Grape and Wine Analysis

Oenologists to exploit advanced test kits

TECHNIQUE

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It is without doubt that testing plays a pivotal role throughout the whole of the vinification process. To produce the best possible quality wine and to minimise process problems, such as "stuck" fermentation or troublesome infections, it is now recognised that if possible testing should begin prior to harvesting of the grapes and continue through to bottling. Traditional methods of wine analysis are often expensive, time consuming, require either elaborate equipment or specialist expertise, and frequently lack accuracy. However, enzymatic bio-analysis enables the accurate measurement of the vast majority of analytes of interest to the winemaker, using just one piece of apparatus, the spectrophotometer *(see previous issue No. 116 for a detailed technical review)*. Grape juice and wine are very amenable to enzymatic testing as being liquids they are homogenous, easy to manipulate, and can generally be analysed without any sample preparation.

Table 1 lists analytes for which enzymatic test kits are available, along with their relevance to the oenologist. Since its introduction, enzymatic bio-analysis has enjoyed an increasing role in the wine industry, offering an attractive alternative to many traditional techniques such as Thin Layer Chromatography, in for instance the determination of L-malic acid, or non-specific chemical tests, as in the case of reducing sugar analyses; indeed, widespread use of the D-glucose / D-fructose enzymatic test kit prompted the OIV to adopt this method in replacement of the traditional chemical method in 2003.

Table 1: Common wine enzymatic test kit analytes and their oenological significance.

Analyte	Oenological Significance
Acetaldehyde	A sensory compound that adds flavour and complexity, but spoils wine at high concentrations. Produced by both fermentation and oxidation.
Acetic Acid	A sensory compound that adds flavour and complexity in small amounts, but spoils wine at high concentrations. Produced naturally by yeast in small amounts and by spoilage organisms such as Acetobacter aceti in large quantities. This is the predominant of the acids comprising volatile acidity (VA).
Ammonia	Most important inorganic source of Yeast Available Nitrogen.
L-Arginine	Most important amino acid in grape juice with respect to being a source of Yeast Available Nitrogen.
L-Ascorbic Acid	Present naturally in grapes and often added as an anti-oxidant.
Carbon Dioxide	Critical level important for sensory perception by the mouth.
Citric Acid	Naturally present in small amounts, large amounts indicate addition for acidification (EU limit is 1 g/L).
Ethanol	Produced during alcoholic fermentation. Amounts > 17.5 % (v/v) indicate supplementation.
D-Fructose	Grape quality indicator. One of the two principle fermentable sugars of grape juice.
D-Gluconic Acid	Grape quality indicator for the production of certain wines such as Champagne.
D-Glucose	Grape quality indicator. One of the two principle fermentable sugars of grape juice.
Glycerol	Quality indicator of finished wine, important for "mouth-feel".
D-Lactic Acid	Produced predominantly by lactic acid spoilage bacteria.
L-Lactic Acid	Produced predominantly from L-malic acid during malolactic fermentation.
D-Sorbitol	High levels indicate addition of fruit.
D-Malic Acid	Only present in significant quantities in adulterated wine.
L-Malic Acid	Grape quality indicator. Very important grape acid, converted to less acidic L-lactic acid during malolactic fermentation.
D-Mannitol	Produced by spoilage organisms from D-fructose, resulting in an undesirable "mannitol taint".
Succinic Acid	Wine acid produced during fermentation.
Sucrose	Added to increase the amount of alcohol. Use only permitted in certain situations, such as in the production of Champagne.
Sulphite	Added to prevent unwanted microbial growth early in the vinification process, and then to stabilise the finished wine by acting as an anti-oxidant/microbial.
Starch	Added to artificially increase wine soluble solids (dry extract), a quality parameter of wine flavour and body.
Urea	Source of Yeast Available Nitrogen and precursor of the human carcinogen ethyl carbamate. Over-supplementation with diammonium phosphate (DAP) can result in elevated levels.

Development of advanced enzymatic test kits for the wine industry

Although the introduction of enzymatic bio-analysis into the wine industry has certainly proved very successful, there has surprisingly been very little development of the kits since their inception some 25 years ago. Although representing the state-of-the-art in terms of specificity and simplicity at that time, these products were designed for general food and beverage analysis and are now somewhat outdated, and in need of re-evaluation especially with respect to the demands of the modern wine industry. This is because during the development of the original range of test kits by the German company Boehringer Mannheim, only a relatively small number of predominantly animal derived enzymes were available, and thus in some cases ideal candidates could not be used. The last 10 years has, however, witnessed a revolution in terms of how biological research is performed, and powerful technologies have emerged such as

molecular biology, that now make it possible to rapidly produce enzymes with tailored specificities for essentially any desired application. This capability prompted innovative test kit manufacturers such as Megazyme to make long-awaited improvements to existing enzymatic bio-analysis products, while also rapidly developing new analytical procedures for either existing analytes or those of emerging importance in the wine industry.

This article will describe the role played by enzymatic analysis throughout the whole of the modern vinification process, with a focus on recent discoveries and innovations that look set to underpin further acceptance of this increasingly powerful technique in the wine industry.

Pre-harvest analyses

L-Malic Acid and D-Glucose / D-Fructose: as grapes ripen prior to harvesting, the level of L-malic acid falls significantly, while levels of D-glucose and D-fructose rise considerably. A balance must be achieved by the vineyard ensuring both sufficient sugar for the desired alcoholic strength, while at the same time optimal acidity for the envisaged character of the wine. Thus rapid and accurate analyses are critical at this time if the best quality grapes are to be achieved at harvest.

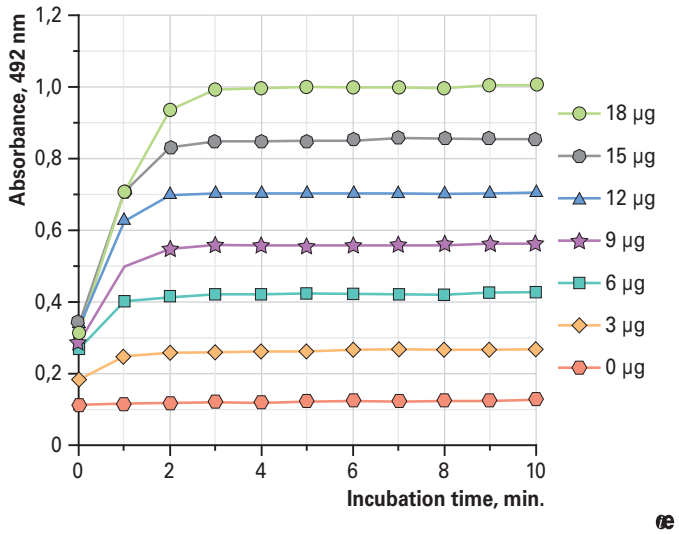
The analysis of L-malic acid is in fact one of the most frequently performed tests in the wine industry, owing predominantly to the role played by this acid during malolactic fermentation (MLF), and its quantification can be achieved very rapidly, in as little as 1 min, using an advanced enzymatic test kit with a spectrophotometer, according to the reaction pathway depicted by ***equations 1 and 2*** *(for a detailed description of how enzymatic test kits work in general, see previous issue No. 116, pages 14-15. This article also describes the enzymatic method for the determination of D-glucose and D-fructose).*

Equation 1 (L-malate dehydrogenase)
L-Malic acid + NAD^+ ➛ oxaloacEtate + NADH

Equation 2 (glutamate oxaloacetate transaminase)
Oxaloacetate + L-glutamate ➛ L-aspartate + 2-oxoglutarate

Equation 3 (diaphorase)
NADH + INT + H^+ ➛ INT-formazan + NAD^+

■ **Figure 1: Rapid performance of the L-malic acid MegaQuant™ test procedure.** The standard procedure was performed with levels of L-malic acid from 0 - 18 µg (as indicated). The end of the reaction is indicated by no further increase in absorbance, i.e. at 4 min with 18 µg of analyte.



However, pre-harvest testing by smaller wineries and grape growers was until recently very difficult, as such companies generally do not even possess a basic spectrophotometer, and thus must rely either on traditional techniques or central laboratories for their analytical requirements. In response to this pressing requirement, a novel product has recently been developed called MegaQuant™, that allows rapid and specific measurement of both L-malic acid and D-glucose plus D-fructose, without the requirement for an expensive spectrophotometer or other specialised laboratory apparatus and skills.

This was achieved by modification of the existing kits by the addition of a further enzyme, diaphorase, that for example in the case of L-malic acid converts the NADH product of ***equation 1*** into a coloured compound, INT-formazan ***(equation 3)***, that can be quantified using an inexpensive hand-held colorimeter, the MegaQuant™ Meter ***(figure 1 and photo 1)***. The MegaQuant™ kits were developed with the small winery especially in mind, and feature simple procedures, highly stable reagents in tablet form, small pack sizes and shelf lives spanning more than two vintages.

Pre-alcoholic (primary) fermentation analyses

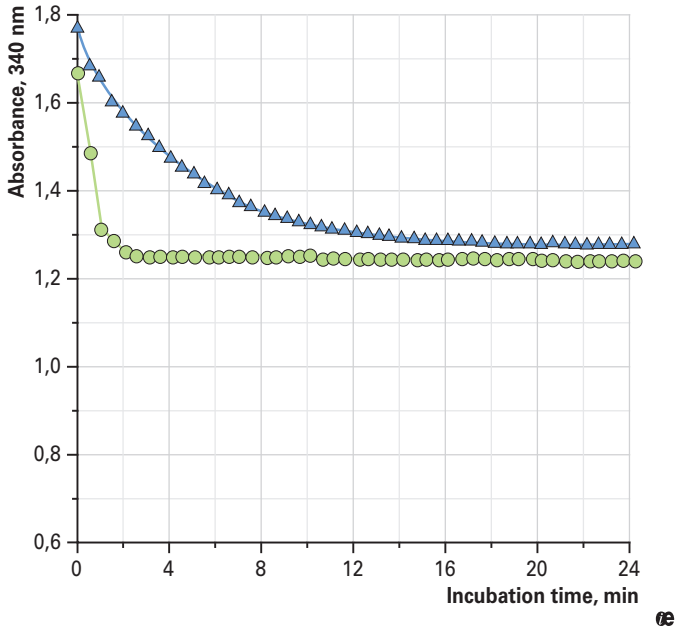
Nutritional Status: after analysis of grape juice for D-glucose and D-fructose, the amount of yeast available nitrogen (YAN) must also be determined, to ensure there is sufficient for efficient fermentation of the sugars. If grape juice contains insufficient YAN, fermentation will slow down or even become "stuck". This is not only inconvenient for the winemaker, but can also result in spoilage microbes becoming established, or more importantly the production of hydrogen sulphide gas, leading to a catastrophic deterioration in the quality of the wine. However, until recently it was not possible to easily and accurately quantify YAN as (a) it was unclear as to exactly what nitrogen sources were available to the yeast during fermentation, and (b) a rapid and specific analytical method was not available for the important YAN component, L-arginine.

■ **Photo 1: The MegaQuant™ Meter and reagents for L-malic acid analysis.**

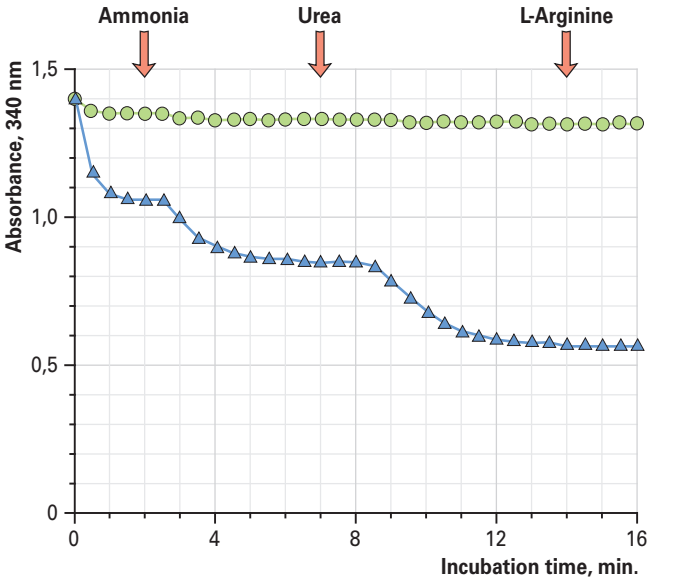
Recent scientific research has, however, now demonstrated that YAN is actually comprised of nitrogen from four basic sources; (1) ammonia, (2) the primary amino group of free amino acids, (3) the side-chain of L-arginine, and (4) urea. Although enzymatic kits have been available for some time for ammonia and urea, the enzyme employed in these kits, glutamate dehydrogenase (GlDH) from beef liver, is actually significantly inhibited by tannins found in grape juice and wine, leading to inconsistent and often inconveniently long reaction times. However, recently a very rapid advanced test kit for ammonia became available, based on a novel microbial GlDH, that is not only uninhibited by tannins, but also has a far superior Km value (affinity) for ammonium ions ($NH4^+$) than the beef liver enzyme.

Figure 2 shows a comparison of the analysis of red wine using an ammonia test kit based on beef liver GlDH with this much more advanced product ***(see equation 4 for reaction)***. This rapid ammonia test kit was subsequently extended by including urease and arginase ***(equations 4-6)***, producing a simple and rapid sequential assay for three of the four YAN components, i.e. L-arginine, urea and ammonia, with a total reaction time of < 16 min ***(figure 3)***. When used in conjunction with a primary amino nitrogen (PAN) kit, YAN in mg N/L can be calculated ***(figure 4)***. This represents a

■ **Figure 2: Analysis of red wine for ammonia using beef liver and microbial GIDH.** The same amount of red wine was analysed using a traditional ammonia test kit employing beef liver GIDH (blue triangles) and a rapid advanced product from Megazyme employing a microbial enzyme (green circles). The end of the reaction is indicated when no further fall in absorbance occurs, i.e. after only ~ 3 min with the Megazyme kit.



■ **Figure 3: Performance of a sequential rapid enzymatic test kit for L-arginine, urea and ammonia.** The sequential reaction shown demonstrates that L-arginine, urea and ammonia can be determined using the same cuvette (blue triangles), with reference to a blank containing no sample (green circles). Individual endpoints for ammonia, urea and L-arginine, after the addition of microbial GIDH, urease and arginase, respectively, are indicated by arrows. The total analysis time is < 16 min.



major improvement over traditional formol titration, that both underestimates the contribution from L-arginine, and includes a significant contribution from proline, an abundant amino acid the secondary nitrogen of which is unavailable during fermentation. Thus although formol titration gives an approximate YAN value, the results can be quite inaccurate depending on the levels of L-arginine and proline.

Similarly, it is not sufficient to estimate YAN by determining just one of the four components such as ammonia, as they are all present in highly variable quantities. Historically, some winemakers didn't even try to estimate YAN, and just added excess nitrogen in the form of the permitted additive diammonium phosphate (DAP). However, it was discovered that this practice frequently leads to the excretion of urea into the wine by the yeast, which after subsequent reaction with ethanol during storage results in the formation of the human carcinogen ethyl carbamate. Thus accurate YAN values should always be determined wherever possible before nitrogen supplementation.

Alcoholic (primary) fermentation analyses

Acetic Acid: during and subsequent to alcoholic fermentation, small amounts of acetic acid are produced, and this is thought to contribute to the "complexity" exhibited by wines. However, it is a very fine balance and spoilage bacteria such as Acetobacter sp. can lead to significant acetic acid production, resulting in very disadvantageous taste/smell characters.

Thus acetic acid is a key quality indicator of wine, and analyses are performed for this acid throughout the vinification process. Test kits based on the enzyme acetyl-coenzyme A synthetase (ACS; ***equations 7-9***) are by far the most common used in the wine industry, but present significant problems to both the small and large winery; like many other test kits, basic acetic acid kits contain vials of lyophilised enzyme (ACS), which after reconstitution with water are only stable for 5 days, not enough time for the average small winery to make adequate use of the enzyme before it must be discarded.

Equation 4 (microbial glutamate dehydrogenase)
2-Oxoglutarate + NADPH + NH_4^+ ➛ L-glutamic acid + $NADP^+$ + H_2O

Equation 5 (urease)
Urea + H_2O ➛ $2NH_4^+$ + CO_2

Equation 6 (arginase)
L-Arginine + H_2O ➛ urea + ornithine

Equation 7 (acetyl-coenzyme A synthetase)
Acetic acid + ATP + CoA ➛ acetyl-CoA + AMP + pyrophosphate

Equation 8 (citrate synthase)
Acetyl-CoA + oxaloacetate ➛ citrate + CoA

Equation 9 (L-malate dehydrogenase)
L-Malic acid + NAD^+ ➛ oxaloacetate + NADH + H^+

The problem experienced by large wineries is that when adapted for auto-analysers, the reagent mixture has a very limited stability, and can only be used for ~ 1 day, while reagents for other kits, such as for L-malic acid prepared in the same way, are stable for ~ 1 week. An advanced acetic acid kit is available however, that employs an ACS presented in a novel highly stable ammonium sulphate suspension, and thus as little as a single reaction can now be performed without affecting the shelf life of the remaining enzyme. The same kit also contains a "stabilisation factor" that dramatically increases the stability of the reagent when prepared for auto-analyser applications ***(figure 5)***. Another limitation of the ACS based assays for acetic acid is that the calculations are complicated, owing to an "indicator reaction" that is in equilibrium ***(equation 9)***. In addition, a slight creep reaction that is a feature of this method may have to be accounted for. These problems have been resolved by development of simple Excel based calculators, such as Mega-Calc™, similar versions to which are also available for other wine test kits and can be downloaded free of charge from suppliers' websites.

Recently, an alternative acetic acid kit based on acetate kinase has also been developed especially for auto-analyser applications in the wine industry ***(equations 10-12)***. This kit offers the key advantages of not only being very stable after preparation for auto-analyser applications, but also a stoichiometric amount of NADH is consumed during the reaction ***(equation 12)***, resulting in linear calibration curves ***(figure 6)***, unlike kits based on ACS, where the equilibrium "indicator reaction" results in non-stoichiometric changes in absorbance relative to the concentration of acetic acid.

Glycerol: like with most foods and other beverages, "mouth feel" is a very important characteristic of wine, and is influenced to a great extent by glycerol content. Testing is thus performed to ensure adequate levels are produced during fermentation and not subsequently degraded by spoilage organisms. Basic test kits are available from a number of suppliers, but the reagents are only stable for 4 days after preparation. However, an advanced test kit was recently developed that is both very rapid ***(figure 7)***, and more importantly presents the labile reagents in highly stable tablet form, resolving the instability issue posed by all other glycerol kits, eliminating wastage and thus minimising cost of analysis.

D-Glucose / D-Fructose: during alcoholic fermentation the content of ethanol rises, while the levels of D-fructose and D-glucose fall from ~ 25 % (w/v) initially, to < 0.2 % (w/v). It is important for fermentation to continue until the residual sugar content is very low, as if not, subsequent fermentation could lead to spoilage through the production of carbon dioxide (CO_2) in the bottle, or growth of spoilage organisms. Where extra sweetness is required, accurate residual sugar levels are very important in order to raise the D-glucose and D-fructose content back up to ~ 8 g/L for instance, by the addition of concentrated grape juice.

Other analytes: the four YAN analytes are also of interest during alcoholic fermentation to ensure continued availability of nitrogen, and also to confirm that significant levels of urea are not accumulating in the must. D-Lactic acid may also be determined when monitoring for infection by lactic acid spoilage bacteria.

Equation 10 (acetate kinase)
Acetic acid + ATP ➛ acetyl-phosphate + ADP

Equation 11 (pyruvate kinase)
PEP + ADP ➛ pyruvate + ATP

Equation 12 (L-lactate dehydrogenase)
Pyruvate + NADH + H^+ ➛ L-lactic acid + NAD^+

Malolactic (secondary) fermentation analyses

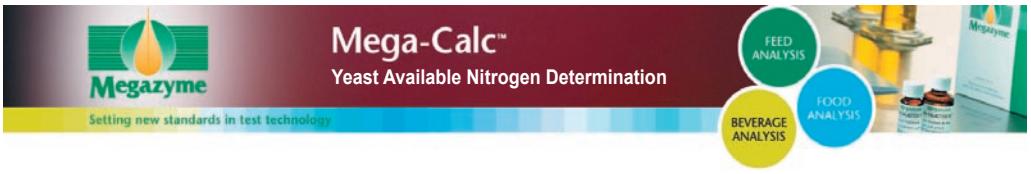
Bacterial MLF is best performed subsequent to alcoholic fermentation, and results in L-malic acid being converted to L-lactic acid and CO_2. It is performed with most red, and some white wines (such as Chardonnay), and results in a significant increase in pH of ~ 0.1 to 0.45 units.

Monitoring for the onset, progress and completion of MLF represents one of the most important tasks in the wine industry; if incomplete at the time of bottling, the CO_2 subsequently produced will at the very least lead to a spoiled effervescent wine, or in the worse case scenario corks being pushed out or even bottles breaking. As described above, rapid test kits are available for larger wineries with a spectrophotometer (or auto-analyser), or for smaller wineries using the MegaQuant™ colorimeter. The ability to perform L-malic acid analyses in-house is certainly very advantageous for the small winery, that would normally not be able to analyse each barrel of wine, and even then would waste much valuable time in simply driving to and from the analytical laboratory. When desired, rising levels of L-lactic acid can also be followed enzymatically.

Finished wine analyses

Many enzymatic test kits are employed in the production and analysis of finished wine:

■ **Figure 4: The Mega-Calc™ raw absorbance data processing aid for YAN determination using the L-arginine / urea / ammonia, and primary amino nitrogen test kits of Megazyme.** Blank absorbance values are entered at the top of the page where indicated. After values are entered for the ammonia, urea, L-arginine and primary amino nitrogen reaction endpoints, the Excel based program automatically calculates values for these individual analytes in g/L and also a total YAN value in mg N/L.



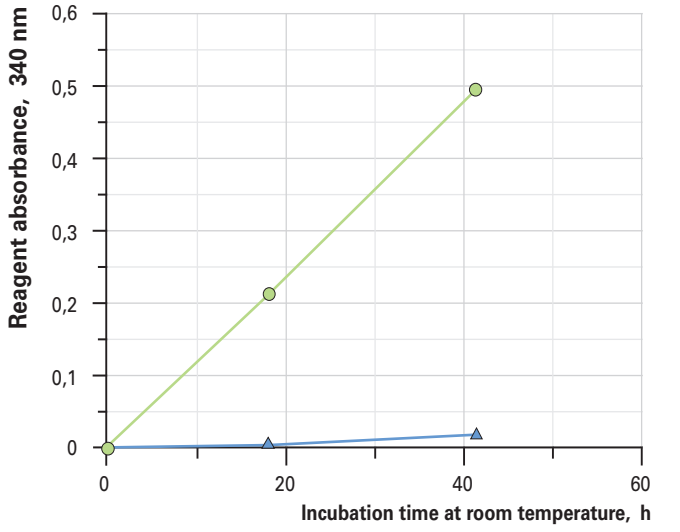
Sample details

Blank absorbance values

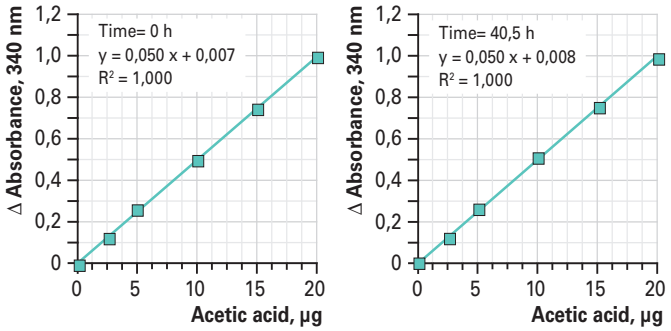
Analyte	A_1	A_2	A_3	A_4
YAN				
PAN				

	Sample identifier	Analyte	Sample absorbance values A_1	A_2	A_3	A_4	Sample volume (mL)	Dilution (-fold)	Results Δ Abs Analyte	Analyte (g/L)	Analyte (mg N/L)
1		$YAN_{Ammonia}$					0,10	1			
		YAN_{Urea}									
		$YAN_{L\text{-}Arginine}$									
		YAN_{AUG}									
		PAN					0,05	1			
		YAN_{Total}									
2		$YAN_{Ammonia}$					0,10	1			
		YAN_{Urea}									
		$YAN_{L\text{-}Arginine}$									
		YAN_{AUG}									
		PAN					0,05	1			
		YAN_{Total}									
3		$YAN_{Ammonia}$					0,10	1			
		YAN_{Urea}									
		$YAN_{L\text{-}Arginine}$									
		YAN_{AUG}									
		PAN					0,05	1			
		YAN_{Total}									

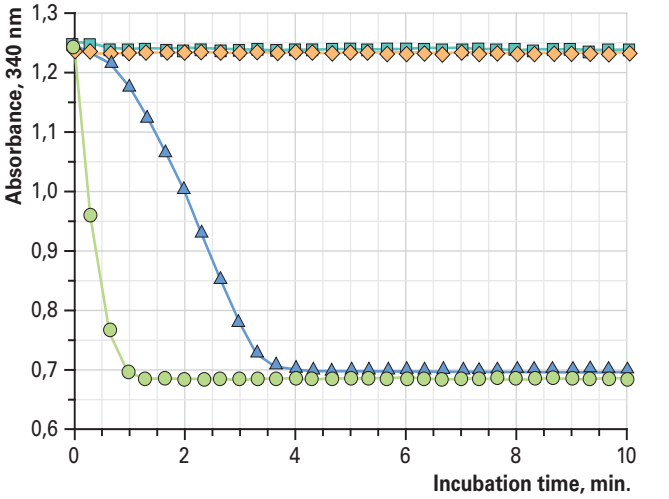
■ **Figure 5: Stabilised advanced acetic acid kit reagent.** Both basic (green circles) and stabilized (blue triangles) acetic acid kit reagents were prepared for auto-analyer applications and incubated at room temperature (~ 22.5 oC). At time points (0 h, 18 h and 41.5 h) the absorbance of the reagents was determin ed. Over this time period, equivalent to ~ 6 days at 4 oC, the absorbance of the stabilized reagent at 340 nm only increased by 0.018, while that of the unstable basic reagent had increased by 0.495.



■ **Figure 6: Linear calibration curves for Megazyme's stable acetate kinase format acetic acid kit.** After incubation of prepared reagents for 40.5 h at room temperature (~ 22.5 oC), Megazyme's advanced acetate kinase format acetic acid kit gave an almost identical linear calibration curve (right graph) to that obtained at time 0 h (left graph).



■ **Figure 7: Rapid performance of Megazyme's advanced glycerol enzymatic test kit as compared to that of a competitor product.** Like many of Megazyme's advanced enzymatic test kits, the reaction time of the glycerol kit (green circles) is significantly less than that of competitor products (blue triangles).



Stability: in order to increase microbial and oxidative stability, the levels of sulphite (SO_2) and L-ascorbic acid must be determined and additions made as necessary. Upper limits for total SO_2 must not be breached (as this could be hazardous to human health), while the presence of L-ascorbic acid in the absence of sufficient SO_2 can actually lead to hydrogen peroxide (H_2O_2) mediated oxidation of the wine. Residual levels of D-glucose, D-fructose and L-malic acid are also determined to estimate microbial stability of the wine, though levels of added SO_2 are usually high enough in finished wine to inhibit the growth of spoilage microbes.

Spoilage: the presence of high levels of acetic acid, acetaldehyde, D-lactic acid, and D-mannitol can all be established using enzymatic test kits, and where present indicate growth of spoilage organisms.

Authenticity: test kits can be used to detect various adulterants; the presence of starch indicates addition in an attempt to artificially increase the dry extract, a quality parameter of wine flavour and body. Citric and D-malic acid are usually present in very low levels, thus elevated levels indicate their addition as acidulants. It is only permitted to use sucrose in the production of certain wines such as Champagne, illegal addition is readily diagnosed using a sucrose / D-fructose / D-glucose test kit. If ethanol is present at levels in excess of ~ 17.5 % (v/v), supplementation is likely to have been performed.

Safety: as already discussed, high levels of urea in finished wine will lead to the production of ethyl carbamate during storage. If present, treatment with urease is permitted to reduce the concentration of this carcinogen precursor to safe levels.

Future of enzymatic bio-analysis in the wine industry

As evidenced by the large number of improved and novel test kits that have been launched in the last 2 years alone, manufacturers of enzymatic bio-analysis products for the wine industry such as Megazyme have clearly been proactive in not only working closely with the oenologist to define exactly what novel products are required or which existing products require updating, but then in also performing the relevant research and development to rapidly find successful solutions.

However, much work still lies ahead for both parties, regarding the development of future products and acceptance of the current ones by the OIV. ■

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