Chapter 2

Nontargeted Unknown LC(ESI-)-Q/TOF MS Approaches for Food Verification

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In this study, an ultrahigh pressure liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization (UHPLC-(ESI)QTOF MS/MS) method for varietal separation of onions was examined. Non-targeted and data mining (unknown analysis) extraction approaches were employed and are discussed. Herein, nineteen flavonoid-based compounds were identified in all onion varieties using accurate mass formula searching. Principal component analysis (PCA) indicated that varietal identification, based upon the content of these 19 flavonoids, might be possible with the collection of a more comprehensive dataset. In addition, a molecular formula extraction algorithm was used to find all compounds in each variety without any attempt in identification. This new approach employes statistical methods to filter out non-differentiating unknown compounds and then PCA to determine if varietal differentiation could be made.

Introduction

Epidemiological studies indicate people who consume diets rich in fruits and vegetables have a reduced risk of chronic diseases (*1*, *2*). Fruits and vegetables are primary dietary sources of vitamins, minerals, fiber and a wide array of non-essential but biologically active phytochemicals including: polyphenolic antioxidants (e.g. flavonoids, carotenoids (e.g. lycopenes, β-carotene), alkaloids, glucosinolates, etc.). Biologically active phytochemicals encompass a wide range

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of chemical structures and chemical activities and have tremendous variability in foods (*3*). To date, there is limited understanding of the influence of cultivar variability, growing season, growing region, processing, storage, formulation and packaging on the chemical composition of bioactives in foods. This lack of knowledge makes the medicinal or functional use of foods difficult, and results in a true manufacturing challenge; that is delivering a food-based product with a consistent level of specific bioactives. Moreover, many of these biologically active phytochemicals are unique in their profiles within a food or species and a more complete understanding of these profiles will lead to analytical advancements in source authentication and in detecting adulteration.

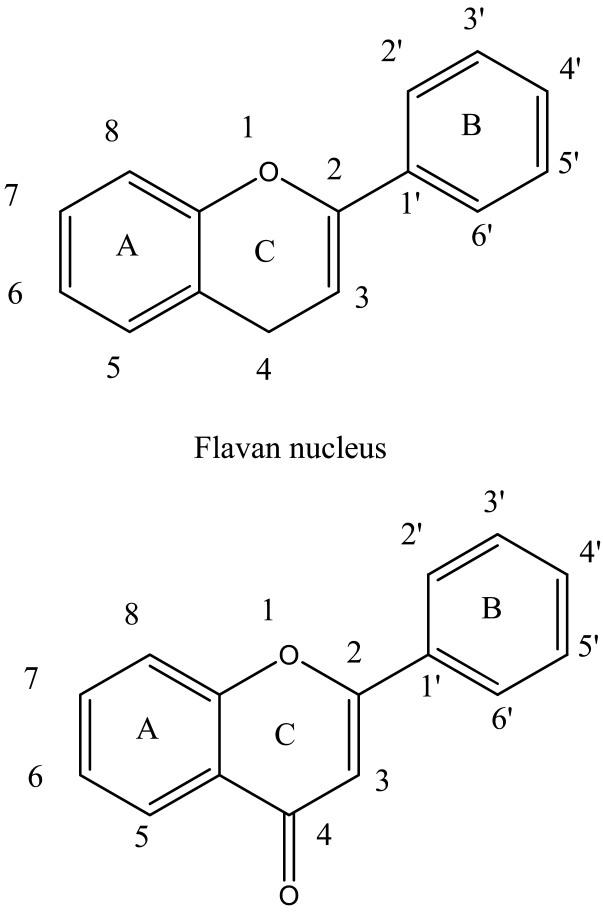

Figure 1. Structure of the Flavonoid Backbone.

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Table 1. Variety Comparisons of Flavonoids in the Outer Layers of Onions (mg/100 g DW)[a]

Variety	*quercetin 3,4′-O-diglucoside*	*quercetin 3-O-glucoside*	*quercetin 4′-O-glucoside*	*isorhamnetin 4′-O-glucoside*	*quercetin aglycone*	*sum*
Cougar	541 ± 4 f	42 ± 1 c	480 ± 4 e	79 ± 0 f	256 ± 2 b	1398 ± 3 e
Don Victor	10 ± 0 a	Below LOQ[b]	56 ± 1 a	7 ± 0 a	20 ± 0 a	93 ± 2 a
Gobi	95 ± 3 d	10 ± 0 b	103 ± 2 c	21 ± 1 d	30 ± 0 a	258 ± 4 c
Milestone	49 ± 0 c	54 ± 1 d	536 ± 3 f	17 ± 0 c	1047 ± 29 c	1703 ± 30 f
Natasha	36 ± 3 b	4 ± 0 a	89 ± 2 b	12 ± 0 b	26 ± 0 a	167 ± 5 b
Warrior	242 ± 1 e	10 ± 0 b	230 ± 2 d	34 ± 0 e	23 ± 0 a	539 ± 3 d

[a] Values are mean ± SD. Mean values followed by the different letters (a-f) within each column are significantly different at $p < 0.05$. [b] Detected but below LOQ (Limit of Quantitation). Source: Reproduced with permission from reference (*2*).

Flavonoids are the most abundant subclass of plant-derived polyphenolic bioactive compounds and more than 6,000 flavonoids have been identified (*1*). Flavonoids are secondary metabolites that play critical roles in plant protection against environmental stress such as solar UV-B radiation (*1*). The flavonoid backbone is composed of two aromatic rings (A and B) connected via a three-member carbon bridge (C6-C3-C6) (Figure 1). The 4-oxo-flavonoids have a carbonyl group on the 4 position of the C-ring. The B-ring is typically attached at the 2 position of the C-ring in most flavonoids. Isoflavones are exceptions to this in that the B ring is attached at the 3 position. Based on the other substitutions and conjugations, flavonoids are subdivided into flavonones, flavonols, isoflavones, anthocyanidins, flavanols, flavanones, and proanthocyanidins. The flavonols most frequently found in plants are those with B-ring hydroxylation in the 3′,4′-positions (quercetin), 4′-position (kaempferol), and 3′,4′,5′-positions (myricetin).

Typically plants convert flavonoids into glycosylated-conjugates as these are more water-soluble and can be stored in aqueous plant compartments (e.g. vacuoles). Glycosides greatly increase the chemical diversity and complexity of the base flavonoid structure. The dominant types of flavonoid glycoside vary among species and cultivars (*2*, *4*). For example, in apples, quercetin is present as a mixture of 3-O-galactoside, 3-O-glucoside, 3-O-rhamnoside, and 3-O-rutinoside, whereas it occurs primarily as the 4′-O-glucoside in onions (*2*, *4*, *5*). The flavonoid glycoside composition affects gastrointestinal absorption and bioavailability in animals (*5*, *6*). For example, enzymatic conversion of hesperidin (hesperetin 7-*O*-glucose-rhamnose) to hesperetin 7-glucoside increases bioavailability two-fold (*7*). Whereas, the consumption of a purified quercetin 4′-*O*-glucoside, or onion dominating in quercetin 4′-*O*-glucoside, presented the same pharmacokinetic parameters over 24 h (*8*), but differed from the quercetin aglycone over 13 h (*6*).

Onions are a primary source of flavonoids in the Western diet (*9*). Flavonoid profiles in onions are relatively simple. Previous studies identified five primary flavonoids in onions by LC-(ESI)MS/MS which include: quercetin 3,4′-*O*-diglucoside, quercetin 3-*O*-glucoside, quercetin 4′-*O*-glucoside, isorhamnetin 4′-*O*-glucoside and quercetin aglycone (*2*). Additionally, studies indicate that there is large variation of flavonoids and their glycosides in different varieties of onions (Table 1). The predominant factor influencing the complement of flavonoids in food is genetics. Therefore, the profile of flavonoids is unique to a species and varieties within that species. Because profiles are unique there is the potential that they may be used as tools for authenticating varietals.

Herein we describe a study in which onions were used as a model to explore the application of UHPLC-(ESI)QTOF MS/MS for the non-targeted analysis (without standards) of onion flavonoids in order to establish varietal differences. Identifying the composition of flavonoids in a particular variety, and how they contrast between varieties, can be accomplished by using chromatography coupled to accurate mass TOF MS. Because TOF acquires mass spectral data by pulsing ions entering the flight tube in an orthogonal beam, full spectra are always collected unlike scanning instruments. The data captured is accurate enough to determine the elemental composition of the flavonoids therefore

allowing identification without standards (*10*). However, even with the data, the true identification may be difficult because there might be isomers and possible other compounds with the same elemental composition. Other techniques such as NMR can be used to increase the probability of identification. In contrast to targeted analysis (*11–13*), which requires the use of analytical standards to determine figures of merit for detection, identification, and quantification, non-targeted analysis employs technology with sufficient analytical 'power' to make a tentative identification from a list of compounds without having standards. An analogous example of this is the use of Kovats indices with gas chromatography (*14–16*). Today, accurate mass TOF MS provides the capability to measure pseudo-molecular ions with accuracy to better than 3 ppm (*17*, *18*). This provides the power to tentatively identify compounds using a library (list), in this case flavonoids.

This initial approach begins with a general survey of these chemically diverse compounds within a sample. This "survey" can then be searched against a database of flavonoids for tentative identification using the molecular formula (based upon exact mass) of each compound in the database. The formula search approach allows comparison of the exact mass, the theoretical isotope spacing, and the relative abundance mass of the adducted molecule (pseudo-molecular ion) to the measured masses found in the data. This procedure makes the assumption that the flavonoid composition can distinguish one variety of onion from another. Once a list of tentatively identified flavonoids is generated from the different varieties, they are further examined by PCA. This statistical approach allows the determination of whether these compounds will provide differentiation of variety. Given a sufficient dataset of varietal replicates, and that these compounds can make these distinctions, a mathematical model (for MS data typically partial least squares discrimination, PLSD, or support vector machine, SVM) (*19*) is constructed to predict variety. The prediction capability of the model truly demonstrates the selected set of compounds ability to establish variety. There are many examples of using both PCA and modeling for food authenticity and verification (*20–24*).

Another approach is to extract all possible compounds found in the single MS LC/ TOF MS data for the different varieties and then filter the found "unknown compounds" based on their presence in one variety and absence in another. The filtering should include not only the presence or absence, but relative concentration as well. This complex extraction or mining of the data may provide differentiation of variety without having to actually identify the actual distinguishing compounds (*25*). This approach, *unknown analysis,* allows one to apply statistical manipulations to compare the general chemical composition of individual varieties for identification. Combining the approach of non-targeted determination (i.e., the database search for flavonoids with the approach of unknowns) gives a varietal differentiation process of non-targeted unknown analysis.

MS/MS providing structural information, is necessary to increase the probability of true identification of the individual flavonoids tentatively identified by single LC/TOF MS; as accurate mass of a pseudo-molecular ion provides only an accurate molecular formula. Compounds that are statistically important

in differentiating samples using this approach, can be searched against larger databases; but again MS/MS is required for improved identification and true structural confirmation. It must be cautioned that the characterization obtained using this approach is limited to only those compounds that 1) are extracted in the sample preparation procedure and 2) those compounds that respond (ionize) to the instrumental technology employed.

Materials and Methods

Samples

Onions were obtained from Gills Onions, LLC (Oxnard, CA) in the summer of 2011. The flavonoid composition was characterized in the four different yellow onion varieties (Cowboy, Chief, Vaquero, and Sommerset) and three red onion varieties (Red Rock, Salsa, and Merenge).

Sample Extraction

The flavonoid extraction was performed following the method of Lee and Mitchell (*2*). Inner layers have limited anthocyanidins (red color). Briefly, after separating inner layers of the onion samples from the outer layer of onions, inner layers were lyophilized and extracted with 80% methanol for 20 minutes. The total extraction process was done in triplicate.

UHPLC-(ESI)QTOF MS Analysis

The instrumental methodology was kept broad to obtain as much "coverage" as possible. A reversed-phase UHPLC gradient, from high water content to high organic modifier, was used to separate polar to less polar compounds. Electrospray ionization was used as it generally can ionize a broad range of compounds, with exception of those that are relatively non-polar. Under these conditions, most flavonoids should respond well, however their relative response is highly impacted by their variation and conjugation. Some may respond only in positive ion mode and some only in negative and those that respond in both may have much higher ionization efficiency in one or the other. In addition, electrospray is subject to ion suppression and enhancement effects, and given the typical complexity of food, these effects are expected (*26*). Thus relative response is a poor indicator of concentration and concentration can only be obtained by comparison to standards of these compounds. To get an accurate measure of concentration, one needs to perform standard addition or use stable isotopes of the compounds to assess matrix effects.

Analysis was performed on an Agilent 1290 Infinity ultra-high pressure liquid chromatography system coupled to a 6530 accurate mass quadrupole time-of-flight mass spectrometer (UHPLC-(ESI)QTOF MS/MS) with electrospray ionization (ESI) via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with integrated vacuum degasser (G4220A), an auto sampler (G4226A) with thermostat (G1330B), and

thermostatted column compartment (G1316C). The 80% methanolic extracts were separated on a Poroshell 120 C_{18} column (2.1 x 100, 2.7 μm, Agilent Technologies). The flow rate was 0.4 mL/min and the injection volume was 5 μL. The mobile phase consisted of a linear gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: 5–10% B, 0–5 min; 10–12% B, 5–8 min; 12–15% B, 8–10 min; 15% B, 10–15 min; 15–55% B, 15–18 min; 55–90% B, 18–20 min. The column was re-equilibrated between injections for 4 min with initial mobile phase.

To identify all possible flavonoids, total ion spectra were collected over a mass range of m/z 100–1000 in negative mode at an acquisition rate of 1.0 spectra/s. The drying gas temperatures and flow rate were 225 °C and 8.0 L/min, respectively. The sheath gas temperature and flow rate were 300 °C and 10.0 L/min, respectively. The nebulizer gas pressure, skimmer voltage, octopole RF, and fragmentor voltage were 45 psi, 65V, 750 V, and 125 V, respectively. The capillary voltage was 2.5 kV. Continuous internal calibration was performed during analysis to achieve the desired mass accuracy of recorded ions with the ions of m/z of 119.0363 (proton abstracted purine) and 966.0007 (formate adduct of protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921).

Data Analysis

Using the open-access databases such as Phenol-Explorer (http://www.phenol-explorer.eu/) and Chemspider (http://www.chemspider.com/), 250 possible flavonoids and flavonoid conjugates were identified. The molecular formula of each flavonoid is imported into a Personal Compound Database and Library (PCDL) manager and used to create a flavonoid database (Agilent Technologies) containing the exact mass calculated from the molecular formula, other useful textual data, and the structure in mol file format (if available). This general flavonoid database can then be customized for a particular food. For example, herein flavonoid database was constricted to the flavonoids that could be plausibly be present in onions (e.g. isoflavones were excluded, etc.,). Using this approach, the list of flavonoids was reduced to 150 compounds. These 150 compounds were used to create a flavonoid database customized for analysis of onions. Using this customized flavonoid database and the "find-by-formula" option in the MassHunter Qualitative Analysis Software (Agilent Technologies), accurate mass tolerances were set and used to search each data file for ions of expected adducts (e.g., H^+, Na^+), dimers, trimers, etc. Potential flavonoids were identified based on a comparison of accurate mass, abundance of the isotopes, and isotope spacing with the calculated theoretical masses and abundances (performed automatically in MassHunter Qualitative Analysis). This is termed a "targeted" search for "non-target" compounds. That is we are looking specifically for all the compounds in the database without having standards. If standards are available, retention times can be also be added to the database and used as figure of merit for determining whether those specific compounds are present in the sample. The total ion chromatogram (TIC), shown of Merenge inner layers appears somewhat non-descript and not complex (Figure 2). However, there are many ions of compounds "hidden" under the TIC and whether these match a

flavonoid of interest can be discovered by specific search of the database using the "find-by-formula" algorithm. This algorithm extracts the ion (m/z) of each adduct specified determined from the exact monoisotopic mass of the formula within the tolerance specified (e.g. 10 ppm), and for as many charge states as specified (e.g. z=1, 2 and 3 but for the flavonoids only a charge state of z = 1 is used) and then integrates the resulting chromatogram. If a peak is found then all ions in that peak are evaluated against the theoretic isotopes expected for the molecular formula in the database. A score is calculated from those results and if that score exceeds a threshold set by the analyst, the compound is listed as found. If many peaks are found each is listed unless a retention time is specified. In that case then only the peak with the matching retention time is listed.

The other approach to the many ions found under the non descript TIC is a data mining (unknown) extraction of the compounds present using a "molecular feature extraction" algorithm. This algorithm takes all ions that represent chromatographic peaks (thus eliminating background ions) and groups them by adduct clusters, possible isotopes, dimers, trimmers etc. all taken together as molecular features without any determination of identity. Each feature is then calculated back to a "molecular mass" again without any identification assigned.

Both approaches provide a "list" of compounds, one tentatively identified from the database search, the other a list of "unknowns." After processing, these lists of compounds are converted into compound exchange format files (.cef files) for each sample and exported to data mining and statistical analysis software. In this case, Mass Profiler Professional (MPP) was used for data mining (Agilent Technologies). For data processing, other commercial software such as MarkerLynx software (Waters, Milford, MA, USA), Metabolic Profiler (Bruker Daltonic & Bruker BioSpin, Billerica, MA, USA), and Sieve (Thermo Fisher Scientific, Waltham, MA, USA) and freely available tool such as XCMS can be used (*27*). Statistical packages such as Minitab, R, and SAS can be used after data mining and data is exported as text or .csv format.

Results and Discussion

Database Mining Results

From the *find by formula* algorithm, the following 19 flavonoids were tentatively identified based upon "find-by-formula" criteria in seven onion varieties: delphinidin3-O-(6″-malonyl-glucoside), dihydromyricetin3-*O*-rhamnoside, dihydroquercetin, isorhamnetin, isorhamnetin 4′-*O*-glucoside, kaempferol, kaempferol 3-*O*-(6"-malonyl-glucoside), kaempferol 3,7-*O*-diglucoside, kaempferol 3-*O*-acetyl-glucoside, kaempferol 3-*O*-rutinoside, kaempferol 3-*O*-xylosyl-rutinoside, quercetin, quercetin 3,7,4′-triglucoside, quercetin -*O*-diglucoside, quercetin 3,4′-*O*-diglucoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, and quercetin 4′-*O*-glucoside. For unequivocal confirmation of identity, further MS/MS analysis and comparisons with MS/MS spectra and retention times of standards would be needed.

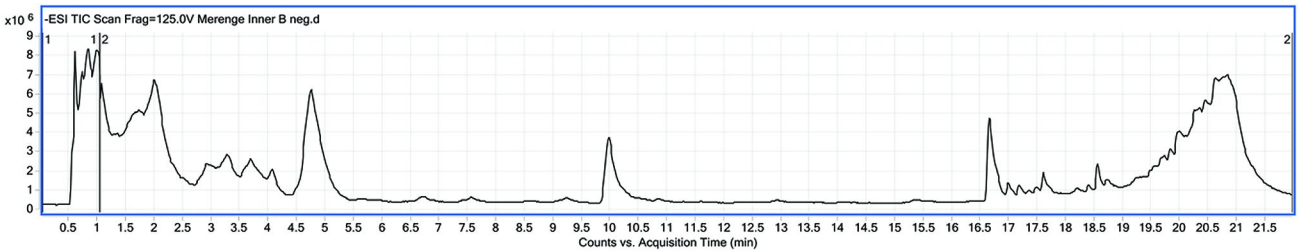

Figure 2. Total Ion Chromatogram of Merenge Onion Variety.

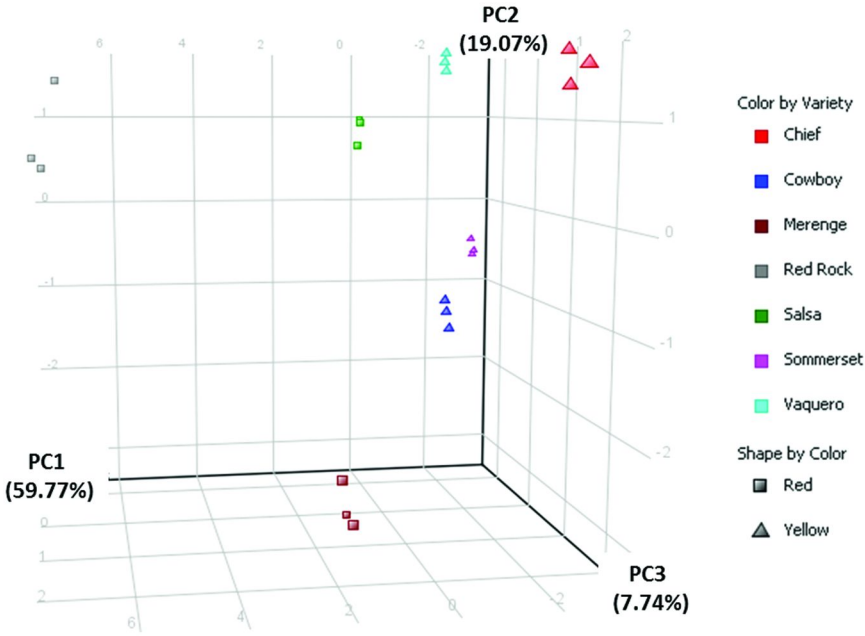

Figure 3. Principal Component Analysis on 19 Targeted Unknowns for Varietal Difference.

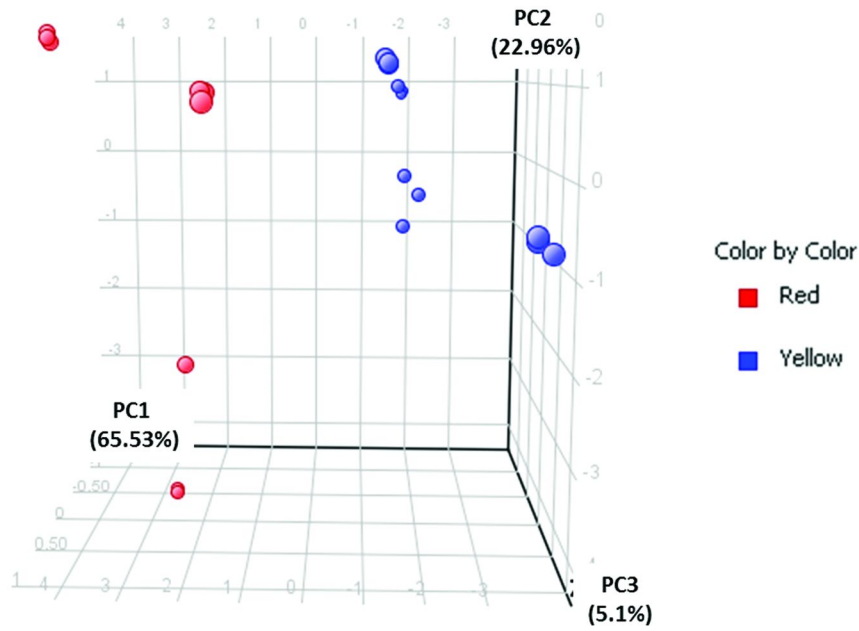

Figure 4. Principal Component Analysis on 19 Target Compounds for Color Difference.

Principal Component Analysis (PCA) on 19 Targeted Unknowns for Varietal and Color Difference

A PCA of the 7 varieties of onions based on the 19 compounds discovered in the database search show good separation of some of the varieties (Figure 3). Because analyses were on the "inner" layers of the onions, and extraction solvents excluded anthocyandins, the "distinction" of variety is not based upon obvious differences in pigment related compounds (e.g. anthocyanidin levels). One could argue that the Cowboy and Summerset varieties are not being separated, and that Chief, Vaquero and Salsa group together. The Redrock and Merenge verities have distinct separation from the other varieties (both are red varieties).

PCA analysis of the 7 varieties (herein 4 yellow and 3 red varieties were analyzed) indicate that there is no distinction between them based upon color although, there is some correlation along the x-axis (note the triplicate analysis of each variety are tightly grouped) (Figure 4).

Principal Component Analysis on Unidentified Compounds for Varietal and Color Difference

It is important to note that PCA of all the unknown compounds found by the unknown approach would be non-descript that is there is no assumption that compounds found may describe a variety. In the case of the flavonoid approach, these compounds were sought as descriptive. Prior to PCA, the data was normalized so that compounds in high concentration do not overly weigh PCA. Then, the list of unknowns must first be filtered to assure consistency within a variety, that is filtering by frequency within a group, and then filtered from variety to variety for distinction. The compounds that are statistically important in differentiating the samples, were filtered using ANOVA and/or fold change (how much that compound changed from one variety to the next), and then PCA was performed. Once compounds that were not common within a variety were eliminated and then those that were common from variety to variety the molecular feature extraction could be used to evaluate the general composition of the different varieties and whether color can be determined by these "unknown" compounds provides interesting and possibly distinguishing results (Figure 5). Again the ability of a model using this data to predict color or variety would be demonstrative in using the "unknown" compound data for differentiation. As Chief and Merenge are well separated in the PCA, the loadings and this list of compounds can be evaluated to find the compounds that can differentiate the two. The top 10 compounds tentatively identified responsible for colored differentiation are 6,8-dihydroxy kaempferol, kaempferol 3-*O*-(6"-malonyl-glucoside), kaempferol diglucoside-1, kaempferol diglucoside-2 (this is an isomer with a different retention time), kaempferol 3-*O*-acetyl-glucoside quercetin, quercetin 3,4′-*O*-diglucoside, quercetin diglucoside-1, quercetin diglucoside-2 (isomer with a different retention time), quercetin 3-*O*-rhamnoside and dihydroquercetin. However, there are a number of possible compounds for each formula generated from the data. Again, MS/MS can help determine which compound is actually present in the samples.

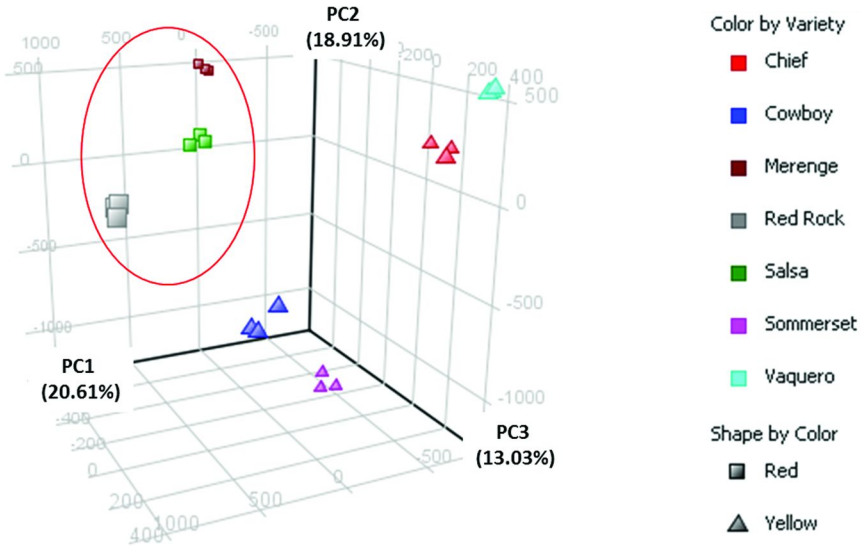

Figure 5. Principal Component Analysis on Unidentified Compounds for Varietal and Color Difference.

Conclusions

A flavonoid library for onions was developed using PCDL manager and included 150 entries. Nineteen flavonoid and flavonoid glycosides were identified in the methanolic extracts of 7 varieties of onions. Principal component analysis on these 19 target compounds demonstrates separation in varietal difference and color difference. Non-target analysis resulted in similar results. Tentative identification of the top 10 flavonoids is associated in the PCA space with color and variety differences. Although, more sampling of varieties grown under different conditions over time are needed to establish clear correlations, it appears that the non-targeted analysis of flavonoids by UHPLC-(ESI)QTOF MS/MS can be used to establish varietal differences.

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