An investigation into how the volume of lipase affects the rate of the hydrolysis of lipids

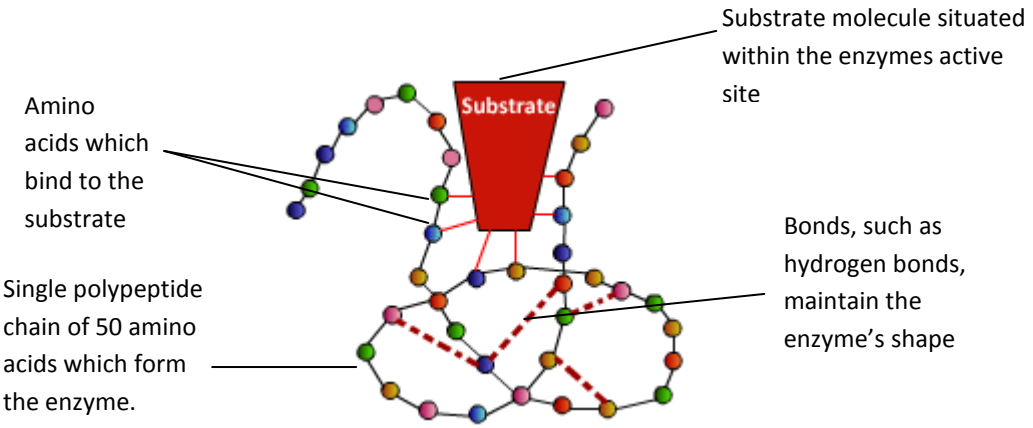
Introduction

In this experiment I shall be investigating how varying the concentration of lipase affects the rate at which lipase catalyses the hydrolysis of the lipids found in Tesco's full fat milk.

Background Information

As globular proteins, enzymes have a specific three-dimensional shape which is determined by their sequence of amino acids. This specific tertiary structure is held together by ionic bonds, hydrogen bonds and disulphide bridges. Despite their generally large size, enzyme molecules have a small region that is functional, known as; the active site. The substrate molecule is held within the active site by bonds that temporarily form between the R groups of the amino acids of the active site and groups on the substrate molecule. The enzyme is then able to break the bonds holding the substrate together, and so , making the substrate molecule break apart into several smaller molecules known as 'products'. This structure is known as the enzyme-substrate complex.

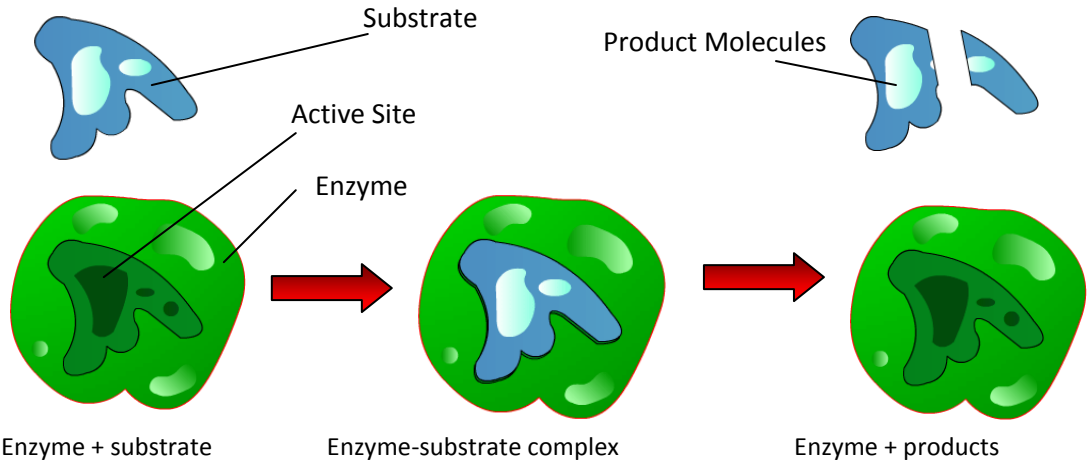
Figure 1 – Enzyme-substrate complex:



Enzymes can influence two reactions: catabolic, the break down of more complex substances into simpler ones, for example, the break down of lipids into fatty acids and glycerol. Or enzymes can influence anabolic reactions, the building up of complex molecules from simpler ones, for example ATP synthetase catalyses the reaction in which ATP is formed from ADP and an inorganic phosphate. Enzymes are said to act as biological catalysts, speeding up chemical reactions without interfering with the reaction itself. In order for either the anabolic or catabolic reaction to occur, the reactants must have enough activation energy for the reaction to continue independently. Enzymes lower this 'energy hill' of activation energy by providing an alternative reaction pathway of lower activation energy so that the reactions can occur more easily, e.g. at lower temperatures. As a result, some metabolic processes occur rapidly at the human body temperature of 37˚C, which is relatively cool in terms of chemical reactions.

The way in which enzymes operate is similar to the way in which a key operates a lock. This analogy, the 'lock and key' theory first postulated in 1894 by Emil Fischer, describes the way in which substrates bind to the active site of the enzyme. The substrate is the key whilst the cleft of the active site is the lock. Only the correct shaped key fits into the key hole of a lock, and so, only specific substrates can bind with the active site of an enzyme. Enzymes are therefore specific in the reactions they catalyse.

Figure 2 – An example of the lock and key theory:



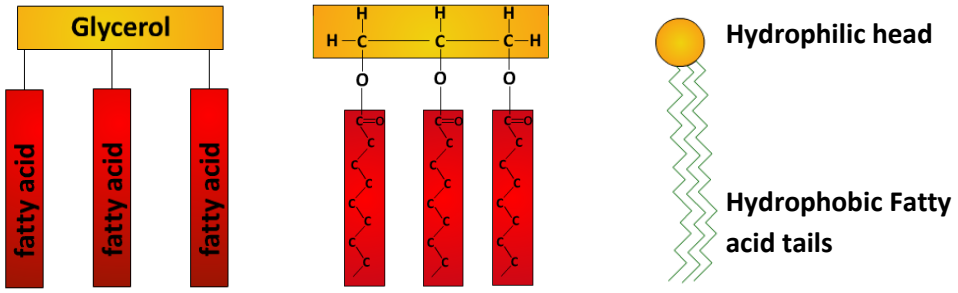
In practice, the process is more refined: it is suggested that, unlike a rigid lock, the enzyme actually changes its form slightly to fit the shape of the substrate. In other words, it is flexible and moulds itself around the substrate, just as a glove moulds itself to the shape of someone's hand. The amino acid side chains of the enzyme can move into very precise positions to allow interaction with the substrate. As the enzyme alters its shape, the enzyme puts strain on the substrate molecule and thereby lowers its activation energy, this process is known as the induced fit theory of enzyme action.

Lipase is a type of enzyme known as a hydrolase and is responsible for catalysing the hydrolysis of triglycerides (the substrate) into fatty acids and glycerol. It is referred to as a hydrolase because the reaction that it catalyses is a hydrolysis reaction – a reaction in which large molecules are broken down into smaller ones with the addition of water.

The molecules being broken down by lipase are lipids. Lipids are organic, non-polar compounds composed of carbon, hydrogen and oxygen which can be extracted using non-polar solvents such as alcohol and ether. Lipids have several roles both inside and outside of the body, including heat insulation, energy storage and the production of steroids and cholesterol. Lipids are also used to produce carotenoids, a photosynthetic pigment found in plants that usually appear orange in colour due to the lack of absorption of light of that particular wavelength. Retinol (Vitamin A) can be synthesised from carotene, which is an important constituent of rhodopsin, the pigment found in the rod cells that make up the retina. It is the breaking up of rhodopsin that allows us to see in low light intensities.

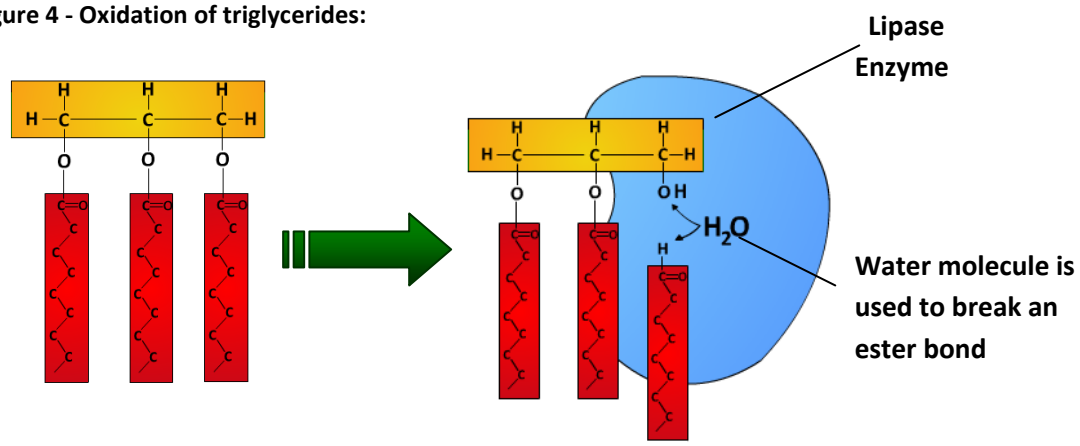
Triglycerides are the most common types of lipids, which consist of one glycerol molecule and three fatty acid molecules bound together by ester bonds. The glycerol molecule in any lipid always remains the same; it is the fatty acids that vary for different lipids. Fatty acids are organic acids consisting of a hydrocarbon tail usually consisting of 14 – 24 carbon atoms with a carboxyl group (-COOH) joined at one end. These fatty acid tails are hydrophobic (water-hating) which is why lipids are insoluble and must be transported in the body by lipoproteins in the blood.

Figure 3 - The structure of triglyceride:



Triglycerides are formed as a result of a condensation reaction (a reaction that produces water) involving the –OH groups of glycerol and the –COOH group of each fatty acid. These condensation reactions produce ester bonds. Lipase however, oxidises triglycerides, using three molecules of water to break these 3 ester bonds and to form one glycerol molecule and three individual fatty acid molecules.

Figure 4 - Oxidation of triglycerides:



The oxidation of lipids can release almost twice the energy that an equal mass of carbohydrate can, which is why lipids are suitable for generating ATP in respiration. As a result of their high energy yield and insolubility in water, lipids make good energy-storage compounds as they do not affect the water potential of the cells in which they are stored (fat is stored in adipose tissue) and having been oxidised they produce many hydrogen ions which can be picked up by NAD and other hydrogen acceptors. Given that oxygen is available, these hydrogen ions can then be transported to the electron transport chain in the cristae of the mitochondria where they can be passed down progressively lower energy levels, releasing energy which can be harnessed for the production of ATP.

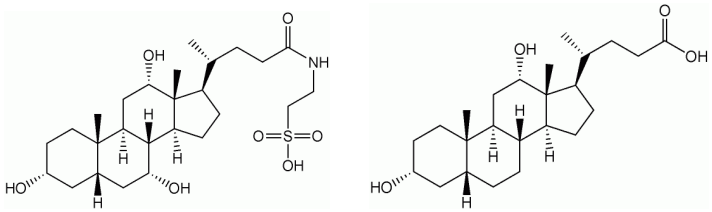
Following the hydrolysis of a lipid, the resulting products – glycerol and three fatty acids are not wasted but can be respired or converted into more useful compounds. For example, the glycerol molecule can be phosphorylated to triose phosphate which is an intermediate in glycolysis, it can either be converted to glucose or enter the Krebs cycle and have its remaining energy released. The three fatty acids produced can be broken down in the mitochondrial matrix to form two carbon acetyl fragments that can combine with coenzyme A to for form acetyl coenzyme A. This acetyl coenzyme A can then enter Krebs cycle and be dehydrogenated, releasing hydrogen ions and energy that can be used to form ATP from ADP and an inorganic phosphate. Many tissues such as cardiac muscle and liver tissue will use fatty acids over glucose as their 'first choice respiratory substrate' however this is not true for red blood cells or nervous tissue which must use glucose.

Fat digestion in the human body takes place mostly in the stomach and the duodenum of the small intestine. Although individually, amino acids are amphoteric and can both accept hydrogen ions and lose hydrogen ions which allows them to resist pH changes, the tertiary structure of the lipase enzyme however is sensitive to changes in pH. Every enzyme has a pH at which it works most efficiently – its optimum pH, this is because the exact arrangement of the active site of an enzyme is partly fixed by hydrogen and ionic bonds between $–NH_2$ and –COOH groups of the polypeptides that make up the enzyme. Even small changes in pH, affect this bonding causing changes of shape in the active site, causing the enzyme to be denatured and to no longer bind to its corresponding substrate.

In the stomach, gastric juice is secreted from gastric pits in response to being stimulated by nerve impulses sent via the vagus nerve (due to the sight/smell of food) or the hormone gastrin (due to the presence of food in the stomach). This contains hydrochloric acid which is secreted by oxyntic cells in the epithelium of the stomach wall, which in turn gives the gastric juice a pH of 1 or less. The optimum pH of gastric lipase is 3 – 6, meaning that gastric lipase does not work at its optimum pH in the stomach (although pepsin – a protease that has an optimum pH of 1.5 to 2 is closer to its optimum pH). However, unlike pancreatic lipase, gastric lipase does not require bile salts to emulsify the fats first and is not denatured by the extremely acidic conditions of the stomach.

Lipase does not work alone in the digestion of fats. Bile is produced by the hepatocytes found in liver tissue and is transported down the bile canaliculi to the gall bladder where it is stored before being released into the duodenum. Bile contains several salts derived from cholesterol including sodium glycocholate and sodium taurocholate. These salts act like detergents and help to emulsify fats, breaking fat droplets in the lumen of the small intestine into tiny globules only 0.5µm to 1.0µm in diameter. This gives the fats a larger surface area over which the enzyme lipase can act on them. Bile also contains sodium hydrogen carbonate which is released in response to the hormone secretin. These hydrogen carbonate ions neutralise the acidic chyme entering the duodenum from the stomach and provides a neutral pH in which many of the enzymes in the small intestine work best. As a result of the hydrogen carbonate ions and sodium carbonate present in the solution, I would anticipate an initial pH of about 9 – 10.

Figure 5 - Sodium glycocholate and sodium taurocholate:



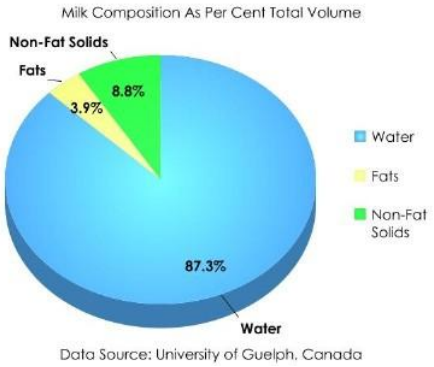
When the cells making up the duodenal wall come into contact with the products of fat and protein digestion this stimulates the secretion of a hormone called cholecystokinin (CCK). This hormone causes the exocrine cells of the pancreas to release a juice rich in enzymes along the pancreatic duct and into the duodenum. CCK also causes the walls of the gall bladder to contract, forcing bile into the duodenum.

One of the many enzymes pancreatic juice contains is lipase. As a result of the alkalinity of the bile salts, the pH of the duodenum is approximately 7.0, which is also the optimum pH for pancreatic lipase. Having been fully digested in the duodenum, the lipid-soluble fatty acids and glycerol diffuse through the phospholipid bilayer of the plasma membranes making up the epithelial cells of the small intestine. They are then converted back to triglycerides and transferred to the Golgi apparatus where they are surrounded by a protein coat to form a chlyomicron and transported along the lymphatic capillaries.

Milk is a white liquid composed mostly of water (87.3%), with small amounts of fats (3.9%), and non-fat solids such as proteins and lactose (8.8%).

Milk appears white to the naked eye because the protein casein and many of the fats contained within the milk do not absorb a wide range of wavelengths of light, therefore reflecting most wavelengths and giving it a white appearance. Fats are usually solids at room temperature, and in milk the lipids form spherical shaped objects called globules varying in size from 0.1 to 15 microns in diameter. Milk contains more fat than most liquids and a majority of these lipids are classed as triglycerides which therefore makes milk a suitable liquid to be used for this experiment. Using solid fat such as lard would be impractical because the enzyme lipase would only be able to bind with lipids on the surface of the lard, meaning there would be an extremely slow reaction rate. The globules of fat found in the milk gives the lipids a larger surface area and provides more 'surfaces' that the lipase enzyme can bind to.

Figure 6 – The composition of milk:



Nutrition Facts

Serving Size 1 cup (8 fl oz/240mL)
Servings Per Container 8

Amount Per Serving	
Calories 160	Calories from Fat 80
	% Daily Value*
Total Fat 9g	14%
Saturated Fat 6g	30%
Trans Fat 0g	0%
Cholesterol 35mg	12%
Sodium 120mg	5%
Total Carbohydrate 12g	4%
Dietary Fiber 0g	0%
Sugars 12g	
Protein 9g	16%
Vitamin A 6% •	Vitamin C 0%
Calcium 30% •	Iron 0%

*Percent Daily Values are based on a 2,000 calorie diet.

An acid is defined as a substance that can donate protons (hydrogen ions) to other substances. Aforementioned, fatty acids contain the carboxylic acid functional group, and the hydrogen in this carboxyl group gives it, its acidic nature. If the carboxylic acid is in solution in water, then this carboxyl group can donate a hydrogen ion to the water, forming a hydroxonium ion, H_3O^+. As a result of the ability of the carboxyl group to give up hydrogen ions, the hydrogen ion concentration in the surrounding solution increases.

The pH of a solution ranges from 0 – 14 and gives a measure of the alkalinity or acidity of a solution. A pH of 1 is very acidic while a pH of 14 is very alkaline, a pH of 7 is neutral, and the pH of full fat milk is most likely to be somewhere between 6.5 – 7.5 as any extremes in pH would cause discomfort to those drinking it. The pH of a solution is defined as the logarithmic measure of hydrogen ion concentration and is given by the following formula:

$$\mathrm{pH} = -\log_{10} a_{\mathrm{H}} = \log_{10} \frac{1}{a_{\mathrm{H}}}$$

The logarithmic relationship shows that a pH of 4 is 10 times more acidic than a pH of 5, and a pH of 9 is 10 times more alkali than a pH of 8. Looking at this relationship, the greater the hydrogen ion concentration or hydrogen ion activity *a*, and therefore the more acidic the solution, the lower its pH, because pH is inversely proportional to the log base 10 of hydrogen ion concentration. Linking this back in with lipids, as the fats and oils present in the milk are hydrolysed to the fatty acids and glycerol, the hydrogen ion concentration of the surrounding solution increases, and therefore the pH of the solution decreases. The rate at which the pH of the surrounding solution decreases gives an indication to the rate at which fatty acids are being released from the triglyceride molecules. The faster the decrease in pH, the faster the triglyceride molecules present in the milk are being hydrolysed to fatty acids and glycerol.

The sensitivity of the enzyme's shape means that any changes in physical and chemical conditions will lead to a denatured enzyme that will be unable to break down the specific substrate that matches the shape of the active sites cleft. If this occurs during the procedure then the lipids will no longer be hydrolysed to fatty acids and no readings will be able to be taken as there will no longer be a change in pH.

If the solution of lipase experiences too high temperatures then the atoms making up the lipase will have more kinetic energy causing them to vibrate vigorously, tearing apart the hydrogen bonds and other bonds holding the protein structure together. Low temperatures however would cause the enzyme to hibernate, as it would have a too minimal amount of kinetic energy to function efficiently.

Despite denaturing enzymes being useful to prevent food spoilage by various enzymes found in food materials, the denaturing of the lipase enzyme would mean that no results could be harvested, and so the experiment would lose its purpose. Many enzymes in the human body have an optimum temperature of approximately 40˚C. I anticipate that the lipase enzyme will hydrolyse fats most efficiently at a temperature of about 37 – 40˚C, as this is close to our human body temperature (37˚C) and matches the temperature of the digestive organs in which lipase acts. It is possible however that the lipase may have an optimum temperature higher than this, as the human body uses 37˚C as additional energy (food) would be needed to maintain a higher temperature. The solution of milk, lipase, bile salts, sodium carbonate and phenolphthalein will be mixed at a range of higher and lower temperatures to test the optimum temperature of the lipase enzyme. The preliminary experiment will be used to verify the optimum temperature in which lipase works best.

Although the pH of the surrounding solution could denature the lipase enzyme, the pH of the solution is a variable that is being measured at regular 1 minute time intervals and so does not need to be fixed at a particular number. Controlling the pH would interfere with recording the change in pH and thus detecting the rate at which fatty acids are being released from the triglycerides.

Sodium carbonate will be added to the mixture of bile salts, lipase, full fat milk and phenolphthalein to give an initial pH of about 10. The change in pH will then be monitored using electronic pH indicators and the change in colour of phenolphthalein.

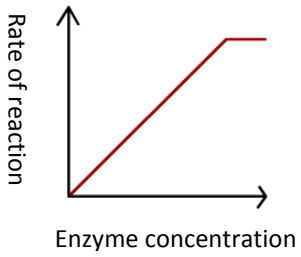
Hypothesis

I anticipate that the greater the concentration of lipase, the greater the rate at which lipids will be hydrolysed to fatty acids and glycerol, and therefore the faster the rate of the reduction in the pH of the surrounding solution.

Provided that conditions such as temperature and pH are suitable and there is an excess of substrate, an increase in the amount of enzyme leads to a proportionate increase in the rate of reaction. As the enzyme concentration increases, there are more active sites occupying the same volume of space, and so there are more opportunities for substrates to bind with the lipases active sites. Therefore the rate of hydrolysis of lipids can occur at a faster rate because there are more active sites available to the substrates – similar to the way in which opening more checkouts at a supermarket means more people can be served. Eventually, increasing the enzyme concentration will have no effect as the number of substrates available to bind with the enzyme's active site will be limited – for example, if all the checkouts are open in a supermarket, the rate of service cannot increase any further if there are fewer shoppers to make use of it. At low enzyme concentration there is great competition for the active sites and the rate of reaction is low.

As the enzyme concentration increases, the rate of reaction proportionally increases, and thus so too should the rate of change of the pH of the surrounding solution. As fatty acids are released from triglycerides increasingly faster, the pH should decline equally as fast.

When the active sites of the enzymes are working at full capacity and the rate of hydrolysis of lipids cannot increase any further, the pH will still continue to fall because hydrogen ions are still being released.

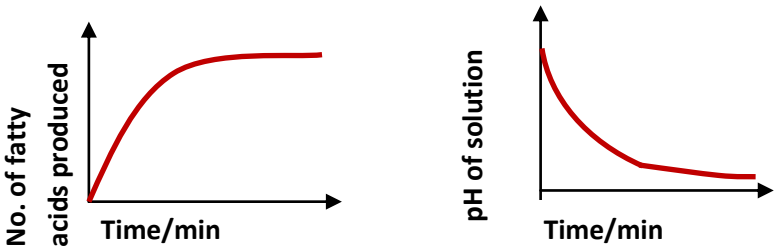


The only change however will be that similar amounts of hydrogen ions will be released each time interval so the rate of reduction in pH will not change, but the pH itself will.

The substrate concentration in each experiment will remain the same as it is anticipated that $5ml^3$ of full fat milk will contain more than enough substrate for the reaction to proceed for 10 minutes without substrate running out and the lipase having no triglycerides to hydrolyse. However, if the substrate concentration were to be increased, this would also increase the rate at which the lipids were hydrolysed to fatty acids and glycerol, until the point at which all of the active sites became filled. I will therefore have to be extremely careful when measuring out the volumes of milk to ensure that each lipase solution will exposed to equal amounts of substrate. For example, it would not be a fair test if $5ml^3$ of lipase was mixed with a smaller volume of milk in comparison to $4ml^3$ of lipase. This could potentially result in the $4ml^3$ of lipase hydrolysing more triglycerides in comparison to the $5ml^3$ of lipase, when in actuality, increasing the number of enzymes should increase the rate of hydrolysis.

For each individual lipase volume I anticipate that the rate at which fatty acids are released from the triglyceride molecules will be rapid at first and then gradually become slower. The reason for this is because initially, there will be a lot of substrate available to the lipase enzymes, so it will be easy for the substrates to come into contact with the empty active sites on the lipase molecules. When all of the lipase enzymes have their active sites filled, the substrates will be rapidly broken down and the amount of substrate will be reduced. As the substrate concentration decreases there will be greater competition for active sites and thus the rate at which the lipids are hydrolysed to fatty acids and glycerol will decrease. There is also the possibility that the product molecules, fatty acids and glycerol, may 'get in the way' of the active sites of the lipase enzymes making it increasingly difficult for the substrate molecules to bind with the lipase. Depending on how many lipids are present in $5ml^3$ of full fat milk, if all of the triglycerides are hydrolysed to fatty acids and glycerol then the pH will cease to change and the reaction will no longer continue.

Figure 7 – Anticipated graphs for the rate of reaction and change in pH for each volume of lipase:



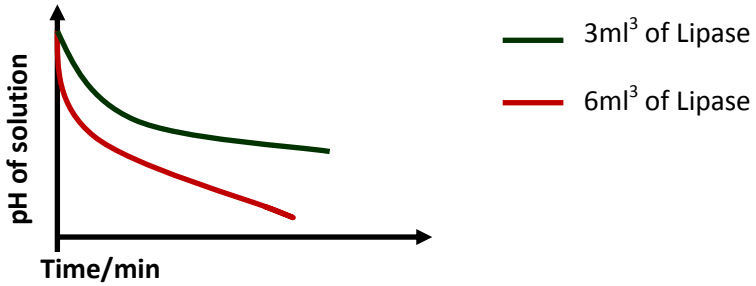
The results obtained will therefore be quantitive; if the concentration of lipase doubles then the number of active sites available will be doubled and thus so too should the number of substrates being broken down into fatty acids and glycerol.

If the number of substrates being broken down doubles, then the rate at which the pH is reduced should also double. However, doubling the number of substrates being hydrolysed, halves the number of remaining substrates available, which could therefore result in a decrease in the rate at which the pH decreases.

I anticipate that using a $6ml^3$ volume of lipase will produce the fastest rate of hydrolysis of lipids into fatty acids and glycerol. This is because the greater the volume of lipase, the more lipase present and therefore the greater the number of active sites present to hydrolyse the fats into fatty acids and glycerol. $6ml^3$ is the maximum volume of lipase being utilised in the experiment, so in turn it should produce the maximum rate of reaction, and thus maximum reduction in pH. As $6ml^3$ is the greatest volume of lipase being utilised, I anticipate that the use of $6ml^3$ will give the fastest time in which the phenolphthalein turns from pink to colourless.

As previously mentioned, doubling the concentration of enzymes should double the rate of reaction, so I anticipate that $3ml^3$ of lipase will hydrolyse the lipids present in the milk at half the rate that $6ml^3$ does. Likewise, the phenolphthalein should take twice as long to turn from pink to colourless when $3ml^3$ of lipase is being used.

Figure 8 – Anticipated results:



In order to certify that the change in pH is solely down to the lipase enzyme and not because of any of the reactions occurring between the chemicals being used, I will be using a control group. This control group will consist of bile salts, sodium carbonate, phenolphthalein and full fat milk. I expect that without any lipase, there will be no change in pH as there will be nothing to catalyse the hydrolysis of fats into fatty acids and glycerol and so the fatty acids will not be released into the solution. Likewise, if lipase was present in the solution, but the milk was not, I would anticipate that there would be no change in pH as there would be no substrate available to be broken down catabolically.

Testing my hypothesis and controlling variables

In order to test my hypothesis I will be filling 5 test tubes each with: $5ml^3$ full fat milk, $3ml^3$ sodium carbonate, $2ml^3$ bile salts and $1ml^3$ phenolphthalein. The volume of lipase added however will vary from $3ml^3$ – $6ml^3$ for each experiment, as well as the control group in which no lipase will be added. The solution of bile salts, phenolphthalein, milk and sodium carbonate will first be mixed together with the addition of the lipase last. This step will ensure that the hydrolysis of fats begins only when all of the substances have been mixed together and that in each experiment the fats have first been emulsified by the bile salts. It would be unfair to add these substances in different orders, because the test tube that had bile salts added subsequent to the addition of lipase would take longer to hydrolyse the triglycerides to fatty acids and glycerol in comparison to one that had, had bile added before the addition of lipase.

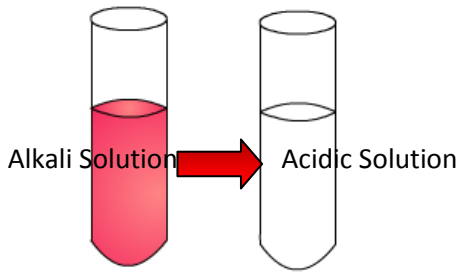
Having mixed all of the substances together for a particular lipase volume, timing will commence and the pH and colour will be noted at regular 1 minute intervals. After ten minutes, the experiment for the particular lipase volume will be terminated and the next volume of lipase will be tested. A graph of pH against time will then be plotted for each experiment, and this will then be analysed to see how the volume of lipase affects the rate of hydrolysis of lipids.

In the experiment I shall be adding sodium carbonate to the solution of bile salts, full fat milk and phenolphthalein. The sodium carbonate should give the solution an initial pH of approximately 10. At a pH of 10, the phenolphthalein solution will appear pink in colour. As triglycerides are hydrolysed to fatty acids and glycerol, the acidity of the solution will increase and at a pH of 8.4 the phenolphthalein should turn colourless. The reason for this change in colour is because the phenolphthalein ion appears pink/purple in colour but the phenolphthalein molecule appears colourless.

When a base, such as sodium carbonate, is added to the phenolphthalein, the molecule to ions equilibrium shifts to the right, leading to more ionization as H^+ ions are removed. The removal of these ions forms the phenolphthalein molecule which is colourless. However, as hydrogen ions are being removed from the phenolphthalein, this will create additional acidity to the solution on top of the acidity being created by the release of fatty acids. I will therefore use small amounts of phenolphthalein so that the change in pH is due to the hydrolysis of fats and not because of the phenolphthalein ions being converted to phenolphthalein molecules. As lipase is able to function in the acidic conditions of the stomach, it is unlikely that as hydrogen ions are released the lipase will become denatured, so the phenolphthalein should pose no threat to the lipase solution.

The fact that the phenolphthalein changes colour gives an indication of the change in pH of the solution. The faster the solution turns from pink to colourless, the faster fatty acids are being released from the triglyceride molecules. The use of phenolphthalein however is not the only form of pH indicator being utilised in this experiment. On its own, the phenolphthalein can only give a measure of how fast the solution is increasing in acidity as it is not really possible to tell the pH of the solution when the phenolphthalein is partway between pink and colourless. This creates difficulty in measuring the pH at given time intervals. The use of phenolphthalein also relies heavily upon human observation which in turn would result in a lot of experimental error.

Figure 9 – The change in colour indicated by phenolphthalein:



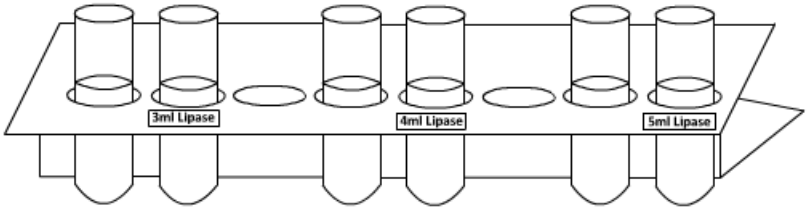
Alongside phenolphthalein I shall also be making use of electronic pH indicators to directly measure the pH at 1 minute intervals. The electronic pH indicators consist of an electrode that monitors hydrogen ion activity and interprets this as a pH value. The LCD screen mounted on the top of the electrode displays the pH value to 2 decimal places and the pH reading changes as the pH of the solution changes. The use of an electronic pH indicator has the advantage that the pH does not need to be calculated but can be read directly. Using litmus paper instead of this device would be extremely inaccurate as this depends on observing a change of colour and the interpretation of the colour depends on human observation. For example where one person might see orange on the litmus paper, another person could interpret it as red, and so there is a loss of accuracy in the measurement.

In order to obtain accurate readings of pH, the pH meters will first be calibrated so that the pH readings they give are known to be correct. Calibration will be performed with two standard buffer solutions with a pH of 3.5 and 9.2. The pH meter has one control to set the metre reading equal to the value of the pH of the first standard buffer solution and a second control which is used to adjust the meter reading to the value of the second buffer. The calibration process correlates the voltage produced by the electrode with the pH scale. After the pH meter has been used for each individual lipase volume being tested, the probe will be rinsed with distilled or deionised water to remove any traces of the previous solution being measured.

The probe will also be blotted with a clean tissue to remove any remaining water which could dilute the next sample and thus alter the reading. When the pH meter is not in use the probe tip will be immersed in an acidic solution of a pH of around 3 to prevent any degrading of the pH meter.

To ensure that the experiment runs smoothly, each test tube being utilised will be labelled with a sticker that denotes which volume of lipase is being utilised in that particular test tube.
Aforementioned, the lipase will be added last to the test tube containing the other substances, so I will therefore have two test tubes for each experiment – one for the particular volume of lipase, and the second containing the phenolphthalein, bile salts, full fat milk and sodium carbonate to which the lipase will be added.

Figure 10 – The labelled test tube set up:



In this experiment I am testing five different concentrations of lipase – $0ml^3$, $3ml^3$, $4ml^3$, $5ml^3$ and $6ml^3$ so I will therefore need nine different suitably sized test tubes (only one is required for the control group). In order to minimalise potential confusion between test tubes, I will make use of two test tube racks. Placed in these test tube racks will be the test tubes required for each experiment. After every two test tubes needed for a particular lipase volume, I will leave a space in the test tube rack so that the test tubes needed for each experiment are spaced apart from one another. Labels will be added to both the test tubes themselves and the test tube rack in case either of the stickers is accidentally removed.

To ensure that my experiment produces consistent results and is a fair test, there are several variables that must be kept constant.

One such variable is the volume of bile salts, full fat milk, phenolphthalein and sodium carbonate.
While obtaining the desired volumes of each substance, great care must be taken to ensure that each experiment utilises exactly the same amounts of each substance. If one experiment were to contain more bile salts than another, then the fat present in the full fat milk may be emulsified to a greater extent than an experiment that contained less bile salts. For example, if the experiment testing $3ml^3$ of lipase was mixed with equal amounts of phenolphthalein, full fat milk and sodium carbonate to the experiment testing $4ml^3$ of lipase, but contained a greater volume of bile, then the fats in the full fat milk could be dispersed over a larger surface area, making it easier for the lipase present in the $3ml^3$ solution to act on the lipids. This in turn could allow the $3ml^3$ solution of lipase to hydrolyse the lipids in the milk at the same rate as or even faster than the $4ml^3$ of lipase. These results would insinuate that the $3ml^3$ of lipase hydrolyses lipids faster than the $4ml^3$ when realistically the $4ml^3$ of lipase should hydrolyse the lipids faster because there is a greater enzyme concentration.

Similarly, if the volume of full fat milk utilised in each experiment did not remain constant then the substrate concentration would vary between experiments. If the substrate concentration were to be increased then the rate of the reaction in which lipids are hydrolysed to fatty acids and glycerol would also be increased.
However the rate of this reaction would only increase up until the point at which all of the lipase's active sites were filled.

Since the rate of reaction is proportional to substrate concentration, it would therefore place different volumes of lipase at an unfair advantage/disadvantage if the volume of milk was increased/decreased between experiments. This experiment is looking only at how the concentration of lipase affects the rate of hydrolysis of lipids, so it is therefore important that any other facts that affect this are stringently controlled. If a lower volume of lipase being tested was exposed to a greater substrate concentration then this could break down lipids at a faster rate than a higher volume of lipase exposed to the correct amount of substrate, giving a faster reduction in pH and producing inconsistent results.

The volume of sodium carbonate and phenolphthalein should not have a great impact on the rate at which lipase hydrolyses fats as these are used mostly as indicators. However for consistency the volume of both of these substances will remain constant for each experiment.

In order to measure out the volumes of each of these substances accurately, I will be using a burette graduated with millilitre marks to which a pipette filler will be attached. Using the pipette filler, I will take up an excess amount of the fluid required, and release the fluid out into a beaker until I have achieved the correct volume of the substance being measured. This process will be done at eye level to reduce any parallax error and the liquid will be assumed to be of the correct volume when the meniscus of the fluid rests exactly on the graduated millilitre mark corresponding to the desired volume. The tip of the burette will be fully immersed in the fluid that is being measured out to prevent any bubbles interfering with the reading of the volume. Bubbles present in the burette could create difficulty in determining where the meniscus is positioned as its shape could become distorted. The liquids will be measured out in close proximity to the test tubes in which they will be stored so that minimal amounts of fluid can drip out of the burette in between storing and depositing the substances. All of these measures should allow accurate volumes of phenolphthalein, bile salts, milk, lipase and sodium carbonate to be obtained.

While measuring out the desired volumes of bile salts, lipase, milk, phenolphthalein and sodium carbonate, the apparatus being utilised will be washed with distilled water before being used. To prevent any unwanted mixing and potential dilution of the substances being used, a different burette will be used for each different substance, i.e. one for the bile salts, one for the lipase, one for the milk and so on. The reason for this measure is to prevent any of the substances that may remain in the burette after the substance has been placed in the test tube from interacting with the other substances. For example, if the same burette were to be utilised for the full fat milk and the lipase, it is possible than any milk that was not removed in washing with distilled water, would be hydrolysed before timing had began and the pH of the solution could also be disrupted.

The use of the same burette each time for each substance should limit the need for repeated washing which in turn will save time and limit the risk of any distilled water left in the burette from diluting any of the solutions. Repeated cleaning is likely to cause anomalous results due to human error and the likelihood of cross contamination occurring between burettes. The pH is a variable being measured and theoretically the initial pH of each solution should be the same, irrespective of the lipase volume being tested. The burettes will be kept away from each other and laid on paper towels to prevent any unwanted substances on the table interacting with the burettes. These unwanted particles could act as inhibitors (molecules that prevent the enzyme from functioning) or cofactors (molecules that help the enzyme to work more efficiently) and put different solutions at unfair advantages/disadvantages. The pipette filler being utilised to extract the desired substances and place them into their given test tubes however will remain the same for each burette as the number of these available is limited. The pipette filler will thus have to be rinsed between each different burette it is attached to, to prevent cross contamination.

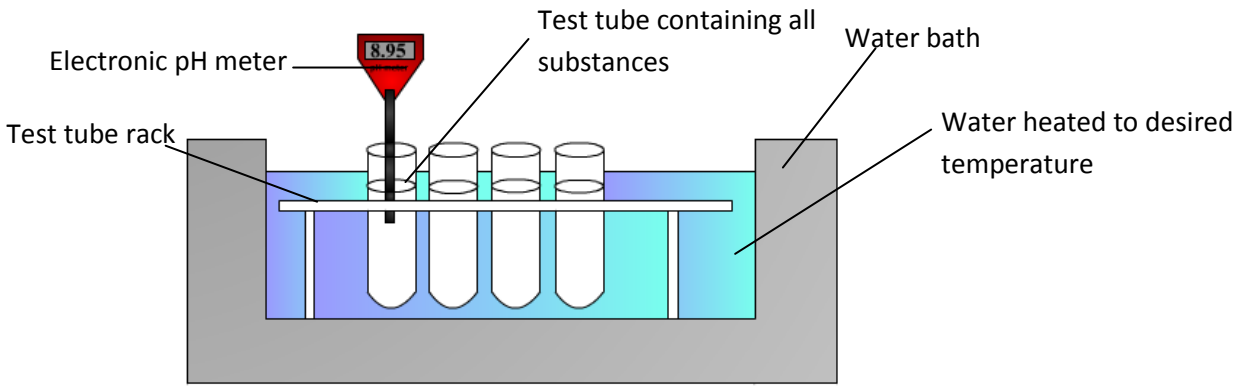
Furthermore, the temperature at which the solution of lipase, bile salts, sodium carbonate, milk and phenolphthalein is kept is another variable that must remain constant. If one test tube containing all of these substances experienced a higher temperature than another, then it is possible that this test tube would experience a greater rate at which the lipids within the milk were hydrolysed to fatty acids. If the temperature was raised for one solution, then the molecules within that solution would have more kinetic energy, causing them to move around and collide more frequently. This would mean that the enzyme and substrate molecules would come together more often in a given time so the rate at which the lipid molecules collided with the lipase would increase. If more lipids collided with more lipase enzymes then the rate at which these lipids were hydrolysed would increase as more active sites become filled and therefore this test tube would be at an unfair advantage.

If one test tube were to experience temperatures of about 60°C then the atoms making up the lipase enzyme would vibrate violently, causing the hydrogen and other bonds that hold it in shape to break. Gradually, the shape of the active site would be disrupted to such an extent that the corresponding substrate would no longer fit in the cleft of the enzyme's active site. This would result in no change of the pH as there would no longer be any active sites able to hydrolyse the lipids and therefore release the fatty acids. In reverse to this, if one solution were to experience lower temperatures, then the lipid molecules and lipase would have less kinetic energy and collide less frequently, reducing the rate at which the lipids were hydrolysed to fatty acids and glycerol.

Having deduced which temperature is the optimum temperature for the lipase enzyme, the test tubes will be stored in a heated water bath that maintains a constant temperature. Each test tube will remain in the water bath for 10 minutes, and timing will begin as soon as the test tubes are held securely in place. In order to hold these test tubes securely in place, a metal test tube rack will be placed inside the water bath. The metal test tube racks are thinner than the wooden test tube racks available and will conduct the heat of the water bath more easily. The metal test tube racks will therefore provide less insulation than the wooden test tube racks and so the test tubes should experience the desired temperature of the water that the water bath has been heated to. Each test tube being measured/observed will remain in the water bath for equal amounts of time, and the water bath will ensure that the temperature remains constant. The time each test tube spends in the water bath will be monitored carefully with a stopwatch.

The use of the water bath gives a more accurate method of maintaining a constant temperature, as room temperature is likely to fluctuate, while storing the test tubes in a beaker filled with water and heating the water with a Bunsen burner would require constant heating and re-heating as the temperature would constantly fall and be hard to control. The use of a water bath means that my hands will be free to control the stop watch and note the recordings with a pen in tables drawn prior to the experiment.

Figure 11 – The apparatus set up in the water bath:



The only variable being changed in this experiment is the volume of lipase. I have decided to use volumes of $0ml^3$, $3ml^3$, $4ml^3$, $5ml^3$ and $6ml^3$ as they are easy to prepare and provide good comparisons from which accurate conclusions can be drawn. The burettes available measure a maximum volume of $20ml^3$, so any volumes being tested that were larger than this would be awkward to prepare. Additionally, the test tubes being used also have a capacity of around $20ml^3$, so once the all of the substances have been mixed together it is important that they are stored safely and cannot overflow from the top of the test tube. For example, when testing the $6ml^3$ of lipase, all of the substances together will give a volume of $17ml^3$, as you can see this is close to the maximum volume that the test tube can store, so using larger volumes of lipase would run the risk of some of the solution being spilt into the water bath and therefore potentially altering its temperature slightly.

Using $5ml^3$ of full fat milk for each experiment should prevent the need for excessive amounts of milk, allowing the same carton of milk being used for each experiment. If a new bottle of milk had to be bought, then it is possible that it would contain slightly more or less fat than the other bottle of milk being tested. This in turn would give unequal substrate concentrations for each experiment making the procedure an unfair test. Using a maximum of $6ml^3$ of lipase and $2ml^3$ of bile salts should also reduce the chances of spilling either of these substances, which could be dangerous as lipids make up a large constituent of my skin (phospholipids make up the cell membranes in each of my cells) and could therefore cause some harm if the lipase were to come into contact with my skin or to the skin of those working close to me.

The Preliminary Experiment

In order to deduce the optimum temperature of the lipase enzymes, and to see if any adjustments needed to be made to my final experiment, I carried out a preliminary experiment. The preliminary experiment was devised of four different temperatures at which the $3ml^3$ of lipase being tested would experience. These temperatures consisted of: freezing - 0°C, room temperature - 20°C, human body temperature - 37°C and 80°C (boiling water that had been left to cool for a short while). The preliminary experiment utilised $3ml^3$ of lipase, $2ml^3$ of bile salts, $5ml^3$ of full fat milk, $1ml^3$ of phenolphthalein and $3ml^3$ of sodium carbonate. $3ml^3$ of lipase was decided as the volume of lipase to be used in the preliminary experiment as it was easy to measure out and would leave enough lipase for the final experiment. This volume of lipase remained constant for each temperature setting, as the effect of temperature on the hydrolysis of lipids was being investigated and not the concentration of lipase.

The bile salts, sodium carbonate, phenolphthalein and full fat milk were measured out using clean burettes and a pipette filler and were mixed together in a single test tube. In a separate test tube the $3ml^3$ of lipase was stored. The lipase was then poured into the test tube containing the other substances and placed within the given temperature setting. The temperature of 0°C consisted of a measuring beaker filled with ice and the temperature of room temperature consisted of a measuring beaker filled with water (this water was the same temperature as the room as it had been left out for quite some while). To obtain a temperature of 37°C the water bath was used and for 80°C water was boiled with a kettle and poured into a measuring beaker. Thermometers were placed in all measuring beakers so that the temperature could be monitored. Using stopwatches and a calibrated pH meter, the pH of each solution was noted each minute and recorded in tables.

Results of the preliminary experiment

Temperature (°C)	0									
Time (min)	1	2	3	4	5	6	7	8	9	10
pH	9.09	9.09	9.09	9.08	9.08	9.07	9.06	9.04	9.04	9.02

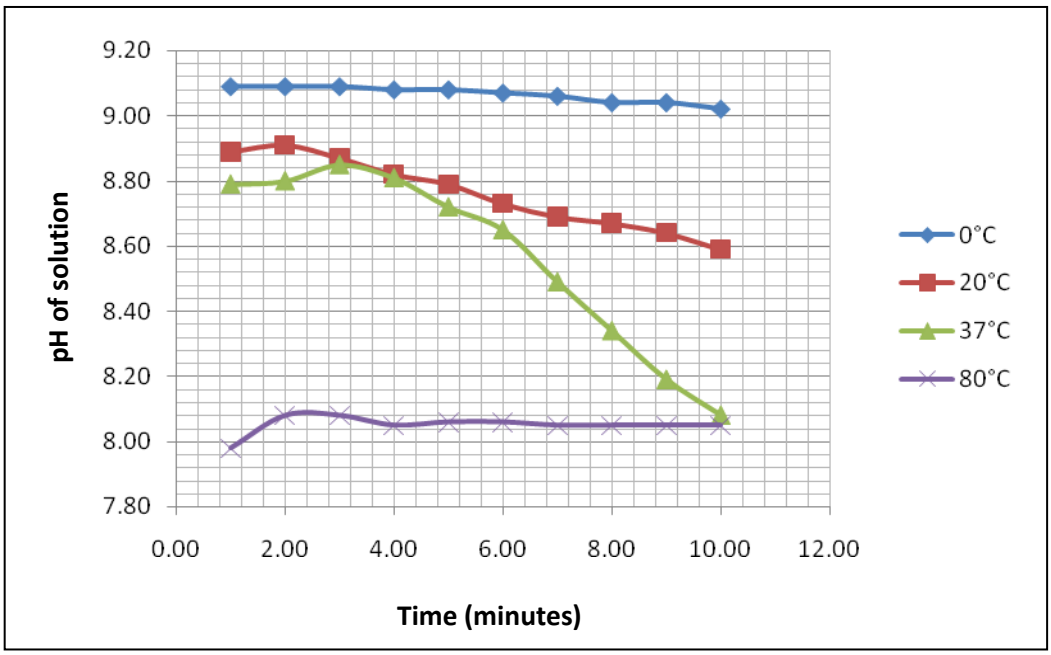
Temperature (°C)	20									
Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.89	8.91	8.87	8.82	8.79	8.73	8.69	8.67	8.64	8.59

Temperature (°C)	37									
Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.79	8.80	8.85	8.81	8.72	8.65	8.49	8.34	8.19	8.08

Temperature (°C)	80°									
Time	1	2	3	4	5	6	7	8	9	10
pH	7.98	8.08	8.08	8.05	8.06	8.06	8.05	8.05	8.05	8.05

**The results highlighted in yellow are highlighted because they could potentially be anomalous.*

A scatter graph comparing the results for each experiment:



Looking at the graph of results for the preliminary experiment it is clear the when the lipase experienced a temperature of 37°C there was the greatest change in pH and most rapid change in pH. If 37°C results in the greatest change of pH, it therefore results in the maximum number of fatty acids being released in the quickest amount of time from the triglycerides present in the milk. It can therefore be said with certainty that 37°C is the optimum temperature for lipase enzyme (the optimum temperature tested in this experiment).

Looking at the graph produced for the lipase solution that experienced a temperature of 0°C, we can see that there was very little change in pH throughout the duration of the 10 minutes. The reason for this would be because at such low temperatures, the triglyceride molecules and lipase enzymes would have very little kinetic energy and the frequency of collisions between the substrate and enzyme would therefore be very low. If the lipase enzymes and substrates did not collide very often then very few hydrolysis reactions would be taking place and so very few fatty acids would be released from the triglyceride molecules. A temperature of 0°C was therefore not the optimum temperature for lipase, and this temperature was not chosen for the final experiment. Using a temperature of 0°C would require the pH to be monitored over several tens of minutes in order for a noticeable change in pH to be observed. This could impose health risks as phenolphthalein can be particularly harmful if a person is exposed to it for long periods of time (see safety measures)

The results for the temperature of 20°C show that this was the second most favourable temperature for the hydrolysis of the lipids present in the full fat milk. At minutes 3 and 4, the change in pH was very similar for the lipase experiencing 37°C and 20°C, and the gradient of the graph displaying the results for the 20°C experiment is relatively similar to that of the 37°C experiment up until the 6th minute. Beyond the 6th minute the rate at which the pH decreases is slower than the 37°C experiment, therefore suggesting that 20°C is not the optimum temperature for the lipase enzyme. I will therefore not be using a temperature of 20°C for the final experiment as the change in pH is somewhat slower and room temperature is likely to fluctuate as the heat produced from the radiator can be lost if a window or door is open etc.

As can be seen from the graph, when the lipase experienced a temperature of 80°C there was very little change in pH and therefore very little enzyme activity. The graph shows that there is initially a very slight rise in pH and then a very small fall before the pH changes no further.

These changes in pH are more likely to be a result of the fluctuating reading on the pH meter and not because of the release of fatty acids from the triglycerides present in the milk. Although the high temperature of 80°C would give the substrates and enzymes present in the solution a lot of kinetic energy, and they would therefore collide more often, at such high temperatures, the molecules holding the enzyme together would vibrate so vigorously that the bonds holding the enzyme's tertiary structure together would be disrupted and the shape of the enzyme would be altered. If the shape of the lipase's active site became altered then the triglycerides would no longer fit in the cleft of the active site of the lipase enzymes and so no fatty acids would be released and there would thus be no change in pH. It therefore follows that 80°C is not a suitable temperature for the final experiment to be carried out at, because the lipase enzymes will be denatured and thus no change in pH will be noted. The use of high temperatures also presents safety hazards as burning could be induced if the measuring beaker were to be knocked over. Using a temperature of 37°C also requires less energy and therefore has less impact on the environment.

Changes made as a result of the preliminary experiment

Although the results produced by the preliminary experiment seemed relatively accurate, looking at the individual results I can see there were a few errors in my procedure and that modifications must be implemented to overcome these in the final experiment.

Theoretically, the initial pH of each solution should be identical for each temperature being tested because the volume of bile salts, sodium carbonate, lipase, full fat milk and phenolphthalein being utilised in each experiment remained constant. Looking at the pHs observed after one minute, we can see that there is a great variation, particularly between the pH of 80°C and 0°C solution. These large differences between the pH after one minute either imply that if both the 0°C and 80°C were at the same initial pH, then the initial pH decreased extremely rapidly for the 80°C for 1 minute and then no longer decreased, or the pH for the 0°C rapidly increased for one minute and then started to slowly decrease. Neither of these scenarios seems very likely as at 80°C the lipase enzymes would have been denatured and thus released very few if any fatty acids from the triglycerides, therefore the change in pH would have been very little. Likewise, using a temperature of 0°C should not have encouraged any reactions that increase the pH of the solution. The most likely explanation is that each of these experiments used solutions of lipase that were at different initial pHs. To reduce the chances of this re-occurring in the final experiment, the pH meters will calibrated again and greater care will be taken when measuring out the volumes of each substance. If the initial pHs of each solution still appears to be different then the rate of change of pH will be examined, rather than the actual values of pH. The pH will not be noted at 0 minutes because the reading on the pH meter is likely to need time to stabilise and so it would be inaccurate to take the first number that appears.

Another inconsistency that was noticed in the procedure was that by placing the bile salts, sodium carbonate, full fat milk and phenolphthalein all in one test tube before adding the lipase meant that the lipids present in the full fat milk were exposed to the bile salts for unequal amounts of time. If the solution experiencing 20°C was exposed to bile salts for longer than the solution experiencing 37°C, then more time would have been allowed for the lipids in the milk to be dispersed over a larger surface area and so it could have been potentially easier for the lipase enzymes to hydrolyse the triglycerides to fatty acids and glycerol in that solution. This could explain why the graphs for 20°C and 37°C are so similar up until the 6^{th} minute, when realistically the 37°C experiment should have produced a greater change in pH from the first minute. To overcome this area of inconsistency, each different substance will be measured out and stored in a different test tube. The contents of each test tube will then be added to one test tube, with the addition of the lipase last. This should ensure that reactions only begin once timing has begun and the initial pH or rate of hydrolysis is unaffected by the duration for which the lipids were exposed to the bile salts.

The only downfall with this measure is that the rate of reaction to begin with could be slower as the lipids present in the full fat milk first need to be emulsified.

In addition, another common theme present in the results of the preliminary experiment is that for the experiments in which no change in pH is expected, i.e. for 0°C and 80°C, there is a change in pH observed on the pH meters at first before the pH reading stops changing. The reason for this could be due to the fact that the containers storing the phenolphthalein, full fat milk, bile salts, lipase and sodium carbonate were not kept at the same temperature as the temperatures they would be later added to. This therefore gave the solution of lipase time to heat up / cool down before reaching the same temperature as the temperature of the water surrounding the test tube. To reduce this source of error in the final experiment, each container storing the phenolphthalein, sodium carbonate, bile salts, lipase and full fat milk will be kept within the water bath so that all of the substances begin the experiment at the same initial temperature.

Moreover, looking at the previous graph, in nearly all experiments the pH rises from the first to second minute before gradually falling. The reason for this could be because it takes time for the lipase enzymes to 'find' the full fat milk and initially there is a lot of competition for the enzyme's active sites.

Finally, for the 37°C experiment, we can see that the rate at which the pH decreases falls after the 9th minute. This could be down to the reduced substrate concentration as the lipids present in the full fat milk are hydrolysed to fatty acids and glycerol. However, another potential cause could be because of the fatty acids accumulating in the solution and blocking the lipases active sites. To reduce the effect of this, the solution will be stirred thoroughly each minute, preventing the different substances from settling (which reduces the chances of the full fat milk coming into contact with the lipase solution) and preventing substances to build up in one region of the test tube.

Apparatus

- **Water bath –** This will provide a constant temperature of 37°C for the entire duration of the experiment. The preliminary experiment confirmed that this was the optimum temperature at which lipase hydrolyses lipids into fatty acids and glycerol. The water bath will heat the water contained within it to a temperature of 37°C and will maintain this temperature despite the water being exposed to the air in the room. The water bath possesses sensors so that the water temperature does not fluctuate and is kept at the 37°C mark. The water in the water bath will be kept at a relatively low level, deep enough to immerse most of the test tubes being stored in the water, but not too deep as to flood into the test tubes themselves. The beakers and flasks containing the bile salts, phenolphthalein, sodium carbonate, lipase and full fat milk will all be stored in the water bath so that when the experiment commences all of the substances are at the same initial temperature. The same water bath that was used in the preliminary experiment will be used in the final experiment.

- **Metal test tube racks x 4 –** To store the test tubes filled with the various different substances. Three of these test tube racks will be used to hold each individual test tube containing the bile salts, sodium carbonate, phenolphthalein, full fat milk and lipase. The fourth test tube rack will be placed inside the water bath so that it too is heated to a temperature of 37°C and can store the substances safely without water entering the solution.

- **An A4 sheet of small stickers** – These stickers will be used to label each test tube, test tube rack and burette so that is clear what substance is present in each container. Labelling each burette will prevent cross contamination as each burette is used for one particular substance only, and this substance can be easily identified from the sticker.

Labelling the test tubes and test tube rack will also prevent confusion, therefore reducing anomalous results – for example, if I were to add $3ml^3$ of lipase to the solution of bile salts, milk, sodium carbonate and phenolphthalein but believed I had picked up the test tube containing $4ml^3$ of lipase, the results produced would therefore be anomalous and contain a lot of error.

- **$568ml^3$ (1 pint) of Tesco's full fat milk –** Full fat milk should contain more fat and therefore more lipids than any other type of milk, so there will be a large substrate concentration available for the lipase present in the volume being tested. $568ml^3$ of milk will provide more than enough from which the 50 ml^3 in total can be obtained. The surplus milk can then be used for repeats if there is an error or an accident occurs in one of the experiments. The bottle of milk being used will be the same as that used in the preliminary experiment so that the optimum temperature determined in the preliminary experiment still holds true and the substrate concentration is the same.

- **'Squirty' bottles of distilled Water –** This will be used to clean the apparatus before and between experiments in order to prevent cross contamination. The electronic pH meters will be cleaned with distilled water between experiments to remove any of the previous solution (but this water will then be dabbed with a clean tissue as the 'ionless' water can suck ions out of the electrode of the pH meter and cause the pH meter to degrade) which could potentially dilute the next solution being tested. The burettes, test tubes and beakers being used will be rinsed thoroughly with distilled water before the experiment begins to ensure that there are no unwanted substances interfering with the results. If any of these containers then need to store a different substance, they will be thoroughly cleaned with distilled water before the new substance is added.

  The pipette filler will also be rinsed with distilled water between experiments, just in case some of the fluid it has drawn in has become accidentally trapped inside.

- **Electronic pH meters –** In order to obtain a direct reading of pH without having to use the logarithmic formula, calibrated pH meters will be added to the solution of bile salts, lipase, sodium carbonate, phenolphthalein and milk. The electronic pH meters will display the pH of the solution to two decimal places, and the value displaced on the LCD screen will be noted each minute for 10 minutes. The pH meters used in the preliminary experiment will be the same as the pH meters used in the final experiment.

- **Buffer solutions x 2 –** To calibrate the pH meters two solutions with different pHs will be used. To give the pH meters a large scope of measureable pH, the buffer solutions being used have a pH of 9.2 and 3.5.

- **Paper towels** - To remove the distilled water from the electrode of the pH meter after each experiment.

- **Screwdriver** – The electronic pH meters have very small buttons which are responsible for calibration. I will therefore need the sharp point of a screw driver in order to use these buttons when calibrating the pH meters.

- **100ml^3 beakers x 4 –** While measuring out the desired volume of each substance, the burettes will be supported above a 100ml^3 beaker and the fluid will slowly be released into the beaker by rotating the stop cock until the correct volume has been achieved. This will allow the volume of the substance to be measured closer to the apparatus so that less of the fluid is lost to gravity in the form of dripping. The use of beakers will also allow substances to be stored in which their original container did not have an opening wide enough for the nozzle of the burette to be placed (for example – the phenolphthalein is stored in a very small bottle).

- **Thermometers x 2** – To verify that the water bath maintains a constant temperature of 37°C thermometers will be placed at each end of the water bath. These thermometers will be held within the test tube rack so that the reading can be read directly without having to lift the thermometers out of the water bath and therefore immediately reduce the temperature that they display. The thermometer used in the preliminary experiment will be used in the final experiment, to reduce any difference in readings between different thermometers.

- **Stop watches x 2 –** These will be used to determine when each minute has passed as the lipase is added to the mixture of bile salts, phenolphthalein, full fat milk and sodium carbonate. The stop watches will have their 'start' button pressed as soon as the last drop of lipase has been added to the test tube containing the aforementioned substances. After each volume of lipase has been tested, the 'stop' and 'reset' button will be pressed so that the process can then be repeated. Two stopwatches will be utilised so that two experiments can be performed simultaneously and therefore to reduce the time taken for the whole procedure. The stopwatches used in the preliminary experiment will also be used in the final experiment.

- **100ml^3 of Lipase –** The solution of Lipase will have been made up by the Science technicians and will be used to hydrolyse the lipids found in the full fat milk. Using burettes, the desired volume of lipase will be obtained and stored in a labelled test tube. 100ml^3 will provide more than the 36ml^3 of lipase required for the experiment and will thus allow extra in case further repeats are needed. The lipase used will be the same lipase utilised in the preliminary experiment so that it is known the optimum temperature of 37°C still applies. Great care will be taken while handling the lipase as any contact with skin or eyes could be harmful (*see safety precautions)*.

- **100ml^3 of bile salts –** The solution of bile salts will be used to first emulsify the fats present in the full fat milk. The emulsification of the triglycerides present in the full fat will disperse the globules of fat into tiny droplets, giving them a larger surface area. Since the lipids will be dispersed over a larger surface area, it will be easier for the lipase enzymes to work on them and so the reaction will proceed a lot quicker, meaning the pH will change at a greater rate. Without the use of bile salts it is possible the rate of reaction would proceed at a rate too slow to harvest reliable data. The solution of 100ml^3 will provide more than the 20ml^3 of the bile required, allowing repeats to be carried out if needed. The time for which each 5ml^3 of milk is exposed to the bile salts will be kept the same so that the lipids in the milk are emulsified equally. The bile salts used in the preliminary experiment will also be used in the final experiment.

- **20ml^3 Phenolphthalein** – The phenolphthalein will be used as an indicator, showing the gradual increase in acidity of the solution. When the phenolphthalein is placed in a basic solution it appears pink in colour, as the hydrogen ion content of the solution increases, the phenolphthalein fades to colourless. Phenolphthalein is insoluble in water and is slightly acidic, so it can lose H^+ ions in solution.

For this reason I shall only be adding $1ml^3$ of phenolphthalein to each volume of lipase being tested. The vial of phenolphthalein containing $20ml^3$ will contain more than enough phenolphthalein needed for the experiment. The vial of phenolphthalein used in the preliminary experiment will also be used in the final experiment, so that its composition is known to be the same. If a different vial of phenolphthalein were to be used, it is possible that it could be more concentrated and thus take longer to decolourise – insinuating that fewer fatty acids were being released.

- **$125ml^3$ Sodium Carbonate –** Sodium Carbonate is a base that can neutralise acids by accepting hydrogen ions (protons) when dissolved in water – milk is mostly water. When sodium carbonate is dissolved in water the following reaction takes place: $Na_2CO_3 + H_2O \rightarrow 2\ Na^+ + HCO_3^- + OH^-$. Therefore the sodium carbonate will be used to give the initial solution of bile salts, phenolphthalein, full fat milk and lipase a pH of approximately 10. The alkali pH will cause the phenolphthalein to appear pink, and so the change in pH can be observed. I will be utilising $3ml^3$ of sodium carbonate for each lipase volume being tested as I do not wish to use excessive amounts that could end up neutralising the solution as fatty acids are released, and thus no results could be obtained since there would be no reduction in pH. The solution of sodium carbonate used will be the same as that used in the preliminary experiment.

- **Clean burettes capable of taking up 10 – $20ml^3$ of solution x 5** – To extract the desired volume of bile salts, phenolphthalein, sodium carbonate, full fat milk and lipase from their original containers, burettes will be used. Burettes graduated with millilitre marks will be utilised as they provide a more accurate form of measurement as opposed to using pipettes which are prone to sucking up bubbles of air. Each different substance will be extracted using a different burette to prevent cross contamination between substances.

- **Pipette fillers** – These will be attached to the top of the burette and will be squeezed, decreasing the air pressure inside, causing the liquid to be sucked up the burette.
  The pipette fillers will be rinsed thoroughly between each burette they are attached to, just in case any of the previous liquid has been trapped inside the spherical squeezer.

- **Sterile test tubes** – To store each individual substance, clean test tubes which fit into the test tube racks will be used. Each individual substance will be stored in an individual labeled test tube so that the time at which the bile is mixed with the full fat milk is the same for each experiment.

- **Glass Rod** – Glass rods are unreactive and so will not interfere with the solutions involved in the experiment. The use of a glass rod allows thorough stirring of the solutions, ensuring that the bile salts, lipase, and full fat milk are fully mixed together. The stirring of the solution should prevent the fatty acids accumulating in one area of the test tube, which in turn could block the active sites of the lipase enzymes. Stirring should also prevent the substances from settling in different layers of the test tube .The concentration of lipase can be kept constant throughout the entire solution through stirring.

- **Goggles** – To avoid potential contact between my eyes and the full fat milk, bile salts, phenolphthalein, lipase and sodium carbonate, goggles will be adorned at all times during the experiment.

Lipase is hazardous if it makes eye contact and is classed as an irritant. The lipase enzymes are designed to hydrolyse fats, and since fats are large constituent of my cells, making up the cell membranes and fats and oils present on my skin, it is therefore important to avoid any eye contact with the lipase solution. Enzymes in cells are usually encapsulated by a membrane-bound sac organelle called a Lysosome, found in the cytoplasm of most living cells. The Lysosome acts as a barrier, stopping the enzyme from digesting the entire cell, it therefore seems sensible to shield my eyes to prevent any damage to the eye cells. Phenolphthalein is also classed as an irritant while bile salts are classed as a mild irritant. Sodium carbonate is a lachrymator – a substance that increases the flow of tears and is known to cause eye irritation. Wearing goggles at all times should reduce any irritation to the eyes that these substances may cause.

- **Latex gloves** – Thin see-through gloves will be worn at all times during the procedure. The gloves being used are thin and thus will not create difficulty in using my hands when using the burettes, stop watches and test tubes etc. Despite being thin, they will create a complete barrier between my skin and the substances being used and they will not be damaged by the substances if they are to come in contact with them. The safety data sheet for lipase states that lipase is slightly hazardous if there is skin contact, so wearing gloves will prevent any irritation that the lipase could induce. Similarly, the data sheet for bile salts and sodium carbonate also states that bile is slightly hazardous if there is any skin contact. Phenolphthalein however is not classed as an irritant, but studies have shown that phenolphthalein is carcinogenic, and if the phenolphthalein is absorbed through the skin, it can cause purging, collapse, a fall in blood pressure, or an itching skin rash that can become ulcerous. Wearing gloves while handling the substances should prevent any skin contact with the substances being used.

Safety Precautions

To aid devising an effective safety plan for this experiment, data sheets have been provided with the substances being used. Below, the relevant information has been quoted from the safety data :

Lipase

"Potential Acute Health Effects:
Hazardous in case of eye contact (irritant) or of ingestion. Slightly hazardous in case of skin contact (irritant), of inhalation.

Potential Chronic Health Effects:
Hazardous in case of eye contact (irritant), of ingestion.
Slightly hazardous in case of skin contact (irritant), of inhalation.
CARCINOGENIC EFFECTS: Not available.
MUTAGENIC EFFECTS: Not available.
TERATOGENIC EFFECTS: Not available.
DEVELOPMENTAL TOXICITY: Not available.

First Aid Measures:

Eye Contact: Check for and remove any contact lenses. Do not use an eye ointment. Seek medical attention.
Skin Contact:
After contact with skin, wash immediately with plenty of water. Gently and thoroughly wash the contaminated skin
with running water and non-abrasive soap. Be particularly careful to clean folds, crevices, creases and groin. Cover the irritated skin with an emollient. If irritation persists, seek medical attention. Wash contaminated clothing before reusing.

Serious Skin Contact: Not available.
Inhalation: Allow the victim to rest in a well ventilated area. Seek immediate medical attention.
Serious Inhalation: Not available.
Ingestion:
Do not induce vomiting. Loosen tight clothing such as a collar, tie, belt or waistband. If the victim is not breathing, perform mouth-to-mouth resuscitation. Seek immediate medical attention.
Serious Ingestion: Not available."

Phenolphthalein

"Potential health effects:

Inhalation:
Nuisance dust. May cause coughing and sneezing.
Ingestion:
Cathartic. Very active, even in small amounts (30-100 mg). May cause purging, collapse, and fall of blood pressure or an itching skin rash that can become ulcerous. Other systemic effects are not well known.
Skin Contact:
Not classified as an irritant but may be absorbed via moist or oily surfaces. Symptoms may resemble those from ingestion exposure.
Eye Contact:
Slight irritant.
Chronic Exposure:
Suspect cancer hazard; contains phenolphthalein which may cause cancer. Risk of cancer depends on level and duration of exposure.
Aggravation of Pre-existing Conditions:
Persons with pre-existing skin disorders or impaired respiratory function may be more susceptible to the effects of the substance.

First Aid Measures:

Inhalation:
Remove to fresh air. Get medical attention for any breathing difficulty.
Ingestion:
Induce vomiting immediately as directed by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention.
Skin Contact:
Wash exposed area with soap and water. Get medical advice if irritation develops.
Eye Contact:
Immediately flush eyes with plenty of water for at least 15 minutes, lifting upper and lower eyelids occasionally. Get medical attention if irritation persists. ‘‘

Bile salts

"**3. Hazard identification:**
Most important hazards: Not classified as hazardous. However this product may cause irritation of the nose, throat andupper respiratory tract if inhaled.
Human health hazards - eyes: May cause mild transient irritation.
Human health hazards - skin: Prolonged or repeated skin contact may cause mild skin rashes to some individuals.
Human health hazards - ingestion: Do not ingest. A large dose may cause diarrhoea, nausea and vomiting.
Human health hazards - inhalation: Inhalation of fine powders at high concentrations may be harmful causing irritation of the nose, throat and upper respiratory tract.

4. First-aid measures
First aid – Eyes: Rinse eyes immediately with water for 10 to 15 minutes with eyelids open. Obtain medical attention if soreness or redness persists.
First aid – Ingestion: Wash mouth out with plenty of water. Have victim drink 250-300ml of water to dilute stomach contents and seek medical advice.
First aid - Skin Contact: Wash skin thoroughly with soap and water. In the event of redness or itching seek medical advice.
First aid - Inhalation: Remove subject from source of exposure. Seek medical advice if you feel unwell."

Sodium Carbonate

"Potential Health Effects
Eye: Causes eye irritation. Lachrymator (substance which increases the flow of tears).
Skin: Causes skin irritation. May be harmful if absorbed through the skin.
Ingestion: May cause irritation of the digestive tract. May be harmful if swallowed.
Inhalation: Harmful if inhaled. May cause respiratory tract irritation.
Chronic: Adverse reproductive effects have been reported in animals.

First Aid Measures
Eyes: Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid.
Skin: Get medical aid. Immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes.
Ingestion: Do not induce vomiting. Get medical aid.
Inhalation: Remove from exposure and move to fresh air immediately. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid.
Notes to Physician: Treat symptomatically and supportively."

The full fat milk being utilised should not cause any irritation if it is accidentally ingested or makes skin contact. Milk is safe to drink from birth and is therefore a safe substance to handle. Obviously eye contact with milk will cause serious discomfort so wearing goggles will limit the likelihood of such discomfort occurring.

In order to reduce the potential health risks that each substance presents, goggles and latex gloves will be adorned at all times during the experiment. Lab coats will also be worn so that there is maximum skin coverage and any spilt substances will not make skin contact. As highlighted in the phenolphthalein's data sheet, phenolphthalein is thought to be carcinogenic, and the likelihood of cancer occurring increases with exposure time. The phenolphthalein being used is in solution and will therefore remain in the test tubes without diffusing into the surrounding air or being ingested or inhaled. The vial of phenolphthalein is very small and so even if this is spilt, there should not be enough to pose a threatening health risk. However, great care will be taken when handling each and every one of the substances to avoid any potential bodily contact, and to prevent any contact with the tables and floor, as the classroom will be used by other students once the experiment has been terminated. A reasonable distance will be kept between me and the test tubes in which the phenolphthalein is contained; I will only approach the solution when I need to observe the pH reading on the pH meter. Lids will be kept on top of the bottles storing the substances when they are not in use.The duration of the experiment is 10 minutes so the exposure to phenolphthalein is not particularly chronic. The reactions occurring within the test tube should not produce any toxic gases and so mouth guards do not need to be worn. The room will however be well ventilated, therefore preventing the build up of any toxic fumes which could cause fainting or headaches.

The burettes, test tubes and measuring beakers are all made from glass, and so these must be handled with care as any shattered glass would present a large health risk to other people using the room.

To reduce the chances of knocking over any of the containers, the worktop I shall be using will be cleared from any clutter and each container will be spaced apart. The burettes will be laid on the table when they are not in use, as balancing them in the measuring beakers would increase the chances of the burettes being knocked out and thus breaking. All containers will be placed away from table edges so that they cannot roll off and fall to the floor. Test tubes will be held securely within the test tube racks.

The substances being used are liquids, or solids dissolved in liquids. It is therefore highly unlikely that these substances will undergo combustion at the temperature of 37°C being used. Despite the highly unlikely risk of a fire, fire extinguishers are present in the room so that if a fire occurs, it can be tackled as quickly as possible by a member of staff who knows how to use the fire extinguisher. The substances will be kept away from direct sunlight in case this provides enough energy for the formation of free-radicals.

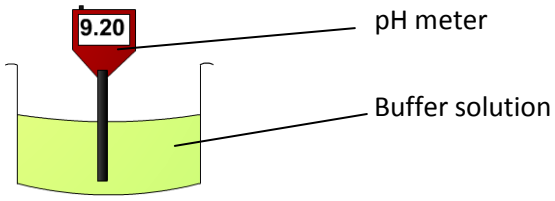
Wearing goggles, latex gloves and a lab coat should minimalise skin and eye contact, therefore minimalising the irritation that lipase, bile salts, phenolphthalein and sodium carbonate are known to cause.

Method

This is the procedure I followed whilst conducting the experiment in order to obtain accurate and reliable results:

1) **Calibrating the pH meters** – In order to verify that the pH meters were giving the correct reading of pH, I first calibrated the pH meters using two buffer solutions. Having turned on the pH meters, using a screw driver, I pushed the mode button and selected the pH mode. The calibration knob was then adjusted to read 100% and the temperature knob was adjusted to 25°C. Having rinsed the electrode of the pH meter with deionised water, and blotted it dry with a paper towel, the electrode was then placed in the buffer solution of pH 9.2. The display on the LCD screen was then allowed to stabilise, before the display reading was set to 9.2 by adjusting the 'cal 1' option. The electrode was then removed from the buffer solution, rinsed with deionised water and dabbed with a paper towel.
   The electrode of the pH meter was then placed in the second solution of pH 3.5 buffer. The pH meter was then given time to stabilise, and then the display was set to read 3.50 by adjusting the 'cal 2' option. The electrode was then removed, rinsed with deionised water and blotted dry using a paper towel. The pH meter was then placed to one side, ready to be added to the solutions once they had been mixed together.

Figure 12 – Calibration of the pH meters:



2) **Setting the water bath to 37°C –** To ensure that the lipase experienced its optimum temperature, the water bath was connected to the mains and set to 37°C, ten minutes before the experiment began. Water was then carefully added to the container of the water bath, being careful not to spill any near plug sockets and onto the floor as this could create a hazardous working environment. Enough water was added so that a majority of the test tubes being stored in the water bath would be covered, but not too much as to flood the test tubes and dilute the solutions being stored. Thermometers were then added to the water bath so that the temperature of the water could be monitored and the 37°C temperature could be confirmed.

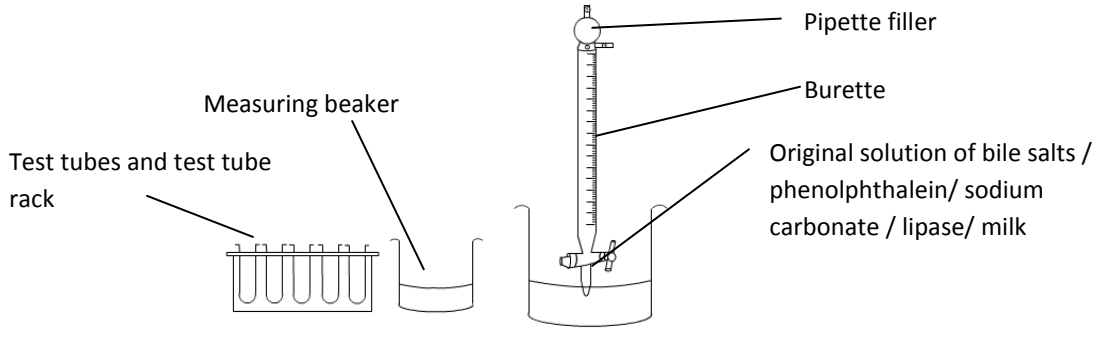
The containers containing the milk, lipase, bile salts, sodium carbonate and phenolphthalein that needed to be measured out with the burettes, were placed inside the water bath, so that all of the substances were at the same initial temperature for the experiment. Placing all of the containers in the water bath meant that both the enzymes and substrates had the same kinetic energy before they were mixed together.

3) **Preparing the volumes of lipase, bile salts, full fat milk, phenolphthalein and sodium carbonate** – Before use, all of the burettes, measuring cylinders and test tubes were rinsed thoroughly with distilled water and left to dry. When cleaning the burettes, the distilled water was squirted into the burette and the burette was rotated slowly in my hands so that the distilled water cleaned the entire surface area of the burette. The apparatus was then left to dry as any left water could dilute the substances being used in the experiment. Using small stickers, each burette was labelled relatively high up its column (so the stickers would not be immersed in the fluids being tested) with the individual substance that it would be used to obtain. These stickers were applied carefully as to not block the measurements on the burette. Measuring beakers capable of storing up to $100ml^3$ were placed close to the original containers in which the substances were being kept. The lid was removed from the vial of phenolphthalein and a small amount was poured into one of the measuring beakers. The reason for this was because the nozzle of the burette was too wide to fit into the opening of the vial of phenolphthalein. The lid was then placed back onto the small bottle of phenolphthalein to prevent any unwanted evaporation. Both the vial of phenolphthalein and measuring beaker were placed back in the water bath so that all of the substances were kept at the same temperature.

4) **Organisation of test tubes** – Before obtaining the necessary volumes of lipase, bile salts, full fat milk, sodium carbonate and phenolphthalein, 25 clean test tubes were collected and arranged into three test tube racks. For each volume of lipase being tested, 5 test tubes were required – one for the volume of lipase being tested, one for the $5ml^3$ of full fat milk, one for the $3ml^3$ sodium carbonate, one for the $2ml^3$ of bile salts and another for the $1ml^3$ of phenolphthalein. This step was introduced because the preliminary experiment had highlighted the fact that by placing all of the substances together, minus lipase, and not recording the time, the lipids in the milk were mixed with the bile salts for unequal amounts of time.

Each test tube was labelled with the substance it would be storing, and each group of 5 test tubes was spaced apart from the next 5 to distinguish the different test tubes needed for different experiments. Stickers were also placed on the test tube racks to limit confusion when identifying the contents of each test tube.

Figue 13 – Showing the use of the burette and the containers involved:

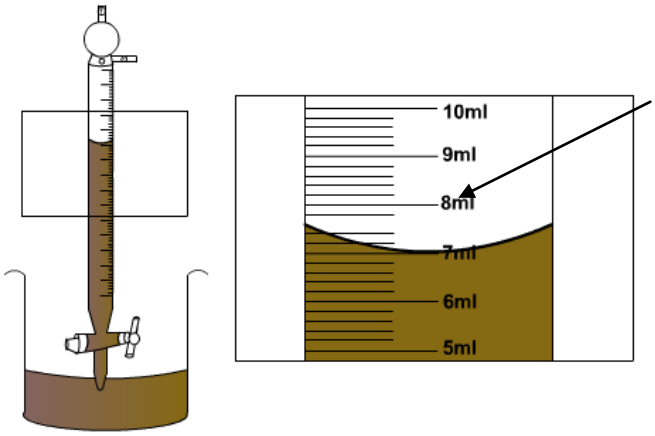


5) **Obtaining the $3ml^3$ sodium carbonate, $1ml^3$ phenolphthalein, $2ml^3$ bile salts and $5ml^3$ full fat milk** – Having collected the burettes and pipette filler, the pipette filler was squeezed, removing the air inside and was attached to the top of the burette. Once the nozzle of the burette was fully immersed in the

substance being obtained, pressure was gently exerted on the '3' mark on the pipette filler and the substance was taken up slowly from the container. This process was performed slowly to prevent any bubbles being created in the solution and therefore affecting the measurement. Following an excess uptake of the substance being measured, the stop cock was rotated so that no fluid could drip away and the burette was then held over a measuring beaker. Slowly rotating the stop cock, the fluid was released gradually by pressing down on the '2' button on the pipette filler, until the meniscus of the fluid sat exactly on the millilitre mark that corresponded to the volume being measured. For example, when measuring out the bile salts, once the meniscus of the bile salt solution rested exactly upon the $2ml^3$, the stop cock was rotated, and the release of fluid was stopped. In most cases, more fluid was stored in the burette than needed, so when obtaining $3ml^3$, the fluid was let out from the $10ml^3$ mark to the $7ml^3$ mark.

The burettes were then carefully transported to the designated test tube and the fluid was deposited. In order to save time, the total volume of bile salts, phenolphthalein, sodium carbonate and full fat milk needed was measured out at once. For example, instead of measuring out $1ml^3$ of phenolphthalein, and then $3ml^3$ of sodium carbonate etc, $5ml^3$ of phenolphthalein was measured out first, and $1ml^3$ was placed in the test tube needed for each experiment. The pipette filler was rinsed with deionised water between each burette it was attached to, to prevent any cross contamination.

Figure 14 – Measuring the volume of each substance accurately:



As a result of the attraction between the molecules of the solution and the glass making up the burette, a concave meniscus occurs.
Meniscus of the solution being measured sits on the graduated millilitre mark.

6) **Measuring out the volume of lipase required –** Similarly, using the pipette filler and the burette allocated for taking up lipase, the burette was immersed in the lipase solution and the lipase was taken up via pressing down on the '3' mark of the pipette filler. Having taken up an excess amount of lipase, the lipase solution was released into a measuring beaker until the desired volume of lipase had been obtained. The correct volume was assumed to be obtained when the meniscus of the lipase solution rested on the millilitre mark of the volume being obtained. The solution of lipase was then released into the test tube labelled with the volume that had just been measured out.

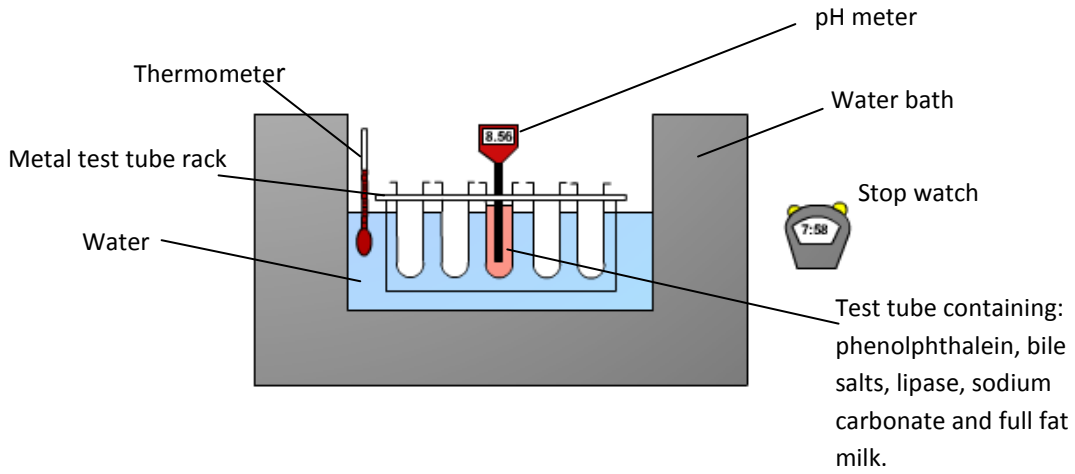
7) **Mixing all five substances together –** Once each test tube was filled with the correct volume of each substance, each of these substances was then poured into a single test tube, with the addition the volume of lipase being tested last. The label on the single test tube was then removed to prevent it from entering the water.

This test tube was then quickly but carefully placed in the test tube rack in the water bath and the pH meter was then added. 1 minute was given for the pH meter to stabilise before the pHs were noted.

8) **Timing the experiment** - The stop watches were then started and timing commenced. The stop watches were placed close to the water bath at all times in the experiment so that there would be a

short delay between reading the time on the stop watch and observing the pH reading on the pH meter. While the solution of lipase, bile salts, phenolphthalein, sodium carbonate and milk were in the single test tube, a glass was rod was inserted and moved around in a clockwise direction, mixing the substances together. After each minute the reading of pH meter was noted in tables drawn prior to the experiment. When the stop watch displayed 10:00 minutes, the pH meters were removed from the solution, cleaned with deionised water and dabbed with a paper towel. Each experiment testing a particular volume of lipase was repeated twice to improve accuracy and eradicate any inconsistent results. Graphs were then drawn from the tables and the data was then analysed.

Figure 15 – The test tubes set up in the water bath:



Results

Volumes of substances used:

Full fat milk: 5ml^3
Sodium Carbonate: 3ml^3
Bile salts: 2ml^3
Phenolphthalein: 1ml^3
Temperature: 37°C

Highlighted yellow = Anomalous result

Experiment 1

Control – 0.00ml^{3}lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.49	8.55	8.59	8.61	8.62	8.62	8.62	8.62	8.62	8.62

3.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10

pH	7.73	8.06	8.05	8.02	7.99	7.97	7.95	7.93	7.91	7.89

4.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.98	9.03	8.97	8.94	8.96	8.98	8.93	8.87	8.82	8.78

5.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	9.73	9.85	9.80	9.69	9.60	9.51	9.46	9.40	9.34	9.28

6.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	7.40	7.10	6.93	6.84	6.81	6.78	6.75	6.74	6.73	6.72

Experiment 2

Control – 0.00ml^{3}lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.25	8.39	8.33	8.30	8.30	8.28	8.28	8.27	8.27	8.27

3.00ml^3 lipase

Time	1	2	3	4	5	6	7	8	9	10

(min)										
pH	8.75	8.80	8.91	8.81	8.77	8.66	8.51	8.35	8.18	8.08

4.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	7.82	7.84	7.70	7.69	7.62	7.51	7.41	7.30	7.20	7.12

5.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	9.21	8.88	8.49	8.30	8.20	8.13	8.10	8.07	8.05	8.04

6.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	7.30	7.04	6.90	6.85	6.80	6.78	6.75	6.74	6.72	6.61

*All values are shown to 2 decimal places because the pH meters only showed values of pH up to 2 decimal places. It should also be noted that a new solution of bile salts and lipase was used when testing the higher volumes of lipase as the amount available from the preliminary experiment was insufficient to carry on with the experiment.

Average results for each volume of lipase – *Rounded to 2 decimal places*

Control

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.37	8.47	8.46	8.46	8.46	8.45	8.45	8.45	8.45	8.45

3.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.24	8.43	8.48	8.42	8.38	8.32	8.23	8.14	8.05	7.99

4.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	8.40	8.44	8.34	8.34	8.29	8.25	8.17	8.09	8.01	7.95

5.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	9.47	9.37	9.15	9.00	8.90	8.82	8.78	8.74	8.70	8.66

6.00ml^3 lipase

Time (min)	1	2	3	4	5	6	7	8	9	10
pH	7.35	7.07	6.92	6.85	6.81	6.78	6.75	6.74	6.73	6.67

Analysis

Interpretation of tables

Firstly, looking at the average maximum change in pH (the largest average value of pH observed minus the smallest average value of pH observed) during the experiment for each volume of lipase, we can see that generally, as the volume of lipase utilised in the experiment was increased, so too was the maximum change in pH. However, as the table depicts, there was a reduced value for the maximum change in pH for 6.00ml^3 of lipase in comparison to the 5.00ml^3 of lipase. Clearly this result is anomalous, as we would expect a greater volume of lipase to contain more enzymes, and thus more active sites would be available to bind with the substrate, and therefore hydrolyse the fats present in the full fat milk more quickly, releasing more hydrogen ions per unit time than the 5ml^3 of lipase could. There is also no change in the maximum change in pH for 3.00ml^3 and 4.00ml^3 of lipase, which aforementioned, we would expect the 4.00ml^3 of lipase to produce a greater change in pH. The reason that this anomalous result is thought to have occurred is because these changes in pH have been calculated using the average pH values for both experiments. The averages are not entirely consistent with the results, as for many repeats, the pHs were different by up to 0.5 for each lipase volume.

Average maximum change in pH

Volume of lipase (ml^3)	Maximum change in pH
0.00	0.02
3.00	0.49
4.00	0.49
5.00	0.81
6.00	0.68

Looking at the control group, we can see that there was an average change of 0.02 in the pH measured. Although this change in pH is somewhat small, realistically there should not have been any change in pH as there was no lipase present, and thus nothing to catalyse the hydrolysis of triglycerides into fatty acids and glycerol, therefore increasing the acidity of the solution. The change in the pH could have been caused by the pH meters taking time to stabilise or potential cross contamination between the equipment. This will be explored further in the evaluation.

If we look at the individual tables for 5.00ml^3 lipase, we can see that after the first minute, in the first experiment the pH is observed as 9.73 whereas in experiment 2 it was noted as 9.21. This gives a difference of 0.52 which is quite significant considering that the pH scale only spans from 0 – 14. Theoretically, both solutions should contain identical amounts of bile salts, full fat milk, sodium carbonate, lipase and phenolphthalein, and so in turn should have exactly the same initial pH. If the initial pHs are equal, and the volume of lipase is equal, then the rate of hydrolysis should be equal in each experiment. Similar differences can be seen for 3.00ml^3 and 4.00ml^3 of lipase. These differences in pH between the experiments is most likely to have stemmed from the fact that the solutions of bile salts and lipase used in the preliminary experiment ran out part way through the experiment and so new solutions had to be sought after. Although the maximum change in pH is lower for 6.00ml^3 than it is for 5.00ml^3, the change in pH for 6.00ml^3 is still larger than the maximum change in pH for the control, 4.00ml^3 and 3.00ml^3. We can therefore say that generally, as the volume of lipase increased, so did the maximum change of pH.

It could be more accurate however to consider the maximum pH changes for the individual experiments rather than the average results, since the averages have used values of pH that are not as close to each other as we would expect.

Experiment 1 **Experiment 2**

Volume of lipase (ml^3)	Maximum change in pH
0.00	0.13
3.00	0.33
4.00	0.25
5.00	0.57
6.00	0.68

Volume of lipase (ml^3)	Maximum change in pH
0.00	0.12
3.00	0.63
4.00	0.72
5.00	1.17
6.00	0.69

Looking at the maximum changes in pH for each individual experiment, it is clearer that as the volume of lipase increases so too does the maximum change in pH. However, as we can see there are some anomalies which in turn have also made the average results calculated slightly anomalous. If we consider experiment 1, then we can see, with the exception of 4.00ml^3 of lipase, as the volume of lipase increases, so too does the maximum change in pH. We would expect that 6.00ml^3 of lipase contains twice as many enzymes as 3.00ml^3 and therefore there are twice as many active sites available, meaning the chances of a substrate colliding with an active site to form an enzyme-substrate complex is twice as high. With twice as many enzyme-substrate complexes, twice as many substrates can be hydrolysed in one go, so that twice the amount of hydrogen ions can be released per unit time. In other words, we would expect that 6.00ml^3 would release twice as many hydrogen ions to 3.00ml^3 of lipase and so the overall change in pH would be double for 6.00ml^3 of lipase compared with the overall pH change for 3.00ml^3 of lipase. Analysing the table above for experiment 1 shows that the change in pH for 6.00ml^3 was just over double that of the pH change for 3.00ml^3, which therefore suggests that the hypothesis is largely correct.

However, for experiment 2, this negative linear relationship is not quite so perfectly explained by the hypothesis. As the table shows, as the volume of lipase increases, so too does the maximum change in pH, yet this does not apply to the maximum change in pH observed for 6.00ml^3 of lipase. In fact the maximum change in pH for 6.00ml^3 is less than the change in pHs observed for 5.00 and 4.00ml^3 of lipase, suggesting that there has been some form of error in experiment 2 when testing the 6.00ml^3 of lipase. As a result of not measuring the pH at the 0.00 minute, I cannot deduce whether there was in fact a large pH change in the first minute of the experiment. The pH after one minute for the 6.00ml^3 of lipase used in both experiments appears to be a lot more acidic in comparison to the other volumes of lipase tested, which could suggest that the solution started at a higher pH but fell quickly in the first minute. This however would seem unlikely as it should take time for the enzymes to come in contact with the substrates and for 3.00ml^3 and 4.00ml^3 of lipase the pH rose slightly before it started to fall. Unlike experiment 1 the pH change for 3.00ml^3 of lipase is not half of the pH change measured for 6.00ml^3 of lipase, in fact the pH change for 3.00ml^3 of lipase and 6.00ml^3 are relatively close, which could suggest there was an error when measuring out the volume of lipase/milk/bile salts in the second experiment.

Similarly, for experiment 2, we can see that the largest maximum change in pH occurred for the 5.00ml^3 and was equal to 1.17. Comparing this value with the maximum change in pH for 5.00ml^3 in experiment 1, we can see that the change in pH for experiment 2 is almost double the pH change in experiment 1. As previously mentioned, if the 5.00ml^3 of lipase was added to equal volumes of bile salts, sodium carbonate, phenolphthalein and full fat milk, then the amount of enzymes present should be equal.

If both solutions contain equal numbers of enzymes then there should be an equal number of active sites available in each solution.

Since the substrate concentration is constant in both experiments, in each experiment testing $5ml^3$ of lipase, the chances of an enzyme colliding with a substrate molecule and forming an enzyme-substrate complex should be equal, and so the rate at which fatty acids are released from the full fat milk should be the same for each experiment. The fact that the changes in pH for $5.00ml^3$ of lipase are not equal highlights the huge impact on the results when a different, more recently made solution of lipase and bile salts were used. There is also the possibility that there were errors in the measuring of volumes of lipase or full fat milk, or human errors in the keeping of time or reading the pH reading on the pH meter screen.

The fact that results for the maximum change in pH for $5.00ml^3$ and $6.00ml^3$ in experiment 1 are quite close could be due to the fact that when increasing the volume from $5.00ml^3$ to $6.00ml^3$, the enzyme concentration may have had less of an effect because there were already enough active sites in the $5.00ml^3$ of lipase to accommodate a large majority of the substrate molecules available in $5.00ml^3$ of the full fat milk. If the number of substrates available in $5.00ml^3$ of the full fat milk became very low very quickly, then the increased number of active sites available would have had less of an impact because there still would have been increased competition between the substrates. If all the substrates were bound to the active sites of the lipase enzymes then increasing the number of enzymes would have no effect on the rate at which the lipids were hydrolysed to fatty acids and glycerol, as all the possible substrate molecules were already bound to the active sites present in the given volume of lipase. There would therefore be no substrate molecules left to bind with the available active sites and be broken down, so the pH would cease to fall once all the substrates had been broken down. Unfortunately it is not known how many exact lipids are present in the full fat milk, nor is it known how many lipase enzymes are present in each volume of lipase, however if the number of enzymes in $5.00ml^3$ of lipase closely matched the number of lipids present in the $5.00ml^3$ of full fat milk, then it would make sense that increasing the volume of lipase, does not increase the change in pH by a large factor, compared to if you were to increase the volume of lipase from 3.00 to $5.00ml^3$.

When the volume of lipase is low, there is increased competition between the substrates to collide with the active site of the lipase enzymes. With fewer active sites available to hydrolyse all of the substrates present, fewer ester bonds are broken and fewer fatty acids are released into the solution, therefore the pH decreases very slowly. However, as the enzyme concentration is increased, the likelihood of a lipid colliding with the active site of a lipase enzyme also increases. If more active sites are available and more substrates are colliding with these active sites, then a greater number of lipids can be hydrolysed to fatty acids and glycerol per unit time, giving a faster rate of change of pH.

Looking at the general trend in the maximum changes in pH, it can be seen quite clearly that as the volume of lipase increases so too does the maximum difference in pH values. Looking at the graphs and performing a statistical analysis will enable me to validate this further.

Although the colour of the phenolphthalein after each minute has not been displayed in the tables, it was apparent that for higher volumes of lipase, the solution faded to colourless at a greater speed than for lower volumes of lipase. Colour is a variable that is hard to quantify, but generally, as the volume of lipase was increased by $1.00ml^3$, the time taken for the solution to appear clear was reduced by approximately a minute. The fact that the phenolphthalein turned from pink to clear faster for higher volumes of lipase shows that the acidity of the solution must have been increasing more for the higher volumes of lipase.

Graphical analysis

The gradients of the scatter graphs showing the average pH each minute for 10 minutes (see page ?)

Volume of Lipase (ml^3)	**Gradient of graph**
0.00	N/A
3.00	-0.071
4.00	-0.067
5.00	-0.080
6.00	-0.066

These two gradients do not correspond with the hypothesis.

* The gradient of the curve for the control group was too small to measure by hand – hence: N/A

Having drawn tangents to the scatter graphs produced using the average pHs, the gradient, and thus the average rate of change of pH for each volume of lipase has been calculated. As the table above displays, the deduced gradients are not as anticipated, as although 5.00ml^3 produces a steeper gradient than both 3.00 and 4.00ml^3, the gradient of 6.00ml^3 is smaller than the gradients found for all the other volumes of lipase. Clearly this does not fit with the hypothesis as we would expect 6.00ml^3 of lipase to possess more enzymes than any of the other volumes of lipase tested. If the number of enzymes was at maximum, then so too would be the number of active sites available and thus the maximum number of triglycerides would be hydrolysed per unit time, giving the maximum rate of decrease in pH.

A potential reason as to why the gradients found do not comply with the gradients expected could be due to the fact the scatter graphs produced do not follow a perfect straight line. In other words, there is not perfect negative linear correlation between the two variables of pH and time. The tangents have been drawn where the shape of the graph is most straight and have therefore ignored any large rises or drops in pH and have only considered the smooth declines in the shape of the graphs. For example, for 5.00ml^3 and 6.00ml^3 of lipase, there is a rapid drop in pH before the pH decreases at a more constant rate. The tangents drawn do not cover all the plots of data making up the graph, for example, the tangent drawn for 4.00ml^3 only considers the pH of the solution between approximately 4.05 minutes and 10 minutes. Therefore the plots of data before 4.05 minutes are not accounted for by the calculated gradient. If the drop in pH had been larger before 4.05 minutes, then the gradient would be expected to be less steep as fewer substrate molecules would be available as more and more are hydrolysed.

The tangents drawn are also do not cover the same amount of time, for example, the tangent for 5.00ml^3 lipase spans from 3 minutes to 8 minutes while the tangent for 3.00ml^3 of lipase spans 3 minutes to 9 minutes. Perhaps what would be a better alternative to this method would be to calculate the equation of the line, taking into consideration that the relationship is not perfectly linear.

Realistically, the general gradient for higher volumes of lipase should always be more negative than the smaller volumes of lipase, because each minute, the higher volumes of lipase should be hydrolysing more ester bonds each minute, releasing more fatty acids, therefore reducing the pH more each minute, giving a steeper graph. The fact that the gradient of the graph is negative shows that as time increases the pH decreases, as expected. If we were to just compare 3.00ml^3 of lipase with 5.00ml^3 of lipase then the gradient would follow the hypothesis more accurately – increasing the volume of lipase increases the rate at which the pH of the surrounding solution falls.

The fact that the gradients of the graph do not show the expected relationship, insinuates that there must be a large source of error or uncertainty somewhere in the procedure and this will be identified in the evaluation where modifications will be suggested.

Looking at the shapes of the graphs, it can be seen that for volumes of lipase of 5.00ml^3 and 6.00ml^3, the decrease in pH is fast at first and then gradually gets less and less as time progresses. Although the gradient of the graph produced for 5.00ml^3 of lipase appears to have a steeper gradient than the gradient of the graph drawn for 6.00ml^3, we can see that in the first 3 minutes, the pH of the solution containing 6.00ml^3 of lipase fell on average from 7.35 to 6.92, giving a change in pH of 0.43. However in the first three minutes of the experiment, the pH of the solution containing 5.00ml^3 of lipase only fell by 0.32. The fact that the pH decreased by a larger amount for 6.00ml^3 of lipase therefore implies that the lipids present in that solution were being hydrolysed more quickly. This corresponds with the fact that in 6.00ml^3 of lipase more enzymes are present and thus more active sites are able to bind with the substrates, therefore hydrolysing more ester bonds per unit time. After 3 minutes however, the rate of decrease in pH for 6.00ml^3 on average seems to be a lot slower, while for the volume of 5.00ml^3 lipase, the rate of decrease in pH continues to fall at faster rate. One such explanation for this could be related to the number of active sites available and the substrate concentration.

Initially, there would have been a lot of substrate available to the lipase enzymes, so it would have been easy for the substrates to come into contact with the many empty active sites on the lipase molecules. When all of the lipase enzymes had their active sites filled, the substrates would have been rapidly broken down (therefore giving a faster decrease in pH) and the amount of substrate left in the full fat milk would have been reduced. As more and more lipids in the milk would have been hydrolysed to fatty acids and glycerol, the substrate concentration would have decreased and so there would have been greater competition for active sites between the substrates remaining. With increased competition between the substrates and fewer of them, the chances of the substrates colliding with the active site of the lipase enzymes would have reduced, and so the rate of hydrolysis and thus decrease in pH would have also decreased. This general trend with a rapid decrease in pH during the first 3 minutes of the experiment, with the rate of decline in pH then decreasing for the rest of the experiment can be seen clearly for volumes of 6.00ml^3 and 5.00ml^3 of lipase (See the graph using the average pHs).

The trend however for 3.00ml^3 and 4.00ml^3 appears to be that the pH first increases before it decreases. This suggests that with fewer enzymes present, more time is needed before the enzymes collide with the substrates available in the full fat milk. With fewer enzymes present in the solution it is less likely that they will collide with a substrate. The shapes of these graphs may have also been related to the fact that the bile salts were added to the solution just before timing began, therefore it took time for the bile salts to collide with the lipids in the full fat milk before they were emulsified and the lipase enzymes could act on them. If the lipids had not been emulsified then it would take longer for the lipase enzymes to act on them, as the substrate would have a reduced surface area available to bind with the active site of the lipase enzymes.

Looking at the graph drawn for the control group using the average pH changes, we can see that although there is some fluctuation of pH, the gradient is far too small to measure accurately by hand. This therefore suggests that the rate of change of pH was extremely small and almost non-existent. For the control group, no lipase was present and so there were no enzymes to catalyse the hydrolysis of lipids into fatty acids and glycerol. In congruence with the shapes of the graphs for the other volume of lipase, we can see that the pH rose before it fell slightly and stopped changing. The fact that the pH rose, despite the fact that no lipase was present, strongly suggests that the pH meters need time to stabilise.

Without lipase, a biological catalyst, the triglycerides would not have had anything to encourage the hydrolysis of the ester bonds and so very few if any fatty acids would have been released into the solution.

The fact that lipase is a catalyst suggests that the reaction could still take place without lipase; however the reaction would be much slower. It is unlikely that the triglycerides would break down into fatty acids and glycerol on their own, and if they were to it would most likely require heating or a great deal of time. The duration of the experiment was only 10 minutes and a temperature of 37°C should not have had any influence in breaking up the lipids. The fluctuations in pH could be due to the pH meter taking time to stabilise, or may have been a result of cross contamination. For example, if a small amount of unnoticed lipase had been left in a test tube and this test tube was then used as the control group, there would have been enzymes present to catalyse the hydrolysis of triglycerides into fatty acids and glycerol. Great care was taken to minimalise such errors so this seems relatively unlikely.

If we look at the graphs produced for experiment 1, we can see that for 3.00, 4.00, and 5.00ml^3 of lipase, the pH rose from the first minute to the second minute before it started to fall. Aforementioned, this may have been related to the fact the bile salts were added to the solution at the 0.00 minute mark, and so time may have been needed to first emulsify the fats. The scatter graph for 4.00ml^3 lipase in experiment 1 shows a lot of fluctuation, for example, the pH rises from 8.98 at one minute to 9.03 at the second minute, the pH then falls from the second minute to the fourth minute from 9.03 to 8.94, and then rises again slightly by 0.04 to the sixth minute. Ideally, we would expect the pH to continually fall throughout the experiment as the hydrolysis of lipids should not be stopping and started but continually happening. One possible root of this anomaly could have been due to movement of the pH meter, if the pH meter was moved in the test tube then it is possible it may have been knocked to an area where more sodium carbonate or bile salts were present and so the pH detected would have risen. The stirring on the solution was implemented to prevent this from happening and the amount of lipase and full fat milk present should have been uniform throughout the test tube. Unfortunately there was insufficient time to repeat this experiment.

Similarly, analysing the graph for experiment 1, we can see that for 3.00ml^3 of lipase there was a significant increase in pH between the first and second minute. The pH rose by 0.33, which suggests something was happening in the test tube that increased the alkalinity of the solution. The hydrolysis of lipids should release fatty acids into the solution, therefore decreasing the pH, so the rise in pH cannot be accounted for by the reaction occurring between the lipase enzyme and the substrate. One possible explanation could be due to an incorrect measurement when trying to obtain 3ml^3 of sodium carbonate and/or 2ml^3 of bile salts. If an excess amount of these had been added to the test tube then the alkalinity of the solution would have been increased. However, the pH should have decreased, regardless of whether or not incorrect volumes of bile salts and sodium carbonate were used because this would have just increased the initial pH. This error may have occurred because of errors in the reading of the pH meter. There is also the possibility that the higher the alkalinity of the solution, the further the lipase enzymes were from their optimum pH, therefore the rate of hydrolysis was much slower. However this has proven not to be the case for the 5.00ml^3 of lipase.

In experiment 1, the graphs clearly show that as the volume of lipase increased, so did the rate of the change of pH. For 6.00ml^3 of lipase we can see that the pH fell each minute, while for the 5.00ml^3 of lipase the pH first rose before falling. This implies that the rate of hydrolysis of the lipids in the full fat milk occurred most quickly for 6.00ml^3 of lipase. Although the shape of the graph for 4.00ml^3 of lipase is not a straight line, overall the change in pH is greater for 4.00ml^3 than it is for 3.00ml^3, so it can be said with some certainty that the 4.00ml^3 of lipase released more hydrogen ions in comparison to the 3.00ml^3 of lipase. It can therefore be said with some certainty, that for experiment 1, increasing the volume of lipase, increases the change in pH and therefore increases the number of triglycerides being broken down to fatty acids and glycerol.

Analysing the graphs produced for experiment 2, we can see that for all experiments, as time progressed, the pH of the solution decreased.

The main differences between experiment 1 and experiment 2 however are that it appears that the solution using 5.00ml^3 of lipase had its pH decrease at a faster rate than the 6.00ml^3 of lipase. For experiment 2 it can also be seen that the change in pH was greater for 3.00ml^3 of lipase in comparison to 4.00ml^3 of lipase. As opposed to experiment 1, there seems to a larger difference between the initial pHs of the solutions between 4.00ml^3 of lipase and 3.00ml^3. This will most likely have stemmed from new solutions of bile salts and lipase being used between those experiments. However, as mentioned, the pH when timing commenced was not noted, so it is possible that in fact 4.00ml^3 and 6.00ml^3 actually experienced a greater pH drop in the first minute, and since more substrates had been broken down in the first minute, fewer were left to be hydrolysed, therefore the rate of change in pH after the first minute was not as fast.

However, comparing 6.00ml^3 with 3.00ml^3 and 4.00ml^3, we can see that the pH started to fall more quickly, and therefore more lipids were being hydrolysed per unit time and less time was taken for the enzymes to collide with the substrates. Similarly, it is clear from the graphs produced in experiment 2, that 5.00ml^3 produces a greater change in pH than 3.00ml^3 and 4.00ml^3 of lipase, and therefore increasing the volume of lipase increases the change in pH. What is not clear from the graphs produced in experiment 2 however is that increasing the volume of lipase from 3.00 to 4.00ml^3 or from 5.00 to 6.00ml^3 does not then lead to another increase in change of pH. There are obviously numerous anomalies in the experiment (as highlighted in yellow in the data tables) which are reducing the consistency of the results.

Statistical analysis (See appendix for calculations)

Using standard deviation and the t-test, I can deduce the validity of my results and calculate the rejection level probability when comparing different volumes of lipase.

In order to use both statistical processes, I first had to calculate the mean value of pH for both experiments. To do this I first found the mean pH observed after each minute for each individual volume of lipase and then found the mean pH observed over the total ten minutes for each volume of lipase.

The mean was calculated using the following formula:

$$\overline{x} = \frac{\Sigma x}{n}$$

$\overline{x}$ = the mean (average)
x = the observation (the pH)
n = the number of observations
Σ = the sum of

The mean value of pH observed through the ten minute period is not really of great use, as the pH changes and we therefore do not want one set value of pH. The standard deviation is used to measure the dispersion of the results and gives a comparison of how far the data increases/decreases away from the mean. We would therefore predict that for higher volumes of lipase, the standard deviation would be greater, since the change in pH would be greater and thus deviate most from the mean value of the pH observed over the ten minute period.

Standard deviation is given by the following formula:

$$S = \sqrt{\frac{\Sigma(X - \overline{X})^2}{(n - 1)}}$$

Volume of lipase (ml^3)	**Standard deviation**
0.00	0.028
3.00	0.168
4.00	0.162
5.00	0.284
6.00	0.204

The t-test

Using the following formula I can calculate the t-value and then see the rejection level probability when comparing different volumes of lipase:

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

As I was comparing 5 volumes of lipase, this therefore means 10 different t-values can be found and compared. Unfortunately, due to time restraints I will not be finding all ten possible t-values, but instead comparing the control with 6.00ml^3 of lipase, 3.00ml^3 with and 6.00ml^3 lipase and 4.00ml^3 and 5.00ml^3 lipase. The following table depicts the t-values calculated:

	Volumes of lipase being compared		
	0.00ml^3 and 6.00ml^3	**3.00ml^3 and 6.00ml^3**	**4.00ml^3 and 5.00ml^3**
t-value	9.93	3.16	7.49
Rejection level probability	<0.01	<0.01	<0.1

Degrees of freedom

$v = (n_1 + n_2) - 2$

n_1 = the number of repeats for a given volume of lipase
n_2= the number of repeats for another volume of lipase

In both cases the value of n was 2, as 2 repeats were carried out for each volume of lipase being tested, limited time and resources meant further repeats were not possible. My value for the degrees of freedom was therefore 2, since (2 + 2) – 2 = 2.

Calculating the degrees of freedom allowed me to check along the second row of the t-table until I reached a corresponding t-value and could therefore see the significant statistical value of the results and the confidence level of each t-value.

If we consider the table displaying the values of standard deviation, we can see that generally, an increase in the volume of lipase also leads to an increase in the value of standard deviation. However, as with the gradients of the graph, there appear to be anomalies in this trend for 6.00ml^3 and 4.00ml^3. Despite these anomalies, the standard deviation for 6.00ml^3 is still larger than the control, 3.00ml^3 and 4.00ml^3 of lipase being used, showing that the pHs observed deviate more from the mean pH observed throughout the whole ten minutes. The greater the value of standard deviation, the greater the plots of data deviate from the mean pH. The greater the results deviate from the mean, the more the pH has changed and the greater the number of fatty acids that have been released in the solution. The fact that 5.00ml^3 and 6.00ml^3 show a greater value of standard deviation than 3.00ml^3 and 4.00ml^3, further supports the claim that increasing the volume of lipase increases the rate at which ester bonds in the lipids are broken down, and thus the rate at which fatty acids are released into the solution, decreasing the pH.

The standard deviation of the control group should theoretically be 0, because the pH should not have changed and so there should not be any difference between the average pH and each pH noted at the one minute intervals. The standard deviation for the control group however is much smaller than the other standard deviations calculated, and shows that the change in pH was very small. The fact that the control group showed virtually no change in pH, verifies that it is the present of lipase that is responsible for the change in pH being observed during the experiment.

The t-test further supports the fact that it must be the increased number of lipase enzymes present in the volumes of lipase being used that is responsible for the increased change in pH. Looking at the comparison of 3.00 and 6.00ml^3 of lipase, and 6.00ml^3 and the control group, the t-values calculated show a confidence level of over 99%, implying there is only a 1 in 100 chance that the results of the experiment are down to chance, and not due to the difference in volumes of lipase being used. It can therefore be said with a high level of confidence that the difference in the change of pH between different volumes of lipase is caused by the fact that there are more enzymes present in higher volumes of lipase. Given that all other factors remained the same – the substrate concentration, the temperature (which was the enzymes optimum temperature), the volume of bile salts being used and the volumes of phenolphthalein and sodium carbonate, the only difference between each experiment was the volumes of lipase being used. It can therefore be said with almost certainty that changing the volume of lipase, changes the rate at which the pH falls. As the volume of lipase is increased more enzymes are present, and so the enzyme to substrate ratio is increased, making it easier for substrates to collide with the active sites of the enzymes. The more often the substrates collide with the enzymes, the greater the number of substrates being broken down (given that the reaction is catabolic).

For the t-value calculated when comparing 4.00 and 5.00ml^3 of lipase, we can see that there is a higher rejection level probability. The rejection level probability of 0.1 suggests there is only a 90% chance the results are significant and not due to chance. This relatively high rejection level probability may have stemmed from the fact when using averages, the values used to calculate them were not concordant. For example, in experiment 1, after the first minute, the pH was observed as 7.82, whereas for experiment 2, after the first minute the pH was observed as: 8.40, giving a difference of 0.58. As mentioned, the pHs should have been very similar after the first minute, which suggests that there has been an error in measuring out the correct volume of lipase, bile salts or full fat milk. Increasing or decreasing any one of these variables would have increased or decreased the rate at which the lipids present in the full fat milk were hydrolysed to the fatty acids and glycerol, because a greater number of substrates or enzymes would have been present, or the lipids may have been emulsified to a greater or lesser extent.

Conclusions

It is clear from the graphs, tables and statistical processes carried out that generally, increasing the volume of lipase increases the change in pH of the solution and also the rate of change of the pH of the solution. This has been proved by the increasing values of standard deviation for higher volumes of lipase, the generally larger maximum change in pH for the higher volumes of lipase and also by the shape and gradients of the graphs drawn. Although there have been anomalies, having used the t-test it can be seen that it is far more likely that the results are not down to chance, but due to the differences between the volumes of lipase being compared. The only differences between each volume of lipase are the number of enzymes and therefore active sites present in the solution. The reason for higher volumes of lipase decreasing the pH more quickly and by a larger amount can be explained by the number of enzymes present and the hydrolysis reaction taking place between the lipids present in the full fat milk.

Lipase oxidises triglycerides, using three molecules of water to break the 3 ester bonds and to produce a glycerol molecule and three fatty acids. Lipids, because of their hydrophobic fatty acid tails do not dissolve in water, and so during fat digestion in the human body, these lipids must first be emulsified by bile salts such as sodium glycocholate and sodium taurocholate. The release of bile into the duodenum is stimulated by the hormone CCK. For human digestion, the bile salts break down the fat droplets present in the lumen of the small intestine into tiny globules helping them to disperse into the watery fluids in the intestine. The lipase enzyme however is made of a globular protein, and because of the hydrophilic 'R' groups on the outside of its structure the enzyme can dissolve in water. In my experiment, the volume of bile salts and full fat milk remained constant, so the amount to which the globules of fat present in the full fat milk were emulsified should have remained the same for each volume of lipase. The bile salts should have enabled the lipase enzymes to act on more lipids present in the solution. As can be seen with some volumes of lipase, the pH first rose before it started to steadily fall, this may have been related to the fact that the bile salts were still emulsifying the fats. If the globules of fat were large, then their surface area available to the active site of the lipase enzymes would have been much smaller and so the rate of the reaction would have proceeded at a much slower rate.

The fact that the bile salts also contains hydrogen carbonate ions can also explain why the pH after one minute was relatively alkali, approximately varying between 7.30 – 9.73. The optimum pH for the lipase acting in the duodenum of the small intestine is approximately 7.00, so theoretically, the 5.00ml^3 of lipase should have been at a slight disadvantage despite the fact that it actually produced the fastest rate of change of pH for experiment 2.

Following the emulsification of the lipids present in the full fat milk, the lipase would have then collided with the tiny globules of fat, forming enzyme-substrate complexes. The lipase would have then broken the 3 ester bond holding the triglyceride together in a hydrolysis reaction requiring three molecules of water. In the human body, the products of this reaction would most likely be converted to other things or stored in adipose tissue, rather than accumulating and creating an acidic environment. In the liver, the fatty acids can be converted to acetyl coenzyme A, which can then be fed into the Krebs cycle, while the glycogen can be phosphorylated to triose phosphate, an intermediate in glycolysis. Clearly in the test tube no such processes would be happening as there are no hepatocytes present in the solution and no respiring organisms (provided the test tube was sterile). The fatty acids and glycerol would have therefore accumulated in the test tube.

Glycerol is an alcohol and looking at its material safety data sheet (see bibliography) it should have a neutral pH. The fatty acids on the other hand possess a carboxyl group (-COOH) at one end of the hydrocarbon tail. If the carboxylic acid is in solution in water, then this carboxyl group can donate a hydrogen ion to the water forming a hydroxonium ion (milk is mostly water). The ability of the carboxyl group to donate hydrogen ions gives it, its acidic nature. The greater the number of fatty acids (and therefore carboxyl groups) being released the more acidic the solution will become, as more and more hydrogen ions are released into the solution.

Provided that there was an excess of substrate present in the $5.00ml^3$ of milk, increasing the volume of lipase and therefore number of lipase enzymes, should have lead to a proportionate increase in the rate of reaction. Assuming that the $5.00ml^3$ of full fat milk possessed an excess of substrate, increasing the number of enzymes would have meant that more of the excess substrate could have been acted upon, and so the rate at which fatty acids were being released into the solution would have increased. If however, the substrate was limiting in the $5.00ml^3$ of full fat milk then increasing the number of enzymes would not have increased the rate at which fatty acids were being released into the solution. The reason for this is because there would not have been sufficient substrate to supply all of the enzyme's active sites at one time, similar to the way in which opening more checkouts at a supermarket will not increase the speed of service if all the customers are already being served. The fact that generally $6.00ml^3$ of lipase produced a much greater change in pH than $3.00ml^3$ of lipase, insinuates that the substrate concentration was in excess, i.e. in $3.00ml^3$ of lipase there were not enough active sites for all of the substrate to bind with in one go.

However, comparing $6.00ml^3$ of lipase with $5.00ml^3$ of lipase, we can see that there is not a great difference between the maximum changes in pH, particularly for experiment 1. This could therefore suggest that in $5.00ml^3$ of full fat milk, there are enough triglycerides to occupy all of the active sites present in the $5.00ml^3$ of lipase all at one time. It is unlikely however, that the triglycerides would all 'find' an active site at the same time, which explains why the reaction didn't come to a direct halt during the ten minutes of the experiment. Assuming that the pie chart in figure 6 *(see planning)* applies to the Tesco full fat milk, if 3.9% of the full fat milk contains lipids, then in $5ml^3$, only 0.195 ml^3 of this contains the substrate. The volume of the substrate is therefore very small but unfortunately it is not known how many enzymes are present in each volume measured. If the number of enzymes was increased, i.e. by increasing the volume, then it is more likely that the lipase enzymes would collide with triglycerides present in the full fat milk, similar to the way in which you're more likely to score a 'bull's-eye' on a dartboard if you throw more darts. With more enzymes colliding with substrates, more fatty acids would be released and so the pH would decrease by a larger amount. As the estimated volume of triglyceride present in the milk shows, the substrate concentration seems relatively small which could explain the fluctuations in the pH as time was needed for the enzymes to collide with the few substrate molecules.

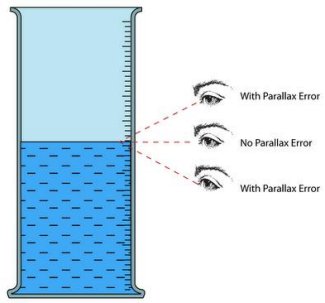
In summary, the catabolic reaction between the lipase enzymes and the substrate was responsible for the decrease in the pH of the solution. As the volume of lipase was increased so too were the number of enzymes and thus active sites capable of hydrolysing the triglycerides to fatty acids and glycerol. With more and more fatty acids being released for larger volumes of lipase, more fatty acids and therefore carboxyl groups were being released into the solution, increasing its hydrogen ion content. The increase in hydrogen ion content/activity was then detected by the electronic pH meter, showing that overall, increasing the volume of lipase increases the change in pH and thus increases the rate at which the substrates are broken down. This common trend is exemplified by the time taken for the phenolphthalein to turn clear, the graphs and statistical processes carried out on all of the data harvested. The trend however is not as perfect as we would anticipate, suggesting that several anomalies have occurred during the procedure.

Evaluation

Although the hypothesis has been verified with reasonable confidence, many anomalous pieces of data were harvested which in turn affected the validity of the results. If I were to be given the opportunity to repeat this experiment then there would be several modifications made to the procedure, to prevent these anomalies from appearing in the data.

A main source of error is most likely to have come from incorrect volumes of lipase, bile salts, full fat milk, sodium carbonate and phenolphthalein being measured out. Despite great care being taken to ensure that the volumes were measured out at eye level, parallax error may still have occurred because there is no direct way of knowing when my eyes are exactly in line with the graduated millilitre mark I was aiming for. If I was to observe the volume of the substance being measured from a slight angle then I could have missed the fact that the meniscus of the substance rested slightly above or below the desired graduated millilitre mark. The fact that for some solutions the meniscus was not a perfect semi-circle also created great difficulty in trying to find the point at which the millilitre mark was touching the bottom of the meniscus. Despite observing the burette at eye level, I was holding the burette with my hands, which may not have positioned the burette perfectly vertically. The wonky positioning of the burette could have also contributed to an error in the measuring out of the correct volumes of any of the substances. One such experiment where an error in measuring out the volume of each substance may have occurred was for the first experiment when measuring out either 3.00ml^3 or 4.00ml^3 of lipase or the volume of bile salts and full fat milk. As can be seen for the scatter graph depicting the results for 4.00ml^3 of lipase in experiment 1, we can see that 3.00ml^3 of lipase seems to have produced a more consistent fall in pH.

Figure 16 – An example of parallax error:



If the 5.00ml^3 of full fat milk was measured out incorrectly, i.e. 5.20ml^3 of full fat milk was measured out, then this would increase the substrate concentration. Increasing the substrate concentration increases the likelihood of the substrates colliding with an enzyme to form an enzyme-substrate complex therefore increasing the rate at which the lipids present in the full fat milk are hydrolysed to fatty acids and glycerol which in turn brings about a faster fall in pH. Therefore if the volume of milk measured out was either too high or too low then this would limit the rate at which the lipase hydrolyses the triglycerides, making the procedure an unfair test.

Looking at the gradients calculated using the average values of pH; we can see that for 3.00ml^3 of lipase, there appears to be a steeper gradient than there is 4.00ml^3 of lipase. It is therefore possible that for the 3.00ml^3 of lipase there was an error in measuring out the volume of lipase, full fat milk or bile salts. Since the gradient is steeper for 3.00ml^3 of lipase, this suggests that either:

- An excess of lipase/bile salts/full fat milk was measured out for the experiment using 3.00ml^3 of lipase – it is possible that a greater volume of bile salts were added to this solution so that the globules of fat were emulsified more quickly or to a greater extent, giving them a larger surface area. It is also possible that the volume of lipase measured out was slightly excessive, so that there were more enzymes present than there were supposed to be. Alternatively, it is possible that there was an excess of substrate due to incorrect measurements of the volume of milk.
- There was too little lipase/bile salts/full fat milk in the experiment using 4.00ml^3 of lipase – if the volume of lipase had been less than 4.00ml^3 of lipase then fewer active sites would have been available in the solution of lipase.

If insufficient bile salts had been measured out then it is likely that the lipids would have required more time be emulsified – this seems the most likely cause as the pH appears to take 2 minutes before it starts to fall. There is also the possibility than insufficient volumes of full fat milk were measured out, reducing the substrate concentration and increasing the competition for active sites.

Similar conclusions can also be applied to the differences in gradient between 5.00 and 6.00ml^3 of lipase.

Overall, for each different substance being measured out, I would give the measuring out of volumes a maximum inaccuracy of ±0.10ml^3.

	Full fat milk (5ml^3)	**Bile salts (2ml^3)**	**Sodium carbonate(3ml^3)**	**Phenolphthalein (1ml^3)**	**Lipase**	
Maximum percentage error (0.10/volume x 100) %	2.00	5.00	3.33’	10.00	0.00ml^3 3.00ml^3 4.00ml^3 5.00ml^3 6.00ml^3	0.00 3.33 2.50 2.00 1.67

As the table above shows, the maximum percentage error occurs for the volume of phenolphthalein being measured out, giving a maximum percentage error of 10%. Although this percentage error seems large, the phenolphthalein itself is just an indicator that should have no impact on the rate at which the lipase enzymes present in the solution hydrolyse the lipids present in the full fat milk. This high percentage error should therefore have little influence over the validity of the results. The percentage error of 5% for the bile salts could explain as to why the initial pHs of the solutions were not the same but varied significantly.

The table above shows that as the volume of lipase being measured out decreases, the maximum percentage error increases. This increasing percentage error for descending volumes of lipase may explain why the results for 4.00ml^3 and 3.00ml^3 of lipase on average are not quite consistent with the hypothesis. On average, the percentage error accounting for all volumes being measured out amounts to: 3.69 % (to 2 d.p.) which shows that the percentage error is reasonably small for this element of the experiment.

In order to prevent these kinds of errors occurring again, firstly I would utilise a clamp stand, to ensure that the burette was always held perfectly vertically. If electronic pipettes were available then these would have been utilised instead of the burettes as these would give a more accurate and consistent measurement of the volumes being measured. Electronic pipettes can be programmed to open for a set amount of time at the push of a button, ensuring that the same amount of the substance is released each time. Having used electronic pipettes would have allowed more accurate volumes of each substance to have been obtained. The use of electronic pipettes would have also eliminated any error occurring as a result of the delay of turning the stop-cock on the burette. For example, in many cases, when measuring out 2ml^3 of bile salts, 6ml^3 of bile salts were taken up and 2ml^3 were released into three different test tubes to save time. Between each test tube the stop cock needed to be rotated to prevent an excess of the substance from being deposited into a single test tube. It is possible that there was a slight delay between seeing the correct volume and then turning the stop cock so an excess of the substance entered the test tube. Alternatively it’s possible that I anticipated the correct volume too early and turned the stop-cock too quickly therefore depositing an insufficient amount of the substance.

The use of electronic pipettes would have eradicated these kinds of errors as they have the ability to detect the volume of a substance in micro-litres, giving a more accurate measurement than the human eye can. The electronic pipettes also feature light-weight ergonomic controls that take the effort out of using the burette and pipette filler and prevent repetitive strain injuries.

Figure 17 – Electronic pipettes:

Another aspect of the experiment that is likely to have caused anomalous data is due to the fact that a new solution of lipase and bile salts had to be sought after part way through the experiment. Unfortunately the solution of bile salts and lipase used in the preliminary experiment was insufficient to provide enough lipase and bile for the experiments testing the 4.00ml^3 of lipase and higher volumes (including repeats). The new solution of bile salts and lipase was freshly made and therefore was not identical to the initial solutions of these substances being used, although chemically it should have been.

It is also possible that when using the older solutions in the final experiment (i.e. the final experiment was performed several days after the preliminary experiment) that these may have been left out in direct sunlight, causing some of the chemicals present to break down (however it is not known whether any of the substances used undergo photochemical decomposition).

Another large source of uncertainty in the results will have most likely come from cross contamination between the equipment. The fact that the control group produced small changes in the pH suggests that lipase must have entered the solution in some form or suggests that it is not the presence of lipase responsible for the decrease in pH. The t-test however counteracts the latter part of the previous argument, because when comparing the control with 6.00ml^3 of lipase, and 3.00ml^3 with 6.00ml^3, the rejection level probability is only 1%, showing that there is only a 1 in 100 chance that the results are down to chance. The only other possible explanation to a change in pH for the control group would have to be due to the use of faulty pH meters.

Great care was taken to prevent cross-contamination. All of the test tubes, burettes and measuring beakers were washed thoroughly with distilled water before the experiment began. This measure should have removed any dirt or potential organisms that might ingest the milk. It is possible however that insufficient time was given for the cleaned equipment to dry. The effect of any distilled water left in the equipment may have diluted some of the substances being measured out, and the volume of the substance being measured out may have appeared to have been increased because of the additional volume that the water would have provided. The pH of the solution using 6.00ml^3 of lipase appeared to be more acidic after the first minute in comparison to the other volumes of lipase, which may suggest that some of the distilled water used to clean the equipment had entered the solution and diluted the solution.

Each substance was obtained with a different burette, so cross contamination between burettes should have been eliminated as only one possible substance entered the burette, therefore any of the substance left in the burette would have been mixed with more of itself. The only way in which this could have occurred would have been because of the use of a contaminated pipette filler. The same pipette filler was used for each burette, and despite being washed with distilled water, it was not possible to see inside the pipette filler, so if too much of a particular substance was sucked up into the pipette filler, this then could have been transferred to the next burette being used.

If lipase had been sucked up into the pipette filler then this could have been transferred to another burette, mixing with the burette's contents, providing the control group with a small amount of lipase. If I were to have utilised electronic pipettes then this would have removed the need to use the pipette filler, and so this form of cross contamination would not have occurred. In terms of cross contamination, the micro-processor based system of the electronic pipette controls all piston movements, therefore preventing any unwanted substances from entering. Electronic pipettes can also have filters added to them to prevent damage and cross contamination between different substances. The sterility of electronic pipettes is also likely to be far better.

The temperature of the test tubes in the experiment is another potential area for anomalous data. Although the temperature of water in the water bath was set to 37°C, for each test tube not all of the test tube was immersed in the water as this would have been impossible to achieve without flooding the test tube and diluting /losing the contents. In order for the whole test tube to be fully immersed in the water, a bung would have had to of been placed in the lumen of the test tube to prevent any of the water from spilling in. The addition of the bung would have then created difficulties in inserting the electronic pH meter into the solution. The tops of the test tube were therefore exposed to the air, meaning heat could have been lost to the surroundings. The majority of the contents of the test tubes however were in the part of the test tube fully immersed by the water, therefore meaning they should have experienced a constant temperature of 37°C. For 6.00ml^3 of lipase, the solution would have been closer to the top of the test tube, which could imply that more heat could have been lost to the surroundings for this particular volume of lipase. Although a thermometer was placed in the water bath to verify that it was at the correct temperature, it is possible that the temperature may have varied by plus or minus 1°C without being noticed. The water bath being used was relatively old and so it is likely that it might not provide an absolute constant temperature.

The original containers of lipase, bile salts, full fat milk, sodium carbonate and phenolphthalein were all stored in the water bath so that they were all kept at a temperature of 37°C. Unfortunately the water bath was not large enough to store all of my test tube racks in while I was trying to measure out the correct volume of each substance, therefore when trying to obtain my desired volumes of each substance, I had to remove the substances from their 37°C container and deposit them in test tubes that were at room temperature. The measuring out of each substance took some time, and so the lipase, full fat milk, bile salts, sodium carbonate and phenolphthalein would have cooled down by the time they were re-inserted into the test tube rack in the water bath. This has the implication that initially, the lipase enzymes were not at their optimum temperature, and so time was needed for the solution to heat to 37°C. As the solution heated up, the molecules gained more kinetic energy, increasing the frequency of collisions between the substrate and enzyme, so the rate at which fatty acids were released into the solution increased. This may explain why for nearly all volumes of lipase, excluding 6.00ml^3 of lipase, the pH took up to two minutes before it started to fall. For 6.00ml^3 of lipase, there may have been enough enzymes to compensate for that the fact they were not working at their optimum temperature, so overall the pH fell after the first minute.

If I were to be given the opportunity to perform this experiment differently, then I would try and make use of a large incubator in which the test tubes could be inserted, and closed off so that the temperature would not be affected by the outside air. The incubator would need to have a window, so that the pH meter could be seen and the pH could be observed at each minute interval. Another alternative to an incubator could be a larger water bath.

A strong point in the method was the labelling of the test tubes which prevented confusion of what was present in each test tube. Although in some cases 3.00ml^3 produced a greater change in pH than 4.00ml^3, and 5.00ml^3 produced a greater change than 6.00ml^3 of lipase, it seems unlikely that this is due to confusing which volume of lipase was being tested.

Each test tube was labelled, and these labelled stayed in place for the entire duration of the experiment. The labelling was done before the volumes were measured out to prevent the volumes being incorrectly labelled. It is possible however, due to human error that the incorrect volume of lipase was placed in the correctly labelled test tube. To reduce these anomalies greater concentration would be required when measuring out different volumes. The use of an electronic pipette would display the volume I had chosen, so if I had entered it incorrectly I could change it accordingly before releasing that volume into the test tube.

In terms of faults with the equipment, the main fault is most likely to be linked with the accuracy of the pH meters. The pH meters were calibrated using two buffer solutions but it was noticed that on entering the test tube filled with a given volume of lipase, bile salts, sodium carbonate, phenolphthalein and full fat milk that the pH fluctuated a great deal for the first 30 seconds. Instead of the pH meter detecting one initial pH the pH would rise and fall until it stabilised at one level. These fluctuations made measuring the initial pH an impossible task as the reading never remained the same long enough to give an accurate reading. The knock on effect of this was that the change in pH in the first minute could not be noted, so if 6.00ml^3 of lipase saw a huge drop in pH in the first minute this would not be known. If there had been a large drop in pH, then the rate of the reaction would proceed more slowly since fewer substrates would be available, however for 5.00ml^3 of lipase, if there had not been such a large drop in pH then this would take longer to fall. Without knowing the initial pH, it can give the impression that lower volumes of lipase produce a greater rate of hydrolysis, which we know should not be true, because there are fewer enzymes present to cause the reaction.

In order to reduce the faulty readings produced by the pH meter, more accurate pH meters would be sought after – such as pH meters that detect the pH to more decimal places. The pH meters used were calibrated in the middle of the day, and because the glass electrode making up the pH meter does not produce a reproduceable e.m.f over long periods of time, it is possible that for the higher volumes of lipase being tested, more time had passed and so the accuracy of the pH reading was reduced. There is also the possibility of cross contamination of the electrode making up the pH meter when being placed in different test tubes. If any of the previous solution the pH meter was inserted to was still attached to the electrode, then this would have affected the reading on the pH meter.

When the pH meter is not being used, then ideally it should be stored in an acidic solution, however, no such acidic solution was available and so the pH meter had to be washed with the distilled water and blotted dry with tissue paper and left to one side in between experiments. The fact that the pH meter was left to one side could have meant that ions were sucked out into the 'ionless' air, degrading its performance. I would estimate that the reading of the pH may have been inaccurate by plus or minus 0.01. Looking at the maximum pH and minimum pH observed during the experiment it can be seen that the percentage error varies from 0.10 – 0.15 %, which suggests that if the pH reading was correct then the number observed each minute was very accurate and should not have had much impact on the final results.

Other difficulties that arose which may have caused anomalies could involve looking at the stop watch and looking at the pH meter at the same time as it was not possible to focus on two objects at once. There was also some discretion when the pH changed exactly on the minute mark, if the pH changed exactly on the minute mark it was uncertain which pH value to take.

In order to improve the accuracy of the pH meter, the pH meter could be calibrated again between each experiment, to ensure that the voltage readings correctly correlate with the pH scale. The use of more advanced pH meters could also involve a larger LCD screen, perhaps one that also records and displays time so that there is no delay between looking at the stop watch and observing the pH reading.

The use of a more accurate pH meter would have also enabled me to note the initial pH of the solution, although a possible way around this could be to revert back to the preliminary experiment in which the bile salts, full fat milk, sodium carbonate and phenolphthalein were all mixed into one test tube before having the lipase added. Theoretically, without the lipase, there should be little if any change in pH, so the initial pH of all the substances minus lipase should be the same as the initial pH of all the substances including lipase, provided that the enzymes do not act immediately and therefore immediately reduce the pH as timing begins.

As can be seen from the averages taken, the standard deviations and gradients of the graph, the general trend in the pattern is not quite what it is expected to be. If more time and equipment have been available, then a further repeat of the experiment would have provided me with more accurate results, and any anomalies could have been more easily identified. Performing a third repeat would have also made the averages more accurate as they would have taken into account more pieces of data. Improving the accuracy of the averages would have then improved the accuracy of the average graphs drawn and the values found for the standard deviation.

Additionally, had I recorded the pH every 30 seconds rather than every minute, this would have increased the number of readings taken and therefore increased the number of plots on the graph. Using more plots of data on the graph would have enabled the line graph to be drawn with greater ease and more accurately, therefore allowing a more accurate gradient to be calculated. The trend in the pH could also be observed more easily since the pH would be known at more time intervals, rather than having been assumed to be at a certain value using the line that crosses between the two points between each minute.

In terms of the time limit used and volumes of lipase utilised in the procedure, given the opportunity to perform the experiment again, a greater time limit would have been used, perhaps up to 20 minutes, and more volumes of lipase would have been used to further support the hypothesis that increasing the volume of lipase increases the rate at which the lipids present in the full fat milk are hydrolysed to fatty acids and glycerol. Using a greater time limit would have enabled me to see how long it takes for the pH stop falling, which would have therefore suggested that all of the substrates present in the full fat milk had been hydrolysed. This could give an indication into just how many substrates are present in the full fat milk.

Similarly using higher volumes of lipase beyond 6.00ml^3 would have enabled me to see at which point increasing the volume of lipase no longer has an effect on how fast or by how much the pH changes. When the pH stops falling any faster, this suggests that the number of enzymes present equals the number of substrates present in the 5.00ml^3 of full fat milk. Utilising higher volumes of lipase should have shown that 7.00ml^3 causes a greater and faster decrease in pH than 6.00ml^3 and 5.00ml^3 and so on, which would have provided yet more evidence supporting the hypothesis. There are limitations however to the amount of lipase being used as the total volume of all of the substances must fit into the test tube without spilling, and it must be possible to measure out the desired volumes using the equipment the school has available. There is also the issue of safety as spilling a higher volume of lipase on skin is more likely to cause irritation.

My research tells me that for pancreatic lipase, the optimum pH is approximately seven and the optimum temperature is approximately 37°C. Had more time been available, I could have investigated this further, exposing the solution of lipase and full fat milk to a greater range of temperatures and pHs in the preliminary experiment. At the temperature or pH in which there was no change in pH, this would have indicated that the lipase enzymes had become denatured and no fatty acids were being released into the solution. I could have then slowly reduced this temperature and pH until I found the maximum change in pH which would have then signified that this was the optimum temperature and pH for the lipase enzyme.

Aforementioned, the pH is the variable that is being measured and it is therefore meant to change. However, the initial pH of the solution could have been altered so that the lipase began its hydrolysis in certified optimum conditions. It makes sense however that the lipase would still work efficiently over a range of pHs because the reaction it catalyses creates a more acidic environment, and it obviously needs to be able to withstand this acidic environment.

The fact that the 6.00ml^3 of lipase was closer to the optimum pH after one minute may have enabled it hydrolyse the triglycerides more efficiently than the other volumes of lipase. If this was true then this could suggest that it was because of its initial pH that it hydrolysed more lipids than the other volumes of lipase and not because more enzymes were present in that solution of lipase. Equal volumes of bile salts and sodium carbonate were added to the solution, so theoretically, providing the lipase enzyme molecule did not affect the pH of the solution, all of the solutions should have started at the same initial pH. Likewise, inaccuracies in the measuring of the pH or volumes of the substances being used may have resulted in the differences between the initial pHs.

Despite difficulties in the procedure and several anomalies appearing in the results, I believe if the improvements suggested above were to be implemented then their would be stronger evidence supporting the hypothesis. Generally I feel that the same results would have occurred, however the relationships would have been clearer as the trends produced in the line graphs would have been more consistent and the number of anomalous data harvested would have been reduced. With fewer anomalies, the t-values calculated would have also corresponded to lower rejection level probabilities which would have further supported the fact that the differences between each volume of lipase and the change in pH is directly related to the fact increased volumes of lipase possess more enzymes. The rejection level probability of 0.1 when comparing 4.00ml^3 and 5.00ml^3 is quite high, however had the improvements above been introduced to the method then it is possible that this rejection level probability would have been reduced somewhat. Having perfected my procedure with the improvements suggested, I still feel my final conclusion would have been the same. The only difference would have been the improved evidence to support my conclusion.

To conclude, my experiment has relatively accurately proved that increasing the volume of lipase, increases the rate at which the pH of the solution decreases when the temperature of the solution is 37°C. If the rate at which the pH decreases is increased, then this implies that the number of triglycerides present in the full fat milk being hydrolysed to fatty acids and glycerol is also being increased. Looking at the graphs, statistical processes and tables of results, it is clear that in general (although not always), increasing the volume of lipase increases the maximum change in pH and the rate at which the pH changes. Perhaps I could have made use of lower volumes of lipase, however it is possible that in lower volumes of lipase the enzyme to substrate ratio would be so low that a great deal of time would be needed to record any significant changes in pH. If there were too few lipase enzymes present to cope with the number of substrates present then the rate of reaction would have been extremely slow. The volumes of lipase I have tested show with some confidence that when the volume of lipase is increased, so too is the rate at which the triglycerides present in the milk are hydrolysed to fatty acids and glycerol. Although anomalies have occurred which could argue otherwise with the hypothesis, in general it can be seen that the hypothesis was largely correct and the aim of the experiment has been successfully achieved. At a temperature of 37°C, increasing the volume of lipase increases the change in pH and the rate at which this change in pH occurs.

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All other figures have been drawn by myself using Adobe Macromedia Flash player

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Appendix