# Methods for predicting and assessing flavour evolution during white wine ageing

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## Abstract

Estimating future flavour evolution in wine generally requires quantitative analysis of a known precursor compound or forcible production using elevated temperature and/or lower pH with analysis of the produced volatiles. While it is understood that these methods are not always accurate, they can provide an indicative assessment of the magnitude of evolving species. However, due to the complex pathways by which some key odorants are formed, these predictive measures may not provide a compositionally relevant outcome, and do not provide any indication of future flavour evolution. Here, extracts derived from the winemaking by-product, grape marc, rich in monoterpene glycosides have been analysed by 'predictive' methods and also added to wine to provide a correlation with real-world evolution profiles. While analysis of a precursor molecule, geraniol glucoside, and acid-catalysed hydrolytic assessment of the extracts both provided good correlations with total flavour production in wine, neither were good predictors for final monoterpene composition in wines. Additionally, a single extract was monitored over 36 months in two different base wines and at two different concentrations to gain a better understanding of hydrolysis kinetics, which was also explored computationally. The generation of first-order rate curves provided a mechanism to determine reaction half-lives for the hydrolysis of geraniol glucoside and related to the monoterpene composition in wines.

*Keywords: white wine, monoterpenes, glycoside hydrolysis, shelf-life, computational*

## Introduction

Wine is an inherently unstable product that is continually changing with time, with additional complexity surrounding the fact that the time from production to consumption is highly varied and can be at the inclination of the consumer. Winemakers hold a wealth of experience in determining the optimum window of time for a certain wine to be consumed, but what happens when the ability to interpolate specific outcomes is replaced by the need to extrapolate?

Throughout the world, grape-growing regions are experiencing climatic extremes and shifts in growing season temperatures that can push grape and wine composition outside that previously experienced, with respect to flavour compounds and the matrix they exist in [1, 2]. Additionally, the growing trend for no- or low-alcohol products [3], can be satisfied by removal of excess alcohol or by supplementation of base grape or wine components into a different matrix [3, 4]. These scenarios equate to a situation where grape or wine components are taken out of their usual context and result in the need for better understanding of flavour evolution to be able to extrapolate known flavour generation pathways into new matrices.

The evolution of monoterpenes has been widely studied due to their ubiquity. In wines, it is well understood that acid-catalysed hydrolysis of a structurally diverse group of saccharide-bound analogues yield the free, odoriferous monoterpenes [5]. Quantitation of the monoterpene potential in grapes can be achieved by either direct analysis of the saccharide-bound monoterpenes, or via catalytic release of the volatiles and subsequent GC-MS analysis [5]. While both methods can provide information as to the magnitude of future monoterpene production, little can be gained about the speed of hydrolysis reactions as it relates to wine ageing and the evolution of monoterpenes, let alone the ability to extrapolate outcomes into different matrices.

Recently, we outlined the use of monoterpene-rich extracts from the winemaking by-product, grape marc, as a latent flavour enhancer when added into wines, giving rise to monoterpenes over the six-month experiment [6]. Here, the storage of grape marc extracts in two different wines described previously has been monitored for 36 months with respect to geraniol glucoside hydrolysis and monoterpene evolution. Additionally, a selection of grape varieties has been accessed to generate similar extracts and investigate their potential as latent flavour additives. The aims were to better understand the role of grape marc-derived extracts in the generation of flavour via 1) specifically investigating mechanistic and kinetic factors of glucoside hydrolysis under different pH conditions; 2) assess the role of extract characterisation methods on predicting flavour generation.

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## Experimental

### *Grapes, wines and marc extraction*

Long-term glycoside extract storage trials in Chardonnay and Riesling wines were initiated as previously described [6], with analysis progressing past the initial six-months to include 18- and 36-month time points. In short, 300 kg of Gewürztraminer grape marc obtained in vintage 2016 was extracted with water followed by purification of the extract on FPX66 resin. The resultant ethanolic extract was dried by low-pressure distillation to yield a brown powder rich in monoterpene glycosides. The extract was added in duplicate to two different wines (Chardonnay, pH 3.40 or Riesling, pH 3.10) at either 0.4 g/L or 0.8 g/L of extract, at pre- and post-fermentation. Here, duplicates at the same concentration but added at different times have been grouped into quadruplicates for simplicity.

Additionally, short-term glycoside storage trials were performed from grape marc extracts isolated from ten different grape varieties collected across South Australia during vintages 2017 or 2018. Isolation and extraction of extracts was performed as described above, but on a laboratory-scale starting with approximately 10 kg of grapes that were hand-pressed to yield approximately 5 kg of marc which was stored at -20 °C until used for isolation of monoterpene glycoside-rich extract. For all short-term trials, each extract was added to 1.2 L of commercially available Chardonnay wine (pH 3.40) at 0.4 g/L, and the wine was then bottled in triplicate (375 mL screwcap sealed glass bottles) and stored at 15 ºC for six months prior to volatile analysis by gas chromatography-mass spectrometry (GC-MS). All wine handling was performed under anaerobic conditions using solid $CO_2$ in vessels, with transfers and bottling performed with laboratory grade nitrogen gas.

### *Marc extracts and research wine analysis*

For quantitation of geraniol glucoside by liquid chromatography (LC)-MS/MS as previously recorded [6], wines were analysed neat and grape marc extracts were dissolved in water, followed by the addition of the internal standard ($d_2$-geraniol glucoside) prior to instrumental analysis. Hydrolysis of grape marc extracts was performed as per Grebneva (2019) [7], where a weighed amount was dissolved in water, acidified to pH 1 and heated at 100 °C for an hour before the hydrolysate was cooled prior to volatiles analysis. Volatiles were quantified from extract hydrolysates or wine samples for a range of monoterpenes and norisoprenoids by SIDA SPME (solid phase microextraction)-GC-MS, as previously described [6].

### *Computational chemistry*

Structures were drawn in Avogadro (version 1.2.0) [8], then optimised (UFF) and systematic rotor conformer search performed, with the resulting geometry used to create initial input files for GAMESS (Linux distribution, version 2019 R2, University of Iowa, USA) [9] running on the University of South Australia's High Performance Compute Cluster. Calculations were first performed for molecules in the gas phase (RHF/3-21G) to provide starting geometries which were then used as the input for density functional theory (B3LYP/6-311G(d)) equilibrium geometry calculations in water (SMD solvent model). Local minima were confirmed by vibrational analysis and the presence of all real frequencies, and all structural outcomes visualised using MacMolPlt (v7.7) [10]. Each calculation was performed in parallel across eight cores.

### *Data, graphing and calculations*

Data handling was performed in R [11], and graphs produced using the package ggplot2 [12]. Rate-order graphs (Figure 2) provided rate constants (k, in months$^{-1}$) from the negative slope of regression lines, estimated initial geraniol glucoside concentration from the y-intercept ($e^{y\text{-intercept}}$), and reaction half-lives ($t_{1/2}$, in months) from the natural logarithm of 2 divided by the rate constant, k ($\log_e(2)/k$). The monoterpene concentration values were converted to geraniol glucoside equivalents using the mass proportion of the monoterpenes in geraniol glucoside (48.735%). Odour activity values were determined using the aroma detection thresholds of 30 μg/L for geraniol, 25 μg/L for linalool and 250 μg/L for α-terpineol [13, 14].

## Results and discussion

Grape marc-derived extracts from ten different varieties were analysed for their geraniol glucoside concentration, and following acid-catalysed hydrolysis as a marker for potential future flavour evolution, and were subsequently stored in a commercial Chardonnay wine for six months followed by quantitation of the evolved odorants. The concentration of the glucosidic monoterpene precursor and the analysis of extract hydrolysates were compared with the measured monoterpene evolution in wine as a benchmarking of predictive methods. The hydrolytic release and measurement of monoterpenes from the extract correlated well with the monoterpene concentration values after six months of storage ($R^2 = 0.99$, $p < 0.0001$). However, the monoterpenes quantified in the extract hydrolysates did not provide a monoterpene profile or yield concentrations that were predictive of that evolved during wine storage after six months. Instead of wine-like monoterpene profiles, hydrolytic release yielded

decreased proportions of geraniol and preferentially promoted subsequent rearrangement to α-terpineol. Similarly, the amount of geraniol glucoside added correlated with the quantity of monoterpenes present in wine after six months of storage ($R^2$ = 0.84, p <0.0001), but again only enabled the ranking of extracts in total magnitude of monoterpene production.

While both methods of estimating future flavour evolution from marc-derived extracts in wine were adequate for ranking the extracts in terms of the relative magnitude, neither provided realistic results in terms of their total monoterpene quantity or profile. Additionally, they provided limited information regarding the extract performance under different conditions and failed to provide an understanding of shelf-life with respect to the evolved volatiles. As such, additional work was undertaken to investigate mechanisms to better predict the speed of flavour evolution, by extending storage trials that have previously been reported [6] to 36 months (Figure 1).

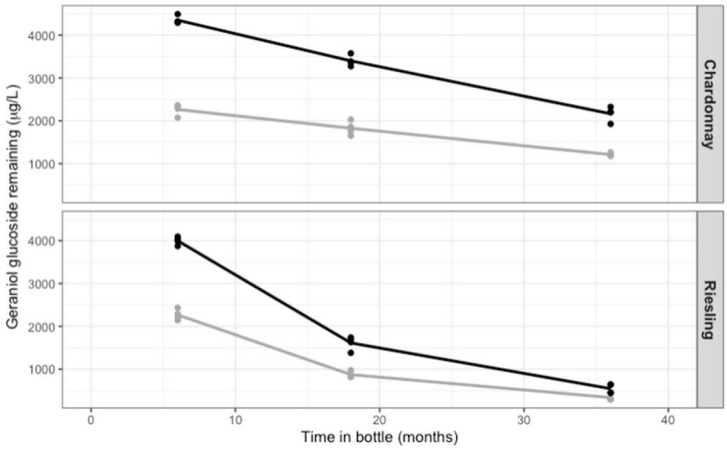


**Figure 1***: Concentration of geraniol glucoside remaining in Chardonnay (pH 3.40) and Riesling wines (pH 3.10) with 0.4 g/L (grey lines/points) or 0.8 g/L (black lines/points) of Gewürztraminer marc extract across 36 months of storage. Points represent individual samples; lines represent mean at each time point (n = 4).*

The wines exhibited expected decreases in geraniol glucoside, which was more rapid for the Riesling wine with a lower pH (3.10) compared with the Chardonnay wine (pH 3.40) [15], albeit with different hydrolysis profiles between the varieties. While the lower pH Riesling wine showed initially rapid decreases which slowed, the Chardonnay wine displayed a linear decrease with time. As expected, the linear relationship of the natural logarithm of the geraniol glucoside concentration against time supported a first-order reaction (Figure 2), in alignment with reported glycoside hydrolysis reaction kinetics [16]. However, the mean rate constant (k) determined across both addition rates for each pH (3.10, 0.0649 $months^{-1}$; 3.40, 0.0222 $months^{-1}$) differed, suggesting a pH effect which could not be accounted for by a single-step first-order hydrolysis reaction alone.

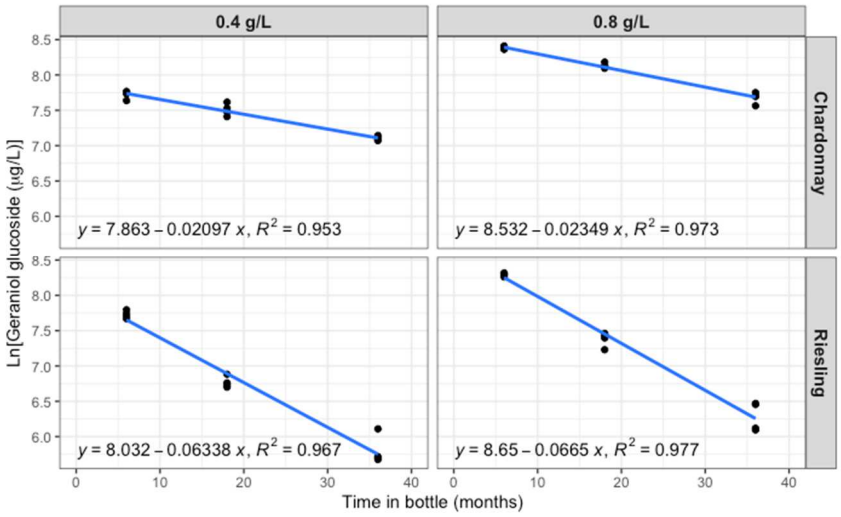


**Figure 2***: Natural logarithm of geraniol glucoside concentration in Chardonnay or Riesling wines (n = 4 at each point), showing regression (blue line) representing the hydrolysis of geraniol glucoside as a first-order reaction.*

The concerted geraniol glucoside hydrolysis mechanism proposed previously [17], explains the over representative formation of linalool from geraniol glucoside [17, 18], via the cationic species, linalyl cation [19] (compound **3**, Figure 3). However, here it failed to explain the changes in hydrolysis with concentration and pH, or a hydrolysis rate that solely relies on the concentration of geraniol glucoside. Additionally, many of the possible acid-catalysed mechanisms proposed for general glucoside hydrolysis involve the aglycone retaining the glycosidic ether oxygen [20], which would fail to yield linalyl cation and would lead to the generation of geraniol only from geraniol glucoside hydrolysis. As such, the findings here can only be explained by hydrolysis involving the initial protonation of the glucoside oxygen (as shown in Figure 3, **1** to **2**) [20], which then proceeds via cleavage of the glycosidic bond to yield **3** [17].

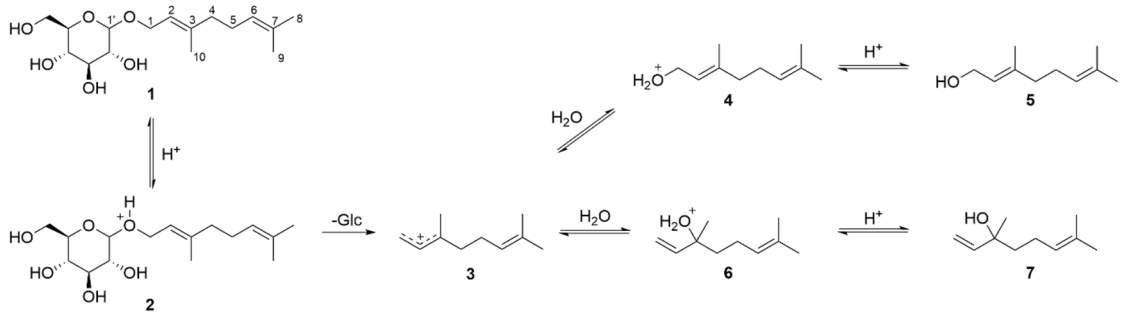


**Figure 3***: Indicative formation of free monoterpenes (**5** and **7**) from non-volatile geraniol glucoside (**1**) via formation of protonated glucoside (**2**) and linalyl cation (**3**), including interchange between geraniol (**5**) and linalool (**7**) via cationic species (**3**, **4** and **6**).*

In the event of a two-step hydrolysis of **1** via the protonated geraniol glucoside (**2**), the cation formed must be stable for the mechanism to be viable. To investigate this, all compounds shown in Figure 3 were studied computationally to determine stability, and to re-confirm previous findings on the transition between geraniol and linalool [19]. The key atomic charges and bond lengths are shown below in Table 1.

**Table 1: Key atom and bond properties of energy minimised structures following equilibrium geometry optimisation using B3LYP/6-311G(d) in water.**

| | Atomic charges (a.u.) | | | | | Bonds, length in Å (order) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Structure | $C_1$ | $C_2$ | $C_3$ | O# | H^ | $C_{1/3}$-O# | $C_1$-$C_2$ | $C_2$-$C_3$ | O-H |
| 1 | -0.22 | -0.26 | 0.07 | -0.41 | N/A | 1.45 (0.88) | 1.50 (1.00) | 1.34 (1.89) | N/A |
| 2 | -0.21 | -0.31 | 0.13 | -0.50 | 0.49 | 1.58 (0.57) | 1.47 (1.04) | 1.35 (1.78) | 0.99 (0.65) |
| 3* | -0.27 | -0.19 | 0.21 | N/A | N/A | N/A | 1.36 (1.64) | 1.42 (1.27) | N/A |
| 4 | -0.17 | -0.35 | 0.15 | -0.66 | 0.54 | 1.55 (0.54) | 1.47 (1.07) | 1.35 (1.77) | 0.98 (0.67) |
| 5 | -0.18 | -0.30 | 0.09 | -0.64 | 0.42 | 1.45 (0.91) | 1.50 (1.00) | 1.34 (1.86) | 0.93 (0.78) |
| 6 | -0.38 | -0.19 | 0.12 | -0.71 | 0.55 | 1.64 (0.44) | 1.33 (1.91) | 1.50 (1.05) | 0.97 (0.67) |
| 7 | -0.45 | -0.19 | 0.18 | -0.65 | 0.41 | 1.44 (0.88) | 1.33 (1.94) | 1.52 (1.00) | 0.97 (0.78) |

*optimisation via both geraniol (**5**) and linalool (**7**) gave the same structure. ^refers to the newly installed hydrogen for protonated species (**2**, **4** and **6**) and only hydrogen for neutral species (**5** and **7**). #refers to the oxygen attached to $C_1$ for geranyl species (**4** and **5**) and C3 for linalyl species (**6** and **7**).

Firstly, the generation of **3** via either **5** or **7** yielded structures that align closely with experimental outcomes with respect to the preferential re-production of **7** from **3** over **5** [19], given the greater positive charge on $C_3$ over $C_1$ due to the stability of the tertiary carbocation. For the generation of **2** from **1**, the formation of a stable intermediate supported the two-step mechanism, and the structural changes were consistent with cleavage of the glucoside between the ether oxygen and $C_1$ of the aglycone, given the increase in bond length between $C_1$-O, and decrease in bond order. As such, the computational structural investigation supported the proposal of an initial protonation of geraniol glucoside, and then cleavage to yield the linalyl cation, which is consistent with previous findings [17,18]. Furthermore, it explains the pH-related change in geraniol glucoside hydrolysis rate. The greater extent of protonation of **1** at the lower pH provides a greater proportion of the hydrolysis reactant, **2**. In essence, the hydrolysis curves showing the removal of **1** (Figure 1) provide a pH-dependant proxy for the actual reactant, **2**.

While the generation of a universal rate constant for the hydrolysis of geraniol glucoside will ultimately rely on determining the pH-dependent equilibrium constant between **1** and **2**, the rate constants estimated above still allowed for the generation of reaction half-lives at each pH. Half-lives of geraniol glucoside hydrolysis of approximately 11 months or 31 months at pH 3.10 or 3.40, respectively, provided an indication of the production of volatiles over time, and information into shelf-life of wines. Shown below (Figure 4) are the key monoterpenes

generated in the Chardonnay and Riesling wines via hydrolysis of geraniol glucoside and subsequent rearrangement, with an indication of the geraniol glucoside half-life.

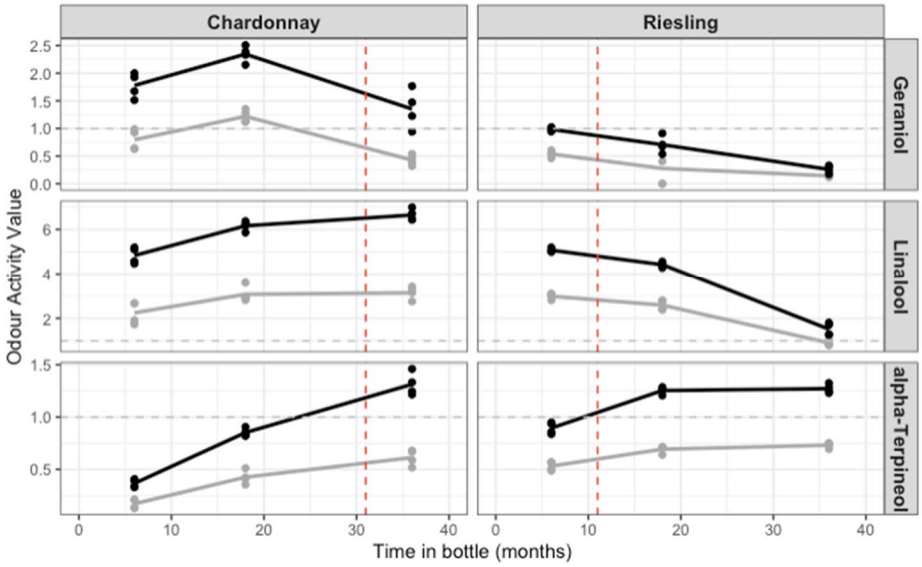


**Figure 4***: Concentration, expressed as odour activity value, of key monoterpenes in Chardonnay and Riesling wines with 0.4 g/L (grey lines/points) or 0.8 g/L (black lines/points) of Gewürztraminer marc extract across 36 months of storage, with red dashed vertical lines indicating the established pH-dependent half-lives of geraniol glucoside in each wine. Horizontal grey dashed lines signify odour activity value of 1. Points represent individual samples; lines represent mean at each time point (n = 4).*

Interestingly, at the half-life of geraniol glucoside hydrolysis, each wine possessed a similar monoterpene concentration and profile, especially when comparing between the 0.4 g/L additions at each pH (grey lines). The formation of linalool, and to some extent geraniol, then rearrangement to α-terpineol (via nerol), and the equilibria that exist between these compounds [19], appear largely dependent only on the hydrolysis of geraniol glucoside. This suggests that determination of a rate constant for a single key step may allow for better shelf-life estimations, which can only be improved by better understanding the formation of protonated species under different conditions (pH and matrix). Importantly, the hydrolysis of geraniol glucoside has not been completely isolated in this experiment with preceding reactions also taking place to yield additional geraniol glucoside as the storage progressed. As such, future investigation will need to be conducted into other significant precursors that yielded geraniol glucoside in these experiments. While non-targeted LC-MS/MS experiments have been conducted on the initial extract that was used, the ability to identify another significant precursor is dependent on correlations between the 'missing' portions and the abundance of mass spectral features. To provide information on the quantity of precursor currently unaccounted for, two different methods were conducted (Table 2).

**Table 2: Estimation of total geraniol glucoside pool including that present in higher precursor forms, using either the sum of free monoterpenes (MTs) and geraniol glucoside (Ger-glc) present at six months of ageing, or using the first-order rate curves.**

| Variety | Extract addition (g/L) | Mean value at 6 months in bottle (μg/L) | | | | From rate curves | |
|---|---|---|---|---|---|---|---|
| | | Sum MTs* | MTs in Ger-Gluc equivs^ | Ger-glc remaining | Estimated starting concentration | y-intercept | Ger-glc (μg/L) |
| **Chardonnay** | 0.4 | 127 | 260 | 2265 | 2525 | 7.863 | 2599 |
| | 0.8 | 278 | 570 | 4351 | 4921 | 8.532 | 5075 |
| **Riesling** | 0.4 | 226 | 464 | 2268 | 2732 | 8.032 | 3078 |
| | 0.8 | 385 | 790 | 3998 | 4788 | 8.650 | 5710 |

*Geraniol, linalool, nerol and α-terpineol. ^converted using mass proportion factor of 0.48735.

Firstly, the concentration of evolved monoterpenes after six-months of storage has been summed and converted to the equivalent concentration if derived from geraniol glucoside. This value was added to the concentration of remaining geraniol glucoside at six-months to give an estimated starting concentration considering formation from

other sources. However, from our analysis of model experiments, the recovery of geraniol glucoside and the four main evolved monoterpenes is not complete (data not shown), so this method is expected to provide an under-estimate. Secondly, the rate-order curves (Figure 2) provided a theoretical estimation of geraniol glucoside starting concentration from the y-intercept, albeit inherently over-estimated. Future efforts will investigate the 'missing' portion of geraniol glucoside and any higher precursors that yield geraniol glucoside as a way of better predicting future flavour evolution from marc-derived extracts.

## Conclusion

Evolution of monoterpenes from winemaking by-product-derived extracts has been followed over 36 months of storage in wines at two different pH values. While the hydrolysis of geraniol glucoside was not an isolated reaction, this system has allowed a deeper understanding of the mechanism and kinetics of hydrolysis. The reaction appears to proceed via a pH-driven protonation, followed by a first-order cleavage, for which the former step requires further investigation. A computational investigation of the proposed intermediates supported the hypothesis and aligns with previous experimental reports.

The chemical investigation into the hydrolysis of geraniol glucoside in wine from odourless extracts has provided an improved understanding of the hydrolysis kinetics, and the estimated rates have proven useful in characterising the flavour generation from these extracts. Additionally, the rate curves provide additional information as to the magnitude of flavour precursors that are currently un-quantitated which will prove useful in further interrogation of non-targeted experimental data.

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