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Omics Forecasting: Predictive Calculations Permit the Rapid Interpretation of High-Resolution Mass Spectral Data from Complex Mixtures

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Supporting Information

ABSTRACT: For some complex mixtures, chromatographic techniques are insufficient to separate the large numbers of compounds present. In addition, these mixtures often contain compounds with similar or identical molecular masses and shared fragmentation transitions. Advancements in mass spectrometry have provided more and more detailed molecular profiles with significant increases in resolution. This has led to a capacity to distinguish a very large number of compounds in complex mixtures, providing overwhelming data sets. The approach of calculating molecular formulas from a mass list has become more and more problematic as the number of signals has increased exponentially, to the point that it has become impossible to manually interpret the thousands of mass signals. The current approach is to calculate a list of possible formulas that fall within a specific mass error of the observed signal. Then, one must look for possible structures that can be derived from each entry on the list of formulas. However, an alternative approach is to anticipate the possible structures of a particular set of compounds, such as red wine pigments, and then compare the ion signals against a predicted list. To that end, starting with known wine pigment types, we have generated a set of expected wine pigment variants based on known derivatives of condensed tannin oligomers, anthocyanins, and fermentation products. After the ability to distinguish compounds by mass spectrometry was accounted for, over 1 million results were generated consisting of known and anticipated wine pigments. A comparison with a small sample of wine phenolic fractions show a large number of matches, suggesting that this approach may be helpful.

KEYWORDS: *wine pigments, mass spectrometry, mass filtering, anthocyanin, tannin, phenolic, high resolution mass spectrometry*

INTRODUCTION

The color exhibited by young red wine comes from grape-derived anthocyanins. Shortly after vinification, monomeric anthocyanin content decreases dramatically; yet, the wine remains red as more complex structures and anthocyanin derivatives are synthesized.[1] New compounds form throughout aging that are the products of reactions between anthocyanins and other wine components including tannins. These reactions are stimulated in part by oxidation reactions as well as acid catalyzed rearrangements.[2]

The condensed tannins of red wine are a complex mixture that includes oligomers and polymers of ±(epi)catechin, ±(epi)gallocatechin, ±(epi)catechin gallate, and ±(epi)-gallocatechin gallate, with degrees of polymerization from 2 to upward of 40 by some reports.[3] To add additional complexity, these compounds can be connected at two or more positions and need not be linear. Previous estimates place the number of possible individual tannin species on the order of 10^7.[4] When anthocyanins react with these tannins by known routes, the resulting number of pigmented products is even more numerous.[5]

There are six anthocyanins in grapes, as well as their glucosides, hydroxycinnamic acid, and acetic acid functionalized derivatives.[6] Proanthocyanidins also comprise a generous portion of the wine matrix. Fermentation products and grape constituents react under the acidic conditions of the wine medium to form new derivatives. Observed thus far, acetaldehyde and pyruvate react with anthocyanins to form the pyranoanthocyanins.[7] Further reaction of the pyranoanthocyanins with grape derived hydroxycinnamates forms pinotins and portisins.[8,9] Primarily acetaldehyde but also other aldehydes link anthocyanins and proanthocyanidins to form bridged oligomeric pigmented tannins.[10]

Further pigments can be found as the result of anthocyanin−proanthocyanidin bridging by aldehydes. The dimers malvidin-3-glucoside-ethyl-catechin and malvidin-3-(6-p-coumaroyl)-glucoside-ethyl-catechin have been identified in wine with the proposed mechanism of several condensation reactions of the carbocation form interaction between acetaldehyde and flavanol.[11] The common model molecule for discussions of pigmented tannin is the direct condensation product of anthocyanin and tannin, yielding the commonly depicted proanthocyanidin chain with a terminal anthocyanin.[12] The products of these reactions are not necessarily similarly pigmented or pigmented at all. Anthocyanin-pyruvate condensation products have also been suggested, forming vitisin A, which is found in wine and grape material[13,14] with the complete structure disputed until NMR[15] and mass spectrometry[10,16] confirmed the Fulcrand structure. Vinylformic adducts

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of pyruvate have also been identified, as have pyruvic acid derivatives of delphinidin, petunidin, and peonidin-3-(6-*p*-coumaroyl)-glucoside.[17] Other pigments have been identified as the results of anthocyanin-4-vinylphenol, and anthocyanin-vinylflavanol condensations. Hayasaka and Kennedy confirmed pigmented polymers up to octamer, but did so utilizing simpler techniques than we now have at our disposal.[12]

Nucleophilic addition of vinylphenols to malvidin-3-glucoside was found to produce some pyranoanthocyanins.[7] Further investigation into the pyranoanthocyanins found such structures as carboxypyranoanthocyanins, methylpyranoanthocyanins, pyranoanthocyanin-flavanols, pyranoanthocyanin-phenols, portisins, oxovitisins, and pyranoanthocyanin dimers.[18] Multitudinous reactants participate in a myriad of reactions leading to a combinatoric problem for which there are many resulting derivatives. The number of similar reactants and reactions also leads to a startling number of isomers. For instance, a simple transfer of a hydroxyl can retain the molecular formula but change the compound's base anthocyanin and residues. Applicable structures are provided in Supporting Information, and reviews on the structures described herein have been published with further details regarding their formation and structure.[19−21]

Classical unknown compound characterization techniques of nuclear magnetic resonance (NMR), and infrared spectroscopy (IR), are fruitless for the investigation of aged red wine pigments as the specific compounds of interest cannot be isolated with sufficient quantity and purity. Because of structural similarities and similar solvation rates even in competing solvents, a complex mixture is made only slightly less complex by solid phase extraction (SPE) or high-performance liquid chromatography (HPLC) with respect to wine pigments. In addition, the number of candidate substances would challenge the capacity of most laboratories.

Deciphering the muddle that is the wine matrix has been significantly improved by innovations in mass spectrometry (MS). Determining or corroborating molecular formulas of unknown and predicted compounds requires high mass accuracy and fragmentation to discriminate isomers. High resolution mass spectrometry currently provides mass accuracy consistently below 1 ppm mass error, and further structural elucidation by tandem mass spectrometry is now routine. The most powerful and increasingly common techniques are quadrupole time of flight (QToF) for fragmentation along with good mass accuracy, Fourier transform ion cyclotron resonance (FTICR) for the greatest mass accuracy but without fragmentation, and Orbitrap for both fragmentation as well as high mass accuracy.

The most basic interpretation of mass spectral data compares neutral mass losses to the parent ion, to build up the molecule by deduction.[22] This method works well for small compounds, but as the molecules grow larger, the number of variations with similar structures becomes too vast to isolate a single compound by chromatography. Thus, most commonly, fragmentation spectra are compared to a spectral library for known compounds.[23] In the case of wine tannin chemistry, and indeed most complex mixtures and natural products, the standards needed to generate such a spectral library do not exist. Likewise, the synthesis for less frequently observed, yet no less abundant, wine compounds is too costly and too time-consuming to be feasible.

To overcome the lack of comparative capacity, many studies circumvent compound identification and instead employ chemometrics. Chemometrics is the application of statistics to large sets of mass spectral data for the purposes of determining correlation between treatments and under the correct circumstances, progress can still be made pursuant to specific hypotheses.[24,25] If compound identification is desired for larger compounds, then it is a time-consuming, expensive, and labor-intensive process.[26]

When working with thousands of compounds for which there are no spectral libraries, and no standards, yet identification is necessary, predictions can be made to bridge the gaps in knowledge based upon known and observed reactions. As certain compounds are more abundant, they are thereby easier to extract and identify. However, lower relative abundance does not necessarily decrease the sensory impact. Wine is a solution of competing reactions, many of which are dependent on pH, oxygen exposure, ethanol concentration, and concentration of competitor molecules, to varying extents. Additional complexity is added when storage conditions are considered as the reactions of interest are also variably accelerated and decelerated by temperature, as well as have different affinities for oxidation as a result of oxygen ingress during storage.

The class of compounds called thearubigins are a recently characterized group of naturally occurring phenolics responsible for the brown pigment in tea, many of which are catechin-like derivatives. These structures have not been observed in wine, but their structural variation and analysis by mass spectrometric means make them relevant. FT-ICR data provided a model for tea fermentation, later confirmed by tandem mass spectrometry and successfully applied to cocoa, roasted coffee, and caramel demonstrating a structural elucidation strategy foundationally based in mass spectrometry.[27,28] They effectively used combustion analysis, IR spectroscopy, NMR spectroscopy, diffusion NMR spectroscopy, UV−vis spectroscopy, circular dichroism spectroscopy and atomic force microscopy, MALDI-TOF-MS, and ESI-FT-ICR-MS to interrogate 15 geographically diverse tea samples, observing 5000 such signals below 2000 Da.[29] In wine, untargeted FT-ICR has been used to delineate the forest origin of the barrels used for wine aging demonstrating the utility of such high mass accuracy techniques in complex mixtures.[30]

Sifting through such dense data sets as those obtained by ultra high resolution mass spectrometry requires exhaustive interrogation, but for the most part metabolomic-like data sets lack qualification and quantification.[31] Particularly of interest would be discriminating compounds, which may help differentiate samples. Indeed, such compounds would often be chosen first for characterization. Another strategy is the use of a candidate molecular structure database wherein a postulated set of compounds is generated and from which the spectral data set can be reduced to a manageable number of outcomes and is interrogated further within the limitations of current technology.[32] This enables the intensive analysis of isolates or extracts comprised of still large groups of compounds, from which we can generate a more refined "chemical space" to confirm postulates based on foreknowledge of the sample composition.[33]

Since malvidin-3-glucoside is the most prevalent anthocyanin in wine, it is often the first compound identified in a given set. Initially identified in wine were vitisin A and B containing malvidin-3-glucoside, but later, vitisins containing the other anthocyanins were found as well.[14,34] It follows that for each observed compound class there may exist many or all possible

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iterations of each anthocyanin, following the same reactions as those observed for malvidin-3-glucoside. The low intensity signals near the noise observed by many researchers may be due to these variants, but as their reactants are in lower abundance, so too will their derivatives be. It is a cogent assumption that higher order derivatives and other known reaction mechanisms behave similarly. Unobserved structures can therefore be calculated by extrapolating observed reaction mechanisms to additional reactants for which the products may be at lower levels. Though they have yet to be observed or may in fact be in too low abundance to distinguish a signal by current methods, it is no less reasonable to suppose that these additional variations of observed compounds are present.

This study provides predicted molecular formulas, mass values, and most probable ions for red wine pigments based upon extrapolation from known, naturally occurring, anthocyanin and tannin derivatives. The enumeration of proanthocyanidin structures as well as a mass spectral database comprising their distinguishable forms was recently completed and is incorporated.[35] Additionally, the total number of entries for each compound type have been calculated using basic combinatorics to ensure that all derivatives have been accounted for. The derived data table is then interrogated for discriminating metrics (molecular masses, number of isomers, m/z separation) which provide insight into the nature of the wine pigments.

MATERIALS AND METHODS

Molecular formulas, molecular masses, and ion signals were all computed using Microsoft Excel 2016 and published atomic masses.[36]

The 42 base anthocyanin variations were mathematically "reacted" with each variation of the reactants (pyruvate, proanthocyanidin oligomers, etc.) so that molecular formulas could be determined by summing the molecular formulas of reactants and subtracting the appropriate net change (representing loss of atoms occurring during bond formation) in CHO from that total (Table 1). In this manner, derivatives were calculated for all possible combinations of anthocyanin and available reactants of the following classes of compounds: proanthocyanidin oligomers, pyranoanthocyanins, anthocyanin-proanthocyanin direct condensation products, and aldehyde bridged anthocyanin-proanthocyanin oligomers.

Table 1. Net Element Change by Class

Class	Reactants	Net Change (C, H, O)
anthocyanin, aglycon, acetyls	anthocyanin, hydroxycinnamic acids	base
proanthocyanidin oligomers	proanthocyanidin	0,2(n−1),0
vitisin	anthocyanin, pyruvate or acetaldehyde	0,4,1
pinotin (hydroxyphenyl-pyranoanthocyanin)	anthocyanin, 4-vinylphenols	1,4,2
flavanyl pyranoanthocyanin	anthocyanin, vinylproanthocyanidin	0,4,0
portisin (phenyl-vinylpyranoanthocyanin), flavanyl vinyl-pyranoanthocyanin	vitisin A, 4-vinylphenols, Vinylproanthocyanidin	1,2,2
oxovitisin	vitisin A, water	1,3,2
anthocyanin-proanthocyanidin direct condensation (AT/TA)	anthocyanin, proanthocyanin	0,1,0 (AT) 0,2,0 (TA)
aldehyde bridged anthocyanin-proanthocyanidin	anthocyanin, aldehyde, proanthocyanidin	0,2,1

Database Construction. Construction of the database of anticipated compounds started by collecting structures of well characterized wine pigments and grouping them into classes (broad categories such as the pyranoanthocyanins, proanthocyanidin oligomers, etc.) and subclasses (refined category such as oxovitisins as a subclass of pyranoanthocyanins). Variants of the reactants for which structural similarities are sufficient to expect equivalent reaction mechanisms were coupled, followed by interpolation of the applicable reaction mechanisms using the various reactants known to be present in wine. Molecular formulas of reactants were modified by the appropriate reaction mechanism and then the resulting molecular formulas, ion formulas, and respective masses were calculated producing a list of predicted wine pigments and proanthocyanidin oligomers. The database enumeration was limited to those compounds (and isomers) which have the potential to be differentiated using mass spectrometry, referred to as mass spectrometrically distinguishable. The four stereoisomers of catechin/epicatechin, for instance, are not distinguishable by mass spectrometry, and sufficient chromatography for the separation of such stereoisomers has not been demonstrated to enough effect as to be useful in identification of complex mixtures such as wine pigments or for separation of oligomers of stereoisomeric variations.

The database was designed to provide the molecular formulas for all possible combinations of reactants processed through their respective reaction mechanism. Therefore, the formulas and masses provided are for the molecule itself. The most probable ion for much of these compounds would be the protonated $[M + H]^+$ or naturally occurring $[M]^+$ in positive mode or the $[M - H]^-$, $[M - 2H]^-$ in negative mode. There will certainly be possible or indeed many sodiated and other adducts observable. They are not included here as they are a linear transformation that adds no additional complexity for discussion and one that is handled by most data analysis programs.

Sample Wine Pigmented Tannin Spectra. Demonstration of the database application to real samples was accomplished using lyophilized wine pigmented tannin extracts prepared on Sephadex LH-20 by previously established methods.[37] Samples were prepared at 2 mg/L in methanol/acetone (87.5:12.5) acidified with formic acid at 2%. Mass spectra were collected in positive mode by direct infusion electrospray ionization on a Varian MS-920 hybrid triple quadrupole Fourier transform ion cyclotron resonance mass spectrometer (ESI FT-ICR MS) with an actively shielded 9.4 T superconducting magnet using established methods.[38] Fragmentation and mass filtering capabilities of the triple quadrupole were not utilized.

RESULTS

Anthocyanins. There are six anthocyanidins in grapes and wine, malvidin-, cyanidin-, delphinidin-, peonidin-, petunidin-, and pelargonidin-3-glucoside. First reported in 2010 in Cabernet Sauvignon and Pinot noir, pelargonidin is present in such low amounts that it was believed to be potentially absent from *Vitis vinifera* until recently.[21] Those anthocyanins can be present in any of seven variations as their aglycon, glucoside, or acyl glucosides (acetyl, coumaroyl, feruloyl, caffeoyl, and sinapoyl). Though unobserved in wine, there appears to be no biosynthetic, kinetic, or steric hindrance preventing sinapic acid from producing a derivative of anthocyanidin glucoside in grapes. Sinapic acid products may just be low in abundance due to competitive reactions that deplete the pool of sinapic acid available to anthocyanins like conversion to 4-vinylsyringol and reaction to form pyranoanthocyanins.[39] Indeed, a study on the reactivity of hydroxycinnamic acids with anthocyanin to form pyranoanthocyanins found sinapic acid to be the most reactive.[40] Also, anthocyanin sinapoyl diglucosides have been reported in broccoli sprouts, and anthocyanin sinapoyl sophoroside glucoside has been reported in radish.[41,42] It is safer to assume that sinapic acid derivatives have yet to be observed in wine, just as was demonstrated with pelargonidin, than to say that they are not present. Sinapic acid is therefore included in this database as a potential anthocyanin glucoside hydroxycinnamic acid deriva-

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Table 2. Compound Enumeration by Class and Ion Type by Polarity

Class/subclass	Positive	Negative	Number	Class/subclass	Positive	Negative	Number
Proanthocyanidin Polymers			**1000**	**Anthocyanin−Proanthocyanidin Bridged**			**924,000**
monomer	$[M + H]^+$	$[M - H]^-$	4	formaldehyde (methanal)	M+	$[M - 2H]^-$	42000
dimer	$[M + H]^+$	$[M - H]^-$	10	acetaldehyde (ethanal)	M+	$[M - 2H]^-$	42000
trimer	$[M + H]^+$	$[M - H]^-$	20	2-methylpropanal (isobutyraldehyde)	M+	$[M - 2H]^-$	42000
tetramer	$[M + H]^+$	$[M - H]^-$	35	2-methylbutanal	M+	$[M - 2H]^-$	42000
pentamer	$[M + H]^+$	$[M - H]^-$	56	3-methylbutanal (isovaleraldehyde)	M+	$[M - 2H]^-$	42000
hexamer	$[M + H]^+$	$[M - H]^-$	84	octanal	M+	$[M - 2H]^-$	42000
heptamer	$[M + H]^+$	$[M - H]^-$	120	nonanal	M+	$[M - 2H]^-$	42000
octamer	$[M + H]^+$	$[M - H]^-$	165	decanal	M+	$[M - 2H]^-$	42000
nonamer	$[M + H]^+$	$[M - H]^-$	220	(*E*)-2-octenal	M+	$[M - 2H]^-$	42000
decamer	$[M + H]^+$	$[M - H]^-$	286	(*E*)-2-nonenal	M+	$[M - 2H]^-$	42000
Anthocyanin			**42**	phenylacetaldehyde	M+	$[M - 2H]^-$	42000
aglycon	M^+	$[M - 2H]^-$	6	diacetyl	M+	$[M - 2H]^-$	42000
glucoside	M^+	$[M - 2H]^-$	36	acetoin	M+	$[M - 2H]^-$	42000
Pyranoanthocyanin			**42462**	furfural	M+	$[M - 2H]^-$	42000
vitisin (B-Type)	M^+	$[M - 2H]^-$	42	sotolon	M+	$[M - 2H]^-$	42000
vitisin (A-Type)	M^+	$[M - 2H]^-$	42	3-methyl-2,4-nonanedione	M+	$[M - 2H]^-$	42000
pinotin	M^+	$[M - 2H]^-$	168	pyruvic acid	M+	$[M - 2H]^-$	42000
portisin	M^+	$[M - 2H]^-$	168	glyoxylic acid	M+	$[M - 2H]^-$	42000
oxovitisin	$[M + H]^+$	$[M - H]^-$	42	glyoxal	M+	$[M - 2H]^-$	42000
flavanyl pyranoanthocyanin	M^+	$[M - 2H]^-$	42000	methylglyoxal	M+	$[M - 2H]^-$	42000
Anthocyanin--Proanthocyanidin Condensation			**84000**	vanillin	M+	$[M - 2H]^-$	42000
AT	$[M + H]^+$	$[M - H]^-$	42000	methional	M+	$[M - 2H]^-$	42000
TA	M^+	$[M - 2H]^-$	42000				

tive. With all possible variations, there are 42 base anthocyanins from which higher order derivatives and oligomers can be made.

Pyranoanthocyanins - Vitisins, Pinotins, Portisins, Pyranones. Simple modifications of the base anthocyanins produce the pyranoanthocyanins. Reaction with acetaldehyde and pyruvic acid produces vitisins B and A respectively, yielding 84 possible vitisins. Anthocyanin reaction with the four hydroxycinnamic acids, coumaric, caffeic, ferulic, and sinapic, generates what appears to be a vitisin with a phenol, catechol, guaiacol, and syringol ring attached, respectively. There are 168 possible of these pinotin-type structures. Likewise, 168 possible portisins are generated by the same hydroxycinnamic acids adding to vitisin A. Decarboxylation of vitisin A can also lead to the formation of 42 pyranone-type compounds. Flavanyl-pyranoanthocyanins, consisting of a pyranoanthocyanins linked to any of the proanthocyanidin oligomers up to decamer produces 42 000 possible variations. All totaled, there were 42 462 pyranoanthocyanins generated.

Proanthocyanidin Oligomers. There are 1000 mass spectrometrically distinguishable proanthocyanidin oligomers from monomer to decamer.[35] The portisins and pinotins can form flavanyl pyranoanthocyanins with oligomeric substituents on the pyran ring numbering 42 000 each assuming oligomers up to decamer. Direct condensation reactions between anthocyanins and proanthocyanidins can take place at three positions. Bonding of the proanthocyanidin chain at the C6 or C8 position of the anthocyanin yields the tannin-anthocyanin (T-A) products which retain the chromophore and result in the loss of 2 net hydrogens, whereas bonding at the C4 position creates the anthocyanin-tannin (A-T) products, only losing 1 net hydrogen while eliminating a double bond on the C-ring, thus eliminating the conjugation responsible for red color. Assuming only terminal anthocyanins, this amounts to 42 000 A-T and 42 000 T-A anthocyanin tannin condensation products. Were we to account for anthocyanins in nonterminal positions, they would be mass spectrometrically indistinguishable from the A-T products of the same degree as bonding at C6 or C8 has the same net change in the molecular formula as proanthocyanidin condensation, but the bond at C4 only loses one hydrogen. The dicyclic form would likewise not be mass spectrometrically distinguishable.

Aldehyde Bridges. Aldehydes such as glyoxylic acid, acetaldehyde, furfural, and vanillin have been shown to form bridges between anthocyanins and proanthocyanidins. It has thus far not been demonstrated that multiple bridges occur, connecting between additional oligomeric subunits of the same molecule, so we will only consider the case of a single bridge. Furthermore, the bridging of subunits other than that of a terminal anthocyanin has not been identified nor would mass spectrometry likely be able to distinguish that position of the bridge, so we will only consider the bridging of an anthocyanin terminal to a proanthocyanidin oligomer for these calculations. With 42 base anthocyanin variations, and 1000 oligomeric substituents, each aldehyde bridge has 42 000 variants for a total of 924 000 from 22 known wine aldehydes and carbonyl compounds with potential aldehydic bridging capability (methanal, acetaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, octanal, nonanal, decanal, (*E*)-2-octenal, (*E*)-2-nonenal, phenylacetaldehyde, diacetyl, acetoin, furfural, sotolon, 3-methyl-2,4-nonanedione, pyruvic acid, glyoxylic acid, glyoxal, methylglyoxal, vanillin, and methional).[43]

Anthocyanin Oligomers. Similarly to the proanthocyanidin oligomers, anthocyanins have been shown to polymerize.[44−46] As few reports exist on anthocyanin oligomerization, only their combinatoric assessment has been made. They are not included in the database as more work is required on their potential structures, though they may be of interest in the future. Given 42 base anthocyanin variants, there would be 903 dimers, 13 244 trimers, and 148 995 tetramers, for the direct

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condensation products using the same formula developed for the proanthocyanidin oligomers to be discussed, also not accounting for order of arrangement. There would be more than 1.58×10^{10} oligomers of anthocyanins up to the decamer.

Complete counts of each compound by class are available in Table 2. Files containing this database are in the Supporting Information for use in further investigation.

DISCUSSION

Accuracy and Precision Requirements (Delta Da). One advantage of creating the entire database is that the database can be interrogated for the relevant mass spectrometric parameters necessary for successful discrimination between ion formulas. The difference in m/z between adjacent signals reveals the required mass accuracy to discriminate between these compounds (Figures 1 and 2). The minimum difference

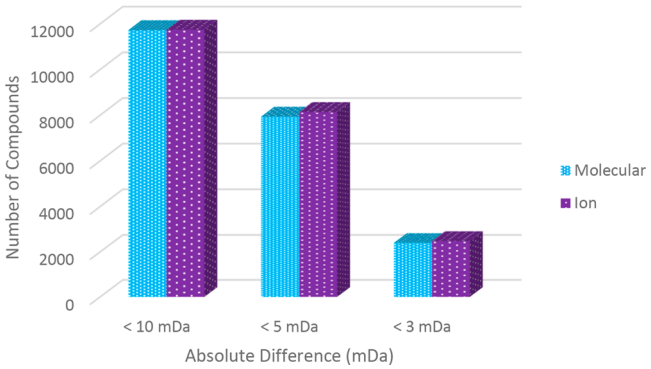

Figure 1. Mass accuracy (mDa) required for discrimination of adjacent molecular and ion formulas.

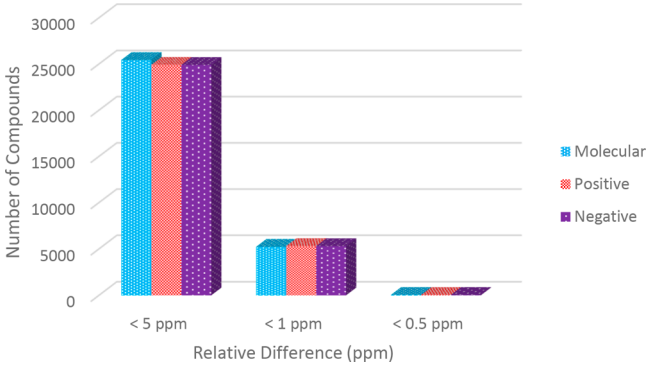

Figure 2. Mass accuracy (ppm) required for discrimination of adjacent molecular and ion formulas.

between molecular and ion formulas in the database is 2.5 mDa or 0.48 ppm, assuming only proton addition or removal. The smallest adjacent signal difference in mDa and ppm represent different compounds since relative mass error decreases with increasing mass.

There are 46 molecular and ion formulas for which signal discrimination requires better than 0.5 ppm mass accuracy (Table 3). Additionally, there are 2401 molecular formulas and 2485 ion formulas for each positive and negative modes near 2.5 mDa. Mass spectrometers are now routinely capable of obtaining spectra with a mass accuracy of less than 1 ppm. Indeed, the capabilities of FT-ICRMS can achieve near 0.05 ppm in some studies.[47]

Table 3. Mass Accuracy (mDa, ppm) Required for Discrimination of Adjacent Molecular and Ion Formulae

	<10 mDa	<5 mDa	<3 mDa	<5 ppm	<1 ppm	<0.5 ppm
Molecular	11728	7937	2401	25435	5223	46
Positive	11745	8143	2485	24920	5311	46
Negative	11745	8143	2485	24900	5304	46

Some of these compounds are already positively charged, as is characteristic of the anthocyanin chromophore, while others are not. There are many compound pairs whose molecular formulas differ by one hydrogen, but upon ionization the signals have identical ion formulas. This occurs when a compound naturally M^+ is one hydrogen greater in the molecular formula than an uncharged proanthocyanidin, which becomes ionized to $[M + H]^+$ during analysis. This is also observed in negative mode when M^+ loses two hydrogens and M loses one hydrogen yielding a signal pair of $[M^+ - 2H]^-$, $[M - H]^-$. $C_{100}H_{88}O_{43}$ (A-T direct condensation of malvidin-3-acetyl glucoside with pentacatechin) and $C_{100}H_{89}O_{43}$ (acetaldehyde bridged malvidin-3-glucoside with a proanthocyanidin pentamer of four catechins and one epigallocatechin), for example, have different molecular formulas, but the same ion signal formula. $C_{100}H_{88}O_{43}$ has no isomers, while $C_{100}H_{89}O_{43}$ has 10 isomers. The ion signal therefore has 11 isomers, making the potential pigment compounds indistinguishable in the molecular ion signal from a nonpigment. There are 6617 such pairs in positive and negative modes.

Structures arising from different compound classes may also have the same ion formula or remarkably similar atomic ratios, making discrimination of compound class by data visualization techniques unsuccessful. Indeed, the prevalence of such similarities cause Van Krevelen diagrams to display no distinct regions separating compound classes within this database from one another (Supporting Information).

Isomers. Compound identification is further complicated by the number of isomers that exist for each molecular formula and ion signal. Of the 1 051 504 compounds in this database, only 47 374 molecular formulas exist. Because of pairing, 6617 of those become signal isomers in positive and negative modes in which each has 40 757 ion formulas. Of the molecular formulas, only 6941 are unique in that they have only a single isomer, and 5217 unique ion signal formulas in positive and negative modes. That means that only 15% of molecular formulas and 13% of ion formulas are compounds that can be identified solely by formula. Greater than 85% of the potential signals therefore require fragmentation for isomeric discrimination. The average molecular formula has approximately 22 isomers, and the average ion signal in both positive and negative modes has 26 isomers. The molecular formula with the most isomers is $C_{212}H_{169}O_{97}$ with 363 isomers.

Disambiguation can be obtained using sufficient fragmentation by tandem MS. However, fragmentation signals associated with one and only one specific isomer, unique transitions, are likely to be few, making the likelihood that all isomers will display fragmentation spectra sufficient for discrimination quite low. To successfully discriminate these compounds from one another, additional work must be done to provide predicted fragmentation patterns. This has been accomplished for a subset of pigments.[48,49] The employment of chromatographic separation will be essential for comprehensive analysis of these

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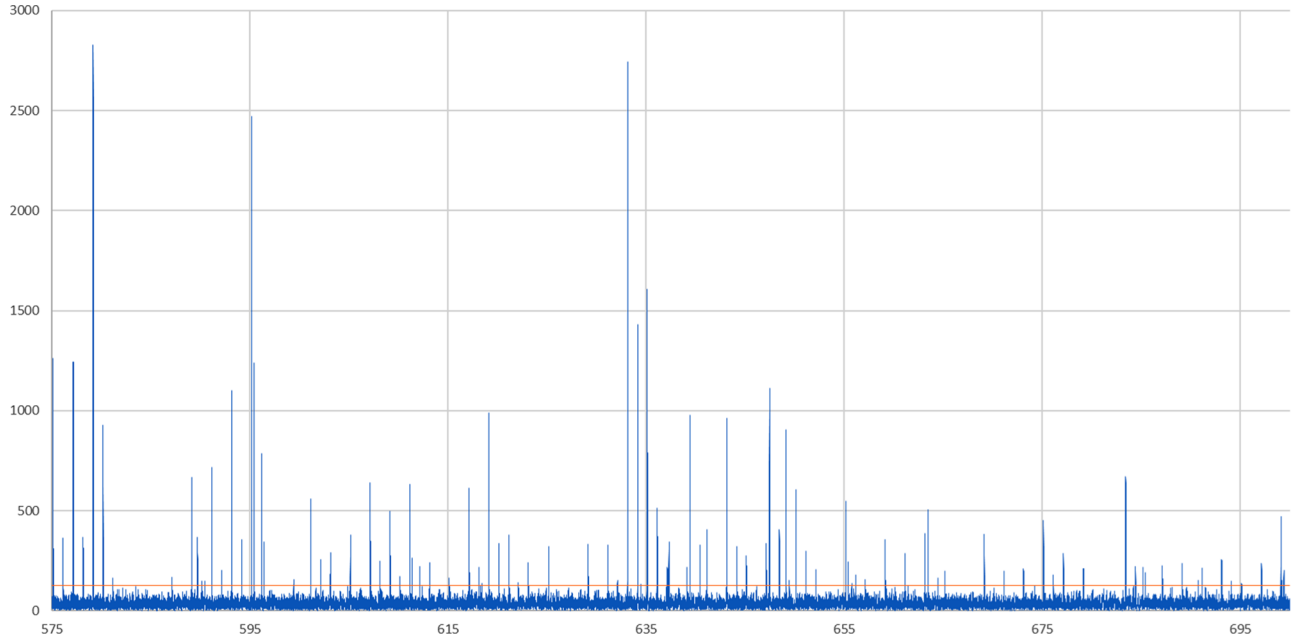

Figure 3. FT-ICR spectrum of third fraction 1990 Cabernet Sauvignon from 575 to 700 *m*/*z*.

Table 4. Positive Ion Formula Matches for a Sample of 1990 Cabernet Sauvignon

Ion Formula	Isomers	Sample Compound	*m*/*z*	Ion Mass	mDa	ppm
$C_{30}H_{23}O_{12}$	2	cyanidin aglycon catechin (TA-M^+)	575.1198	575.1184	1.4	2.4
$C_{30}H_{25}O_{12}$	2	cyanidin aglycon catechin (AT-$[M + H]^+$)	577.1353	577.1341	1.2	2.2
$C_{30}H_{27}O_{12}$	4	catechin dimer ($[M + H]^+$)	579.1508	579.1497	1.1	1.9
$C_{31}H_{25}O_{12}$	3	peonidin aglycon catechin (TA-M^+)	589.1359	589.1341	1.8	3.1
$C_{30}H_{23}O_{13}$	2	cyanidin aglycon epigallocatechin (TA-M^+)	591.1153	591.1133	2.0	3.4
$C_{31}H_{27}O_{12}$	4	pelargonidin acetyl-glucoside coumaric acid (pinotin-M+)	591.1507	591.1497	1.0	1.7
$C_{30}H_{25}O_{13}$	2	cyanidin aglycon epigallocatechin (AT-$[M + H]^+$)	593.1306	593.1290	1.6	2.8
$C_{30}H_{27}O_{13}$	6	catechin epigallocatechin ($[M + H]^+$)	595.1458	595.1446	1.2	2.0
$C_{32}H_{25}O_{12}$	1	glyoxal bridged pelargonidin aglycon catechin (M+)	601.1351	601.1341	1.0	1.7
$C_{31}H_{25}O_{13}$	4	peonidin aglycon epigallocatechin (TA-M+)	605.1302	605.1290	1.2	2.0
$C_{32}H_{29}O_{12}$	2	peonidin acetyl-glucoside coumaric acid (portisin-M^+)	605.1656	605.1654	0.2	0.4
$C_{30}H_{23}O_{14}$	1	delphinidin aglycon epigallocatechin (TA-M^+)	607.1096	607.1082	1.4	2.3
$C_{31}H_{27}O_{13}$	6	cyanidin acetyl-glucoside coumaric acid (pinotin-M^+)	607.1455	607.1446	0.9	1.5
$C_{31}H_{29}O_{13}$	5	peonidin coumaroyl-glucoside (M^+)	609.1618	609.1603	1.5	2.5
$C_{30}H_{27}O_{14}$	5	epigallocatechin dimer ($[M + H]^+$)	611.1404	611.1395	0.9	1.4
$C_{33}H_{25}O_{12}$	1	peonidin vitisin B-type catechin (flavanyl-pyrano-M^+)	613.1352	613.1341	1.1	1.9
$C_{32}H_{25}O_{13}$	3	glyoxylic acid bridged pelargonidin aglycon catechin (M^+)	617.1301	617.1290	1.1	1.8
$C_{32}H_{27}O_{13}$	8	cyanidin coumaroyl-glucoside vitisin B-Type (M^+)	619.1459	619.1446	1.3	2.1
$C_{33}H_{25}O_{13}$	2	peonidin vitisin B-type epigalloatechin (flavanyl-pyrano-M^+)	629.1309	629.1290	1.9	3.1
$C_{32}H_{27}O_{14}$	7	delphinidin coumaroyl-glucoside vitisin B-type (M^+)	635.1415	635.1395	2.0	3.1
$C_{35}H_{27}O_{12}$	1	furfural bridged pelargonidin aglycon catechin (M^+)	639.1508	639.1497	1.1	1.7
$C_{34}H_{27}O_{13}$	1	malvidin aglycon vitisin B-type catechin (flavanyl-pyrano-M^+)	643.1465	643.1446	1.9	2.9
$C_{33}H_{27}O_{14}$	8	pelargonidin coumaroyl-glucoside vitisin A-Type (M^+)	647.1415	647.1395	2.0	3.0
$C_{33}H_{29}O_{14}$	9	petunidin coumaroyl-glucoside vitisin B-Type (M^+)	649.1562	649.1552	1.0	1.6
$C_{34}H_{27}O_{14}$	1	malvidin aglycon vitisin B-type epigallocatechin (flavanyl-pyrano-M^+)	659.1409	659.1395	1.4	2.1
$C_{33}H_{27}O_{15}$	8	cyanidin coumaroyl glucoside vitisin A-Type (M^+)	663.1359	663.1344	1.5	2.2
$C_{34}H_{31}O_{14}$	10	malvidin coumaroyl glucoside vitisin B-Type (M^+)	663.1728	663.1708	2.0	3.0
$C_{36}H_{29}O_{13}$	1	furfural bridged peonidin aglycon catechin (M^+)	669.1615	669.1603	1.2	1.8
$C_{35}H_{27}O_{15}$	1	furfural bridged delphinidin aglycon epigallocatechin (M^+)	687.1347	687.1344	0.3	0.4

compounds. Analysis is made more difficult by the anticipated low abundances of unobserved possible structures.

Comparison to FTICR-MS Samples. By calculating the masses of predictable products, it is possible to anticipate mass spectrometric observations and provide tentative assignments that can be confirmed or rejected. With mixtures containing millions of possible structures, it is not possible to assess each structure de novo by hand, but comparing the list of predicted formulas against observed data greatly decreases its complexity for a targeted interrogation. High accuracy mass spectrometry, such as FTICR, ToF, and orbitrap, can establish lists of molecular formulas to be compared to the database of

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anticipated compounds for a preliminary filtering of the signals and provide candidates for continued fragmentation work by MS^n examination.

In a previous study, phenolic extract of a 1990 Cabernet Sauvignon was obtained by fractionation on Sephadex LH-20.[37] Remaining sample was analyzed by FT-ICR as a demonstration of the use of this candidate molecular structure database. Presented is the third and final fraction narrowed to a high signal density mass window of 575−700 m/z and compared to the pigment and tannin database discussed herein (Figure 3). Matches were considered for a maximum difference of 2.0 mDa (roughly 3.5 to 2.9 ppm in that range). Of the 63 ion signals accepted above the noise, 29 matches were made to molecular formulas in the database (Table 4). This constitutes 46% of signals matched, and 50% of signal intensity matched. A positive match does not necessarily constitute a positive qualification but does provide candidates for targeted compound identification by fragmentation analysis, an essential step given the number of isomers observed for these matches, an average of 3.8 for this data. High enough mass accuracy can identify molecular formulas for greater confidence in candidate structures. The sample contains many more compounds than those in our database. We are not looking at general phenolics, though we know the samples analyzed here contain more phenolics we are not considering; however, to see a 50% match to the database indicates a significant utility for this type of comparative filtering.

CONCLUSION

As more is learned about wine matrix components, and the associative effects on flavor and aroma perception, it becomes more important for wine quality and health implications to identify specific compounds and their amounts. Wine science has ventured beyond bulk assays such as the Harbertson−Adams assay or methyl cellulose precipitation. Numerous derivatives have been observed in the literature; yet (numbering less than 10 000), they comprise less than one percent of those possible.

Greater understanding cannot be accomplished without a more comprehensive qualification and quantification of these trace components of the wine matrix. As the discipline moves in that direction, a larger set of analytes must be considered. Reduction of this list to those sensorially relevant cannot currently be done because few compounds of this set have been characterized as to their sensory perception, and even fewer have been characterized comprehensively enough to extrapolate a reasonable predicted sensory impact as to be useful with more specificity than astringency implications of broad classes. Certainly, those products with the most abundant reactants are most likely to be of sensorially relevance, but predicting sensory importance in such a way without accounting for reaction kinetics would be a likely unrepresentative statistical prediction. Consideration of the reaction kinetics leading to creation of the unobserved, predicted compounds described here would be speculative and would not accomplish a reduction in the processing of these data sets, as the competition of the multiple reactions occurring simultaneously has not been sufficiently assessed.

When considering the identification of compounds in complex mixtures, all possible anticipated structures arising from high mass accuracy signals must be considered, narrowed only by parameters based on foreknowledge of the sample to avoid mischaracterization. This database provides an upper bound for the possible compounds responsible for mass spectral signals extrapolated from currently characterized pigmented tannin. Assuredly, many of these compounds will never be observed, but as of now they must be considered until knowledge precluding their existence can rule them out. That is not to say that it is unreasonable to assert high confidence in some compound assignments over others if considering such factors as precursor abundance, sample age, varietal composition, inhibitory or promotive production practices.

As demonstrated by interrogation of the database, comprehensive elucidation of these pigments can only be achieved by employing multiple techniques of analysis. Though accurate mass can identify many compounds, fragmentation is required to differentiate isomers. Chromatographic separation must also be applied to enhance this differentiation.

The benefit of constructing a database, rather than a deconstructive algorithm, is the ability to interrogate the database for specific properties and instrumental requirements for identification and discrimination. Starting from published pigment structures, over 1 million mass spectrometrically distinguishable variations on those structures were formulated based on the similarly structured precursors of the same compound classes.

Given the structural similarity of these compounds, it was expected that no discriminating factors between subclasses exist based on the molecular formula, making high resolution mass spectrometry valuable for the development of molecular formulas but incomplete for compound identification in most cases. Fragmentation profiles are essential to compound identification by mass spectrometry within this set of analytes. This database was constructed in such a way that fragment signals may be postulated based on subunit composition directly from the compositional naming scheme.[49]

Examining the data from a real wine tannin sample, approximately 50% of HS-MS signals were matched to the database of predicted compounds. This does not mean that each matched signal has been effectively narrowed to the few isomers displayed. It is possible and indeed probable that currently unknown structures are responsible for some of the signals for which matches were made and that those yet to be discovered compounds are isomers of compounds in the database. Those signals that match compounds in the database can be represented as tentative assignments. Those compounds in the database for which no match is observed may be assessed as too low in abundance to meet sensitivity thresholds and over the course of enough studies may be asserted to not be present with confidence. MS signals for which no match can be made are excellent candidates for MS^n studies, leading to new compound identities.

MS signals having a unique molecular ion (no isomers) are significant in that high mass accuracy can trace the signal to a single molecular source. In the case of wine research, this has several implications. For instance, treatment effects resulting from various sources can be determined. The exclusive source (grape, fermentation product, barrel, etc.) may be helpful for treatment markers. Unfortunately, of those unique formulas, many or most will undoubtedly be in extremely low abundance.

It is important to remember the limitations of mass spectrometry so as not to allow assumptions that lead to misidentification. The calculations provided here demonstrate the vast number of possible pigment compounds and the extent to which their discrimination can be difficult. The data

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do suggest that current mass spectrometry is technologically capable of measuring these compounds if in sufficient abundance, but also that the qualification of pigmented tannin is far more complex than is generally discussed. Automated analysis of mass spectral fragmentation is needed to distinguish among the many substances present.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b04384.

Pigmented tannin database - Table of molecular mass by compositional name (XLSX)

Table of negative ion type and formula (XLSX)

Table of positive ion type and formula (XLSX)

Table of ion pairs (XLSX)

Pigmented tannin isomers by molecular mass and formula (XLSX)

Van Krevelen plots, structures (PDF)

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Notes

The authors declare no competing financial interest.

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