Enzymes: Industrial and Analytical Applications

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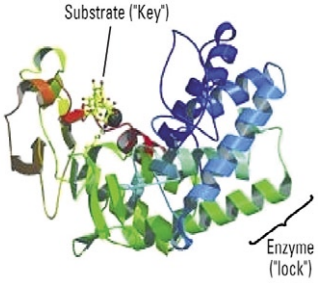
All living organisms from bacteria to Man are built and maintained by biological catalysts called enzymes. These micro-machines, that are made from protein, have each been evolved over millions of years to perform very specific biochemical tasks. Some enzymes have been designed by Nature to build chemical compounds up, while others are responsible for either breaking them down or modifying them. These "reactions" involve the making and breaking of chemical bonds, and enzymes make such reactions occur much faster, often millions of times faster, than they would do normally, hence their description as "catalysts". However, their ability to speed chemical reactions up is only one of the many useful properties possessed by enzymes. Probably the most notable and intriguing feature of enzymes is their so called "specificity", or in simple terms, their ability to recognise only the chemical compound, or "substrate", that they were designed to act on, and ignore everything else. Although this property remained a mystery for a long time, namely because enzymes were far too small to be seen by even the most powerful microscope, scientific techniques now enable enzymes to be observed in fine detail. It was revealed that enzymes are actually shaped like biological "locks", while the substrates they act upon are shaped like "keys". Enzymes work analogously to the opening of a locked door, a reaction will only take place if the correct key (substrate) is placed in the correct lock (enzyme). For example, ***figure 1*** shows a pectinase enzyme actually caught in the act of catalysis, where the substrate, pectate (shown in yellow), has diffused into the active-site (the lock) of the enzyme, and catalysis is just about to take place. Like a skilled locksmith, Nature possesses the ability to make slight changes to the shape of its locks, and this results in the generation of new, or "evolved", enzymes. Indeed, Nature has been evolving enzymes for so long, that specific enzymes now exist to make, modify, or breakdown every naturally occurring compound!

Table 1: Industrial enzymes and their use.

Enzyme	Application	Sector
Protease	Degradation of proteins	Detergent
Cellulase	Degradation of cellulose	Detergent
Lipase	Degradation of fats	Detergent
Amylase	Degradation of starch	Detergent
Amylase	Conversion of starch to glucose	Starch Processing
Glucoamylase	Conversion of starch to glucose	Starch Processing
Glucose Isomerase	Production of high fructose syrup	Starch Processing
Xylanase	Improve nutrient uptake of poultry	Animal Feed
Phytases	Improve nutrient availability	Animal Feed
Proteases	Improve digestion of proteins	Animal Feed
Xylanase	Removal of lignin "bio-bleaching"	Paper and Pulp
Arabinanase	Removal of post maceration haze	Fruit/Vegetable Processing
Amylase	Removal of juice starch haze	Fruit/Vegetable Processing
Polygalacturonanase	Increased juice yields	Fruit/Vegetable Processing
Hydrolases	Breakers for biopolymer gels	Gas and Oil
Chymosin	Clotting in cheese manufacture	Dairy
Urease	Removal of urea	Wine
Pectinase	Increased yields	Wine
Protease	Tenderisation	Meat
Amylase	Desizing	Textiles
Amylase	Process control	Baking
Beta-glucanase	Avoid filtration problems	Brewing
Protease	Increased area yield	Tanning

Figure 1: A pectate lyase enzyme in the act of catalysis.



The substrate, coloured in yellow, has the correct shape to fit deep inside the active-site cleft of the enzyme, where, protected from the aqueous environment outside, enzymatic catalysis can take place efficiently.

Industrial Application of Enzymes

Not surprisingly, Industrialists were among the first to recognise and exploit the great potential of enzymes, for they realised if reactions could be speeded up, production processes could be performed in only a fraction of the normal time, or at lower temperatures or pressures, or using cheaper starting materials. In other situations, enzymes made certain reactions commercially viable for the first time. Today the global market for industrial enzymes is growing rapidly, and is currently worth more than €2 billion per annum, the principle sectors being in the detergent, starch processing, and animal feed industries, though as can be seen from ***table 1***, enzymes are now finding many and widespread applications, from the "bio-bleaching" of paper, to the more efficient recovery of oil and gas. In general, the use of enzymes is very safe, cost effective and is regarded as a "green" or environmentally friendly technology.

Table 2: Enzymatic test kits and their use in bio-analysis today.

Test Kit	Field of Application		
	Medical	Food	Beverage
Acetaldehyde		•	•
Acetic Acid		•	•
Alanine aminotransferase	•		
Alpha-amylase	•	•	
Ammonia	•	•	•
Amylose/Amylopectin		•	
Arabinan		•	•
L-Arabinose/D-Galactose	•	•	
L-Arginine/Urea/Ammonia			•
L-Ascorbic Acid		•	•
L-Asparagine/Ammonia		•	
L-Asparagine/L-Aspartic Acid		•	
Aspartate aminotransferase	•		
Aspartame		•	•
Beta-Glucan			•
Beta-amylase		•	•
Carbon Dioxide	•		•
Cholesterol	•	•	
Citric Acid		•	•
Creatine Kinase	•		
Ethanol	•	•	•
Fructan	•	•	
D-Fructose/D-Glucose		•	•
Formic Acid		•	•
Galactomannan		•	
Glucomannan		•	
D-Gluconic Acid/D-Glucono-δ-lactone		•	•
D-Glucose	•	•	•
Glucose-6-phosphate dehydrogenase	•		
Glucose Oxidase		•	
L-Glutamic Acid		•	
Glycerol		•	•
D-3-Hydroxybutyric Acid	•	•	
D-Isocitric Acid		•	•
D-/L-Lactic Acid		•	•
L-Lactic Acid	•	•	•
Sorbitol/Xylitol/Lactitol		•	•
Lactose/D-Galactose		•	•
Lactulose			•
Leucine aminopeptidase	•		
D-Malic Acid			•
L-Malic Acid		•	•
Maltose/Sucrose/D-Glucose		•	•
D-Mannitol/Isomalt/L-Arabitol		•	•
D-Mannose/D-Fructose/D-Glucose		•	
Nitrate		•	•
Oxalic Acid		•	•
Pectin Identification		•	
Raffinose/D-Galactose		•	
Raffinose/D-Glucose		•	
Resistant Starch		•	
Starch Damage		•	
Succinic Acid		•	•
Sucrose/D-Fructose/D-Glucose		•	•
Sucrose/D-Glucose		•	
Sucrose/Lactose/D-Glucose		•	
Sulphite		•	•
Total Dietary Fibre		•	
Total Starch		•	
Trehalose/D-Glucose		•	
Triglycerides	•	•	
Urea/Ammonia	•	•	•
Uric Acid	•		
Yeast/Mushroom Beta-Glucan		•	

The Food Industry

Today most applications for industrial enzymes are found in the food industry. The application of enzyme technology began in the 1960s with the use of glucoamylase to produce greater yields, higher purity and easier crystallisation of glucose from starch. This application rapidly replaced inefficient traditional acid hydrolysis methods, and led to steam costs being instantly reduced by 30 %, ash production by 50 % and by-product production by 90 %. The next major development was the invention of immobilised glucose isomerase in 1973, enabling for the first time the industrial production of high-fructose corn syrups from starch. Other major applications include the use of amylase to extend the shelf life of bread and cakes, protease, amylase and glucanase in the efficient production of beer, animal and non-animal derived proteases (rennets), lipase, lysozyme, and beta-galactosidase in the production of cheese and other dairy products, pectin lyase and cellulase in the production of carrot juice and purée, and protease for the tenderisation of meat, and improvements to the solubility, emulsification, gelling or foaming properties of various other foods.

The Beverage Industry

The use of industrial enzymes in the beverage industry goes back as far as the 1930s, when RöhM & Haas launched the pectinase "Pectinol K" onto the market for the clarification of apple juice haze. Today, in addition to numerous clarification applications, enzyme preparations are used to both increase juice yields and decrease energy consumption, by enzymatically breaking down pectins, and other components of the plant cell wall, resulting in either efficient partial maceration, or complete liquefaction. Pectinase preparations comprise primary enzymes, such as pectin lyase, pectinesterase and polygalacturonanase, and lower concentrations of secondary components, such as cellulase and xylanase. In the wine industry, the use of pectinase preparations is permitted to increase juice yields, and beta-glucanase can be employed as a clarification and filtration aid. Currently, there is significant concern regarding the occurrence of ethyl carbamate in wine; this known carcinogen is formed from ethanol and urea (if present), during wine storage. As a precautionary measure, the use of the enzyme urease to remove urea from wine, and thus prevent ethyl carbamate formation, has recently been approved by the Office International de la Vigne et du Vin (OIV).

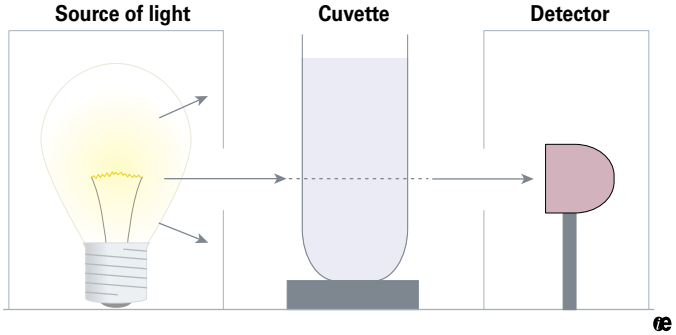
The Detergent Industry

The detergent industry is currently the largest user of industrial enzymes, and employs proteases, lipases, amylases, and cellulases predominantly in the production of "biological" washing powders. These enzymes quickly breakdown or release the dirt that normally can only be removed at much higher temperatures, or by using larger quantities of chemical detergents over a longer time period.

The Age of Enzymatic Bio-Analysis

As early as the 1960s, research

Figure 2: A simple representation of a spectrophotometer.



scientists realised the great potential for the application of enzymes in medical diagnostics, and food and beverage analysis. However, for an enzyme to be used analytically, it must be of exceptional purity, as although individual enzymes are exquisitely specific, they are generally found along with many other enzymes in Nature, and these "contaminants" must be removed before the enzyme is of any use. Additionally, analytical enzymes are often required in large amounts, and in the 1960s, preparing the necessary quantities was either simply not viable, or very time consuming and thus expensive. Finally, the apparatus required to monitor the course of enzymatic reactions, the spectrophotometer, although invented in the 1940s was still very much in its infancy and far too expensive for routine analysis applications.

However, by the 1980s, advances in the large-scale production of industrial enzymes, in addition to a series of related scientific breakthroughs, namely methods that enabled scientists for the first time to efficiently screen for (find) enzymes that had not yet been discovered, resulted in a large number of pure enzymes being commercially available and in good quantity. Coupled with the now widespread commercial availability of spectrophotometers, the age of "Enzymatic Bio-Analysis" was born. At this time, a German company, Boehringer Mannheim, pioneered the development of enzymatic "test kits", and went on to dominate the market as the supplier of "Gold Standard" products for the next 20 years. Today, the same technology as developed in the 1980s by Boehringer Mannheim is still available from the diagnostics division of the multinational company Roche, who acquired the renowned German company in 1997.

Table 3: Companies actively developing and manufacturing enzymatic test kits and related products.

Products	Applications	Company	Website
Reagents	Medical Diagnostics	Roche	www.roche-diagnostics.com
	Dairy Analysis	(Originaly developed by Boehringer)	
	Food Analysis		
	Beverage (wine) Analysis		
Test Kits and Reagents	Dairy Analysis	Megazyme	www.megazyme.com
	Food Analysis		
	Feed Analysis		
	Beverage (wine) Analysis		
Test Kits and Reagents	Medical Diagnostics	Merck	www.merck.de
	Beverage (wine) Analysis		
Test Kits and Reagents	Medical Diagnostics	Trinity Biotech	www.trinitybiotech.com

Enzymatic bio-analysis today

The past 25 years has seen the real price of both spectrophotometers and enzymatic test kits fall significantly, while the expertise required to perform these tests has become less demanding and at the same time more widespread. Additionally, not only is there now a number of test kit manufacturers, driving competition, but some of them are actually expanding the range of enzymatic test kits and related reagents that are available, or are systematically improving the disadvantageous properties of many of the old products. Indeed, at the moment there is a continual expansion of the range of enzymatic test kits that are available for medical diagnosis, and food and beverage analysis. The enormous potential of enzymatic bio-analysis can be appreciated from the long list of test kits presented in ***table 2***. Companies actively developing and manufacturing enzymatic test kits and related products are listed in ***table 3***.

Properties and selection of suitable enzymes for industrial and analytical applications

Like all other proteins, enzymes are sensitive to environmental factors such as temperature, pH, and ionic strength, and under certain conditions, an enzyme can even lose its catalytic activity permanently through "precipitation" or "denaturation". Also, all enzymes exhibit different biochemical characteristics, such as variable affinities for their substrate (the "Km value"), variable speed of reaction (the "Vmax value"), variable pH optima, variable cofactor requirements, and variable optimal ionic strengths. Enzymatic reactions can also be significantly affected by "inhibitors", such as heavy metal ions, or other biological compounds, that manipulate the biochemical properties of an enzyme, and slow the reaction down. Sometimes this inhibition is intended by Nature as a control mechanism for the activity of a particular enzyme, while other times this phenomenon results from the simple fact that a particular compound just happens to fit into the active-site of the enzyme in question. Thus, when developing industrial or analytical applications, after selecting an enzyme simply based on the reaction that it performs, research scientists must then carefully check that the enzyme will be adequately stable under the conditions that it will be used in, and that in those conditions, catalysis is still performed efficiently. Furthermore, a final challenge must be overcome for analytical enzymes, in that a way must also be found to accurately quantify the products of the reaction, and it is for this reason that a spectrophotometer must be employed.

Figure 3: A typical enzymatic test kit.

In this case, test kit reagents are supplied in individual bottles protected by a polystyrene container, that also enables easy storage. This commercial L-Malic Acid Test Kit comprises 5 reagents; bottle 1 contains 1 M glycylglycine buffer (pH10.0) plus 1 M L-glutamate, bottle 2 contains 180 mg of NAD +, bottle 3 contains 1.25 mL of the enzyme glutamate-oxaloacetate transaminase, bottle 4 contains 1.25 mL of the enzyme L-malate dehydrogenase, and bottle 5 contains 5 mL of L-malic acid standard solution (0.15 mg/mL).

What is a spectrophotometer and how does it work?

A spectrophotometer is a machine that passes a very well defined amount of light through a solution that is placed in a special plastic or glass container called a cuvette. The solution will absorb some of the light, and the rest emerges from the opposite side of the cuvette and is accurately quantified by the spectrophotometer's detector ***(figure 2)***. The difference between the amount of light entering the cuvette, and the amount of light that emerges from the cuvette, is called the "change in absorbance", and is measured in "absorbance" units, i.e. the greater the amount of light that is absorbed, the larger the absorbance value will be.

What does a typical enzymatic test kit comprise?

A test kit contains all the reagents in convenient form and quantity required to perform a set number of analyses, in the optimal conditions for the enzymes employed. In addition to the enzymes themselves, test kits usually contain a buffer solution (to ensure pH control of the reaction and thus optimal activity of the enzymes), a cofactor such as NADP + or NADPH (to enable monitoring of a change in absorbance), and a standard material, generally in a convenient liquid form ***(figure 3)***. Test kits are stored at 4oC,

and the shelf life varies between manufacturers, with the better quality products being stable for greater than 2 years.

What laboratory equipment is required?

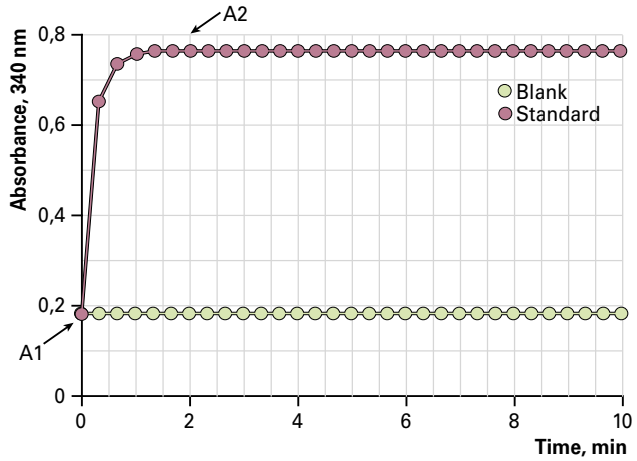
In addition to a spectrophotometer, very little laboratory equipment is actually required to perform enzymatic bio-analyses; a set of pipettes (e.g. from Gilson®) with disposable plastic tips to accurately dispense liquid volumes between 20 µL and 1000 µL will be required, along with disposable plastic cuvettes or test-tubes (depending on the type of spectrophotometer used). Basic filtering or other simple sample treatment apparatus may also be necessary, depending on the nature of the samples being analysed.

How is the test actually performed?

An assay mixture is first prepared by mixing a small volume of the buffer, distilled water, cofactor(s) and sample in a cuvette. The final reagent to be added is the enzyme specific for the analyte of interest, and the subsequent change in absorbance due to the reaction is then determined. This is achieved practically by measuring the absorbance of the assay solution before the addition of the enzyme (A1), and then again after the reaction has finished, or come to an "end-point" (A2), and then simply calculating the change in absorbance that occurred (A2-A1; ***figure 4***).

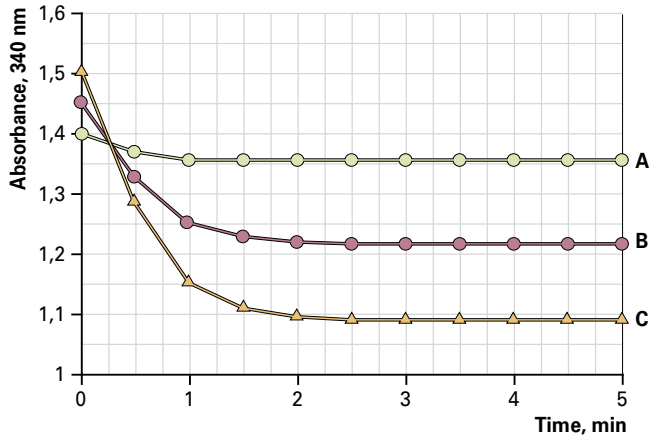
An additional "blank" reaction is also performed that does not contain any sample. The difference in absorbance changes between the two reactions, i.e. the "sample" and "blank" reactions, is then used in a simple equation supplied with each test kit to calculate the amount of analyte in g/L or percentage terms. Detailed but simple instructions are supplied with all kits, and some companies offer free on-line software aids for the automatic calculation of results from raw absorbance data. For a more detailed explanation of how test kits work, see the following examples:

Figure 4: The increase in absorbance over time during a typical L-malic acid test.



After the addition of the enzyme L-malate dehydrogenase (at time = 0 min; A1) to start the reaction, the absorbance (closed circles) rapidly increases from 0.19 to 0.77. As can be seen, no further increase in absorbance occurs after approximately 1.5 minutes (A2), i.e. this was the end-point of this particular L-malic acid test reaction. The open circles represent a "blank" reaction, that did not contain any sample.

Figure 5: The decrease in absorbance over time during a typical ammonia test.



The decrease in absorbance at 340 nm was followed on incubation of untreated red must preparation using a commercial Ammonia (Rapid) Test Kit. A, blank reaction; B, 25 µL of red must sample; C, 50 µL of red must sample.

Example 1: determination of ammonia (NH_4^+)

The quantitative determination of ammonia is very important in medical diagnostics, and food and beverage analysis. For instance, in the wine industry accurate ammonia determination is critical in both checking optimal levels of yeast available nitrogen (YAN) is present for efficient fermentation, yielding good quality wine, and in the prevention of over-supplementation with diammonium phosphate (DAP), that can lead to the formation of ethyl carbamate.

The enzyme catalysed reaction in the case of ammonia determination actually leads to a reduction in absorbance, rather than an increase ***(figure 5)***. Only a single enzyme, glutamate dehydrogenase, is required to catalyse the reaction ***(equation 1)***:

During the reaction, NADPH which absorbs light strongly, is converted to NADP +, that does not absorb light, and thus the absorbance falls during the reaction, until the end-point is reached. 2-Oxoglutarate is an additional substrate required by the enzyme and is present in excess levels, to ensure the reaction proceeds as rapidly as possible.

When both an ammonia and urea determination is required, such as in the wine industry, the ammonia test kit reaction can be extended by the subsequent addition of urease, according to ***equation 2***. Practically, this is achieved by performing two reactions, i.e. after the first "ammonia" reaction has reached its end-point, a second reaction is then initiated by the addition of urease, and a further reduction in absorbance occurs ***(figure 6)***.

In this case, the urease produces two molecules of ammonia (as ammonium ions; NH_4^+), and this is taken into account in the equation when calculating the results.

Example 2: determination of D-glucose and D-fructose

In the food and beverage industries, the quantification of D-glucose and D-fructose is very important, as these sugars are present in many food ingredients, or are added in the form of high-fructose syrup sweeteners. In the wine industry, D-glucose and D-fructose are the principle fermentable sugars utilised by the yeast, and represent approximately 25 % of fresh grape juice by weight. After fermentation is complete, the residual levels of these sugars are also determined prior to supplementation.

Similarly to the urea and ammonia

Equation 1

(glutamate dehydrogenase)

$$\text{2-Oxoglutarate} + \text{NADPH} + NH_4^+ \rightarrow \text{L-glutamic acid} + \text{NADP}^+ + H_2O$$

Equation 2

(urease)

$$\text{Urea} + H_2O \rightarrow 2\,NH_4^+ + CO_2$$

example above, test kits for D-glucose and D-fructose are also designed to independently measure both analytes in the same cuvette, however, in this case, it is the products of the reactions that absorb light, and thus absorbance increases are measured. Also, in the case of D-glucose and D-fructose analysis, four chemical reactions are actually involved in the final reaction scheme as follows:

The first reaction involves the conversion of D-glucose and D-fructose into D-glucose-6-phosphate (G-6-P) and D-fructose-6-phosphate (F-6-P), respectively, by the enzyme hexokinase ***(equations 3 and 4)***. No change in absorbance takes place during this stage of the reaction scheme, but these conversions must take place before the reactions that do result in an absorbance change can occur.

In the next step, after an initial absorbance reading (A1) has been taken, the enzyme glucose-6-phosphate dehydrogenase is added to the cuvette, and G-6-P is quickly converted into gluconate-6-phosphate, while at the same time NADP +, that does not absorb light, is converted into NADPH, that absorbs light strongly ***(equation 5)***. The result is an increase in absorbance that is representative, or "stoichiometric", to the amount of D-glucose present in the sample. During this reaction nothing happens to the F-6-P, as glucose-6-phosphate dehydrogenase, as its name suggests, is absolutely specific for only G-6-P.

When the conversion of G-6-P is complete, an absorbance reading is taken (A2), and then the final enzyme, phosphoglucose isomerase, is added to the cuvette. This enzyme converts F-6-P into G-6-P ***(equation 6)***, the latter of which is immediately converted to gluconate-6-phosphate according to ***equation 5***, leading to a further increase in absorbance, stoichiometric with the amount of D-fructose in the sample. When the end-point is reached, a final absorbance reading is recorded (A3).

As can be seen from ***figure 7***, both reactions are very rapid, with a typical combined D-glucose and D-fructose test taking only approximately 10 minutes to perform.

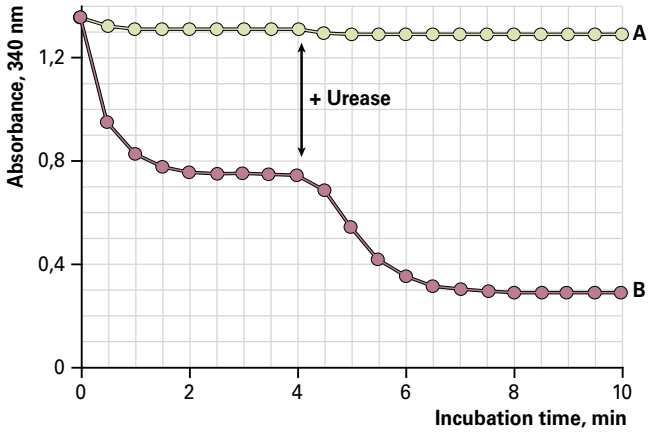
What is the Future of Enzymatic Bio-Analysis

The falling prices of both spectrophotometers and test kits, coupled with more readily available expertise to use them, have generated an expanding enzymatic bio-analysis market. The growth of this market is, however, now entering a new and more dynamic phase, as innovative companies are rapidly expanding the number of test kits available.

Not only are new kits being launched, but existing kits are being improved to make them faster, more stable and increasingly flexible for the analyst. In the last two years alone, the number of test kits for food and beverage analysis has risen from approximately 40, to almost 60, and this number is expected to increase further to more than 80 within the next two years!

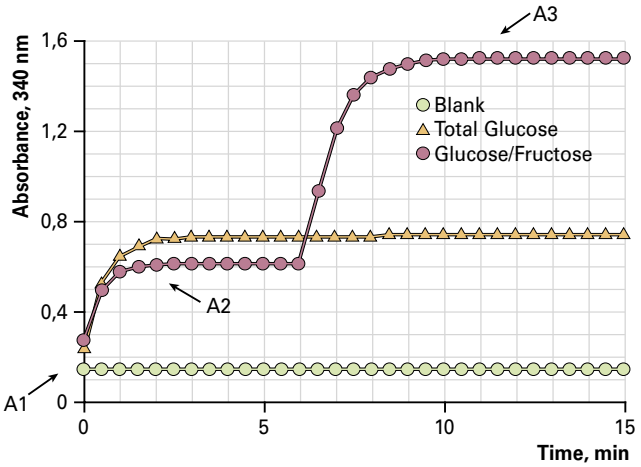
With companies now possessing the ability to rapidly develop new test kits in response to new legislation or developments in the food industry, such as in labelling, the future is clearly very bright for enzymatic bio-analysis. ■

■ Figure 6: The sequential decrease in absorbance over time during a typical ammonia and urea test.



The decrease in absorbance at 340 nm on incubation of a urea/ammonia standard solution using a commercial Urea/Ammonia (Rapid) Test Kit. A, blank reaction; B, 4 µg of ammonia plus 7 µg of urea. The urease was added at the point shown by the arrow.

■ Figure 7: The sequential increase in absorbance over time during a typical D-glucose and D-fructose test.



After the addition of the enzyme glucose-6-phosphate dehydrogenase (at time = 0 min; A1) to start the reaction, the absorbance (closed circles) rapidly increases from 0.13 to 0.61. As can be seen, no further increase in absorbance occurs after approximately 2 min (A2), i.e. this was the end-point of the D-glucose reaction. The point of addition of phosphoglucose isomerase is indicated (+ PGI), and the absorbance then increases further to 1.53. As can be seen, no further increase in absorbance occurs after approximately 10 min (A3), i.e. this was the end-point of the D-fructose reaction. The open circles represent a "blank" reaction, that did not contain any sample, while the closed triangles represents a reaction performed using the D-glucose control solution that comes with the kit.

■ Equation 3	(hexokinase)	**D-Glucose + ATP → G-6-P + ADP**
■ Equation 4	(hexokinase)	**D-Fructose + ATP → F-6-P + ADP**
■ Equation 5	(glucose-6-phosphate dehydrogenase)	**G-6-P + $NADP^+$ → gluconate-6-phosphate + NADPH + H^+**
■ Equation 6	(phosphoglucose isomerase)	**F-6-P → G-6-P**

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