Application Note: 30173

Direct Analysis of Red Wine Using Ultra-Fast Chromatography and High Resolution Mass Spectrometry

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Key Words
- LTQ Orbitrap™
- U-HPLC
- Flavonoids
- Red Wine

Overview
Red wine is a very complex mixture and a rich source of beneficial anti-oxidants. Identification and quantitation of these natural products is challenging. Ultra High Pressure Liquid Chromatography (U-HPLC) coupled to the Thermo Scientific LTQ Orbitrap XL mass spectrometer was used for analysis of French red wine, which enabled simultaneous detection and relative quantitation of the wine's anti-oxidant constituents. The phenolic compounds (such as quercetin) responsible for most of the health benefits associated with the consumption of red wine were identified and their variable content across two different harvest years was observed. Direct wine analysis approach was then applied to monitor the progressive changes in red wine after its exposure to air. This work demonstrated the feasibility of analyzing complex mixtures without any prior sample preparation by making use of the high resolving power of both U-HPLC and the Orbitrap™ mass analyzer detector.

Introduction
Free radicals derived from molecular oxygen are considered major causative agents of tissue damage.[1,2] Both recent and historical evidence suggests that regular drinking of wine ***in moderation*** has a positive impact on human health thanks to its high content of anti-oxidants.[3,4] Red wine in particular contains a complex mixture of phenolic compounds which are important contributors to the organoleptic quality of wines as well as essential components in the evolution of wine. Quercetin is of special interest for its commercial use as an anti-oxidant food supplement with a proven record of promoting vascular relaxation, inhibiting human platelet aggregation *in vitro*, and modulating eicosanoid synthesis towards a pattern likely to be protective against coronary heart disease.[5]

Reversed-phase HPLC is well established for the analysis of flavonoids in red wine, including quantitative analysis.[6,7,8] Coupling reversed-phase HPLC to a mass spectrometer adds considerable benefits such as the ability to:

1) analyze complex mixtures without much sample fractionation
2) monitor hundreds of compounds in a single analysis over a wide dynamic range of concentrations
3) provide an unambiguous identification and structural characterization of the compounds based on accurate mass measurement and informative fragmentation spectra.

Recent advances in both HPLC and mass spectrometry techniques are having a significant impact on the analyses of complex mixtures such as those represented by food and agricultural products. First, the use of small particles (< 2 µm) in HPLC columns can provide remarkable increase in speed of analysis while maintaining or even improving the separation efficiency. Second, the new generation of powerful but easy-to-use hybrid mass spectrometers, like the LTQ Orbitrap XL, combines extremely high mass accuracy and resolution with the capability of multiple levels of fragmentation.[9] The combination of these powerful techniques provides a robust and confident means of profiling complex mixtures as well as successful identification and advanced structural characterization of detected compounds. As a result, we are seeing rapidly growing interest in the area of metabolomic analysis being applied in nutrition and health research.[10,11]

We investigated the potential of a direct analysis of red wine using U-HPLC coupled to a linear ion trap – Orbitrap hybrid mass spectrometer. Of particular interest was the ability of the designed workflow to pinpoint statistically significant differences between individual harvest years for wines of a specific origin (area, label). In addition to that, we used the developed methodology to monitor the trend in oxidative changes of red wine after exposure to air.

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Methods

Two bottles of French red wine Les Charmes de Kirwan, Margaux (cuvee, Bordeaux region, France), years 2003 and 2005, were obtained from a specialized wine merchant. The wine was stored at room temperature in the dark until analyzed. Immediately after opening the bottle, a glass vial (20 mL) was filled with the wine to the very top, quickly closed to ensure minimum oxidation, and stored at 4°C in the dark. This sample was collected just in case there was a need for repeated analysis of the profiling experiments or structural elucidation studies. A second 20 mL aliquot of wine was poured from the original bottle into a glass beaker. From this beaker a sample vial was immediately filled to the rim and placed in the chilled (4° C) Thermo Scientific Accela autosampler tray, awaiting analysis. For a wine oxidation trend analysis, further samples were taken from this open beaker 1, 5 and 24 hours after the bottle opening.

Chromatography was performed using an Accela U-HPLC injecting 20 µL sample from a cooled tray (4°C) directly onto a Thermo Scientific Hypersil GOLD column (2.1 mm x 100 mm, 1.9 µm particles, equilibrated in 95% solvent A (0.1% aqueous solution of formic acid), 5% solvent B (acetonitrile containing 0.1% formic acid). The compounds were eluted using flow rate 300 µL/min by linearly increasing solvent B concentration from 5% to final 40% over 15 min, and from 40% to 95% over 1 min. The column was then washed with 95% solvent B (2 min) and re-equilibrated in 95% solvent A, 5% solvent B. The total run time, including column wash and equilibration, was 20 min.

A Thermo Scientific LTQ Orbitrap XL mass spectrometer was operated in positive ion mode at 30,000 resolving power (defined as FWHM @ *m/z* 400) for full scan analysis (mass range 150 – 1500 u) followed by data dependent MS/MS on the most intense ion from the full scan at 7,500 resolving power (~0.3 sec per scan). The measurements were done in triplicate with external calibration. The settings for the higher energy collisional dissociation (HCD) fragmentation mode were 65% normalized collision energy, isolation width 3 u.

Thermo Scientific SIEVE 1.2 software was used for comparative and trend analyses. The software allows for processing a large number of samples, presenting the statistically significant differences between populations and various time points. Data were normalized on total spectral ion current. Results were filtered using pValue < 0.001 and at the same time requiring a minimum 2-fold change in peak height.

The results from SIEVE™ were further subjected to multivariate analysis with SIMCA P+™, version 11 (Umetrics, Umea, Sweden).

Mass Frontier™ (HighChem, Slovakia) software was used to confirm a suggested compound identity and structure based on observed fragmentation patterns.

Results

Due to the large number and the chemical complexity of phenolic compounds in wine matrix, analytical methods in the past involved sometimes difficult and complicated traditional chromatographic techniques. One of the major problems underlying separation of the phenolic compounds is their similarity in chemical characteristics. As many phenolics show similar UV spectra with maxima in a narrow range of 280-320 nm, extensive fractionation steps might be needed prior to HPLC analysis. Rather large initial volumes required and variable losses occuring due to incomplete extraction or oxidation can be an issue. The use of modern chromatographic techniques coupled to mass spectrometric detection can alleviate these problems.

Our approach avoids entirely the sample fractionation step: red wine is injected directly on the reverse phase column. Moreover, the use of small particles (< 2 µm) and relatively high flow rates (300 µL/min) enable swift analysis with excellent chromatographic resolution. The observed peak width for individual compounds was, on average, 7 sec, back pressure not exceeding 350 bar. With 20 min total cycle time per injection, this setup allows for high throughput analysis while the total sample consumption remains negligible (20 µL per injection). U-HPLC coupled to the LTQ Orbitrap XL proved to be very robust, allowing for an uninterrupted analysis of 24 untreated red wine samples which corresponds to an 8-hour continuous analysis without any requirement for a system cleanup or column change.

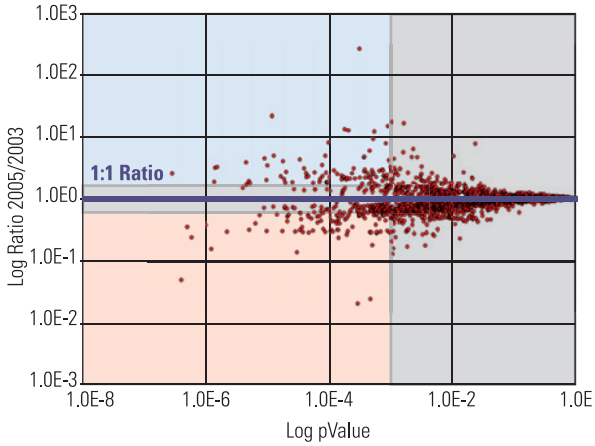

Figure 1: Overview of differences between harvest years 2003 and 2005. The result from differential analysis software (SIEVE 1.2) highlights the compounds having at least two-fold higher concentration in year 2005 compared to year 2003 (blue shaded area) and compounds whose concentration in year 2005 was less than a half of that in year 2003 (red shaded area). The purple horizontal line represents 1:1 ratio between concentrations in the year 2005 and 2003. The grayed area covers the features with less pronounced concentration difference and those with low statistical significance, i.e. pValue > 0.001.

Variables like wine varietals, soil composition, and harvest year will play an important role by providing the basic pool of compounds for these biotransformations. With accurate mass acting as a highly selective filter we could monitor hundreds of compounds across multiple samples, enabling advanced comparative studies and trend analyses. Initially, we were interested in comparing the wine of the same origin (area, label) but harvested in different years.

Our differential analysis of the Les Charmes de Kirwan, Margaux, contrasted wine from production years 2003 and 2005 using SIEVE software. The features (peaks) were filtered for their statistical significance (pValue < 0.001) and significant change defined as a minimum 2-fold concentration difference between the two harvest years (Figure 1). We observed 75 individual compounds which showed at least 2-fold higher content in year 2005 compared to year 2003 (blue shaded area in Figure 1). Kaempferol and quercetin concentration increased 25- and 8-fold, respectively, in year 2005 compared to 2003 (Figure 2). On the other hand, there were 36 other compounds whose concentration in the 2005 sample was significantly less than in the 2003 sample (red shaded area in Figure 1). Some flavonoids (myricetin) showed no change in concentration between the two harvest years.

Total anti-oxidant status refers to overall antioxidant properties of wine, and can be largely ascribed to a group of compounds comprising vanillic acid, *trans*-polydatin, catechin, *m*-coumaric acid, epicatechin, quercetin, *cis*-polydatin and *trans*-resveratrol.[12] In our analysis we detected vanillic acid, (epi)catechin, coumaric acid, and quercetin. When compared to wine produced in 2003, the wine produced in 2005 contained 50, 40 and 20% less coumaric acid, vanillic acid and (epi)catechin, respectively, while the amount of quercetin increased 8-fold (Table 1).

Calc *m/z*	Formula MW	Name	Change 24h/0h	Change 2003/2005
165.0546	$C_9H_8O_3$	Coumaric acid	0.32	0.51
169.0495	$C_8H_8O_4$	Vanillic acid	0.40	0.61
199.0601	$C_9H_{10}O_5$	Syringic acid	0.59	0.92
391.1387	$C_{20}H_{22}O_8$	Polydatin	Not found	Not found
229.0859	$C_{14}H_{12}O_3$	Resveratrol	Not found	Not found
291.0863	$C_{15}H_{14}O_6$	(Epi)catechin	0.66	0.82
303.0499	$C_{15}H_{10}O_7$	Quercetin	0.78	7.96
319.0448	$C_{15}H_{10}O_8$	Myricetin	0.69	1.00
287.0550	$C_{15}H_{10}O_6$	Kaempferol	1.00	20.96

Table 1: Overview of some compounds of interest and the changes in their content between year 2003 and 2005 (column **Change 2003/2005**), and after 24 hours following exposure to air (column **Change 24h/0h**). The compounds highlighted are the major contributors to the total anti-oxidant status.[12]

The remarkable difference in the content of quercetin between the two harvest years is interesting. Quercetin is one of the most abundant natural flavonoids found in fruits, vegetables and wine.

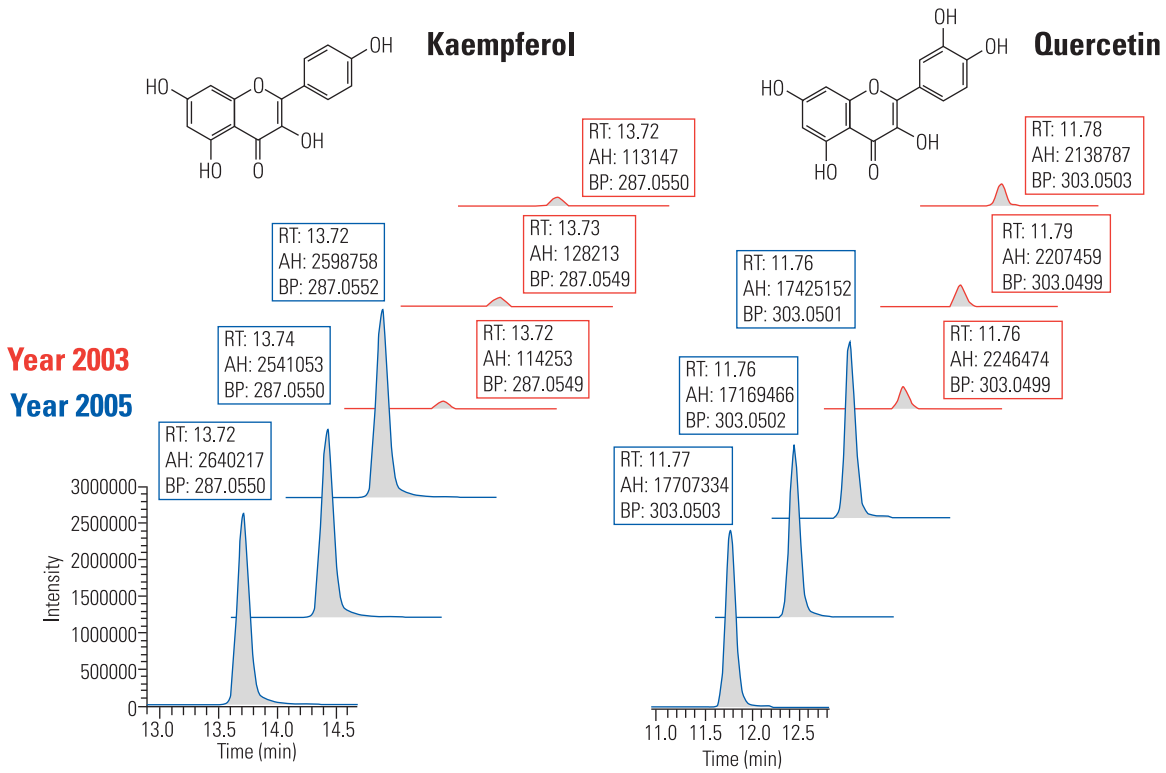

Figure 2: Extracted ion chromatogram for kaempferol and quercetin (left and right pane, respectively; 3 injections each) shows remarkable difference in concentration of these compound in wine harvested in year 2005 (blue trace) and 2003 (red trace). The mass deviation did not exceed 0.7 ppm for kaempherol (calculated *m/z* = 287.0550) and 1.3 ppm for quercetin (calculated *m/z* = 303.0499). Note the reproducibility of the retention time (RT) values and peak height calculations (AH) for 3 replicate injections.

At present, labeling requirements for red wine are far from comprehensive, basically limited to listing the total alcohol content and the comment that it contains sulfites. Including more specific information about compounds with strong anti-oxidant properties would improve a general public awareness and be helpful in the current climate of debate on healthy balanced diet. A fast but highly informative analysis of wines as described herein can thus help maintain consistency and quality, and provide useful information about product's nutritional value.

Reliable accurate mass measurements over a broad dynamic range of concentration are helpful for unambiguous identification of compounds of interest. The mass deviation of our measurements did not exceed 2 ppm using external calibration. Such an accuracy supported by reliably measured isotope abundancies in the LTQ Orbitrap XL enabled a confident assignment of elemental composition to individual peaks.

For confident identification of a compound, the elemental composition suggestions based on mass accuracy need to be complemented with the evidence from the fragmentation spectra. Our method was set up to collect higher energy collision dissociation (HCD) spectra. On average 700 such spectra were collected during each 20-minute LC-MS run. The MS/MS spectrum acquired in the multipole collision cell of the LTQ Orbitrap XL serves for confirming identity of a known compound or even determining identity of an unknown. Such an approach was demonstrated for the analysis of antioxidant compounds in olive oil.[13] Rich fragmentation, accurate mass measurement of both parent and fragment ions, and spectrum interpretation provided by Mass Frontier software were all crucial for this challenging task (Figure 3).

The anti-oxidant properties of wine are clearly beneficial to a consumer. On the other hand, wines with higher polyphenolic concentration are more susceptible to oxidation. We were interested to observe a trend of changes in the wine samples over the period of 24 hours after opening the bottle. The groups of samples from time points 0, 1, 5, and 24 hours (triplicate injections) were processed with SIEVE and further subjected to principal component analysis. The progressive changes caused by exposure to air are well observable and statistically significant (Figure 4).

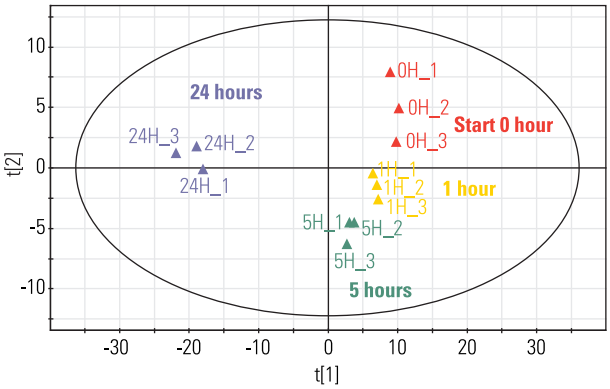

Figure 4: Wine sampled in triplicate at 0, 1, 5, and 24 hours after exposure to air. The sample groups are easily separated by the first two principal components.

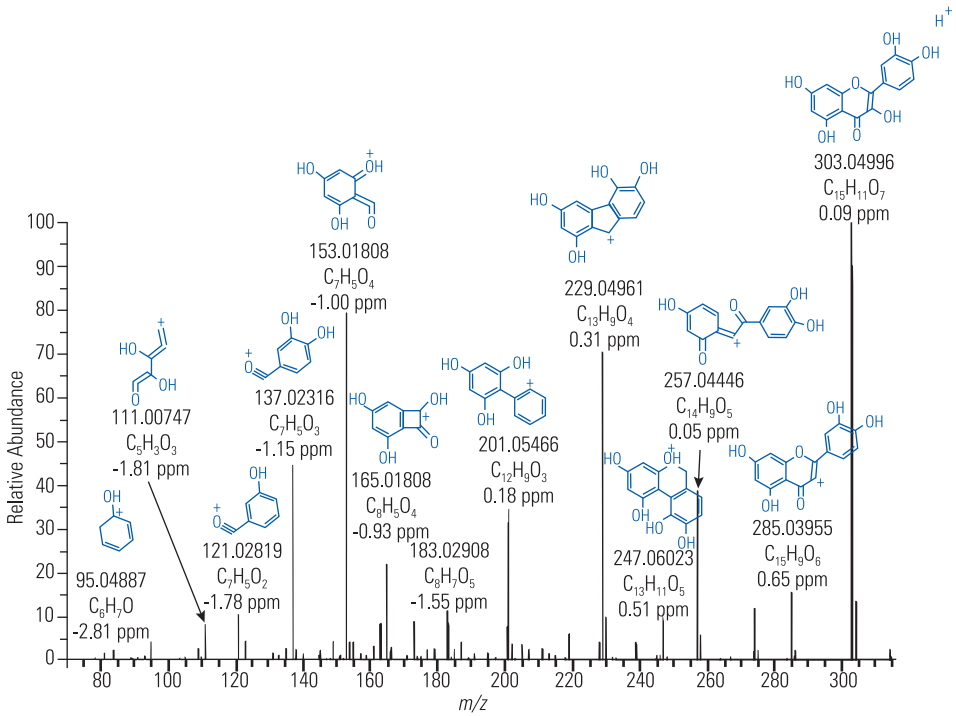

Figure 3: Confirmation and structural characterization of quercetin. Assignment of fragments in HCD spectrum using the Mass Frontier software relying on its extensive database of fragmentation mechanisms.

At this point, a potential effect of evaporation of more volatile constituents of wine has to be considered. In general, the partial pressure for compounds with molecular weight 300 and higher is considered negligible – such compounds should not be lost to evaporation at room temperature. Kaempferol (MW 286), (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302) would fall into such a category. Kaempferol showed no change over this period. Thus the decrease of 20% for quercetin and 30% for (epi)catechin and myricetin observed over the period of 24 hours following the bottle opening could be confidently ascribed to oxidation. For coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acids we observed a more pronounced drop in concentration (60, 70 and 40%, respectively) after 24 hours following the exposure to air (Figure 5). It might prove difficult, however, to distinguish between the effect of evaporation and oxidation under the employed experimental conditions.

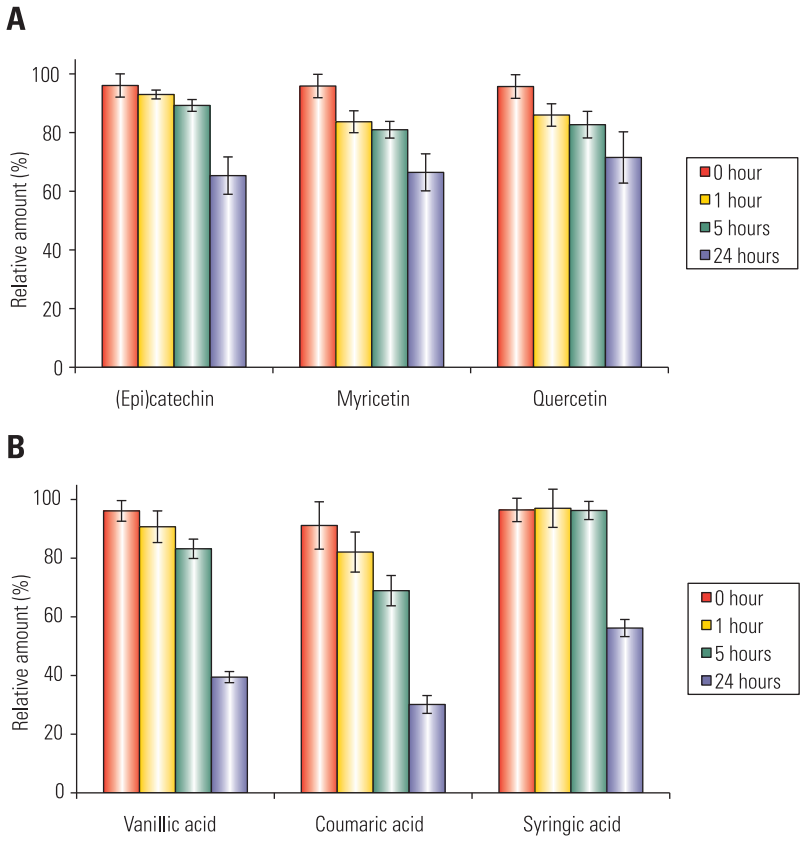

Figure 5: Changes over a 24-hour period of air exposure. The amount of a given compound at time 0 h defined as 100%. Panel A shows decrease in content of higher molecular weight compounds such as (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302). Lower molecular weight components including coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acid display much higher rate of disappearance (panel B). Error bars show the standard deviation for three repetitive analyses of samples at each time point.

Conclusions

As consumers are becoming increasingly aware of the harmful as well as helpful content of what they eat and drink, modern powerful analytical tools will undoubtedly play a crucial role to supply that information more accurately and quickly. Albeit a very complex mixture, red wine is perfectly suitable for mass spectrometric supported by SIEVE differential expression software. Such 'fingerprinting' analysis can be applied in quality control and process monitoring, and for highlighting relevant nutritional value to consumers.

- U-HPLC affords fast analysis times while maintaining very high chromatographic resolution (peak width 7 seconds at half height).
- The mass deviation of the LTQ Orbitrap XL measurements was always smaller than 2 ppm using external calibration up to one day old.
- Higher collision energy dissociation MS/MS spectra confirm the identity and structure of compounds in complex mixtures.
- Accurate mass measurements also significantly improve the precision of quantitation by eliminating nearly isobaric interferences. This is a particularly important aspect for complex mixture analyses, which red wine undoubtedly is.
- The methodology described here is extremely robust, allowing for an uninterrupted analysis of 24 untreated red wine samples (continued analysis over an 8-hour period).

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