). After preprocessing, data tables from positive and negative ionization mode acquisition were exported from the different softwares and imported into Microsoft Excel. Excel was used to match MRE compounds, if present, that had been spiked into the samples at different concentrations (20, 10, and 5 μmol/L) based upon theoretical *m/z* or neutral mass and experimental RT across the different acquisition modes and software. Results were validated through annotations from database matching performed during preprocessing and positive correlation between peak height and known MRE concentration. Number of MRE compounds picked by each method, average compound mass or *m/z*, individual compounds reported mass error (ppmR) and average mass error (ppmA), the compound standard deviation of the mass error in ppm, and each compounds fold change between sample concentrations was then calculated. Statistics were performed only on 10 μmol/L samples. An outline of this screening approach can be found in Fig. S2.

$$ppmR = \frac{|(exact\ mass\ (Da) - \boldsymbol{reported\ mass}\ (Da))|}{exact\ mass\ (Da)} \times 1E6$$

$$ppmA = \frac{|(exact\ mass\ (Da) - \boldsymbol{\bar{x}\ acurate\ mass}\ (Da))|}{exact\ mass\ (Da)} \times 1E6$$

$$S\ mass\ error\ (ppm) = \frac{S\ (|(exact\ mass\ (Da) - \bar{x}\ accurate\ mass\ (Da))|)}{exact\ mass\ (Da)} \times 1E6$$

2.5.2. Sample preparation

2.5.2.1. Ultrafiltration versus non-filtered. Data from the Amicon filtration test of experiment samples was analyzed using the MS DIAL preprocessing method with the MS FINDER database applied. Data was analyzed in one batch and the alignment file was exported to Excel. Average fold change differences in peak heights for MRE standards were calculated and compared in Excel.

2.5.2.2. First extraction versus second extraction. For the dual extraction method, the first aqueous sample extraction was compared to the second methanolic sample extraction on both the single column method and the dual column method using data from experiments 1, 2, and 4 (Fig. 1: **orange pathway**). Data was preprocessed in MS DIAL with a method respective MS FINDER in-house database and the screening approach outlined above was performed. For experiments 1 and 2, the retention time RSD across samples for extracted MRE compounds was also calculated in Excel.

Additionally, alignment files from QC samples for experiments 1, 2, and 4 were exported from MS DIAL and further processed in R (Fig. S3). The number of compounds observed in each extraction was counted and the chemical space of the samples was compared by plotting RT and *m/z* in a feature plot. MRE compounds and their LogP values were annotated in feature plots.

2.5.3. Chromatography

Single versus dual column data was compared using both MRE samples which were reconstituted in a solvent standard and which were reconstituted in extractions from experiment 0 and 3 (Fig. 1: **purple pathway**). Data was preprocessed with the aforementioned MassHunter Qual targeted method with the PCDL database and the MS DIAL non-targeted method utilizing the MS FINDER in-house MRE databases, and the screening approach outlined above was performed.

Data from a QC sample from the second extraction of experiment 4 with MRE compounds spiked in at 10 μmol/L was run on both single and dual column method in negative and positive ionization mode. This data was preprocessed in MS DIAL and then in R as described in the previous section.

3. Results & discussion

3.1. Open source versus Agilent MassHunter profinder

Data conversion is not a prerequisite of proprietary data analysis, which is a benefit since different conversion software has been shown to generate different results [41] and to cause a variety of issues with the data [30]. Additionally, by using the raw data directly, there is full trackability of the reported results back to the raw data. However, these software, from acquisition to data analysis, have certain "black box" calculations tailored to their data structure (e.g., the Thermo software automatically processes Orbitrap data using Fourier transformation without the ability to change processing parameters).

In the solvent standard, the MassHunter Qual targeted analysis was able to find 43 of the 58 compounds in the MRE mixture (26 % false negative rate). I.e., 15 of the standards either did not ionize well or eluted before the void volume and could not be detected by the instrument. Less of these compounds were expected to be reported with the non-targeted analysis as the preprocessing strategies had a 1E3 peak height cutoff to filter out noise. Of the 43 detectable compounds, Profinder picked 31 in non-targeted mode (total spike compound false negative rate of 47 % and instrument detectable compound false negative rate of 28 %). Of the 31 compounds which were shared across all preprocessing methods (Fig. 4A), the average ppmR value aligned across samples was 1.09 ppm for Profinder. The average ppmA calculated from of these 31 compounds at a concentration of 10 μmol/L (n = 9) was within the same range for all MassHunter preprocessing methods at 0.97 ± 0.75 for MassHunter Qual targeted screening with no peak saturation filter, 0.94 ± 0.84 for targeted screening with 60 % peak saturation filter, 1.10 ± 0.85 ppm for Profinder. For Profinder, the similarity of the ppmR and ppmA values to one another and the targeted analysis values highlights the success of the algorithm to generate and report high accuracy mass values across many samples to the end user.

The distinction between ppmR and ppmA is that the first is the error of the "reported mass" in Daltons. This is the mass value generated by the software (a median neutral mass value for Profinder and a mean *m/z* value for MS DIAL) for a given feature after data preprocessing and alignment. This is what most users will utilize in their workflows. The ppmA first calculates the average of the accurate mass from aligned 10 μmol/L samples (n = 9) and then calculates the error of this average value. I.e., each spiked standard compound reported after sample alignment for an experiment has one overarching ppmR and one ppmA value generated by each of the NTA preprocessing strategies.

In a high salt sample matrix, the targeted MassHunter analysis detected 42 of the MRE compounds which had been spiked into matrix, showing that matrix interference had resulted in the suppression of one more compound (arachidic acid, which was also poorly ionized within the solvent standard samples) beyond instrument detectability when compared to solvent standard samples. This is in addition to the 15 standards which either did not ionize well or eluted before the void volume. The non-targeted Profinder method picked only 24 compounds in the high salt matrix (Fig. 4B). The ppmR of 23 shared compounds was 1.44 ppm for Profinder, with the ppmA being 1.37 ± 0.73 for MassHunter Qual with no saturation filter, 1.15 ± 0.78 for MassHunter Qual with a 60 % saturation filter, and 1.45 ± 0.77 for Profinder. The fact that fewer compounds could be detected in the sample matrix than in solvent standard when spiked into each at equal concentrations highlights the issue of matrix effects. This is because high salt matrix, such as Hoagland's sample matrix, lowers the intensities of the protonated or deprotonated ion species due to the presence of nonvolatile solutes [42,43]. Indeed, Fig. S4 highlights catechin, epicatechin, and glutamic acid in solvent standard matrix and in sample matrix at a concentration of 5–20 μmol/L, showing that the signal intensity lowers due to the matrix effects from the sample. This lower signal intensity, in turn, negatively impacts mass accuracy [44,45]. This is important to keep in mind when benchmarking methods using standards as the solvent standard samples will be slightly more accurate but not as representative of actual sample behavior.

Alternatively, open-source software is becoming more popular. This software is free of charge and allows users to become familiar with one data analysis pipeline instead of needing to learn multiple interfaces across different vendors' instruments and data sets. Additionally, many users prefer less "black box" analysis and having access to the source code for auditing purposes.

For solvent standard samples, MS DIAL picked 38 compounds (Fig. 4A). There are 58 total MRE compounds within the sample and 43 instrument detectable compounds as highlighted above, resulting in a total spike compound false negative rate of 34 % and instrument detectable compound false negative rate of 15 %. For the aforementioned 31 compounds observed by all pre-processing methods, there was an average ppmR of 1.80 ppm and a ppmA (n = 9) of 1.27 ± 1.23 ppm for the MS DIAL dataset. Data was manually checked, but as only one possible result was returned for each spiked MRE compound for each software by the excel data analysis matching method (based on known retention time, *m/z*, fragmentation pattern, and/or peak intensity matching spiked concentration), there were no observed false positives. In sample matrix, MS DIAL method picked 31 of the 42 detectable peaks (Fig. 4B). The ppmR of the 23 shared compounds was 2.22 ppm and the

ppmA was 1.43 ± 1.98. While the ppmA of the MS DIAL software is similar to data from the MassHunter suite, the ppmR of the MS DIAL was the highest. For all ppmA values, the error was calculated only from 10 μmol/L samples. ppmR values came from samples where an analyte has passed certain thresholds set by the software. This indicates that MS DIAL, while picking more of the desired standards (7 compounds), it also picked more peaks in general, including those with higher mass error. This decreased the mass accuracy of the reported values that users work with. This indicates that there is a balance between the selectivity of the preprocessing algorithm, the number of features incorporated, and the accuracy of the data.

While MS DIAL reported *m/z* values that were the least accurate, the mass error was still well below 5 ppm. Nevertheless, MS DIAL does not remove the *m/z* value information for individual samples and the reported *m/z* value does not need to be the ones used. It may be interesting to see everything with low preprocessing filters and selectivity and then statistically filter out low quality data points at a later step. Then, the average *m/z* value from these filtered data points can be calculated instead. However, this process would require external scripting to implement.

Most importantly, for Profinder specifically, there is one major issue: MS2 data is not kept when performing batch recursive feature extraction, Profinders non-targeted feature picking algorithm. Thus, no MS2 database can be used when performing NTA analysis with Profinder. The fragmentation pattern cannot be referenced later for validation. If the goal is to identify compounds after NTA analysis, there is no straightforward way to easily feed Profinder data into an identification pipeline for up to version 10.

3.2. Sample preparation

3.2.1. Effect of ultrafiltration on compound recovery in single extracts

During the concentration process, samples with large amounts of Hoagland's solution in the matrix generated precipitate that needed to be removed due to increased system backpressure over several sample injections. Hence, impact of ultrafiltration on metabolites recovery was assessed by comparing filtered and unfiltered samples from the same extraction. For all compound classes, the unfiltered sample had a larger peak height (though not always significantly higher) when compared to samples that underwent ultrafiltration. For the ultrafiltration, there was no significant difference between amino acids and organic acids from the unfiltered sample. Fatty acids were ~1.7 fold lower in ultrafiltered samples. For flavonoids, the ultrafiltration samples were ~13-fold lower than unfiltered samples. However, flavonoids were heavily skewed by myricetin (~35-fold signal loss) and quercetin (~25-fold signal loss). Excluding those two, flavonoids showed a ~1.5-fold loss in signal when processed with an Amicon filter. This agrees with previous research, which has observed that lipoproteins and hydrophobic compounds are also removed along with the proteins during ultrafiltration [16,17]. The metabolites may be bound to either the membrane of the filter or to larger compounds (e.g., proteins) which are unable to pass through the filter. For this reason, ultrafiltration was not used for further experiments and ultracentrifugation was utilized when precipitate was an issue.

3.2.2. Expected matrix and metabolite composition of the dual extracts

The composition of the first aqueous extraction of exudates was compared to the methanolic extraction performed after a 24-h re-exudation period. While each extraction uses a different solvent based on the limitations of the method (e.g., the first extraction must be aqueous to prevent damage to the plant so that the second extract can occur 24 h later), the comparison made is more about assessing the matrix effect arising from the high concentration of Hoagland's salt in the first extract versus the comparatively lower amount of Hoagland's salt in the second extract. The first aqueous extract of the sample is considered closer to the biological condition of the plant as it contains the full 2 weeks of exudation, breakdown, transformed and intermediate compounds found within the substrate. I.e., it represents more accurately what one would observe in an undisturbed soil substrate community in chemical flux. Moreover, the plant has not risked additional stress that comes from washing off the soil solution and adding H_2O. This could cause nutrient and osmotic shock to the plant [46,47] which could, in turn, impact the plant's metabolome and exudation. Additionally, the first extract, using H_2O as the extraction solvent, would be limited in the number of nonpolar metabolites in favor of more polar metabolites. Methanol was not viable for the first extract due to toxicity to the plant, which would make the subsequent 24-h untenable.

Though this was not the focus of the comparisons made in this study and thus was not tested, the second methanolic extract performed after the 24-h re-exudation period should logically contain fewer intermediate or breakdown products or compounds coming from a microbial community which has theoretically been partially washed away by the first extraction. This is because the plant growth and sampling protocols were not performed under sterile conditions. Petri dishes with culture media swabbed with the different samples generated microbial colonies (data not shown). It should be noted that the blank sample did not result in microbial growth. It has been established that microbes which live in soil solution break down, consume, and transform compounds exudates by plants [5]. It is reasonable to extrapolate that a larger proportion of the compounds in this soil solution should come directly from root exudates in the second extract since a portion of the microbes have been removed by the first extract, thus making the second extract more favorable in this sense. The second extraction also allowed the plant to have a "reset" point to allow for a snapshot of the plant exudate profiles at two-week post-gemination life stage. This included a 24-h re-exudation period which was necessary as plant metabolism is affected by their circadian rhythm [48].

However, the major benefit to the second extract is the removal of Hoagland's solution from the sample matrix. Plants of different species, nutrient needs, size, and strength will uptake the Hoagland's nutrients (Table S1) the plants are supplied with at different rates [49]. Hoagland's solution not being taken up uniformly by plants results in a variation in matrix among samples. Not only does the second extract reduce the concentration of salts in the matrix, but it also results in a more uniform ionic strength across samples as it gives the plants less time to uptake limited nutrients disproportionally.

The use of 30 mL of methanol to extract ~15 mL of exudate sample solution (minus the volume taken up by the plants in 24 h) would result in an extraction of more nonpolar metabolites in comparison to the first extract. Indeed, previous studies have shown that fully aqueous extractions miss nonpolar compounds, such as phenolics [50], which are known to be important root exudates [51].

It should also be noted that, while there was a concern that the MeOH could lyse cells and release intracellular contents to contaminate the root exudate extractions and artificially inflate numbers, the extraction procedure was explicitly limited to 1 min to ameliorate this issue. Additionally, imaging was performed on roots post MeOH extraction to confirm there was no significant damage to the surface of root tissue (data not shown). This is in agreement with previous studies that 1 min of contact with diluted methanol does not have detectable effects on root cell integrity [52] which indicates that intracellular metabolite leakage was unlikely.

The ability of the dual extraction method to provide samples which could both cover the need for undisturbed root exudate experiments and those in which the Hoagland's solution was removed resulted in exclusively using the dual extraction method over the single extraction method for further experiments.

3.2.3. Impact of matrix ionic strength on ionization suppression

A series of buckwheat samples from experiment 1 and black oat samples from experiment 2 were extracted using the dual extraction method and samples from each were measured to assess the impact of

the different extraction matrices (the high ionic strength Hoagland's solution extracted with water versus the low ionic strength re-exudation solution extracted with methanol) on the dual column method.

Compounds were considered "weakly retained" if they eluted after the void volume (2.5 min for the dual column setup) but before 1 min after the gradient began switching from 5 % organic to 40 % organic (minute 8.0 on the dual column method (Fig. 3B)). This time point is also after the last standard elutes on the PGC column on the dual column method (isocitric acid at 6.2 min).

A buckwheat pooled QC from experiment 1 across all experimental conditions of the first aqueous extract samples contained 166 compounds (Fig. S5A) whereas the second extract had 412 (Fig. S5B) in negative ionization mode. For positive ionization mode, there were 76 compounds (Fig. S5C) and 377 observable compounds (Fig. S5D), respectively. In these samples, 2 MRE compounds which were extracted from the plant that could be ionized in the aqueous Hoagland's QC versus 17 in the second (methanolic) extract. In the weak retention region, below the 8-min mark, ~16 % of observable compounds were eluted (16 compounds in positive ionization and 17 in negative) for the first extract. For the second extract, ~42 % of compounds eluted in this same region (125 compounds in positive ionization and 202 in negative). The second extract revealed both more metabolites overall and within the weakly retained region for both known and unknown compounds.

For the QC sample from experiment 2 (black oat), 211 compounds (Fig. S5E) were observed in negative ionization mode for the first aqueous extract and 491 were in the second extract (Fig. S5F). In positive ionization mode, 137 (Fig. S5G) and 336 (Fig. S5H) compounds were observed, respectively. 1 MRE compound was successfully extracted, retained, ionized, and picked from the first Hoagland's QC whereas 17 MRE compounds were in the second extract. In the first extract, ~43 % of compounds (45 in negative and 40 in positive mode) eluted in the weak retention region. ~60 % of compounds (276 in negative and 224 in positive) eluted in this region for the second extract. The results confirm the conclusion from the previous experiment that the second extract observes more compounds overall and within the weakly retained region of the dual column method.

The same pattern was observed with the single column method when analyzing pooled QC samples from black oat and pigweed from experiment 4 in which pigweed metabolites were also sampled and included. 402 compounds were observed in negative ionization mode (Fig. S5I) for the first aqueous extract of Hoagland's solution and 776 were observed in the second methanolic extract (Fig. S5J). In positive ionization mode, 306 compounds were observed in the first extract (Fig. S5K) and 669 compounds were observed in the second (Fig. S5L). ~6 % of compounds (19 in positive ionization and 22 in negative) eluted in the weak retention region, below 5.5 min for the single column method, for the first extract and ~20 % retained (177 in positive ionization mode and 113 in negative) in this region for the second extract. This shows that the pattern observed displaying more compounds in the second extracts is not influenced by the analytical method (i.e., the single column approach using the PFP column versus the dual column using a PFP by PGC column approach).

The focus was to assess how the results of the first aqueous extracts compared to the second re-exudation extract. The first extraction contains two weeks of exudate accumulation, an excess amount of Hoagland's solution (Table S1), and uses water as an extraction solvent which should result in bias in favor of small polar metabolites. However, salt in the Hoagland's solution can create ion suppression of compounds [42, 53] for the polar metabolites eluting within the same time range as salts. Indeed, even though there should be more compounds accumulated over a two-week span when compared to 24 h, there is overall less signal for the first extract when compared to the second extract. This could be somewhat accounted for by the increase in non-polar compounds in the second extract due to the use of MeOH. However, both chromatographic methods observe a higher total number and larger percentage of compounds eluting in the weak retention (i.e., polar) region for the second extraction. I.e., there are more small polar metabolites in the second extract, which uses methanol, even though one would expect more of these compounds in the first extract. The first extract has both more time to accumulate metabolites and uses water as the extraction solvent which has a higher affinity for small polar metabolites. This is in spite of the fact that the second extract utilized MeOH instead of water and should theoretically have a lower affinity for extracting small polar metabolites. This indicates that Hoagland's solution is suppressing these polar metabolites and causing a loss of signal.

3.2.4. *Ruggedness of analytical method against matrix variation*

An issue with the direct extraction of Hoagland's sample is the variation in matrix (i.e., a variation in ionic strength) among samples. By imparting a partial charge on the column with mobile phase and then disrupting that charge, the PGC column can theoretically be influenced by the sample matrix if there is enough variation in ionic strength (see section 3.3.1). Ergo, this variation in matrix can practically result in issues both with the analytical method for data acquisition and with data preprocessing by impacting compound ionization and retention time shifting in a non-uniform way.

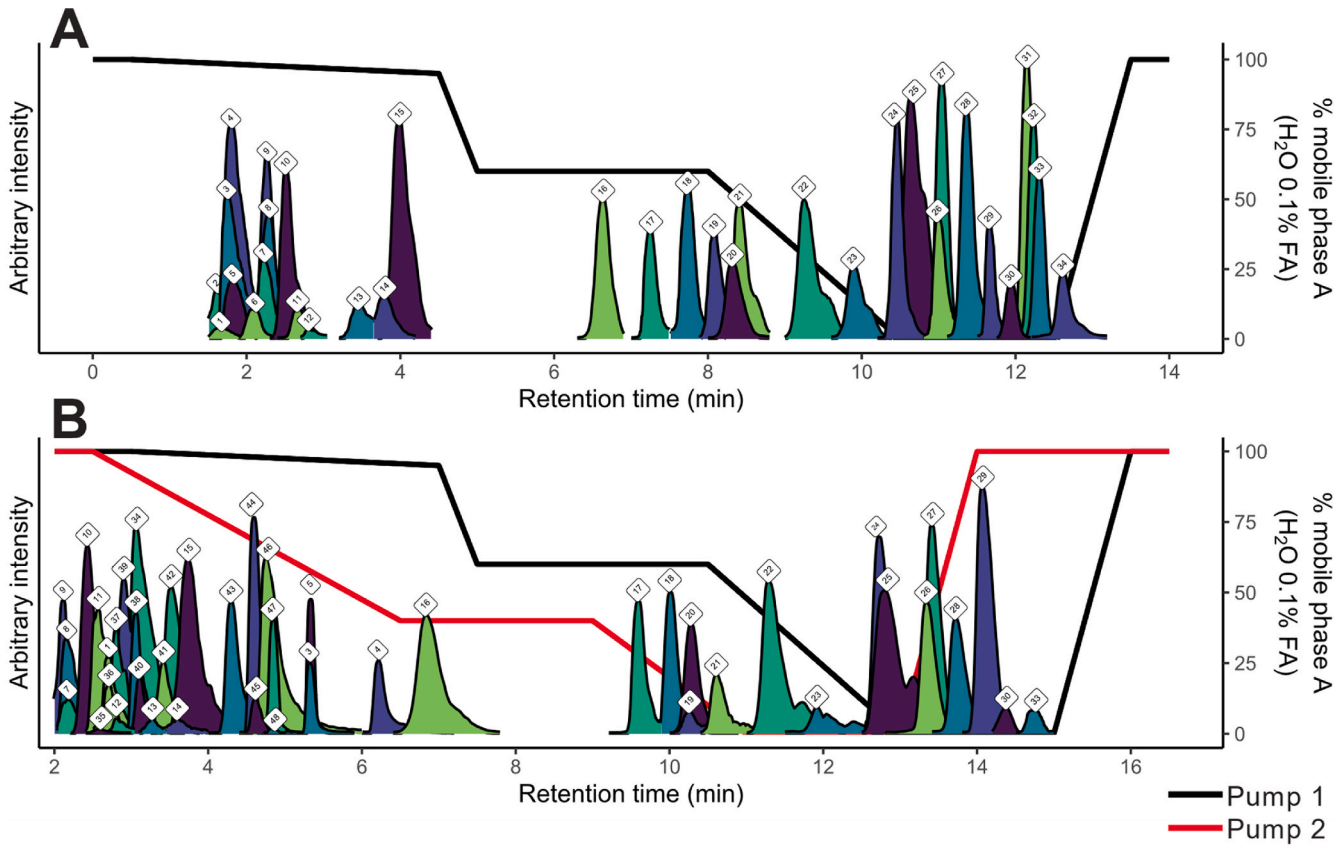
This is supported by the results of this study when looking at results across all samples from experiment 3 and 4 to assess retention time shifting. While most of the compounds which elute on the PGC column were suppressed by the matrix of the Hoagland's extract, threonic acid and arginine could be observed in both matrices on the dual column method. Threonic acid had a RT RSD of 1.2 % when present in the Hoagland's samples (Fig. S6A) on the dual column method and 0.6 % when present in the second extract (Fig. S6B) and arginine had a RSD decrease from 0.44 % to 0.28 %. For compounds eluting between 5.0 and 6.5 min, the higher the ionic strength of the sample, the less the compound is retained. This is supported by the fact that threonic acid elutes earlier on the column at 5.48 min for the first extract versus 5.72 min for the second. Additionally, several unknown compounds which elute in the same range of the PGC column follow a similar pattern where the compound is retained more in the low ionic strength samples and RT is unstable in the high Hoagland's sample and then stabilized in the second extract (Table S8). In fact, some compounds such as cystathionine are so chromatographically sensitive to matrix effects that they experience repeatable and reproducible retention time shifting across injections and days based on the different concentrations of the MRE compounds, even when the matrix is otherwise the same (solvent standard of 10 % MeOH and 0.1 % FA) across samples (Fig. S7).

While this retention time shifting is different across samples with different matrices, the retention time does not experience this level of shifting when the sample matrix remains consistent. This can be observed by the results of selected compounds in Table S8. The observed retention time shifting for samples with matrix variation might be seen as a drawback for the dual column configuration, especially since a retention time shift correction is not possible. In the described dual column setup, both columns and performing separation and joining their flows before introducing analytes to the source, but only one of the two columns (the PCG column) experiences shifting issues. However, the second extract effectively resolves these concerns and eliminates the need for a correction during data analysis. Moreover, the second extract is not only advantageous for the dual column method, but it is also beneficial for any chromatographic method as the lowered matrix fluctuations between samples also lowers fluctuations in ion suppression between samples, resulting in overall improved data consistency.

3.3. *Chromatography*

3.3.1. *Mechanisms of improved retention when utilizing a dual column system*

Reversed phase columns generically function as a non-polar stationary phase retains non-polar and semi-polar compounds in an



Compound	Peak #	Single Column RT	Dual Column RT	LogP	Compound	Peak #	Single Column RT	Dual Column RT	LogP
Amino butyric acid	1	1.64	2.7	-2.77	Salicylic acid	25	10.63	12.89	2.19
Proline	2	1.64	3.11	-2.41	Myricetin	26	11	13.34	0.84
Malic acid	3	1.76	5.28	-1.52	Jasmonic acid	27	11.04	13.45	2.00
Isocitric acid	4	1.79	6.2	-2.00	Quercetin	28	11.36	13.72	1.50
Shikimic acid	5	1.8	5.34	-1.16	3,5-Di-tert-butyl-4-hydroxybenzoic acid**	29	11.67	14.08	5.01
Valine	6	2.09	**2.00***	-2.07	Lauric acid	30	11.9	14.35	5.10
Uracil	7	2.23	**2.14***	-1.06	Palmitic acid	31	12.12	14.41***	7.21
Methionine	8	2.29	**2.20***	-1.73	Stearic acid	32	12.24	14.67***	8.27
Citric acid	9	2.24	**2.20***	-2.07	Arachidic acid	33	12.31	14.78	9.33
Pyroglutamic acid	10	2.51	2.50	-1.15	α tocopherol	34	12.6	15.00***	12.05
Succinic acid	11	2.64	2.60	-0.53	Serine	35	**< 1.60**	2.62	-2.81
Fumaric acid	12	2.91	2.91	-0.17	Threonine	36	**< 1.60**	2.73	-2.50
Isoleucine	13	3.45	3.27	-1.67	Asparagine	37	**< 1.60**	2.81	-3.54
Leucine	14	3.78	3.65	-1.76	Glutamine	38	**< 1.60**	3.08	-3.37
Tyrosine	15	3.99	3.75	-2.22	Lysine	39	**< 1.60**	3.16	-3.42
Phenylalanine	16	6.63	6.84	-1.56	Aspartic acid	40	**< 1.60**	3.11	-2.41
Catechin	17	7.23	9.59	0.53	Glutamic acid	41	**< 1.60**	3.45	-2.69
Epicatechin	18	7.72	10.02	0.53	Cystathionine	42	**< 1.60**	3.56	-4.54
Vanillic acid	19	8.07	10.20	1.36	Threonic acid	43	**< 1.60**	4.30	-2.77
Tryptophan	20	8.29	10.28	-1.57	Histidine	44	**< 1.60**	4.64	-3.73
Syringic acid	21	8.4	10.63	1.07	N-acetyl-D-mannosamine	45	**< 1.60**	4.67	-3.01
Coumaric acid	22	9.26	11.33	1.57	Arginine	46	**< 1.60**	4.8	-3.52
Ferulic acid	23	9.89	11.97	1.42	Dihexose	47	**< 1.60**	4.84	-4.40
Rutin	24	10.45	12.72	-1.36	Hexose	48	**< 1.60**	3.55	-2.90

Fig. 3. Gradients and chromatography

Two different chromatography methods were compared to one another with a set of 58 mock root exudate (MRE) standard compounds. A) A single column chromatography method separated retained, separated, and ionized 33 compounds using a Discovery HSF5 (2.1 × 150 mm, 3 μm particle size, Sigma-Aldrich) pentafluorophenyl (PFP) column. The black line shows the mobile phase A (0.1 % formic acid (FA) in H_2O) gradient (mobile phase B: 0.1 % FA in methanol (MeOH)). B) The dual column method, utilizing both the PFP column and a Hypercarb porous graphitic carbon (PGC) column (2.1 × 150 mm, 5 μm particle size, Thermo Scientific) utilized two different pumps and gradients and observed 39 compounds. Pump 1 led to the PFP column with the gradient shown in black (mobile phase A: 0.1 % FA, mobile phase B: 0.1 % FA in MeOH) and pump 2 led to PGC gradient shown in red (mobile phase A: 0.1 % FA, mobile phase B: 0.1 % FA in acetonitrile).

* These compounds exist within the void volume cutoff for our analysis, but could be recovered in analysis if the void volume for the second PGC column was "sliced out" instead of using a hard cutoff

** This is not a known root exudate but a synthetic standard

*** Poor ionization made these signals unreliable. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

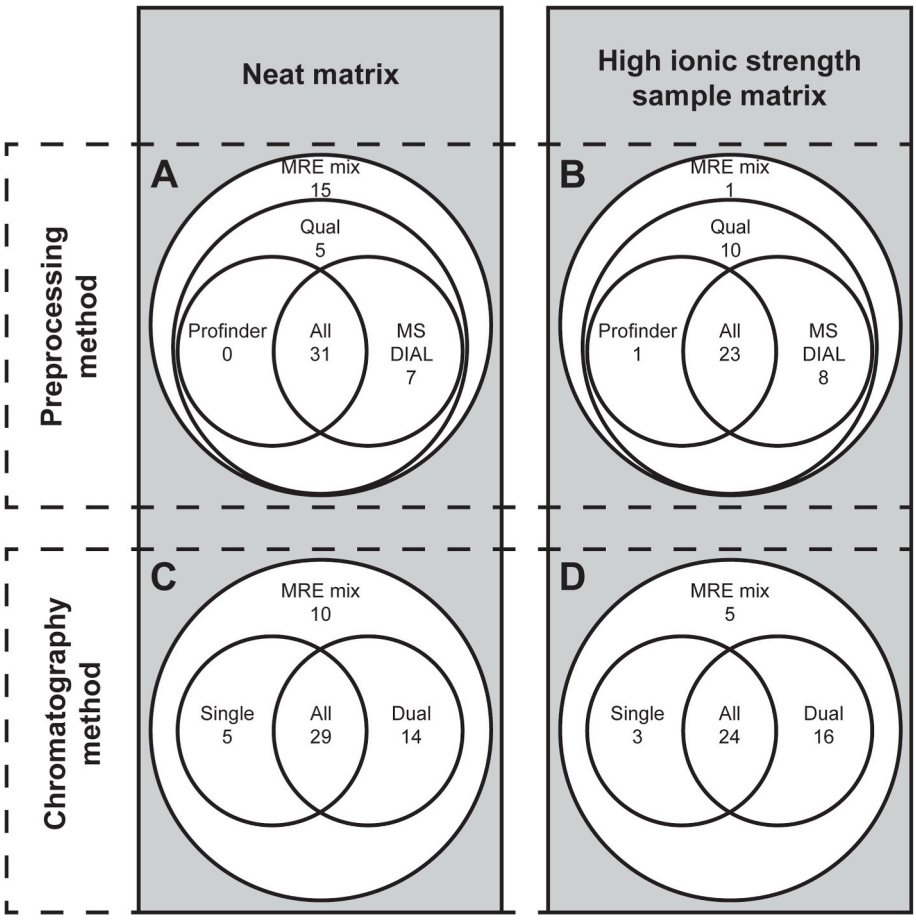


Fig. 4. Venn diagrams

Venn diagrams A and B show the number of compounds which were picked by each software (MassHunter Qualitative analysis for suspect screening, MassHunter Profinder for proprietary NTA, or MS DIAL for open-source NTA) using the dual column method for mock root exudate (MRE) samples containing 58 authentic standards. Standards were reconstituted in either A) a solvent standard (10 % methanol and 0.1 % formic acid) and B) sample matrix with high ionic strength (exudates in Hoagland's extracted with methanol, see experiment 0 and 3). In diagram B, only the 43 compounds which were detected by one of the preprocessing methods in diagram A were considered.

The second set of Venn diagrams (C and D) highlight the number of compounds which were retained and ionized on each chromatographic method (either the single column method or the dual column method) using the MassHunter Qualitative targeted preprocessing for MRE compounds reconstituted in C) the solvent standard and D) the high ionic strength sample matrix. Only the 48 compounds which were observed in solvent standard (C) were considered in the sample matrix analysis (D).

aqueous mobile phase as these compounds have low affinity for polar solvents. The PFP column works both through these traditional means and additionally through hydrogen bonding, and π-π, dipole-dipole, and ion-exchange interactions [54]. This allows for its stronger retention of small polar, especially basic, and aromatic compounds when compared to a traditional C18 column. This is supported by a study which observed more compounds retained on a PFP column when compared to C18 [21]. However, the PFP column is still unable to retain many small polar metabolites which have a higher affinity for the polar mobile phase, i.e., water, than they do for the non-polar stationary phase. This can be shown by the lack of retention of compounds with low LogP values. It should be noted that while LogP, or octanol-water partition coefficient, is an imperfect measure of polarity, it serves as a relatively good proxy for measuring how hydrophilic or hydrophobic a compound is. Compounds with low LogP values could only be observed in the void volume of the single column method.

A PGC column works fundamentally differently from reversed phase columns. The stationary phase material has a partial charge imparted upon it by the water in mobile phase A (likely via electron lone-pair interaction with the graphite [55]). This partial charge makes the stationary phase polar, and thus it can interact with small polar metabolites even in an aqueous mobile phase. The second mobile phase is then used to disrupt the partial charge on the column, thus removing its affinity for small polar metabolites. This is why acetonitrile is used instead of methanol as mobile phase B. Methanol would continue to maintain the partial charge of the stationary phase. This is also why, after analyte elution on the PGC column, a maintenance phase is required to re-establish the column charge before beginning the next sample run. Incomplete establishment of this charge could result in poor method repeatability and retention time shifting of analytes on the PGC column [55].

With the dual column method developed, the mobile phase can be fully aqueous for both columns at the beginning of the method. This would not be possible if coupling a reversed phase column with a hydrophilic interaction liquid chromatography (HILIC) column as the H_2O would prevent metabolites from retaining on the HILIC column. Even if a HILIC method was used on a secondary method run, the sample composition would need to be tailored to each method as highly aqueous matrices have performance issues on HILIC methods [56].

When the PFP column is coupled to the PGC column, the dual column method benefits from the combined modes of action of both columns. Indeed, the results from the MRE samples, whether in neat and high ionic strength sample matrix, support this. Not only were standards with lower LogP values retained on the dual column method, but there was an increase in weakly retained compounds both in the MRE samples and in real extracts, resulting in a retention of a wider variety of compounds.

3.3.2. Single versus dual column method ability to retain MRE compounds in solvent standard

The ability of each chromatographic method to retain different MRE compounds was compared using both the MassHunter Qualitative method and the non-targeted MS DIAL method.

Of the 58 MRE compounds, 48 could be retained and ionized by one or both chromatographic methods in a solvent standard. Of these 48, the single column method was able to retain, ionize, and pick 34 compounds when using the targeted workflow while the dual column method was able to do so for 43 (Figs. 3 and 4C). There were 14 compounds which could be measured by the dual column method that were not retained by the single column method. For MRE compounds specifically, the single column method could not retain anything with a logP value below −2.77. However, the average logP value of the compounds gained by the dual column method was −3.25 and this method was able to retain compounds with a logP as low as −4.54. This emphasizes the capability of the dual column method to increase coverage of small polar metabolites.

Additionally, there were, depending on the preprocessing method, 5 (MassHunter Qual) or 10 (MS DIAL) compounds which were picked by the single column method and not on the dual column method. There were three reasons why the single column method observed compounds which the dual column method did not. First, when the NTA workflow was used, one compound (vanillic acid) was simply not picked by MS DIAL algorithm for the dual column method in solvent standard, even though it was present. Manual assessment of the peak in MassHunter Qualitative software shows that it should have passed the MS DIAL thresholds and filters (group presence, peak height, and number of points across the peak). These algorithms are imperfect and can miss compounds during peak picking.

Four compounds, valine, uracil, methionine, and citric acid, eluted before the dual column void volume even though they separated on the PFP column. Since the valve connecting the PFP column to the PGC column (Fig. 2A) switches at minute 1.8 (and then sends the flow from the PFP column to the MS source (Fig. 2B)), there are metabolites coming off the PFP column and going to the MS source starting at minute 1.8 while the void volume is traveling through the PGC column. The void volume finally makes it through the PGC column and elutes to the MS source between minute 2.4 and 2.5. Many compounds could be kept for analysis if the PGC void volume is sliced out of the analysis instead of using a rigid void volume cutoff of 2.5 min. As in, instead of a hard void volume cutoff of 2.5 min being filtered from the data, a cutoff of 1.8 min with an additional range from 2.4 to 2.5 min could have been removed.

The dual column method resulted in approximately a 3-fold decrease in peak height for compounds shared between methods. There are two reasons why this could happen, both of which probably resulted in a compounding effect. 1) The dual column method had two streams coming together at a T joint, thus functionally increasing the flow rate at the source from 0.35 mL min-1 to 0.70 mL min-1 and decreasing ionization efficiency at the source. 2) The second pump going to the PGC column begins to elute water to equilibrate and reestablish a partial charge before the PFP column is finished eluting its more non-polar compounds (I.e., minute 14 to 15 is 100 % organic on the PFP column and 100 % aqueous of the PGC column (Fig. 3B)), resulting in a reduced ionization efficiency of nonpolar compounds due to the presence of water. These factors combined resulted in five compounds (fumaric acid, palmitic acid, lauric acid, stearic acid, and α-tocopherol) being below the MS DIAL preprocessing cutoffs. While the methods applied in this study were not developed with lipidomics in mind, the dual column method could be altered to be more amenable to nonpolar compounds by adjusting the gradients of each pump so that the required maintenance phase of the PGC column does not overlap with the nonpolar elution phase of the PFP column. However, this would significantly elongate the gradients and acquisition time.

Of the 24 reliably detectable compounds shared by each method at 10 μmol/L, the RSD of the retention times was 0.53 % for the single column method and 0.35 % for the dual column method. When looking at all compounds for each method, the RSD was in the same range of 0.5–0.6 %. This shows that retention times are stable between the methods for solvent standard samples.

3.3.3. Impact of high ionic strength matrix on standard compounds for each chromatographic method

The high ionic strength matrix resulted in an overall loss in signal for both chromatographic methods when observing MRE standards that had been spiked into the Hoagland's matrix. The single column method was able to retain and ionize 27 of the MRE compounds while the dual column method was able to retain and ionize 40 (Fig. 4D). In line with the solvent standard MRE sample results, the single column method observed 3 compounds which the dual column method could not, and the dual column method could measure 16 compounds which the single column method could not. As with the solvent standard analysis, the single column method failed to retain as many low logP value metabolites (could not observe anything with a logP value below −2.22). The dual column method could not observe some compounds based on the same reasons outlined in the previous section. Again, the overall loss in signal for high ionic strength samples in comparison to solvent standard results was likely due to matrix ion suppression which mainly affected the weakly retained compounds which coeluted with the Hoagland's salt.

Of the 17 compounds shared by each method, the RT RSD was 0.44 % for the single column method and 0.17 % for the dual column method. When looking at all compounds, the RSD was 0.88 % and 0.35 % respectively. For this analysis, the high salt matrix was the same for the single column sample group and the same for the dual column sample group, thus the within group retention times remained stable because the within group matrix was stable. The results show that the dual column method is robust in comparison to the single column method when sample matrix is consistent, even when the sample has a high ionic strength.

3.3.4. Overall observable compounds in a methanolic extraction

When analyzing a low ionic strength methanolic extract of a black oat pooled QC sample spiked with MRE, the performance of the single versus dual column approach is mixed. As with the previous spiked sample, more of the standards could be observed by the dual column method overall (18 for single, 32 for dual, Fig. 5A–B). The single column method was able to retain and pick 775 in negative ionization mode (Fig. 5A) and the dual column method picked 654 (Fig. 5B). In positive ionization mode, 669 (Figs. 5C) and 396 (Fig. 5D) compounds were picked, respectively. Thus, the single column method was able to outperform the dual column method in positive ionization mode for the specific root exudate sample observed in this study.

For the single column method, ~20 % compounds eluted before 5.5 min (112 in negative ionization mode and 177 compounds in positive ionization mode). For the dual column method, ~35 % of compounds eluted before 8.0 min (182 in negative ionization mode and 169 compounds in positive). This shows that the dual column method excels more with small polar metabolites at the expense of the aforementioned loss in signal intensity for later eluting compounds.

4. Conclusions

In this study, dual extraction and chromatography methods for analysis of root exudates were developed to increase the coverage of compounds observed during non-targeted mass spectrometric analysis. MassHunter Profinder, while more accurate in some ways (e.g., ~1 ppm gain in mass accuracy), has issues in that it drops features of interest and loses MS2 data association. The better accuracy may be beneficial in identification, but the hardly accessible MS2 data that is kept during the MS DIAL workflow is beneficial in future identification. While the dual column system does lose sensitivity as highlighted by the fewer observed

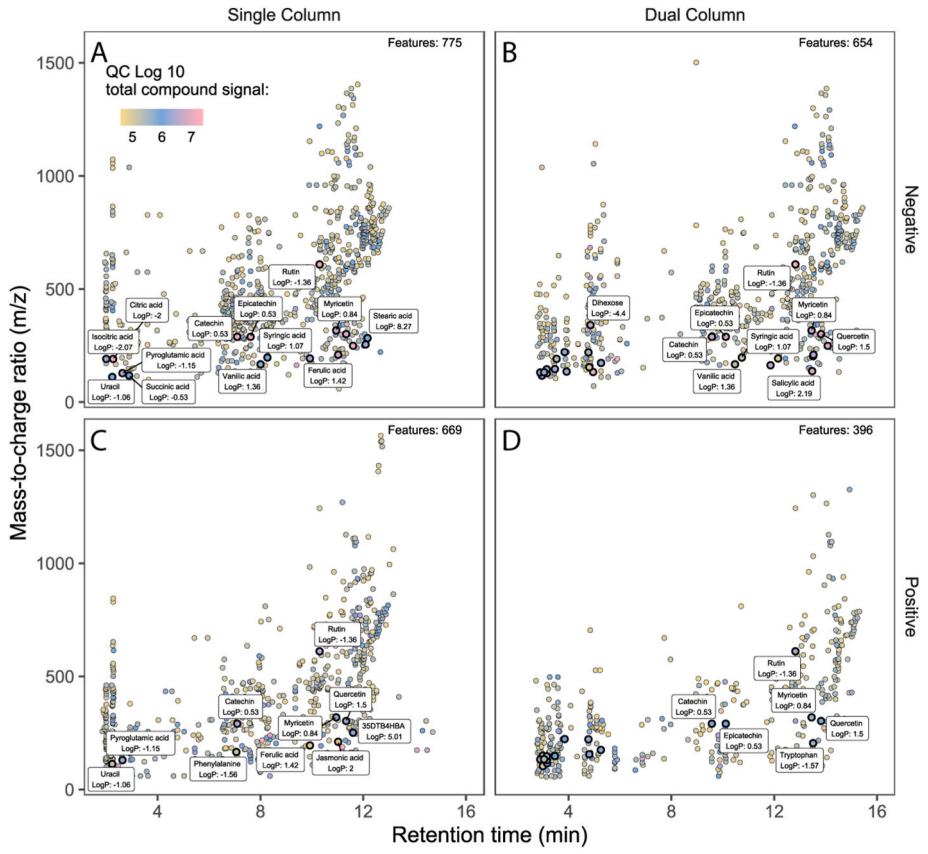


Fig. 5. Feature plots

A set of samples from a split root experiment 4 of black oat grown with pigweed on glass sand were also extracted twice. The second extract was then pooled into a QC sample and used to reconstitute a MRE mixture. This was then run on both the single and dual column chromatography method in both positive and negative ionization mode and preprocessed in MS DIAL. Feature plots of retention time (min) by *m/z* highlight the number of compounds and their chemical space for A) the single and B) dual column method in negative ionization mode and the C) single and D) dual column method in positive ionization mode. MRE compounds spiked into sample and their logP values are highlighted.

features when performing NTA of root exudate samples, it gains small polar metabolites missed by the single column method as shown by the spiked MRE samples. If loss of signal intensity is a concern, then it is recommended to increase the preconcentration factor of the sample to re-gain sensitivity (i.e., only 10 of the 30 mL of extract was used and concentrated 30-fold when 20 or all 30 mL could be concentrated up to 100-fold). However, if high matrix variation is unavoidable or low abundance compounds are more interesting than diverse compounds and stronger preconcentration is not possible, then this dual column method is not viable. The second extract of the dual extraction method results in lower overall ion suppression, and it stabilizes the matrix which improves issues with retention time shifting and fluctuations in analyte ionization.

The conclusion of these results is that the cumulative effects, strengths, and weaknesses of each individual step should be carefully considered when generating the overall method as different extraction methods could perform differently on each chromatographic system. For example, an extraction with many high abundant small polar metabolites could result in overall more compounds retaining and ionizing on the dual column method when compared to the single column method. Or, if a dual column system is desirable for both coverage of diverse and low abundant compounds, a higher pre-concentration is easily achievable by using a larger volume of the original extract. Additionally, if nonpolar compounds are of more interest, then the maintenance phase of PGC column on the dual column method can be altered to generate a longer but more accommodating method. This highlights how the term "non-targeted analysis" is a misnomer and shows the importance of tailoring methods based upon the experimental needs and assessing the scope and limitations of the selected methods used in NTA experiments.

CRediT authorship contribution statement

Alexandra A. Bennett: Writing – original draft, Visualization, Validation, Software, Methodology, Data curation, Conceptualization. **Teresa Steininger-Mairinger:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Çağla Görkem Eroğlu:** Resources, Methodology, Investigation. **Aurélie Gfeller:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Judith Wirth:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Markus Puschenreiter:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Stephan Hann:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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