Application Note

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Identification and Semiquantitation of Monoterpene Glycosides in Ripening Muscat of Alexandria Grapes

Application of UHPLC-ESI Accurate-Mass Q-TOF LC/MS and MS/MS

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Abstract

This Application Note presents the workflow and software used to study monoterpene glycosides in Muscat of Alexandria grapes at different stages of ripening. An Agilent 1290 Infinity LC with an Agilent 6530 Accurate-Mass Q-TOF LC/MS was used to identify and semiquantitate the abundance of 18 different monoterpene glycosides found in the grapes. The process starts with the creation of a Personal Compound Database Library (PCDL) and the search for potential compounds with untargeted single mass spectrometry. Second, is the generation of fragmentation spectra through auto MS/MS. From the fragments, the correlation and identification of structures are done using Agilent Molecular Structure Correlator (MSC). The process finishes with an update of the PCDL with the found retention times and the creation of an Agilent MassHunter quantitative analysis method that will auto-integrate chromatograms for semiquantitation. This process allows for the identification and semiquantitation of nonvolatile molecules in the absence of chemical standards and without derivatization.

Introduction

The original report for this study on monoterpene glycosides can be found in the journal Analytica Chimica Acta[1]. Monoterpene glycosides are of interest due to their high relevance in the potential flavor of many grape and wine varieties[1–4]. Monoterpenes are plant metabolites that allow the plant to communicate with the surrounding environment to attract or deter specific pests, pollinators, or herbivores[1]. The monoterpenes in grapes are largely found in the skin and mesocarp cells[2]. High terpene varieties of wine, such as Muscat, Riesling, and Gewurztraminer rely on the flavors of terpenes such as linalool, geraniol, and nerol for their floral characteristics. The glycosides of these compounds serve as reservoirs of terpenic flavor that are slowly released over time during fermentation and storage of the wine[5].

Monoterpene glycosides consist of an aglycone monoterpenol bound to a glycone group of one, two, or three sugar residues. Figure 1 displays the general form of a monoterpene glycoside with a single glycosyl residue. The monoterpene glycosides serve as a reserve of potential aroma compounds in wines. The hydrolysis of monoterpene glycosides in grapes and wines may occur because of enzymatic or acid hydrolysis after crushing, during fermentation and wine storage, and within saliva during consumption[3].

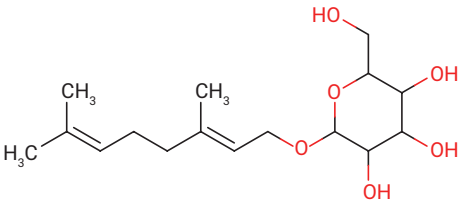

Figure 1. An example monoterpene glycoside with a single hexose sugar.

Traditionally, the analysis of free monoterpenes is accomplished using gas chromatography (GC). Studies primarily use enzymatic or acid hydrolysis to release monoterpenes from the sugars so the glycosides may be measured indirectly as the volatile aglycone[4]. These hydrolysis processes are largely effective in freeing the monoterpenes but have limitations. Acid conditions speed up hydrolysis, but cause degradation and rearrangements of the terpenic compounds. Enzyme analysis favors the hydrolysis of specific terpenes over others, and GC methods offer no information on the glycone portion of the glycoside[1,4]. In addition, the free monoterpenes need to be analyzed before hydrolysis to distinguish the free volatile fraction from the bound monoterpenes. This study develops a method to measure the glycosides directly.

Ultrahigh performance liquid chromatography (UHPLC) paired with electrospray ionization (ESI) is used with accurate mass quadrupole time-of-flight mass spectrometry (Q-TOF MS) to overcome the limitations of monoterpene glycoside analysis. UHPLC-ESI/Q-TOF MS provides the appropriate sensitivity and selectivity to identify monoterpene glycosides. However, standards for monoterpene glycosides are largely unavailable. Therefore, tentative identification of compounds is based on predictive databases and structural verification through tandem mass spectrometry (MS/MS).

This Application Note outlines the software used for data analysis and quantitation of monoterpene glycosides in grapes. Specifically, these tasks are done using Agilent MSC, Agilent PCDL manager, Agilent MassHunter qualitative analysis, and MassHunter quantitative analysis. Since standards are not available for the terpenic glycosides, an internal standard was used for semiquantitation. The original study used Agilent Profinder for quantitation[1]. However, in this report, data analysis has been completed with MassHunter quantitative analysis, and the current quantitation process is outlined within this Application Note.

Experimental

Instrumentation

Monoterpene glycoside extracts were obtained as described in reference[1]. These extracts were then analyzed using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6530 Accurate Mass Q-TOF LC/MS with Dual Agilent Jet Stream Electrospray Ionization. The UHPLC system used an Agilent 1290 Infinity binary pump (G4220A), a temperature-controlled Agilent autosampler (G4226A), an Agilent infinity isocratic pump (G1310B), and a temperature-controlled column compartment (G1316C). Table 1 gives the settings and parameters for the UHPLC analysis.

Negative polarity Q-TOF MS was used for identification and semiquantitation of monoterpene glycosides after separation with UHPLC. Analysis was performed under three separate time segments. During the first and third time segments, before five minutes and after 25 minutes, the LC system diverted the eluent to waste. The eluent was diverted to the Q-TOF MS during the second-time segment where mass spectra were obtained for a mass range of *m/z* 100 to 1,000 at a rate of three spectra/s. Precursor ions with a minimum of 200 counts were selected for auto MS/MS. The MS/MS acquisition mass range was between *m/z* 50 and 1,000. Table 2 shows the settings used for single MS and MS/MS analysis.

Continuous internal calibration was performed during the analysis. The reference masses for the calibration were deprotonated purine (*m/z* 119.0362) and the acetate adduct of hexakis (^{1}H, ^{1}H, ^{3}H-tetraflouropropoxy) phosphazine (*m/z* 980.016375). Semiquantitation was done using the internal standard decyl-β-D-glucopyranoside. The internal standard was spiked into each sample before solid phase extraction to account for any variations during sample preparation.

Data analysis

The identification of the terpenic glycosides began by determining the molecular formulas of potential compounds. The molecular formulas included the mass of a monoterpenol bound to glycosyl residues such as pentose, hexose, or deoxyhexose sugars. Each combination of the glycosyl sugars was considered for the various disaccharides or trisaccharides as well. From there, the molecular formulas were added and named as compounds within the PCDL Manager. The PCDL Manager then automatically generated the exact mass of each compound based on the molecular formula. Alternatively, both the formula and the exact mass may be derived from a chemical structure if it is added to the Personal Compound Database (PCD) as a mol file. The structures may be obtained from online sources such as Chemspider, or drawn through various molecular structure programs such as Chemdraw and Chemsketch. The database created was then saved and applied to an obtained LC/MS chromatogram within MassHunter qualitative analysis software, find-by-formula algorithm. To tentatively determine the compounds present in the chromatogram, each hit from the database search was then analyzed for mass accuracy, isotope spacing, and relative abundance.

Table 1. UHPLC parameters.

Parameter	Value
Instrument	Agilent 1290 Infinity Binary LC
Mobile phase	A) 0.1 % acetic acid in water B) 0.1 % acetic acid in acetonitrile
Gradient	Linear Time (min) %B 0–5 5–20 5–18 Hold 20 18–22 20 to 90 22–25 90 to 5 25–28 Hold 5
Flow rate	0.420 mL/min
Column	Agilent InfinityLab Poroshell 120 Phenyl Hexyl, 2.1 × 150 mm, 2.7 µm, LC column (p/n 693775-912) with an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl 2.1 × 5 mm, 2.7 µm Guard Column (p/n 821725-914)
Temperature	40 °C
Injection volume	10 µL

Table 2. Q-TOF LC/MS parameters.

Parameter	Value
Instrument	6530 accurate-mass Q-TOF LC/MS
Ionization mode	Negative electrospray with Dual Agilent Jet Stream Technology
Single MS acquisition rate	3.0 spectra/s
Single mass range	100 to 1,700 *m/z*
MS1 acquisition rate	3.00 spectra/s
MS2 acquisition rate	4.00 spectra/s
Precursor per MS1 spectrum	3
Active exclusion	On
Collision energy	20.00 eV
Drying gas temperature	150 °C
Drying gas flow rate	10 L/min
Sheath gas temperature	350 °C
Sheath gas flow rate	11 L/min
Nebulizer gas	35 psi
Skimmer voltage	65 V
Octopole RF	750 V
Fragmentor	120 V
Capillary	3.5 kV

Hits found with a mass of ±10 ppm difference to the target exact mass were exported from qualitative analysis as an auto MS/MS preferred list. Auto MS/MS was then performed to generate fragmentation spectra that were used for structure matching and compound identification. In accordance with the structure found in the PCD, auto MS/MS spectra were exported into MassHunter MSC where they were analyzed to generate molecular fragment structures. Figure 2 shows an example of generated fragments. In addition, Figure 2 shows the auto MS/MS spectrum used to generate the structures in MassHunter MSC. To determine the likelihood of the compound being an analyte of interest, each auto MS/MS spectrum was analyzed within MassHunter MSC. If the compound was verified with fragments and putatively identified, the PCDL Manager was updated with its respective retention time and MS/MS spectra.

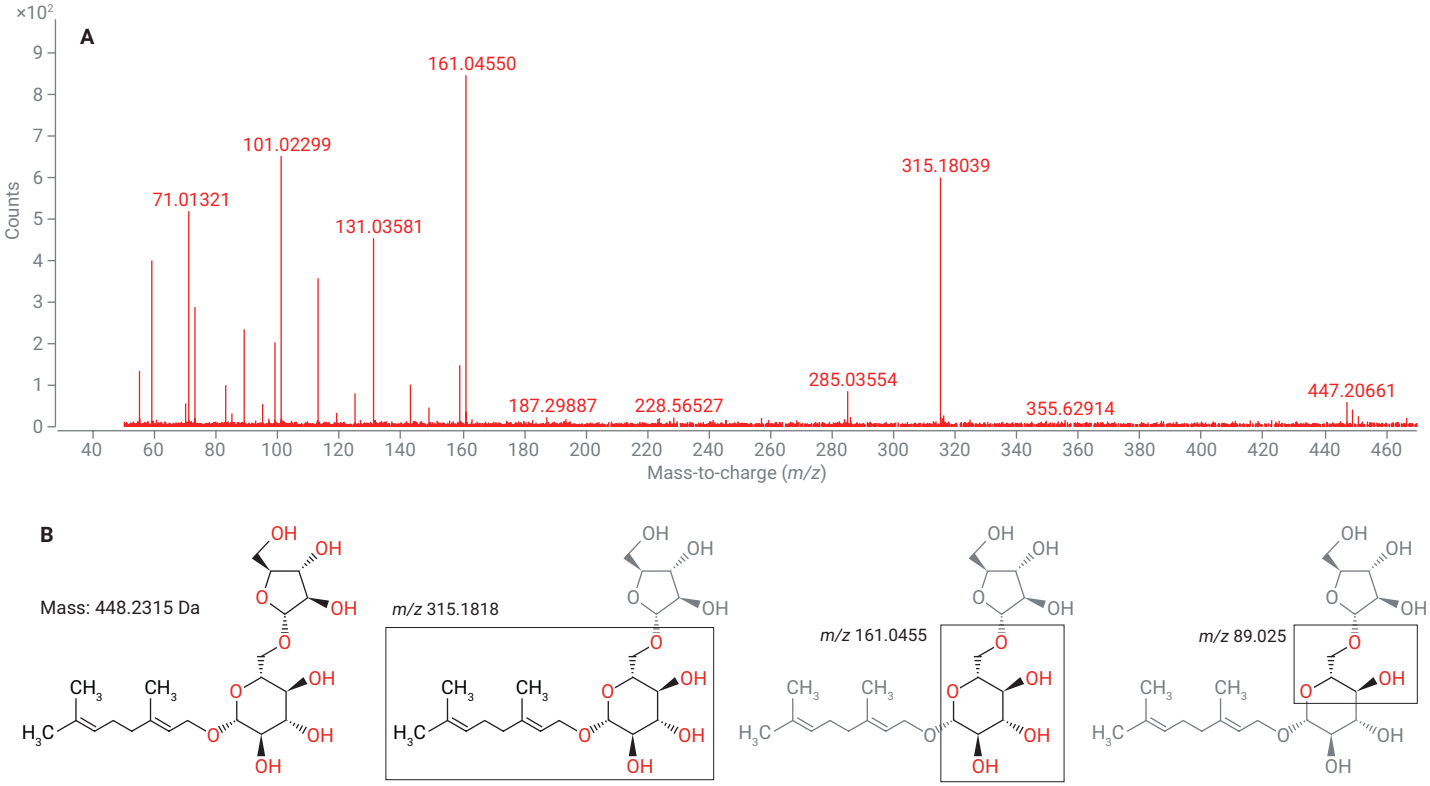

Figure 2. A) An Auto MS/MS spectrum of a monoterpene hexose pentose. B) Potential fragment structures and proposed structure generated through MSC based on the spectrum in A. Each fragment ion is shown with the corresponding *m/z* found in A. Fragmentation losses through collision-induced dissociation are shown in grey.

MassHunter quantitative analysis software was implemented for semiquantitative analysis. To set up a method in the quantitative analysis software, a single MS data file was loaded into qualitative analysis. Compound peaks were identified within the data file by searching for compounds using the PCDL, as described previously. The same data were loaded into quantitative analysis, and a new method was created to analyze the remaining data files. Figure 3 shows the process of adding a compound. Quantitative analysis outlier setup was used to help with the data analysis process by identifying when the integrator found a peak outside of the retention time window ±0.5 minutes, or if the peak was integrated with a signal-to-noise ratio (S/N) of less than three.

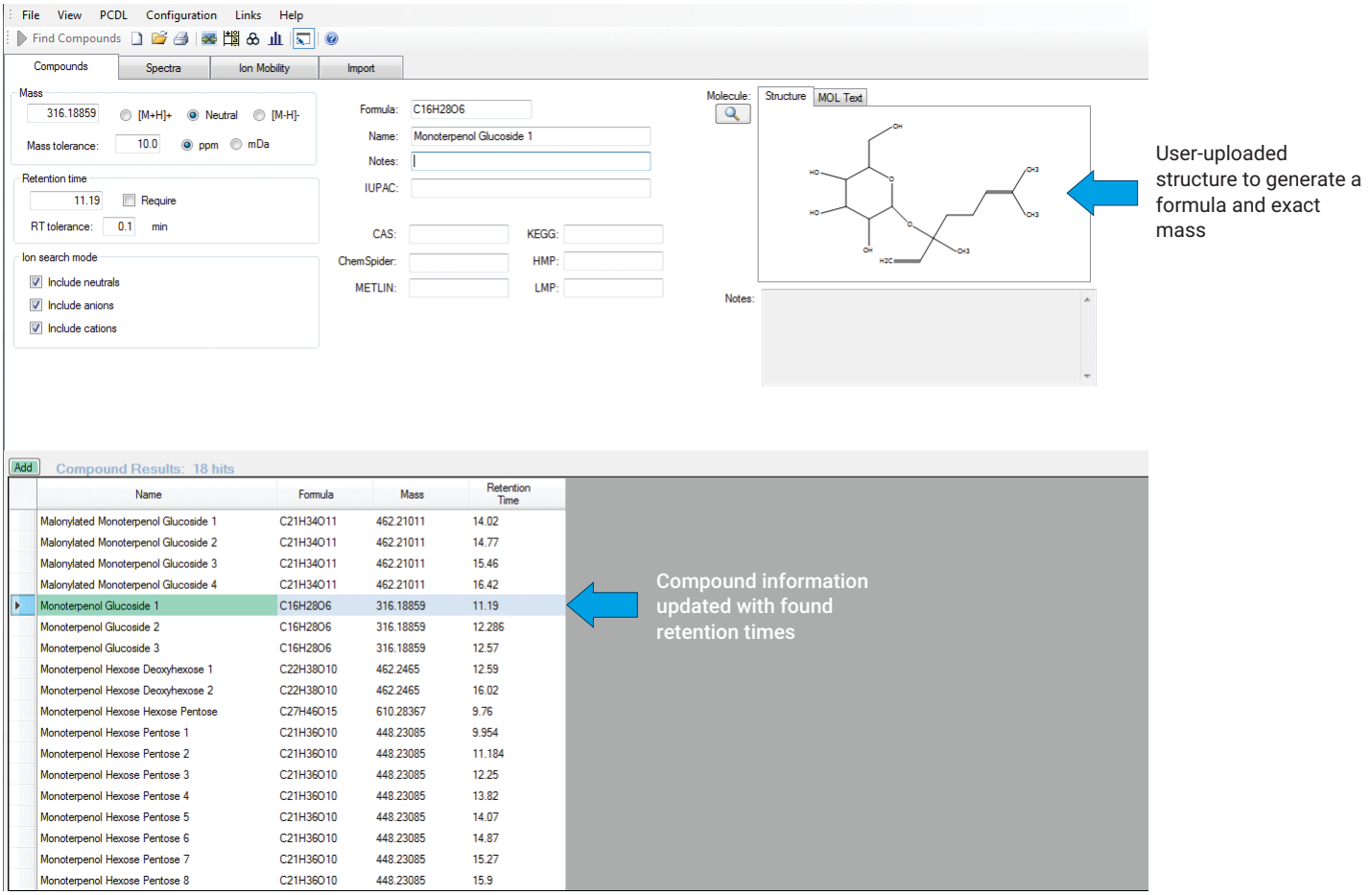

Figure 3. MassHunter PCDL manager was used to create a custom library to search for potential monoterpene glycosides in an untargeted MS study. Once compounds were identified, the database and library were updated with the respective retention times and MS/MS spectra.

Noise regions for each compound were manually determined for the S/N. Figure 4 displays the auto-integration of the extracted ion chromatograms along with the method used for the integrations. To calculate peak intensities relative to the internal standard, integrations were then exported from quantitative analysis into Microsoft Excel where the values were analyzed further. R Foundation for Statistical Computing, 2011 (R) was used to perform a one-way analysis of variance (ANOVA) followed by student's t-tests to determine statistical significance between ripening stages based on an α of 0.05.

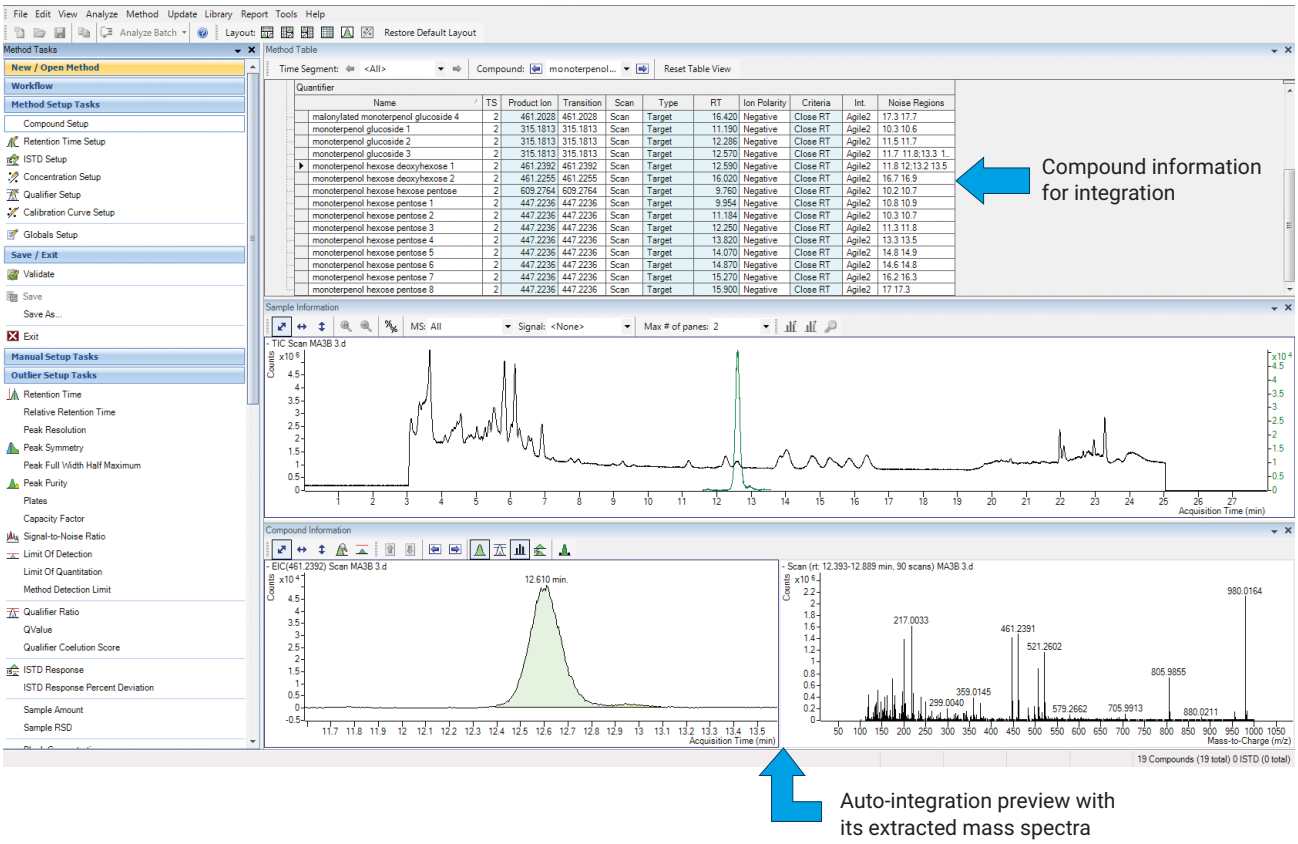

Figure 4A. Compounds from the PCDL were input into MassHunter quantitative analysis for an auto-integration method. Each compound was given a name, retention time, noise region, and *m/z* ratio(s). A representative spectrum was used to ensure that the software finds the correct peaks with the given parameters.

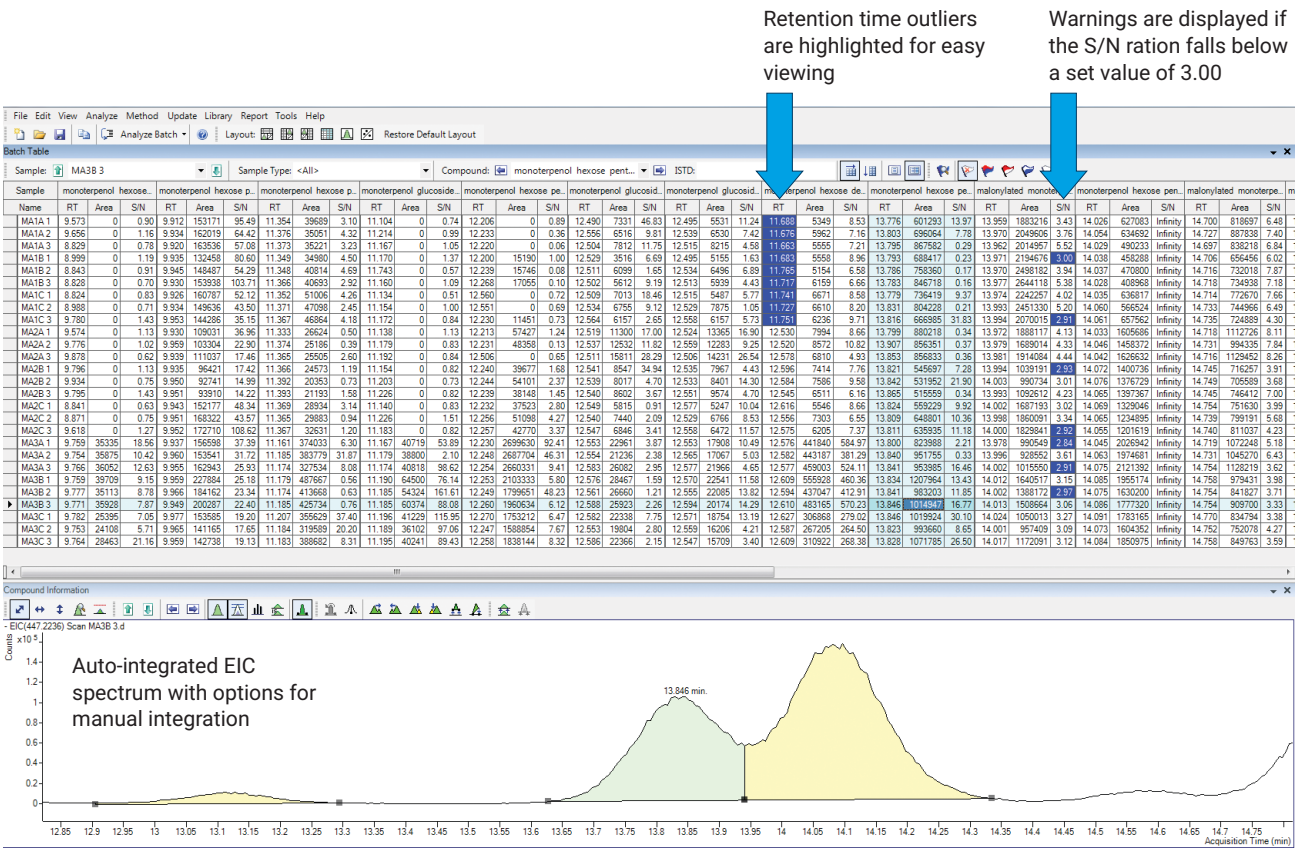

Figure 4B. MassHunter quantitative analysis auto-integrated each spectrum based on the method used. The software displays the peak area, retention time, and S/N. Cells that are outliers for retention time and S/N are highlighted in blue.

Results and discussion

A representative extracted ion chromatogram of monoterpene glycosides in Muscat of Alexandria grapes was generated from the data in qualitative analysis, and is shown in Figure 5. The compounds were found through a find-by-formula search using the generated PCDL. Table 3 shows the possible monoterpene glycosides along with their retention time, formula, expected and observed *m/z* values, and mass errors.

The compounds were identified through an auto MS/MS analysis. The collision-induced dissociation of monoterpene glycosides produced various charged fragments depending on the number of sugars in the glycone. The identification of a compound through MS/MS depends on the presence of glycone fragments along with the pseudomolecular ion. Monoterpenes were not identifiable as fragments since they were a neutral loss during dissociation. Figure 2 displays the MSC-generated structures from an MS/MS spectrum of a monoterpenol hexose pentose. MSC displays the pseudomolecular ion (*m/z* 447.2236) and fragments of the molecule. MSC outlines the loss of a pentose sugar (*m/z* 315.1381), a hexose (*m/z* 161.0472), and ring fragments characteristic of sugars (*m/z* 59.0139, 71.0139, 89.0244, 101.0244, and 113.0244). The identification process was repeated, giving the list of compounds in Table 3.

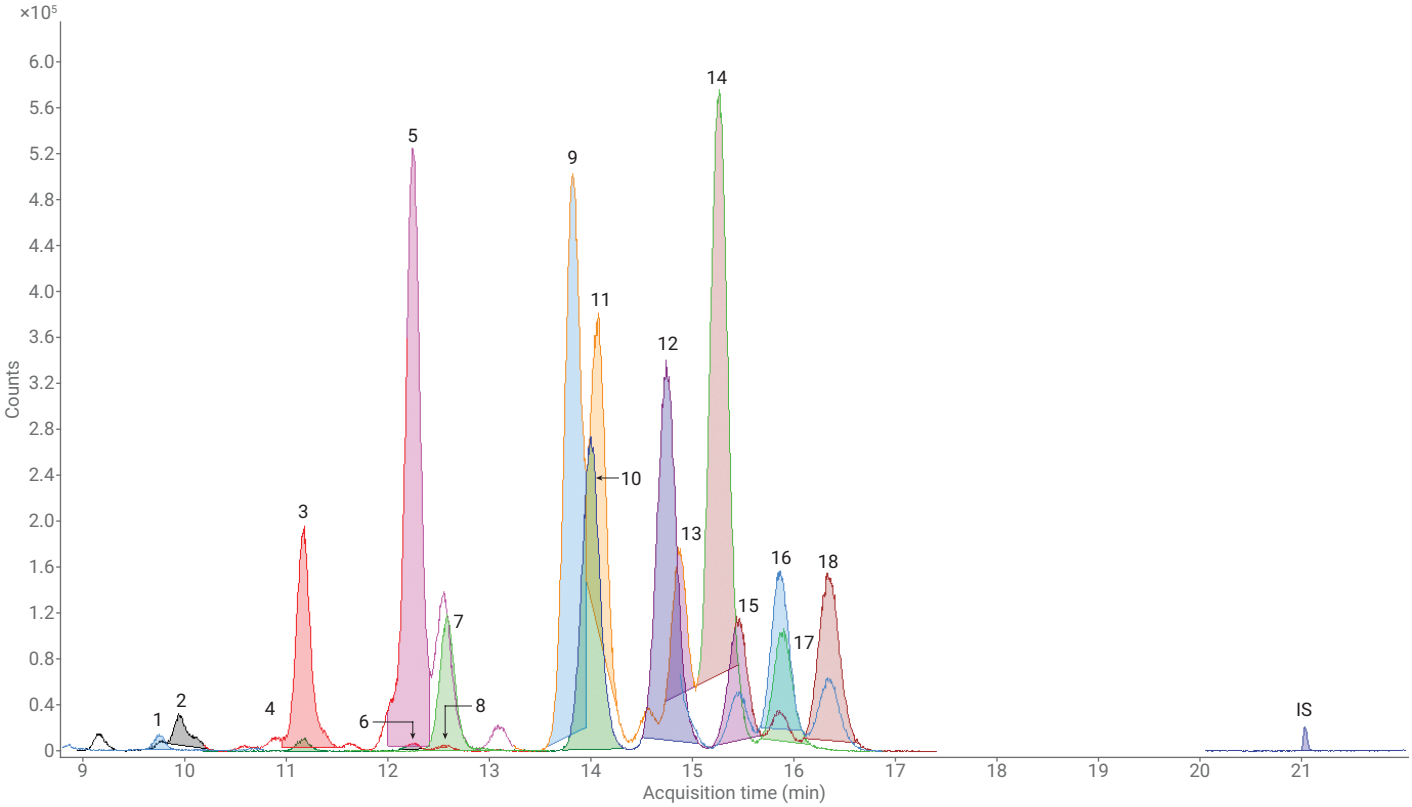

Figure 5. An extracted ion chromatogram of monoterpene glycosides found by searching with the custom PCDL library. Peak numbers correspond to the monoterpene glycosides in Table 3.

Table 3. Identified monoterpene glycosides with their corresponding retention times, masses, and mass errors.

Glycoside	RT (min)	Compound	Formula	Predicted *m/z*	Observed *m/z*	Mass error (ppm)
1	9.76	Monoterpenol dihexose pentose 1	$C_{27}H_{46}O_{15}$	609.27639	609.27603	0.59
2	9.95	Monoterpenol hexose pentose 1	$C_{21}H_{36}O_{10}$	447.22357	447.22326	0.69
3	11.18	Monoterpenol hexose pentose 2	$C_{21}H_{36}O_{10}$	447.22357	447.22330	0.60
4	11.19	Monoterpenol glucoside 1	$C_{16}H_{28}O_{6}$	315.18131	315.18118	0.41
5	12.25	Monoterpenol hexose pentose 3	$C_{21}H_{36}O_{10}$	447.22357	447.22389	−0.72
6	12.27	Monoterpenol glucoside 2	$C_{16}H_{28}O_{6}$	315.18131	315.18112	0.60
7	12.57	Monoterpenol glucoside 3	$C_{16}H_{28}O_{6}$	315.18131	315.18141	−0.32
8	12.59	Monoterpenol hexose deoxyhexose 1	$C_{22}H_{38}O_{10}$	461.23922	461.23917	0.11
9	13.82	Monoterpenol hexose pentose 4	$C_{21}H_{36}O_{10}$	447.22357	447.22362	−0.11
10	14.02	Malonylated monoterpenol glucoside 1	$C_{21}H_{34}O_{11}$	461.20284	461.20262	0.48
11	14.07	Monoterpenol hexose pentose 5	$C_{21}H_{36}O_{10}$	447.22357	447.22376	−0.42
12	14.77	Malonylated monoterpenol glucoside 2	$C_{21}H_{34}O_{11}$	461.20284	461.20341	−1.24
13	14.87	Monoterpenol hexose pentose 6	$C_{21}H_{36}O_{10}$	447.22357	447.22347	0.22
14	15.27	Monoterpenol hexose pentose 7	$C_{21}H_{36}O_{10}$	447.22357	447.22398	−0.92
15	15.46	Malonylated monoterpenol glucoside 3	$C_{21}H_{34}O_{11}$	461.20284	461.20321	−0.80
16	15.9	Monoterpenol hexose pentose 8	$C_{21}H_{36}O_{10}$	447.22357	447.22356	0.02
17	16.02	Monoterpenol hexose deoxyhexose 2	$C_{22}H_{38}O_{10}$	461.23922	461.23822	2.17
18	16.42	Malonylated monoterpenol glucoside 4	$C_{21}H_{34}O_{11}$	461.20284	461.20274	0.22
I.S.	21.03	Decyl-*Beta*-D- Glucopyranoside	$C_{16}H_{32}O_{6}$	319.21261	319.2127	–0.28

MassHunter quantitative analysis was used for the semiquantitation of each identified compound over the grape ripening process. The software autogenerates an extracted ion chromatogram for each compound and integrates peaks based on their compliance to measured retention times and S/Ns. Relative values for each glycoside are generated by comparing the peak areas of each compound to the peak area of the internal standard, decyl-β-D-glucopyranoside.

Muscat of Alexandria grapes harvested at commercial maturity were found to have the highest abundance and diversity of monoterpenol glycosides compared to earlier maturation time points. Of the 18 identified compounds, 15 showed a statistically significant increase in relative abundance after the onset of ripening. This increase indicates that the ripening stage of fruit development is crucial for the development of monoterpenes and their respective glycosides[1].

Conclusion

Grape monoterpene glycosides were identified and semiquantitated, giving relative concentrations at three ripening stages through accurate mass UHPLC-Q-TOF MS. A workflow was developed by building a database of potential monoterpene glycosides with possible structures in the PCDL Manager. Untargeted searches of Muscat of Alexandria grape samples were done through the MassHunter qualitative analysis find-by-formula algorithm using the custom PCDL. Once potential matches were found, auto MS/MS studies were performed to generate fragmentation spectra for structure elucidation and identification in MSC.

After identification of the structures, a custom quantitative analysis method was created to auto-integrate peaks based on the ions present and retention times. Peak integrations were then related to the internal standard to determine the relative amounts of each of the 18 glycosides in the grapes at various growth stages. For most of the compounds, the key event for their accumulation is the onset of ripening. This is possibly due to an increase in synthesis and storage within the grape.

References

1. Hjelmeland, A.; Zweigenbaum, J.; Ebeler, S. E. Profiling Monoterpenol Glycoconjugation in Vitis vinifera L. cv. Muscat of Alexandria using Novel Compound Database Approach, High Resolution Mass Spectrometry and Collision Induced Dissociation Fragmentation Analysis. *Analytica Chimica Acta* **2015**, *887*, 138–147.
2. Flamini, R.; *et al.* Profiling of Grape Monoterpene Glycosides (Aroma Precursors) by Ultra-High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC/QTOF). *Journal of Mass Spectrometry* **2014**, *49*, 1214–1222.
3. Parker, M.; *et al.* The Contribution of Wine-Derived Monoterpene Glycosides to Retronasal Odour During Tasting. *Food Chemistry* **2017**, *232*, 413–424
4. Dziadas, A.; Jelen, H. Comparison of Enzymatic and Acid Hydrolysis of Bound Flavor Compounds in Model Systems and Grapes. *Food Chemistry* **2016**, *190*, 412–418.
5. Wilson, B.; Strauss, C. R.; Williams, P. J. The Distribution of Free and Glycosidically-Bound Monoterpenes Among Skin, Juice, and Pulp Fractions of Some White Grape Varieties. *American Journal Enololgy and Viticulture* **1986**, *37*, 107–111.

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