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Using Kendrick Defect Mass Analysis and Chemical Informatics to Enhance Annotation in Soybean Metabolomics

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Amongst abiotic stresses to agricultural crops, water deficiency is amongst the most prolific and has worldwide detrimental impacts. The soybean (*Glycine max*) is one of the most important sources of nutrition to both livestock and humans, and different plant introductions (PI) of soybeans have been developed which have different water tolerances. Here, extracts from two different cultivars of soybeans (Pana, a drought susceptible cultivar, and PI 567731, a drought-tolerant cultivar) are analyzed by direct infusion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. The high mass resolution and accuracy of the method allows for identification of ions from hundreds of different compounds in each cultivar. The exact *m/z* of these species are determined and filtered through SoyCyc and Human Metabolome Database to identify possible molecular formulas of the ions. Based on the SoyCyc matches, the metabolomes of each cultivar are compared and contrasted and assessed within the context of metabolic mapping. Next, the exact *m/z* values are converted into Kendrick masses and their Kendrick mass defects (KMD) computed, which are then sorted from high to low KMD. This latter process assists in identifying many additional molecular formulas so that more than 460 unique ions are identified in Pana, and more than 340 unique ions are identified in PI 567731; many of these metabolites are reported as being derived from soybean for the first time.

Key words: Kendrick mass defect, metabolomics, soybean, drought tolerance, electrospray ionization, FT-ICR, chemical informatics

Introduction

Abiotic stresses to agricultural crops can have a significant impact on crop yields, with water deficiency or drought being one of the most prolific.[1] Legumes such as soybeans (*Glycine max*) are particularly susceptible to water deficiency in early growth stages, which may have dramatic impacts on crop yield.[2] Reduced yields due to drought are the result of alterations in homeostasis, impacting composition of plant tissues at the molecular level.[3] Recent advances in agronomy have led to the identification of slow canopy wilting (SW) phenotypes in soybeans which exhibit a tolerance to water stress.[4] The plant introduction (PI) cultivar PI 567731, an exotic soybean germplasm, has been shown to consistently possess the SW phenotype and also utilizes less water and produces a greater crop yield under drought conditions.[4, 5] Unfortunately, many of the underlying metabolomic mechanisms for the drought tolerance of the PI 567731 cultivar are not clear.[6] Increased knowledge of the molecular composition of components of soybean plants would enhance present understanding of the mechanisms by which certain cultivars might have enhanced resistance to abiotic stress.

Mass spectrometry is a rapidly growing technique for fingerprinting in soybean metabolomics.[7, 8] A report which examined metabolic profiles of soybean leaves using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) found that a number of important pathways related to nitrogen and sugar metabolism under drought and heat stress were impacted.[3] Another study used GC-MS and ultrahigh perfomance liquid chromatography (UPLC) to demonstrate more than 160 metabolites from seeds of different soybean cultivars.[9] The impact of flooding stress on soybean plants was investigated by capillary electrophoresis coupled to MS, revealing numerous metabolites sensitive to flooding, including increased levels of gamma-aminobutyric acid, glycine, NADH2, and phosphoenol pyruvate.[10] A recent paper used UPLC and tandem mass spectrometry to investigate soybean metabolomics under drought stress, and revealed that amino acid metabolism and lipid metabolism both play a key role in drought resistance with the tricarboxylic acid (TCA) cycle being one of the core pathways enabling drought resistance.[11] Our group has promoted the utility of high resolution mass spectrometry independent of chromatographic methods for metabolomic studies of soybeans, showing clear distinction of

soybean leaves due to senescence[12] and for drought-stressed soybean leaves, particularly with respect to chlorophyll and its related metabolites.[13] Indeed, the utility of mass spectrometry in soybean metabolomics is so high that a recent review covered its use in application to underused parts of the soybean plant.[14]

While chromatographic methods in combination with mass spectrometry are widely used in metabolomics, it is also important to recognize that with high mass resolving power and high mass accuracy measurements, it is possible to assign molecular formulas to very complex mixtures. Kendrick recognized that by rescaling a mass spectrum from the IUPAC mass scale (^{12}C is exactly 12 Da) to a mass scale based on methylene units enables ready identification of a homologous series of compounds of the same class and type, but with different extents of alkylation.[15] Effectively, the IUPAC mass is converted into a Kendrick mass:

$$\text{Kendrick mass} = \text{IUPAC mass} \times (14/14.01565) \quad (1)$$

By rescaling the mass spectrum, compounds with identical numbers of heteroatoms and double bonds + rings possess identical Kendrick mass defects (KMD):

$$\text{KMD} = (\text{nominal Kendrick mass} - \text{exact Kendrick mass}) \quad (2)$$

Obtaining KMD has shown great utility in identification of molecular formulas from highly complex mixtures of hydrocarbons,[16-20] synthetic polymers,[21-29] and specimens of biologic origin for metabolomics.[30-37] Of these, only one study using KMD has been employed for phytochemical assessment within plants.[36]

Expanding upon an earlier study using direct infusion electrospray ionization (ESI) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry to examine phytochemical composition of different soybean cultivars, here we filter measured *m/z* values through the SoyCyc database (https://soycyc.soybase.org/), which was populated with data from SoyBase (https://soybase.org),[38] and the Human Metabolome Database (HMDB) (https://hmdb.ca/)[39] for initial assignments of molecular formulas. Subsequently, KMD analysis is conducted, which is used to assign additional molecular formulas. The results are used to compare and contrast differences in the metabolome between two differet soybean cultivars with different levels of water deficiency tolerance, with a discussion of the implications.

Experimental

Materials

HPLC grade methanol was from Sigma-Aldrich (St. Louis, MO). For vacuum filtration of particulate matter, Cytiva Whatman filtration papers with 11 micron pore size (Little Chalfont, Buckinghamshire, UK, Cat. 1001-055) were used.

Plant Material

Two cultivars of soybean (*Glycine max*) were grown in the field at the University of Missouri, the drought-sensitive cultivar Pana and the drought-tolerant cultivar PI 567731. Here, plants were grown for three weeks in the field under irrigation with watering two days prior to plant harvest, and leaves had reached the R2 growth stage. After collection, leaves were flash frozen and transported at -80°C to the University at Buffalo, and stored in polycarbonate petri-dishes at -20°C until extractions were performed.

Extraction and Sample Preparation

Flash frozen leaves from multiple plants of each cultivar were pooled together, and then each group was individually macerated manually for five minutes in methanol using mortar and pestle. To remove particulates, vacuum filtration was performed. The samples were subsequently dried in a vacuum oven, and then the dried residue was reconstituted into 2 mL of HPLC grade methanol prior. These samples were diluted by 50x prior to ESI FT-ICR analysis.

ESI FT-ICR mass spectrometry

Direct infusion ESI FT-ICR mass spectrometry was conducted using three replicates from each cultivar, the details of which are described in detail previously.[13] Once the mass spectra were collected, the data sets were processed as follows using Bruker Daltonics (Bremen, Germany) Data Analysis 4.0 software. Software was instructed to find all peaks with a signal-to-noise ratio ≥ 3 to produce a peak list. Next, the peak list was subjected to the deconvolution process such that isotopic envelopes were determined, and each individual ionic species was then grouped as part of the given isotopic cluster. A threshold of 0.1% peak area relative to the most intense peak (*m/z* 1073.506 in each cultivar list, corresponding to ion $C_{67}H_{94}NaN_4O_6$) was used. The peak list was reduced to the monoisotopic isotope of each isotopic cluster, and this was the *m/z* value used in compiling lists for each cultivar.

Data Processing and Analysis

After compilation of the *m/z* list for each cultivar, it was first passed through the SoyCyc database of metabolites (https://soycyc.soybase.org/); matches of either protonated, sodiated, or potassiated ions to the known metabolites within 3 ppm mass error was considered a confirmation of the ionic formula. Each list was then filtered through HMDB to discover matches to either protonated, sodiated, or potassiated ions in the database. For endogenous compounds, the 3 ppm mass error was again used to constitute a match. For non-natural compounds, however, a stricter limit of 1 ppm was used to constitute a match between the database and the *m/z* list. To further annotate the *m/z* with ionic formulas, each list was converted to the corresponding Kendrick mass and KMD calculated for each ion; ions were then sorted by KMD and plotted as nominal Kendrick mass vs. KMD in order to assist in identification of ionic formulas to those *m/z* which did not yet have an identified formula. Final lists of ionic formulas from each cultivar were then compared, with similaries and differences recorded. For those m/z values which matched entries in the SoyCyc database, an examination of the metabolic pathways involved was also performed to obtain context on how the cultivars might respond to drought at a molecular level.

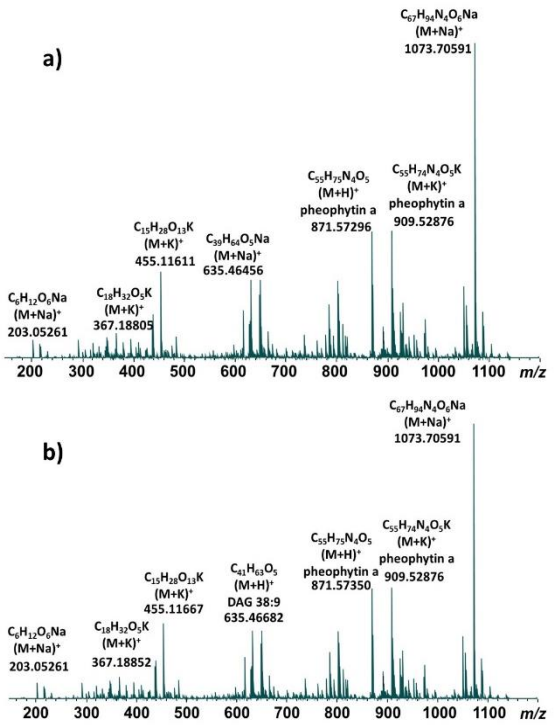

Figure 1. Direct infusion ESI FT-ICR mass spetrometry of methanolic soybean leaf extracts of the cultivars a) PANA and b) PI 567731.

Results and Discussion

ESI FT-ICR of Soybean Leaf Extracts

Representative direct infusion ESI FT-ICR mass spectra of methanolic leaf extracts are shown for the Pana cultivar (Figure 1a) and the PI 567731 cultivar (Figure 1b). The major components are highly similar for both cultivars, and the same base peak is observed for each at *m/z* 1073.706; our previous study indicates this molecule is derived from the sodiated ionic species of pheophytin a, possessing an additional $C_{12}H_{20}O$ moiety.[13] All of the detected ions are singly-charged. After deconvolution of each mass spectrum in Data Analysis 4.0, a total of 612 distinct isotopic clusters were identified for the Pana methanolic extract, while 528 distinct isotopic clusters were identified for the PI 567731 methanolic extract. Lists of *m/z* values using the monoisotopic peak for each cluster were compiled for each cultivar for subsequent comparison with databases.

Data Processing to Identify Matches in SoyCyc

The *m/z* peak list from each cultivar was initially passed through the SoyCyc database to find potential matches to known constituents within 3 ppm mass error. With the Pana cultivar, 84 unique *m/z* values were matched to protonated, sodiated, or potassiated ions from known soybean metabolite components; in addition, sodiated and potassiated dimers of $C_6H_{12}O_6$ were also detected. Ionic formulas were assigned based on the matches. For the PI 567731 cultivar, 65 unique *m/z* values matched protonated, sodiated, or potassiated ions of known soybean metabolites in the database; in addition, the sodiated dimer of $C_6H_{12}O_6$ was also detected. Of the ions detected in the extracts, 24 corresponded to formulas of soybean metabolites found only in Pana. These are listed in Table 1. Five metabolites were detected uniquely in PI 567731, and these are listed in Table 2. Implications of these findings will be discussed next.

Table 1. Measured *m/z* Matching Entries to SoyCyc Exclusively in Pana Methanolic Extracts

Measured *m/z*	Ion Formula	Mass Error (ppm)	Possible ID (molecule or class)
309.20358	$C_{16}H_{30}NaO_4$	-0.16	hexdecanedioic acid
325.17755	$C_{19}H_{26}NaO_3$	0.41	carlactone
351.17563	$C_{18}H_{32}KO_2S$	0.49	carboxylic acid
353.22983	$C_{18}H_{34}NaO_5$	-0.04	2 stearic acid isomers
359.01734	$C_{15}H_{12}KO_8$	2.69	carboxylic acid
365.06327	$C_{15}H_{18}KO8$	-0.15	carboxylic acid
367.10843	$C_{10}H_{23}O_{14}$	0.54	carboxylic acid
395.17342	$C_{21}H_{28}KN_2O_3$	0.68	galactopinitols
435.25066	$C23H_{40}KO_5$	-0.17	5 isomers
471.25074	$C_{26}H_{40}KO_5$	0.02	glucoside
497.18664	$C_{20}H_{33}O_{14}$	0.32	3 isomers
585.37007	$C_{40}H_{50}NaO_2$	-0.40	15-cis-phytoene
609.27067	$C_{29}H_{46}NaO_{10}S$	0.46	3 isomers
647.46492	$C_{46}H66NaO2$	0.27	epoxypheophorbide a
649.18953	$C_{29}H_{38}KO_{14}$	0.33	glucoside
651.43852	$C_{39}H_{64}KO_5$	-0.02	glutathione disulfide
675.49608	$C_{51}H_{96}KO_6$	0.05	2 isomers
741.57946	$C_{17}H_{25}NaNO_6$	0.10	menaquinol-8
771.60506	$C_{56}H_{96}KO_3$	0.01	2 isomers 34:5 MGDG
893.55467	$C_{55}H_{74}NaN_4O_5$	-0.53	pheophytin a + Na
907.5214	$C_{55}H_{71}MgN_4O_6$	-0.50	chlorophyll b + H
911.52523	$C_{46}H_{81}NaO_{14}P$	-0.42	pheophytin a + K
923.50859	$C_{55}H_{72}KN_4O_6$	0.27	pheophytin b + K
945.47643	$C_{55}H_{70}KMgN_4O_6$	-1.38	chlorphyll b + K

Table 2. Measured *m/z* Matching Entries to SoyCyc Exclusively in PI 567731 Methanolic Extracts

Measured *m/z*	Ion Formula	Mass Error (ppm)	Possible ID
277.08988	$C_9H_{18}NaO_8$	1.77	galactosyl glycerol
481.3652	$C_{30}H_{50}NaO_3$	-0.12	soyasapogenol B
527.1585	$C_{18}H_{32}NaO_{16}$	0.54	trisaccharides + Na
543.1325	$C_{18}H_{32}KO_{16}$	0.58	trisaccharides + K
771.6055	$C_{50}H_{84}KO_3$	0.34	plastoquinone

Leaf extracts from the two cultivars grown under control conditions share 60 ionic formulas which are matched to the SoyCyc database. Prominent amongst these are mono- and diacylglycerols, pheophytin a and chlorophyll a, monosaccharides, disaccharides, xanthins, and vicenin-2 (a flavonoid diglucosylation product). Notable also is the simultaneous presence of plastaquinone, detected with products echinone and plastoquinol, essential components of photosynthetic electron transfer. Likewise, ubiquinol-8 and -9 are detected along with 3-demethylubiquinol-9 and demethylmenaquinol-8, key components of aerobic respiration and photosynthethic electron transfer. The metabolite cycloeucalenone is involved in phytosterol biosynethesis.

Pana is a drought-sensitive soybean cultivar. Using the known soybean metabolites putatively identified in Table 1, there are several carboxylic acid molecules present in Pana that were not detected in PI 567731; these are essential precursors to lipids. Carlactone is an oxidation product of cartenal, possibly indicating oxidative stress in Pana even in the control which has not experienced drought. This is further supported by the presence of glutathione disulfide, the oxidized dimer of glutathione. Galactopinitols are required substrates and products of galactosylcyclitol biosynthesis. The compound 15-cis-phytoene is needed for production of plastoquinol and carotenes. Likewise, the substance menoquinol-8 is a polyprenyl quinone required for electron transport. A richer complement of pheophytins and chlorophylls are detected in Pana in comparison to PI 567731 (e.g., chlorophyll b was only detected in Pana). However, our earlier work showed that PI 567731 maintains greater levels of pheophytins and chlorophylls during drought.[13]

In contrast, PI 567731 is a drought-tolerant soybean cultivar. As shown in Table 2, the metabolites uniquely detected in the methanolic extract of PI 567731. The galactosyl glycerol compound is 3-β-D-galactosyl-sn-glycerol, formed from the degradation of diacyl glycerols. Soyasapogenol B is a key precursor in the formation of its glucuronide. There are many possible structures for the trisaccharides, so anabolism of more complex saccharides from mono- and disaccharides might explain the appearance of trisaccharides here. Plastoquinones are electron carriers that are necessary building blocks for plastoquinol, and are found in chloroplasts, thus playing a central role in the photosynthetic electron transport chain. Therefore, PI 567731 may adapt better to drought conditions because of how it processes sugar molecules and builds a reservoir of electron transport carriers.

Kendrick Mass Defect Analysis

Although soybean metabolites are not human metabolites, it was recognized that they may share molecular formulas with common human metabolites. Thus, the peak lists were imported into HMDB and searched against possible matches to protonated, sodiated, or potassiated molecular formulas with known components of the human metabolome and exposome. In each case ionic formulas were matched within 3 ppm; in a few cases where a metabolite was not natural in humans, and is instead due to contact with a component in the environment, and therefore part of the human exposome, a stricter setting of 1 ppm mass error was tolerated to be a match; it should be noted that the vast majority of matches indicated less than 1 ppm error. This analysis yielded more than 300 matches between *m/z* and ionic formula for each cultivar.

Once these lists had been compiled subsequent to HMDB import, KMD were computed for each individual *m/z* value in each cultivar, then sorted according to their calculated KMD in Excel. This step, coupled with plotting KMD vs. nominal Kendrick mass for each cultivar, was essential for the annotation of several additional ionic formulas. The utility of Kendrick plots is highlighted by Figure 2, which is a Kendrick plot for the Pana leaf methanolic extract over the Kendrick nominal mass range of 550-750. Several features become apparent when displayed in this way that are valuable for annotation of complex mixtures. First, as shown by the solid lines, species which differ by two hydrogen atoms form diagonals with parallel slopes, enabling determination of many ionic formulas graphically when one member of the class is known. Second, horizontal lines represent chemical classes that differ only in the number of alkyl units. In metabolomics, this is often represented by ethylene (C_2H_4) differences common to fatty acids and acylglycerols; one example is shown in Figure 2 as a dashed line.

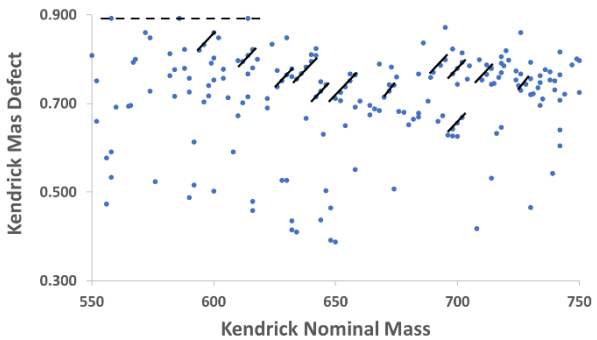

Figure 2. Kendrick mass plot from 550-750 indicating the metabolites from the Pana extracts. The solid lines represent speceis differening by only two hydrogen atoms. The dashed line represents metabolites with identical KMD, and in this case differing by C_2H_4 units.

The net result after KMD analysis yields assignment of 469 ionic formulas to the metabolites in the Pana leaf extracts (Supplementary Table 1), and 345 in the PI 567731 leaf extracts (Supplementary Table 2). The vast majority of these formulas do not correlate with any compounds currently cataloged in SoyCyc. Only a single previous example of application of KMD for the analysis of plant metabolites has been reported;[36] clearly, application of KMD after filtering *m/z* lists through databases considerably expands the total number of ionic formulas identified. Because many of these are identified for the first time, the results indicate that this process of filtering the high mass accuracy spectra through databases for initial formula identification followed by KMD analysis generates an expanded catalog of leaf metabolites from different soybean cultivars.

Conclusions

High mass accuracy mass spectrometry is shown here to identify literally hundreds of different components from leaves of two soybean cultivars with different levels of tolerance to drought as their control conditions. The *m/z* data, when filtered through databases, identifies many ionized molecular formulas and compounds that part of metabolic pathways. Upon KMD analysis of the data coupled with graphical display of Kendrick mass plots, additional ionized molecular formulas are identified, with a particular note to those compounds differing in mass by only two hydrogen atoms, which are readily visualized on Kendrick mass plots. Clearly, the use of KMD analysis in expanding annotations of plant metabolites is a methodology that should have expanded use in metabolomics research.

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Notes and references

† Electronic Supplementary Information (ESI) available: Supplementary Table 1 (KMD Analysis of Pana Metabolites) and Supplementary Table 2 (KMD Aanlysis of PI 567731 Metabolites). See DOI: xx.xxxx/xxxxx/

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