The Analysis of Biogenic Amines by standard and Novel Methods.

Thesis

Presented for the Degree of

Master of Science by Research

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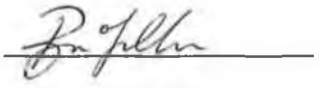
I hereby declare that this material, which I now submit for assessment on the programme of study leading to the award of Masters degree by Research described is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: 

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Abbreviations

AOAC	Association of Official Analytical Chemists
C_{18}	Carbon 18
DAO	Diamine oxidase
DCU	Dublin City University
ELISA	Enzyme-linked immunosorbent assay
GC	Gas Chromatography
HPLC	High performance liquid chromatography
LB	Luria Bertani
MAO	monoamine oxidase
mM	Millimolar
MRS	de Man, Rogosa, Sharpe broth
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NaOH	Sodium Hydroxide
NH_4	Ammonia
nm	nanometer
OPT	o-phthaldialdehyde
ppm	parts per million
PVC	poly vinyl chloride
TFIB	Tuna fish infusion broth
TLC	Thin layer chromatography
TMA	trimethylamine
UV	ultraviolet

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Abstract

Biologically active amines in cheese and fish arising from metabolic activities of food-borne microorganisms have been implicated as the causative agents in many food poisoning outbreaks. An awareness of amine levels in foods today is therefore important in relation to food spoilage and safety.

In recent years there has been increased consumer awareness about food composition and safety and a corresponding increase in regulatory action. The food industry requires reliable and cost effective analytical methods for process and quality control to meet the needs of the consumer. Therefore, this study focussed on comparing conventional analytical methods involving HPLC and fluorimetry, for histamine analysis in food.

Irish cheeses and canned tuna samples were selected for analysis of histamine content by a standard fluorometric technique. The cheeses, Cheddar, Cooleney, Edam, Emmental and Brie were analysed over a three-week period and observations of any changes in histamine levels were made. All samples were stored at 4°C until analysis to replicate the retail outlet storage conditions. The histamine levels found in cheese in this study were low in comparison to levels in reported literature, ranging from 0.2 to 4.3 mg/100g and were non hazardous for consumption. Canned tuna was analysed by the same technique. Results from this study showed that the tuna contained high levels of histamine, 20 mg/100g that increased to hazardous levels upon putrefaction after a 96 hour period. A HPLC method of detection was established based on the derivitisation of histamine with dansyl chloride and UV detection with the aim of detecting histamine quantitatively in food samples and to facilitate correlation studies with the fluorometric method of detection. A novel method, based on an amine oxidase system coupled to an ammonia sensing calixarene, was investigated as an alternative and improved method of histamine detection.

1.1 Introduction

Biogenic amines are chemically defined as low molecular weight aliphatic, alicyclic, or heterocyclic organic bases (Table 1.1) formed from the decarboxylation of amino acids by the metabolic activity of bacteria, plants and animals. The decarboxylation process can proceed through two biochemical pathways: decarboxylation through endogenous (naturally occurring) decarboxylase enzymes or by exogenous decomposition through enzymes released by microflora. The production of amines by the exogenous process is considered far more significant. (Rawles and Flick, 1996).

Biogenic amines are naturally present in a wide variety of foods in low concentrations. As they are derived from amino acids they can be found in practically all protein-containing foods such as meat and fish and as a result of bacterial action are found in fermentation products such as beer and sauerkraut.

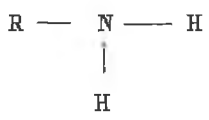
The number of R groups bound to the nitrogen moiety is used in the characterisation of amines, i.e. primary, secondary or tertiary (Figure 1.1).

Table 1.1: Biogenic amines and their different structural classes.

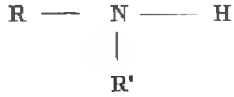
Aliphatic	*Aromatic*	*Heterocyclic*
Putrescine	Tyramine	Histamine
Cadaverine	Phenyletyylamine	Tryptamine
Spermine		
Spermidine		

Figure 1.1: Amines are characterised by the number of R groups bound to the nitrogen

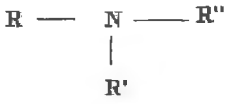
Primary amine:



Secondary amine:

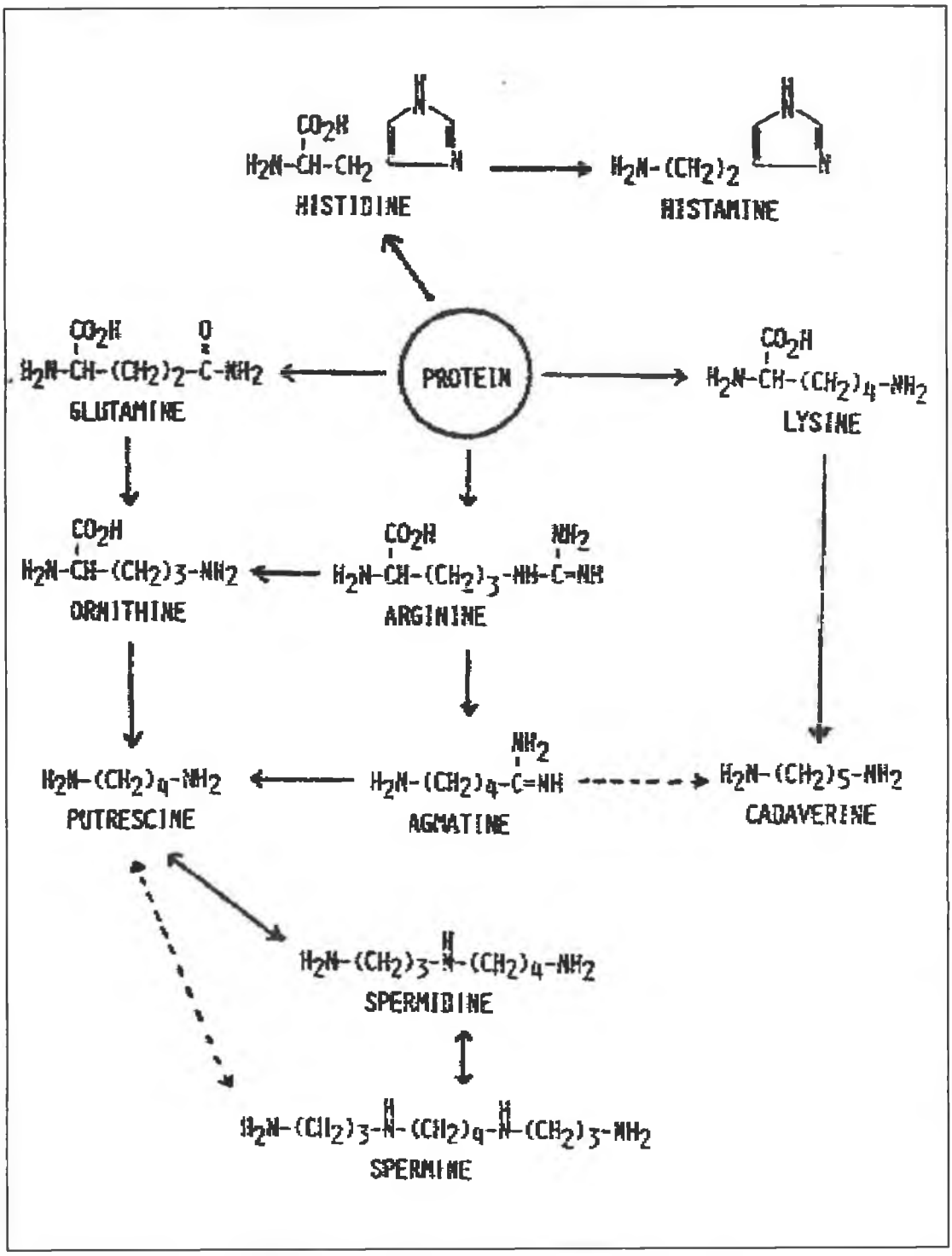


Tertiary amine:



Histamine, 4-(2-aminoethyl)imidazole) is a primary heterocyclic amine derived from the decarboxylation of the amino acid L-Histidine (Figure 1.2). It plays an important role in biological processes (vasodilation and gastric acid secretion) but also occurs exogenously in the food supply and can cross the intestinal barrier. Intoxication can result if significant quantities cross the barrier and enter the bloodstream (Taylor, 1988). There have been many reported incidences of food poisoning involving histamine/other amines and as a consequence determination of these levels in foods is an important aspect of food safety.

Figure 1.2 Biological pathways for the formation of amines



(Rawles and Flick, 1996)

1.2 Biogenic Amines in Food

Biogenic amines are present in a wide variety of foods from non-fermented foods such as fish, meat, chocolate, milk and fruit to fermented products like wine, cheese, beer and sauerkraut. There is a vast amount of reported data of amine levels in these foods (Maga, 1978, Smith, 1981 and Santos, 1996) which outlines the necessity to be able to identify possible contributors to histamine poisoning.

1.2.1 Non-Fermented Foods

The presence of biogenic amines above a certain level in non-fermented foods arises as a consequence of undesired microbial activity and indicates some level of food spoilage. Foods with a high protein content such as fish and meat are candidates for amine production by such microbial activity.

1.2.1.1 Fish

Scombrotoxin poisoning (histamine poisoning) is caused by the ingestion of foods containing high levels of histamine and may also include other vasoactive compounds such as cadaverine and putrescine. Scombroid fish poisoning was so called as it was historically associated with the ingestion of spoiled fish from the scromboid families which include tuna, mackerel, skipjack and bonito. Non-scromboid fish such as mahi-mahi, herring, sardines and anchovies have also been implicated with outbreaks. (Morrow *et al.* 1991). These fish all contain high levels of free histidine and other amino acids in the fish muscle, which serves as an excellent medium for the growth of invading bacteria. (Karmas, 1981). Proteolysis, either autolytic or bacterial, may play a role in the release of free histidine from tissue proteins. (Taylor, 1986). Histidine can be catabolized in two ways in fish muscle: Amino-acid deamination to obtain urocanic acid or histidine decarboxylation to produce histamine. (Santos, 1996). Any food with the appropriate amino acids and that is subjected to certain bacterial contaminants and growth may lead to scombroid poisoning if ingested. (Food and Drug Administration, 1998).

The illness is an intoxication, so the incubation period is rather short ranging from immediate to 30 minutes after ingestion of the spoiled food. (Taylor, 1986)

The duration of the illness is usually three hours but may last several days (Food and Drug Administration, 1998). Histamine poisoning is most often confused diagnostically with food allergies as they share identical symptoms and can be treated by antihistamines. It can be distinguished from food allergy on the basis of; a) no previous history of a reaction to the food, b) high attack rate in group outbreaks and c) high histamine levels detected in incriminated foods. (Taylor, 1986, Taylor, 1988).

Symptoms include flushing, nausea, vomiting, diarrhoea, headaches, dizziness, rash and sometimes swelling of the face and tongue (Morrow, *et al.* 1991). There has been considerable doubt as to whether histamine is the causative agent in scombroid fish poisoning. Evidence supporting this doubt is the fact that when individuals are administered histamine orally no significant symptoms associated with scombrotoxism are observed. Luthy and Schlatter (1983) in a placebo-controlled double blind experiment showed that histamine (25mg) administered orally in apple juice to 27 healthy volunteers did not show any significant effect. This was also true of wines containing natural amounts of histamine (non-detectable to 21 ppm). (Luthy and Schlatter, 1983).

However Morrow *et al.* (1991) provided evidence that histamine is the causative toxin of scombroid fish poisoning. Poisoned individuals who had ingested fish containing high levels of histamine began to exhibit symptoms ten to thirty minutes after ingestion. Their urine was analysed one to four hours afterwards and showed histamine and N-methylhistamine levels of 9-20 times and 15-20 times the normal mean, respectively. Levels dropped with time and after 14 days they were back to normal. It was concluded that the histamine in the urine was most likely derived from the spoiled fish and the results showed histamine to be the toxin responsible. (Morrow *et al.* 1991).

Two hypotheses have been proposed to explain this paradox between the toxicity of histamine when consumed in conjunction with spoiled fish and the lack of toxicity when reagent-grade histamine is ingested. The biogenic amines cadaverine and putrescine, which have been shown to be present in spoiled fish (Mietz and Karmas 1977), acting as potentiators, are at the basis of both hypotheses. Since the oral ingestion of toxic levels of histamine alone does not lead to fatality, it was proposed that a barrier to histamine absorption exists in the gut. Large amounts of mucin bind to histamine hindering its absorption

while detoxification occurs by enzymatic processes as it is released from the mucin.

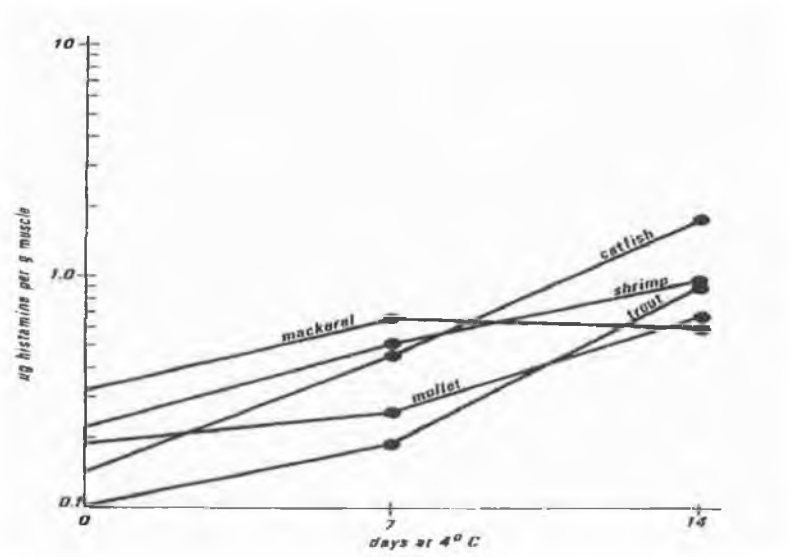
The inhibitor hypothesis proposes that the potentiators act by inhibiting intestinal metabolism of histamine, i.e. inhibiting histamine-metabolising enzymes diamine oxidase (DAO) and histamine-N-methyltransferase (HMT), therefore allowing more histamine, relative to its less toxic metabolites reaching circulation (Lyons *et al.* 1983).

The barrier diffusion hypothesis proposes that through inhibition of the binding of histamine to mucus which lines the epithelial cells, the potentiators alter the barrier function of the small intestine and allow greater quantities of histamine to diffuse across into circulation. Histamine binding was decreased 46.5% by spermine, 9.5% by cadaverine and 11.1% by putrescine. (Chu and Bjeldanes, 1981).

Temperature and storage

The toxin, once produced is incredibly resistant to degradation so to prevent its formation immediate packing at 0°C is essential as soon as possible after the fish is caught. If fish are allowed to remain at room temperature, tissue concentrations of histamine rise rapidly due to the bacterial action on free histidine. Figure 1.3 shows that at 4°C no significant rise in histamine levels occured after 14 days storage, although the appearance and odour deteriorated indicating psychrophilic spoilage had occurred.

Figure 1.3: Rise in histamine levels with decomposition in various fish.



(Edmunds and Eitenmiller, 1975)

Histamine-forming bacteria are capable of growing and producing histamine over a wide temperature range, but growth is more rapid at temperatures above 20°C. Once the histidine decarboxylase has been formed, it can continue to produce histamine even if the bacteria are not stable. (Food and Drug Administration, 1998). The enzyme can be active at or near refrigeration temperatures. After cooking, recontamination of the fish with the enzyme-forming bacteria is necessary for additional histamine to form, therefore histamine development is more likely in raw, unfrozen fish.

In the fishing industry, good handling practices are used to control histamine formation. They include icing or rapid immersion of the catch in chilled water (-1°C), followed by uninterrupted frozen storage. At high storage temperatures histamine can form long before other indicators of decomposition are detected such as odour and physical appearance of decomposition. The rate of histamine formation at low temperatures is much slower and the other indicators of decomposition are evident in the same time frame. (Food and Drug Administration, 1998).

A new problem has been the increased duration of fishing voyages to obtain greater catch quantities and less than adequate handling and storage facilities onboard ships which results in less fresh fish for the processor and increases the potential for histamine formation. (Karmas, 1981).

Canned fish are of considerable concern in relation to food safety. The fish is prepared from previously frozen fish, which is then thawed before processing and thus is subjected to additional handling, which may result in higher histamine levels. Karmas and Mietz showed that histamine exhibited a marked increase in concentration with canning where levels were twice that of uncanned fish. (Karmas and Mietz, 1978).

Table 1.2: Approximate Safe Shelf life at Various Storage Temperatures

Product Temperature	Safe Shelf life(days) with Rapid Cooling	Safe Shelf life(days) with Delayed Cooling
0°F (-17.8°C)	No limit	No limit
32°F (0°C)	14	8
38°F (3.3°C)	10	7
40°F (4.4°C)	7	5
50°F (10°C)	3	0
70°F (21.1°C)	0	0
90°F (32.2°C)	0	0

(Food and Drug Administration, 1998).

As can be seen from Table 1.2 the safe shelf life is significantly reduced above 4.4°C and fish should not be subjected to this temperature for more than four hours.

In fish, the most important histamine-producing bacteria are *Morganella morganii*, *Klebsiella pneumonia* and perhaps a few other enteric bacteria. (Taylor, 1986).

Figure 1.4: Microbial Isolates showing Histidine-decarboxylase activity.

Identification of isolate	Decomposition temp on which found	Source
Acineiobacter Iwoffi	0, 15	
Aeromonas hydrophelia	0, 15	Beef
Citrobacter freundii	15	Skipjack tuna
Clostridium perfringens	15, 30	Skipjack tuna
Enterobacter sp.	30	Food, tuna
Escherichia coli	15, 30	Tuna
Hafnia alvei	15, 30	Tuna, mackeral
Klebsiella sp.	15	Tuna, mackeral
Morganella morganii	0,15,30	Scombroid fish
Proteus vulgaris	30	Beef, pork, turkey
Proteus sp.	15	Fish, tuna, mackeral
Vibrio sp.	15	Mackeral

(Rawles and Flick, 1996)

Histamine in raw fish is not uniformly distributed in the tissue muscle. It is usually higher in tissue close to the gills or intestines that are the main areas for histamine-producing bacteria. The bacteria naturally exist here with no harm to the fish but when death occurs, the fish's defence mechanisms cease to inhibit bacterial growth and so histamine levels increase with the growth of these histamine-producing species. The levels also vary within a species of fish. It is for these reasons that histamine by itself does not serve as a reliable index of spoilage. (Karmas and Mietz, 1978). A level of 50ppm in one section may accompany a level in excess of 1000 ppm elsewhere in the same fillet (Fish and fishery products hazards and control guide, 1998). The Food and Drug Administration (FDA) have set a hazard action level for histamine in tuna of

50mg/100g(500ppm) of fish. A level of 20mg/100g(200ppm) is considered evidence of decomposition. There is uncertainty about the level of histamine that is considered toxic due to the fact that potentiators of toxicity which lower the effective dosage may be present in the fish (Taylor, 1985), and the variance of detoxification mechanisms in individuals.

Incidence

Fish is the major source of protein in the Japanese diet and therefore it is not surprising that this country has a very high incidence of food poisoning outbreaks.

Most outbreaks in Japan involve a large number of people with the largest outbreak occurring in 1973 where 2656 people were effected. Given that raw fish is the preference in Japan one would expect it to be responsible in the majority of cases, however most illnesses are associated with cooked fish. One argument to explain this is that only the highest quality fish is used for consumption in this manner.

Since the 1970's the countries with the most reported cases of histamine poisoning have been Japan, U.S.A. and Britain but better reporting may account for such figures.

The type of fish caught and methods of harvesting are important determinants in histamine poisoning. In Scandinavian countries where there is a high consumption of fish, one would expect many outbreaks like Japan but there have been very few incidents of poisoning. The type of fish consumed is not prone to histamine formation and catching and storage temperatures are low which decrease the possibility of such formation.

Table 1.3. Reported Levels of Biogenic Amines in Fish.

Fish source	Histamine (mg/100g)	Cadaverine (mg/100g)	Putrescine (mg/100g)	Reference
Canned tuna	1.97	0.8	0.12	Kim and Bjeldanes 1979
Canned tuna *	118	10.8	1.25	
Canned tuna	0.38	0.15	0.12	Mietz and Karmas (1978)
Canned tuna*	25.3	1.93	0.25	
Canned tuna	3.46	-	-	Taylor, 1978
Sardines	0.79	-	-	

1.2.2 Fermented Foods

The use of micro-organisms in food by the food and drinks industry has supplied us with many products today especially in brewing and wine production where the production of alcohol is derived from yeast's and lactic acid bacteria. However these micro-organisms do not just produce beneficial substances as part of their metabolism but also present are unwanted and potentially hazardous products like amines.

1.2.2.1 Cheese

After fish, cheese is the next food product responsible for food poisoning outbreaks, and numerous cases have been reported. (Sumner *et al.* 1985). Cheese acts as a perfect environment for amine production supplying substrates, the presence and activity of bacteria and enzymes, proteolysis, water availability and ideal ripening and storage conditions. (Edwards and Sandine, 1981). The amine content in fresh milk is quite small which is why proteolysis may play

such an important role in the formation of histamine by the release of free histidine. The precursor amino acid, tyrosine is present at high concentrations in cheese and may give rise to tyramine, which has the effect of causing the release of noradrenaline from the sympathetic nervous system. This in turn causes an increase in blood pressure and can cause serious headaches and even induce a brain haemorrhage or heart failure. (Smith, 1981).

The body possesses a natural detoxification system to eliminate amines by conversion to an aldehyde through the action of monoamine oxidase (MAO). However, drugs known as monoamine oxidase inhibitors used for the treatment of depression and other psychiatric illness hinder the detoxification process. When these drugs are taken along with the ingestion of tyramine containing cheese, the "cheese reaction" occurs where a hypertensive crises is provoked. (Marley and Blackwell, 1970).

Studies have shown that in 4-6 month Cheddar cheese 2μmol. of histidine, tyrosine and tryptophan are present per gram of dry weight. (Voigt *et al.* 1974). For histidine this accounts for 12g/100g cheese at 36% moisture. Only 0.83% of this would be required to produce a toxic level of 100mg/100g.This suggests that substrate availability is not a limiting factor in amine production in cheese. Edwards *et al.* confirm this in experiments where bacterial isolates from various cheeses were tested for amine- producing potential by measuring carbon dioxide production from the amino acids.

Amino acid decarboxylases require pyridol phosphate for activity and they found that concentrations ranged from 42-215μg/100g, which appears to be sufficient to saturate the decarboxylases needed for amine production. (Edwards *et al.* 1981).

Outbreaks of histamine poisoning have occurred following the consumption of cheese containing high levels of histamine. (Chambers and Starusziewicz, 1978, Sumner *et al.* 1985).

Table 1.4. Reported Levels of Biogenic Amines in Cheese

Cheese	Histamine (mg/100g)	Tyramine (mg/100g)	Reference
Cheddar 1	26.5	108.5	De Vust *et al.* 1976
Cheddar 2	4.6	65.0	
Edam	8.8	Trace	
Camenbert	2.4	Trace	
Brie	1.4	39.8	
Cheddar	14.0	24.0	Voigt *et al.* 1974
Edam	N.D.	31.0	
Camenbert	7.0	12.0	
Gouda	7.5	29.0	
Cheddar 1	5.8	-	Chambers and Staruszkiewicz 1978
Cheddar 2	1.2	-	
Camenbert	0.7	-	
Swiss	116	-	

1.2.2.2 Wine

Wine, as it is a product of the fermentation process, is a candidate for high biogenic amine content. Headaches from red wine consumption are a common complaint and can be induced by histamine in wine in people with histamine intolerance. Histamine intolerance is due to impaired histamine degradation based on decreased activity of the detoxification enzyme diamine oxidase whether it be natural deficiencies or inhibition from drugs (MAO inhibitors), other amines (cadaverine and putrescine) or competitive inhibition from

alcohol. It is believed that 4 to 7 per cent of the population has difficulty in eliminating histamine from their bodies.

Classic red wine headache occurs within two hours of consuming less than two ounces of wine. The headaches are migraine in nature and are accompanied by flushing and congestion, symptoms of histamine poisoning. (Jarisch and Wantke, 1996).

There are several types of headache, vascular, tension, pressure and inflammatory. Wine headaches are usually vascular in nature due to the presence of vascular compounds causing the dilation of blood vessels when ingested. (Shore, 1996).

Red wines in general contain higher levels of histamine than white wines. In a study by Baucom et al, they reported that while histamine levels were higher in red wines, cadaverine and putrescine levels where higher in white wines and that a great deal of variation in amine content existed among grape varieties and wines produced by different vintners. (Baucom *et al.* 1986). Red wine contains about 20-200 fold more histamine than white wine. Jarisch and Wantke reported a range of 60-3,800μg/L (6-380mg/100g) in red wine and 3-120μg/L in white wine (Jarisch and Wantke, 1996).

Table 1.5. Histamine in Austrian red and white wines.

Wine	Level (μg/L)	Wine	Level (μg/L)
Red wine		*White wine*	
Cuvee 1989	3,776	Riesling 1989	120
Bordeaux 1989	2,197	Riesling 1988	42
Zweigelt 1991	281	Chardonnay 1988	35
Goldeck 1988	133	Messwein 1991	9
Cuvee 1987	92	Langenloiser 1988	3

1.3. Production by Micro-organisms

Amino acid decarboxylases are not widely distributed among the bacterial population but species such as *Bacillus, Proteus, Klebsiella, Pseudomonas, Salmonella* and the lactic acid bacteria *Lactobacillus* are capable of decarboxylating one or more amino acids.

However not all are prolific histamine producers. Taylor *et al* (1978a) tested 112 bacterial strains for their potential to produce histamine levels capable of causing food poisoning. Of the organisms tested only *Proteus morganni* and *Enterobacter aerogenes* had the capability of causing an outbreak of food poisoning. The strains were capable of producing over 200nmoles/ml (4.0mg/100g tuna) of histamine in TFIB, trypticase-soy broth-histidine medium, which is generated from raw tuna fish. There are a lot of differences between the media and solid tuna flesh but the growth and histamine formation in the media should demonstrate an organisms potential to cause histamine accumulations in fish. The average histamine production for *P. morganni* and *E. aerogenes* following 7-hour incubation amounts to 500 and 133mg/100g tuna, which is enough for illness to occur. (Taylor *et al.* 1978a).

As discussed in section 2.1.1 low storage temperatures are used in the fishing industry to control bacterial histamine formation. The lower temperature limits for production of toxicologically significant levels of histamine in tuna fish infusion broth were 7°C for *Klebsiella pneumonia*; 15°C for both *Proteus morganni* strains and 30°C for *Hafnia alvei, Citrobacter freundii* and *Escherichia coli*. The abilities of the *Proteus* and *Klebsiella* species to produce significant levels of histamine at these low temperatures is critical to the freezing and storage conditions on board fishing vessels.

P. morganni and *K. pneumoniae* produced large quantities of histamine in a short period of time (<24 hours) with *K. pneumoniae* producing a maximum amount (42μmoles/ml by 24 hours of incubation at 37°C and then declining. The levels of histamine production with *P. morganni* accumulated up to 72 hours incubation. This is a significant result as fish containing these strains may

accumulate high levels of histamine in a short amount of time but yet show no appearance of spoilage. (Behling and Taylor, 1982).

Detection of histidine-decarboxylating bacteria may be difficult as often they account for a minority of bacterial species present in fresh fish. To facilitate detection, a histidine-containing agar medium was developed for the quantitative detection of these organisms. The organisms will decarboxylate the histidine incorporated into the medium converting it to histamine, which corresponds to a marked pH change in the agar. Also incorporated is a pH indicator, so any colonies positive for histamine producing bacteria appear purple with a purple halo on a yellow background. (Niven JR. *et al.* 1981).

Histamine production can be confirmed in the cultures by a modified fluorometric method (Shore *et al.* 1959).

This plating method does not work with most histamine-producing, dairy-related bacteria due to lactic acid being produced simultaneously with histamine and so a pH change cannot be observed as histidine is converted to histamine. An alternative method involving a two step enzyme system was developed by Sumner *et al* (1988).

Bacterial isolates are incubated on histidine incorporated de Man, Rogosa and Shapre (MRS) broth, which is then reacted with diamine oxidase. Histamine is oxidised by the enzyme, producing imidazole acetaldehyde, ammonia and hydrogen peroxide. The hydrogen peroxide is detected by the formation of crystal violet from the leuco base in the presence of horseradish peroxidase. A purple colour results if the bacteria have produced more than 1200nmole histamine per millilitre. Other amines will not interfere in the assay. (Sumner and Taylor, 1988).

1.4 Amine Oxidases

Amine Oxidases are enzymes which catalyse the oxidative deamination of mono-, di- and polyamines with the formation of an aldehyde, ammonia and hydrogen peroxide.

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

In the case of Histamine:

Histamine + O_2 + H_2O → imidazoleacetaldehyde + NH_3 + H_2O_2

The enzymes are divided into two separate groups. The first group is the flavin co-factor dependent enzymes known as monoamine oxidases (MAO), EC 1.4.3.4. These oxidases are located in the outer mitrocondrial membrane and metabolise neuroactive amines. Inhibitors of these enzymes are effective in the treatment of Parkinson's disease, schizophrenia and clinical depression.

The second group is the copper amine oxidases or diamine oxidases, EC 1.4.3.6, which are found in animal tissue and plasma, plants, yeast's, fungi and bacteria. (Malmström *et al.* 1975). The metabolic function of these enzymes is the breakdown of a number of biologically active amines.

Highly purified enzymes have been obtained from the fungus *Aspergillus niger*, pea seedlings, bovine blood plasma, pig plasma and pig kidney cortex.

The amines most rapidly oxidised are the aliphatic monoamines with chain lengths C_3-C_6 including agmatine and histamine. Tyramine and tryptamine are oxidised at a slower rate. Pig kidney oxidases oxidise alkyl diamines such as cadaverine and putrescine most rapidly but histamine is also a good substrate. The purification of the enzyme from this source has been used in a number of studies (Bouvrette *et al.* 1997) because of its specificity for these diamines which are very important from a toxicological point of view.

The optimal substrate concentration for enzyme activity is 34mM for cadaverine and 1mM for histamine in the pH range 6.3 and 7.4. Histamine inhibits activity at concentrations greater than 1mM and hydrogen peroxide produced during the reaction of a diamine and the enzyme inhibits activity at neutral pH. (Mondovì *et al.* 1971).

Copper is the only metal found in significant quantity in the amine oxidases and is an essential component for activity. The copper ions are firmly bound but can be partially removed by treating with diethyldithiocarbamate (Yamada and Adachi, 1971). Activity is lost on removal of the copper and reactivation can be achieved by addition of suitable amounts of Cu^{2+}.

1.5 Methods Of Analysis

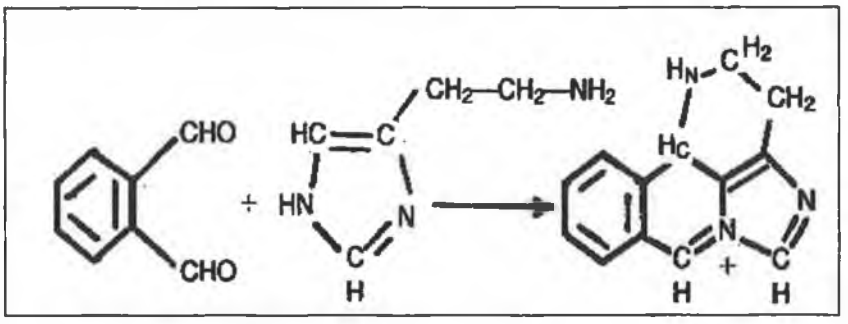
Many methods are available for the detection of histamine but only a few have been specially developed for detection in foods. The most popular methods are the fluorometric assay, enzymatic and chromatographic techniques and more recently immunological detection and the use of biosensors.

The fluorometric procedure is the official AOAC method and is most commonly used for histamine determination. (AOAC, 1990). The method involves extraction with methanol followed by selecting for histamine over other amines and amino acids by passing the sample through an ion-exchange column and reacting with o-phthaldialdehyde and fluorometric measurement. It is an accurate and sensitive method but tends to be slow and laborious.

1.5.1 Fluorometric Method for Histamine Analysis

Fluorimetry as a technique offers a number of distinct advantages over other conventional techniques, primarily its sensitivity. It is greater than 100 times more sensitive than colourimetry and offers a limit of detection down to the nanomolar range. (Roth, 1971). The fluorometric method is based on the coupling of histamine to a compound called o-phthaldialdehyde (OPT/OPA) at a highly alkaline pH to form a fluorescent product. The complex is then acidified which further enhances stability and a signal is obtained using a fluorimeter. Many other primary amines and amino acids react with OPT in the initial alkaline conditions but there are relatively few compounds which can form an acid-stable fluorophore and are broken down upon acidification. (Shore, 1971b). However, there are a small number of interfering reaction products, which must be removed by purification/clean-up procedures such as ion-exchange chromatography and organic extractions. (Shore, 1971a, Taylor *et al.* 1978b). The reagent rapidly forms fluorescent derivatives at room temperature, is non-fluorescent itself and does not breakdown and form fluorescent by-products when present in excess. (Alvarez-Coque *et al.* 1989).

Figure 1.5: Condensation of OPT with Histamine.



(Goutgou *et al.* 1987)

Optimum fluorophore formation occurs at pH 12.5 after 10 hours under nitrogen at -20°C. Under these conditions histamine as low as 1ng/ml can be measured and is therefore much more sensitive than the conventional assay. Fluorometric intensities at these conditions are almost twice that of standard conditions (20°C for 4 minutes). (Håkanson and Rönnberg, 1974). Obviously this method would severely limit the amount of samples analysed due to the length of time involved in the reaction.

The acid-stable fluorescence obtained following condensation of histamine with OPT at an alkaline pH is reasonably specific for N-unsubstituted imidazol-ethylamines. Compounds such as histidine, histidylhistidine and other hisdtidyl end group peptides can react with OPT in the same way as histamine but the purification procedures mentioned above eliminate them. (Shore, 1971a).

Since biological fluids are composed of complex matrices and can include histamine mixed with other compounds it is essential to see if they can effect the fluorometric assay to any degree.

Kownatzki *et al.* (1987) analysed low weight molecular amines and amino acids for interference with the assay and they observed three mechanisms of interference.

- Mimicking of histamine.
- Suppression of histamine fluorescence
- Generation of increased histamine fluorescence during the excitation of the OPT-histamine complex with daylight or UVA light.

Table 1.6: Substances producing histamine-like fluorescence after butanol extraction and condensation with OPT.

	mol/l[1]
Spermidine	4×10^{-5}
Noradrenaline	5×10^{-4}
Spermine	4×10^{-4}
Putrescine	3×10^{-3}
Tryptamine	5×10^{-3}
5-OH-Tryptamine	5×10^{-3}
Adrenaline	10^{-2}
3-OH-Tyramine	10^{-2}
Methyl histamine	2×10^{-2}
Adenine	6×10^{-2}

[1] Concentrations giving the same fluorescence as 5×10^{-7} mol/l of histamine.

(Kownatzki *et al.* 1987)

Histamine fluorescence was suppressed by methylhistamine, 3-OH-tyramine and 5-OH-tryptamine. These only were effective when added before the OPT to the sample and no suppression occurred on addition after OPT condensation had proceeded.

In order to show the generation of added fluorescence the histamine-OPT product was left sitting in a cuvette and a constant increase in signal, seven times the starting signal was observed after 25 minutes. This time could be reduced if exposed to daylight or a 20W UVA fluorescent lamp. Samples kept in the dark for the same length of time did not show this increase in fluorescence. This phenonomen was experienced by other amino acids but no biogenic amines including histamine experienced the increase in fluorescence. Staruszkiewicz *et al*, observed a similar increase in fluorescence when conducting stability experiments on the OPT-histamine derivative. They noticed that fluorescence intensity decreased 5 and 7% after 1 and 5 hours respectively. When the samples were stored in fluorimeter tubes in room light, minute gas bubbles formed in the tubes adding to fluorescence but intensities returned to normal when the solution were transferred to new tubes. (Starusziewicz *et al.*

1977). Interferences by other compounds were remarkably decreased by the butanol extraction step showing its selectivity for histamine over other interfering compounds.

A number of important precautions were outlined in order to determine the presence of interferences.

1. By determining the recovery of pure histamine from a spiked sample, the presence of histamine fluorescence-suppressing compounds can be determined.
2. Digesting with either diamine oxidase or histamine methyl transferase allows the determination of histamine-mimicking substances in a sample.
3. Any increase in fluorescence on exposure to light indicates the presence of histidine or other amino acids. (Kownatzki *et al.* 1987)

The major difficulty with histamine analysis in foods is the need to selectively extract the compound from the biological matrix and eliminate all possible interfering compounds. This is why the sample treatment step prior to the actual fluorometric assay is so important. Taylor *et al* made some efficient and improved modifications to the method of Shore (1971) and that of Rice *et al* (1975). Firstly the initial extraction of amines from the food was investigated. Methanol proved more efficient compared to 10% trichloroacetic acid (TCA), (Lerke and Bell, 1976) and 0.4M perchloric acid (Rice, 1975). Recoveries were 103, 86% and 43% respectively.

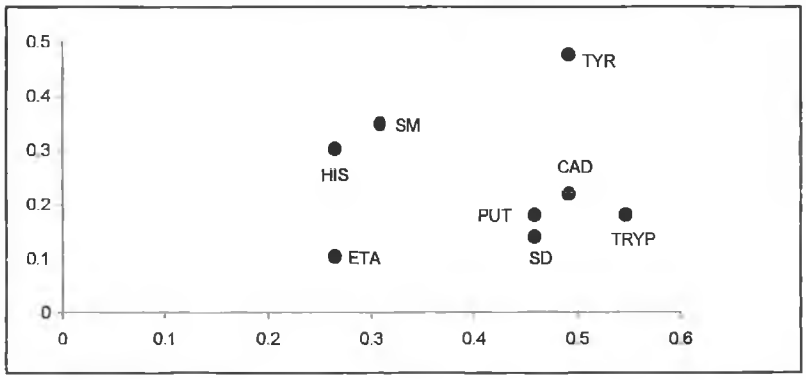
To determine the specificity of the second organic solvent extraction other potentially interfering amines were added to an aqueous phase and n-butanol added. Following separation of the phases amines were assayed with fluorescamine, (a general amine detection reagent). The experiment showed that the method selectively concentrates histamine into the n-butanol phase and leaves other amines in the aqueous phase with the exception of spermidine and histidyl-L-leucine but these levels are reduced considerably.

Other important factors are the use of sodium carbonate in saturating amounts as a salt and the presence of sodium hydroxide. The extraction procedure eliminates the need for a chromatographic step that is cumbersome and increases the analysis time. (Taylor et al. 1978).

1.5.2 Thin Layer Chromatography Analysis

Thin layer chromatography (TLC) has been reported as an efficient chromatographic technique for amine identification. TLC consists of migration of a sample extract on silica gel and detection of histamine by various spray reagents such as fluorescamine or ninhydrin. A method was devised and used for determining the amines tyramine, tryptamine, ethanolamine and histamine in pork bellies by Spinelli *et al.* (1974). The dansylated derivatives were separated by a combination of two solvents in two directions and finally viewed under long-wave ultra violet light. Levels were found in the range of 0.07-1.49mg/100g histamine and 0.03-1.27mg/100g cadaverine.

Figure 1.6: Two-dimensional thin-layer separation of dansyl-derivatives of various amines.



SM: Spermine, SD: Spermidine, PUT: Putrescine, CAD: Cadaverine, TRYP: Tryptamine, TYR: Tyramine, HIS: Histamine, ETA: Ethanolamine.

Abdel-Monem *et al.* (1975) successfully identified spermine, spermidine, cadaverine and putrescine by this method which appeared as well-defined and well-separated spots on both silica gel and alumina plates. The amines were derivatised with dansyl chloride and separated using chloro-isopropanol and chloro-dioxane-isopropanol as solvents. Confirmation of the individual amine spots was achieved by high-performance liquid chromatography. (Abdel-Monem and Ohno, 1975).

1.5.3 High-Performance Liquid Chromatography (HPLC)

HPLC is widely used as a method for amine analysis both by UV and by fluorescent detection. With the exception of the aromatic amines, most amines do not possess any significant chromophores or fluorophores. This necessitates the use of derivitization in order to confer fluorescene or UV activity on the molecules. Amines are usually quantified by pre-column or post-column derivitization with dansyl chloride (5-(dimethylamino)-1-naphtalene) or o-phthaldialdehyde.

The most widely used HPLC detectors are generally not very selective of highly sensitive (UV/vis and refractive index) for trace analysis while others tend to be very expensive (Mass spectrometry and infrared). The ideal system would be a combination of a general detection system and a sensitive method of compound identification. Most derivitization reactions for HPLC involve the sample or the column effluent being mixed with a derivitization solution in off-line or on-line (pre- or post-column) procedure before detection.

Fluorescence detection coupled with HPLC offers possibilities to improve specificity. Gouygou *et al.* (1987) used a reverse phase system with pre-column derivitization with o-phthaldialdehyde to detect histamine in fish. Extraction of histamine from the fish was achieved by TCA extraction followed by centrifugation and filtering. The sample pre-treatment is simple and non-selective as interfering compounds are eliminated following acidification of the histamine-OPT condensation product. (Shore, 1971a). It was very important to choose a mobile phase with a high resolution for the histamine fluorescence without quenching the fluorescence. A mobile phase of 40% acetonitrile in water with monosodium phosphate gave good separation in less than 15 minutes. The detection limit was 100pg histamine per 20μl loop injection. (Gouygou *et al.* 1987).

Pre-column derivitization with dansyl chloride with reverse phase linear gradient elution was used to establish a chemical quality index for canned tuna. The index can determine the extent of putrefaction in fish prior to canning by the putrefactive amines. From the results of fish analysis it was observed that in fresh fish the levels of cadaverine, putrescine and histamine were low and increased upon decomposition while the opposite was observed for spermine

and spermidine. These observations formed the basis of the equation for the quality index. (Mietz and Karmas, 1977).

$$\text{Index} = \frac{\text{histamine + ppm cadaverine + ppm putrescine}}{\text{1 + ppm spermine + ppm spermidine}}$$

Class 1: 0-1 good

Class 2: 1-10 borderline

Class 3: > 10 decomposed

There are several other methods based on derivitization with dansyl chloride for the simultaneous determination of biogenic amines in fish, cheese and beer and have reasonably quick elution times of 24 minutes or less. (Wei *et al.* 1995, Buiatti *et al.* 1994, Vallé and Malle, 1997 and Vallé and Gloria 1997).

A group of reagents (polymeric benzotriazoles) are known to derivatize most amino acids/peptides rapidly and efficiently and previously were only used for amino acid protection and peptide synthesis. Gao *et al.* (1998) developed new polymeric reagents based on the polymeric benzotriazole with an o-acetylsalicyl or 9-fluoroenyl labelling group that provided increased UV absorptivity and fluorescent properties for derivatized amines. Derivatization is performed off-line (pre-column) at 60°C for only 10 minutes and the limit of detection is 1 to 2 picomoles for polyamines using the fluorenyl label and fluorescence detection. (Gao *et al.* 1988).

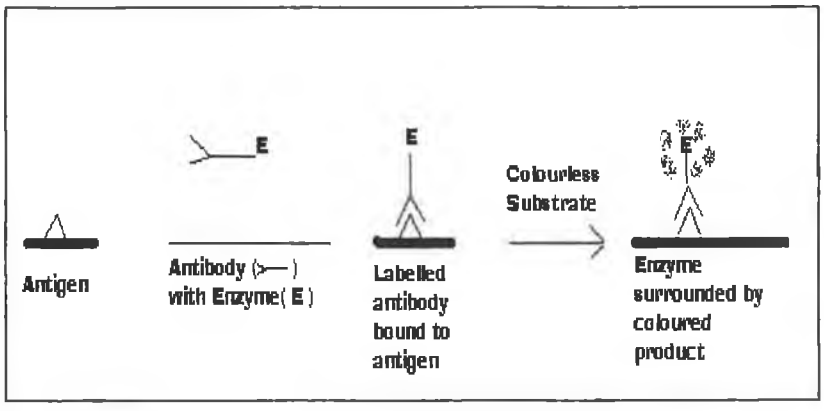
A drawback in these derivatisation procedures in the analysis of food samples is that many of the derivatization reagents will react toward undesired compounds present in the biological matrix, thus necessitating a sample clean-up step or re-extraction of the derivatized samples to remove the unreacted reagent. Herráez-Hernández *et al.* devised a detection system based on on-line derivatization with 9-fluorenylmethyl chloroformate (FMOC), separation by reverse phase chromatography and fluorescence detection. The reaction between the FMOC and the amines is completed within a few minutes but the total analysis time takes approximately 28 minutes, the limiting step being the chromatographic

separation. The limitation of the method is that is only responsive to primary and secondary amines. (Herráez-Hernández *et al.* 1996).

1.5.4 ELISA Analysis

Enzyme-linked immunosorbent assay (ELISA) is a detection system based on the binding of an antibody to an antigen and detection using an enzyme label. The enzyme acts on a colourless substrate to give a coloured product, which is readily detectable.

Figure 1.7: The presence of an antigen is detected using the enzyme-labelled antibody. The amount of coloured product is proportional to the antigen concentration.



Research Diagnostics Incorporated (RDI) have developed a kit for the quantitative measurement of acylated histamine in food. Sample preparation involves the acylation of histamine to form N-acylhistamine. The kit functions as a competitive ELISA, whereby there is competition between a peroxidase-conjugated and a non-conjugated antibody for a fixed number of antibody-binding sites (rabbit-anti-histamine). The peroxide conjugated antibody-antigen complexes bind to the well of the plates, which are coated with goat-anti-rabbit antibodies. Any unbound antigen is then removed by washing. The plates are read and the O.D. (complexes bound) is inversely proportional to the histamine

concentration in the sample, ie: the greater the concentration of the antigen in the sample the lower the amount of labelled antigen binding to the antibody. The sensitivity of the assay is 0.8µg/L.

Serrar *et al*, developed a monoclonal antibody-based ELISA based on the same competitive system. In order to obtain antibodies specific for histamine it is necessary to provoke an immune response from an animal against the compound. However, histamine is a small molecule and alone is not immunogenic. It must be coupled to a large immunogenic carrier molecule usually a protein to stimulate antibody production. Once the monoclonal antibodies (mAbs) were obtained and purified they were retested against histamine-protein conjugates, other amines and negative controls to investigate their antigenic specificity. It was found that they recognised histamine only and showed high affinity to histamine after chemical derivitisation. The lower limit of detection was found to be 10ng/ml. (Serrar *et al.*1995).

1.5.5 Screening Test for Histamine

This method involves a two step sequential enzyme system and is similar to the assay for the detection of decarboxylase activity in dairy-related bacteria (Sumner and Taylor, 1989).

1. DAO catalyses the breakdown of histamine forming H_2O_2 as a product.
2. H_2O_2 is then detected by the formation of crystal violet from the leuco base in the presence of oxidase.
3. Colour formation is read at an O.D. of 596nm.

Specificity of the test: DAO also catalyses the breakdown of other amines associated with decomposition. The amines spermine and spermidine are found to decrease with spoilage and are usually present in non-interfering amounts but cadaverine and putrescine increase with spoilage, (Mietz and Karmas, 1977), so they may add to positive reactions with histamine in the screening test.

However the procedure would fail to detect any non-histamine type spoilage in fish.

Samples are absorbed onto filter paper strips by inserting them into a cut in the dorsal are of the fish for 5 minutes. The strips are then placed into reaction tubes

for colourimetric detection. The strip results were correlated with a standard method of detection by excising the same fish sample, homogenising and analysing by the AOAC fluorometric method. Poor correlation was found which was due to a broad range of saturation intensities after 5 minutes on the strips, which led to variations in sample size. The uneven distribution of histamine in tuna was also a contributing factor.

To overcome these problems, samples are usually taken from the area of highest histamine content, the gills and a smaller amount of absorbent is used so that the samples can become saturated quickly. (Lerke *et al.* 1983).

The sensitivity of the test can be adjusted to test samples with a particular histamine level by altering the horseradish peroxidase concentration.

1.5.6 Biosensors

Currently the food industry is not very receptive to biosensor technology but in the long run it is hoped that a niche in the market can be developed. Biosensors have to compete with other analytical methods in terms of cost, performance and reliability. Chromatography is the method of choice for multiple sample analysis and recently HPLC and GC instrumentation has been improving making them more cost effective. The fact that they can analyse several compounds simultaneously increases their reliability and speed of analysis. Also simple dipstick tests are easier and more user friendly than portable biosensors for field testing and these account for why there is only limited acceptance of biosensors in the food industry.

The food industry spends 1.5-2% of its total value sales on quality control and appraisal. Recent trends in increased regulatory action and consumer awareness about food safety has created a need for reliable and cost effective analytical methods. New legislation requires the extensively labelling of all major and minor constituents present in the food. (Luong *et al.* 1997)

The functioning of a biosensor involves the combination of biochemical and electrical interactions. It consists of a receptor, which is an immobilised, biologically responsive material linked in close contact to a suitable transducing element.

The interaction of the analyte and the biological element produces a biochemical signal, which is converted into an electrical response by the transducer. (Graham and Moo-Young, 1985). There are many materials that can be utilised in biosensors:

- Enzymes
- Microbial cells
- Monoclonal antibodies
- Whole sections of plant
- Animal tissues
- Organelles

(Turner, Karbue and Wilson, 1987)

The food industry has a need for simple, rapid and inexpensive methods that are ideally automated. Process control in food is more complicated than other industries due to the complicated biochemical matrices involved. Electrochemical sensors allow for the measurement of many substances such as those outlined in Table 1.7 (Schaertel and Firstenberg-Eden, 1988).

Table 1.7: Substances determined by enzyme sensors.

Substance Category	Examples
Carbohydrates	Glucose, Lactose, Starch.
Amino Acids	L-arginine, D-alanine.
Alcohols, Phenols	Ethanol, Glycerol, Cholesterol.
Gases	NH_3, H_2, SO_2, NO.
Amines, Amides	Urea, Histamine, Creatine.
Inorganic Ions	Nitrite, Nitrate, Sulphite.

In order to be sucessful to industry a biosensor must possess the following characteristics:

1. The biocatalyst must be highly specific for the purpose of the analyte and be stable under normal storage conditions.
2. The response should be accurate, precise, reproducible and linear.
3. It should be cheap, small, portable and simple to use.
4. The biochemical reaction should be independent of physical parameters such as pH, temperature and stirring.

(Karube, 1992).

The critical factor for biosensor design is maximal retention of bioactivity and biostability of the biological molecule. By increasing these factors, a more cost effective biosensor can be designed to compete with existing analytical methods.

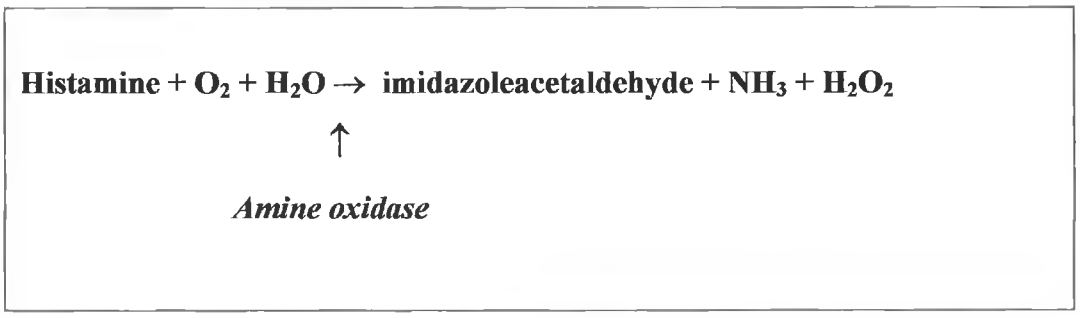
The AOAC fluorometric assay, HPLC or TLC has traditionally determined the analysis of biogenic amines in the food industry. However due to extensive sample pre-treatment and time consumption, the methods are not as efficient as desired.

Biosensors owing to their many advantages have become more and more prominent as analytical devices in this industry and much research is being put into further applications of them.

Trimethylamine (TMA) is an important component of the smell of spoiled fish and so lends itself as a good detection element for such spoilage. A sensor has been developed for TMA detection based on an ammonia-sensing electrode, which consists of a glass (pH) electrode and a AgCl reference electrode in an internal filling solution of ammonium chloride, neutral salts and a dye. The filling solution is separated from the analyte by a gas-permeable, ion-permeable membrane. Any dissolved ammonia in the sample diffuses across the membrane leading to an increase in pH, which is detected. In order to detect TMA, the internal filing solution has been replaced with 0.01M TMA hydrchloride and 0.04M Potassium chloride. Formaldehyde is added to the sample solution in order to decrease the response of the electrode to ammonia. The analyte concentration range is 0.1-10mM. (Chang *et al.* 1976).

Ammonia is another compound responsible for the off-flavours and odours associated with spoiled fish and the ammonia-sensing electrode is a very popular device for these determinations. (Ward *et al.* 1979)

Using an amine oxidase enzyme as a biological component a biosensor was constructed to quantify histamine levels in fish. In a closed reaction cell of the sensor the following reaction proceeded:



This reaction can be followed by several methods including, substrate disappearance, oxygen consumption and the production of ammonia, hydrogen peroxide and an aldehyde.

Oxygen consumption was measured by an oxygen sensor following the reaction of amine oxidase with fish extracts. One mole of dissolved oxygen was consumed upon oxidation of one mole of histamine, so based on this relationship of consumption histamine could be determined selectively. The assay requires no sample pre-treatment to remove interfering compounds which allows analysis to proceed rapidly. The enzyme is a fungal amine oxidase, which was purified from the mycelium extract of *Aspergillus niger* AKU-3302 strain. The enzyme from this source most rapidly oxidises the aliphatic monoamines of C_2-C_6, benzylamine, phenylethylamine, histamine and agmatine. Aliphatic diamines, C_4-C_6, are oxidised at a lower rate. (Yamada and Adachi, 1971).

The method correlated well with the official AOAC method, (AOAC, 1990) and histamine recoveries were 100%. (Ohashi *et al.* 1994).

Male *et al.* (1996) also used this amine oxidase system in the development of a amperometric biosensor for determining histamine, cadaverine and putrescine. The system utilised an amperometric electrode for the detection of the product hydrogen peroxide and was linear up to 6mM with a lower limit of detection of

25μM for the three substrates. The enzyme was a diamine oxidase purified from porcine kidney, which exhibits specificity towards diamines like cadaverine and putrescine but also deaminates histamine. (Male *et al.* 1996).

The influence of biosensors will rise in the food and beverage industry in the near future. Advances in improving the stability of the biological component, simultaneous analysis of multiple analytes and mass production of biosensors will in the long run create a more cost-effective analytical device.

The aims of the thesis are to study the different levels of histamine in both tuna and five varieties of Irish cheeses by conventional methods of detection. These methods will be High performance liquid chromatography and fluorimetric detection. The production of histamine by food microorganisms will also be monitored over time by the above methods. A new method of detection utilising a group of compounds called calixarenes will be investigated and its potential use as an analytical tool evaluated.

Chapter 2
Materials and Methods

2.1 Fluorometric Assay for Histamine analysis in food

(Taylor *et al.* 1978b)

Reagents. **Sigma:** o-phthaldialdehyde (OPT), methanol, n-butanol, diethyl ether, sodium carbonate, hydrochloric acid, histamine dihydrochloride.

Food samples were purchased from two separate local retail markets and stored at 4° C during the experiment.

The procedure according to Taylor *et al.* (1978b) was followed with a few modifications carried out. Ten grams of cheese were homogenised with 50 ml of reagent grade methanol for 10 min in a Waring Blender. The blender cup was rinsed with methanol and added to the homogenised sample in a 100 ml stoppered volumetric flask. The flasks were heated in a water bath for 30 min at 60°C and allowed to cool before adjusting the contents to 100 ml with additional methanol. The samples were transferred to capped polypropylene tubes and centrifuged at 2000 rpm in an IEC PR-6000 centrifuge for 8 min. A 2ml portion of the supernatant was diluted 1/20 (v/v) with deionized water and a 5 ml aliquot of this dilution was added to a test tube containing 1ml of 5M NaOH. Saturating amounts of Na_2CO_3 were added and the samples were vortexed. Six ml of water-saturated n-butanol were added followed by vigorous shaking in order to extract histamine into the organic phase. A 3 ml aliquot of the organic phase was then added to 3ml of 0.1N HCl and vortexed well.

The upper organic layer was removed and 2 ml of the acid phase was used for the fluorometric assay (Shore, 1971b). A reagent blank was carried out by substituting 5 ml of deionized water in place of the 1/20 diluted sample before proceeding as described. An external standard (5 ml of a 25 μM histamine solution) was added at this same stage and treated likewise. The standard was used to calculate the unknown samples by comparison of fluorescence intensities.

2.2 Fluorometric Assay

A 400 µl aliquot of 3M NaOH was added to 2 ml of the acid phase followed by 100 µl of 1% (w/v) o-phthaldialdehyde (OPT). The reaction proceeded for 4 min. exactly and was terminated by addition of 200 µl 3M HCl.

Fluorescent intensities were read on a Perkin Elmer spectrophotometer with excitation and emission wavelengths set at 360 nm and 450 nm respectively. The excitation and emission slit widths were set at 10.0 and 5.0 respectively.

2.3 Fluorometric Assay for Histamine production in bacteria

A 5 ml broth culture of *Providencia retgerri* 865 was grown overnight and was used to inoculate one 100 ml culture of LB broth and one 100 ml L-histidine supplemented culture of LB broth. A growth curve was constructed by taking O.D. readings at 550 nm at regular intervals. Simultaneously samples were taken for histamine quantification by a fluorometric method as follows.

A 1.0 ml aliquot of broth culture was added to 9.0 ml of methanol and heated at 60°C for 15min and allowed to cool. 1.0 ml of the methanol extract was run through an ion exchange column (Dowex 1X-8 in the hydroxide form, 80 x 5 mm). The column resin was prepared as described (AOAC, 1980). The column was washed with 35 ml of distilled water and the eluant was collected in a 50 ml volumetric flask and the volume raised to 50 ml with distilled water. (Behling and Taylor, 1982).

Histamine content was determined using a fluorometric assay (Shore,1971b),

Column eluant (2 ml) was placed into a test tube followed by 0.4ml of 3M NaOH and the tube shaken. A 100 µl aliquot of 1% (w/v) o-phthaldialdehyde (Roth, 1971) was added, mixed and the reaction was let to proceed for 4 min exactly. The reaction was stopped by addition of 200 µl 3M HCl. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm (slit widths: excitation 10.0, emission 5.0).

2.4 Microbiological Media

All media constituents were obtained from Oxoid unless otherwise stated. Media were solidified with 1.2% agar No.3 where necessary. Distilled water was used in all preparations. Sterility was ensured by autoclaving at 15lb/in^2 for 20 minutes.

Luria Bertani (LB)

Tryptone	10g
Yeast Extract	5g
NaCl (Sigma)	10g
H_20	1 Litre

pH 7.5

Differential Plating Medium for Quantitative Detection of Histamine-Producing bacteria.
(Niven *et al.* 1981)

Tryptone	0.5%
Yeast Extract	0.5%
L-Histidine.2HCl	2.7%
NaCl	0.5%
$CaCO_3$	0.1%
Agar	2.0%
Bromocresol purple	0.006%

pH 5.3

2.5 Bacterial strains

- *Providencia retgerri 865* (obtained from NCIMB Aberdeen, Scotland)
- *Escherichia coli* DH5α *(DCU stocks)*
- *Proteus vulgaris (DCU stocks)*

2.6 Differential Plating Media for Quantitative Detection of Histamine-Producing bacteria

(Niven *et al.* 1981)

A loopful of a single bacterial colony from a streaked plate was transferred to 5ml of LB broth and grown overnight at 37°C. Decimal dilutions (10^{-1} to 10^{-9}) were prepared from the broth by diluting with sterile Ringer's reagent. Duplicate pour plates were prepared for each dilution with the solidified agar being overlaid with ~5ml of the same medium to suppress spreading colonies. Plates were incubated for 72 hours at 37°C. Controls of bacterial free media and definite non-histamine producing strains were run in parallel. Plates were examined for purple colonies with a purple halo on a yellow background indicating positive histidine decarboxylating colonies.

2.7 HPLC Analysis of Histamine

2.7.1 Derivitisation with Benzoyl Chloride

(Yen and Hsieh,1991)

Reagents: Histamine dihydrochloride, methanol, diethyl ether, sodium bicarbonate, ammonium acetate and magnesium sulphate. (Sigma), benzoyl chloride (Aldrich), HPLC grade acetonitrile, acetone and Mili-Q water. Sodium hydroxide, perchloric acid, toluene, ammonia, sulphuric acid, sodium chloride, phosphoric acid, hydrochloric acid.

All analysis was carried out on a Varian liquid chromatograph which consisted of a Varian 9012 solvent delivery system, a Rheodyne model 7125 syringe loading sample injector, a Varian 9050 variable wavelength uv-vis detector and a Hichrom lichrospher RP-18-5 reversed phase column (5μm x 12.5cm x 4.6mm i.d.).

Method

Histamine dihydrochloride (165.7 mg) was dissolved in 10 ml of de-ionised water. This gave a concentration of 10mg/ml.

To the histamine solution (50 μl) an aliquot of 2M NaOH (1ml) was added, followed by the addition of 10 μl of benzoyl chloride. The solution was then vortexed for 30 seconds and then left standing for a further 20 minutes. Saturated sodium chloride (2ml) was then added, followed by extraction with 4mls of diethyl ether.

Chromatographic Conditions

A gradient elution system was employed. The gradient elution program was set at 1.1 ml min^{-1} at room temperature, starting with a methanol: water mixture (55:45, v/v) for 2.5 minutes. The program proceeded linearly to methanol: water 88:22 (v/v), with a flow rate increasing from 1.1 ml min^{-1} to 1.3 ml min^{-1} over 3.5 minutes. This was followed by the same composition and flow rate for 2 minutes, then decreased over 7 minutes to methanol: water (55:45 v/v) at 1.1 ml min^{-1}. Finally the system was re-equilibrated at methanol: water (55:45, v/v) for 10 minutes before the next injection.

2.7.2 Derivitisation with Dansyl Chloride

(Wei *et al.* 1995)

Reagents:

Sigma. Sodium carbonate, dansyl chloride, histamine dihydrochloride, n-butanol, methanol, diethyl ether.

Labscan. Acetonitrile.

The method of analysis is based on a derivatization of histamine samples followed by HPLC separation and detection by UV.

Derivatization Reaction

A concentrated solution of histamine dihydrochloride (16.57 mg/100 mL water and diluted 1/100 v/v) was prepared.

A 200 µl aliquot of this solution was placed in a round-bottomed flask to which 2.0 ml dansyl chloride (10.0 mg/ml acetone) and 3.0 ml of 4% (w/v) sodium carbonate was added. All solutions were freshly prepared before each experiment. The flask was foil covered, as the dansyl chloride is light sensitive. A blank (containing no histamine) was derivatized along with the standard. This solution was placed in a water bath at 40°C with constant stirring overnight. The following day, 15.0 ml of MiliQ water was added to the reaction mixture,

washing the sides of the flask thoroughly. The solution was then extracted with three 5.0 ml portions of diethyl ether, the ether layers were then combined and evaporated using a rotary evaporator set at 60°C. The residue in the flask after evaporation was dissolved in acetonitrile and made up to a volume of 10ml. From this stock solution, other dilutions were made up with acetonitrile (80, 60, 40 and 20%). A 20 µl sample of each solution was injected into HPLC (Waters) by a fixed loop. A 200 µl aliquot of extracted sample was treated in the same way as the standards.

Chromatographic Conditions

Separating Column: Inertsil 5ODS-2 (250mm x 4.6mm, 5µm)

Mobile Phase: Acetonitrile: Methanol: Water (3:10:3)

Flow Rate: 1.2 mL/min

λ_{MAX}: 254nm

Chapter Three.
Fluorometric Method of Analysis

3.1 Introduction

A simplified fluorometric assay for food analysis, as described by Taylor *et al.* (1978b), was established as the standard method for determining levels of histamine in various foods. The method involved extraction of histamine from the biological matrix into methanol followed by protein denaturation and precipitation and then further extraction into n-butanol. The organic extraction step was selective for histamine and was dependent on the presence of a salt (sodium carbonate), its concentration and the type of organic solvent used. Prior to analysis it was important to assess the specificity and accuracy of the method and also to investigate possible interference's in the assay. The amount of histamine that is recovered from the sample clean-up process is an essential factor and so spiking samples with known amounts of histamine should show the efficiency of the procedure. A linear correlation between fluorescence and histamine concentration is necessary in order to determine histamine levels present in samples and this is demonstrated by Fig. 3.1 which shows a high degree of linearity with a R^2 value of 0.997.

3.2.1 Specificity of Organic extraction for histamine

3.80 ppm solutions of histamine and other amines were prepared with 1 ml sodium hydroxide and saturating amounts of sodium carbonate prior to the addition of n-butanol. Following phase separation the fluorometric assay was performed on the solutions and recoveries calculated (Table 3.1). A variety of amines, amino acids and histidine were assayed fluorimetrically to show the specificity of the assay for histamine and to show the interference, if any of other amines. An amino acid solution containing lysine, leucine, proline and glutamine were investigated as possible interferants in the assay also. (Table 3.3). Both Figure 3.7 and Table 3.3 show that histidine can be detacted by the fluorimetric assay to some degree but a the use of ion chromatography can totally eliminate this interference.

Table 3.1 Recoveries of various amines following butanol extraction.

Solution	Concentration Measured (ppm)	Recovery
Histamine	3.80	100%
Histamine + Histidine (a)	3.94	104%
Histamine + Histidine (b)	3.91	103%
Histamine, histidine, putrescine, cadaverine, glutamine and tryptophan. (a)	4.14	109%
Histamine, histidine, putrescine, cadaverine, glutamine and tryptophan. (b)	4.03	106%

(a) and (b): duplicate analysis

3.2.2 Estimation of interfering compounds in the assay/specificity of the fluorometric assay for histamine

Two food samples (canned tuna) were extracted and treated according to the method described by Taylor *et al* (1978b) and subjected to the fluorometric assay. The assay was performed with and without the acidification step by 3N hydrochloric acid and the results were read fluorometrically. Table 3.2 shows the differences in amine levels in tuna samples.

Table 3.2 Levels of amines in tuna with and without acidification of the OPT-histamine condensation complex.

	+ Acid	- Acid
Tuna sample (a)	214.7 ppm	218.0 ppm
Tuna sample (b)	197.4 ppm	199.8 ppm

Figure 3.1 Linear Range for Fluorometric Assay for Histamine

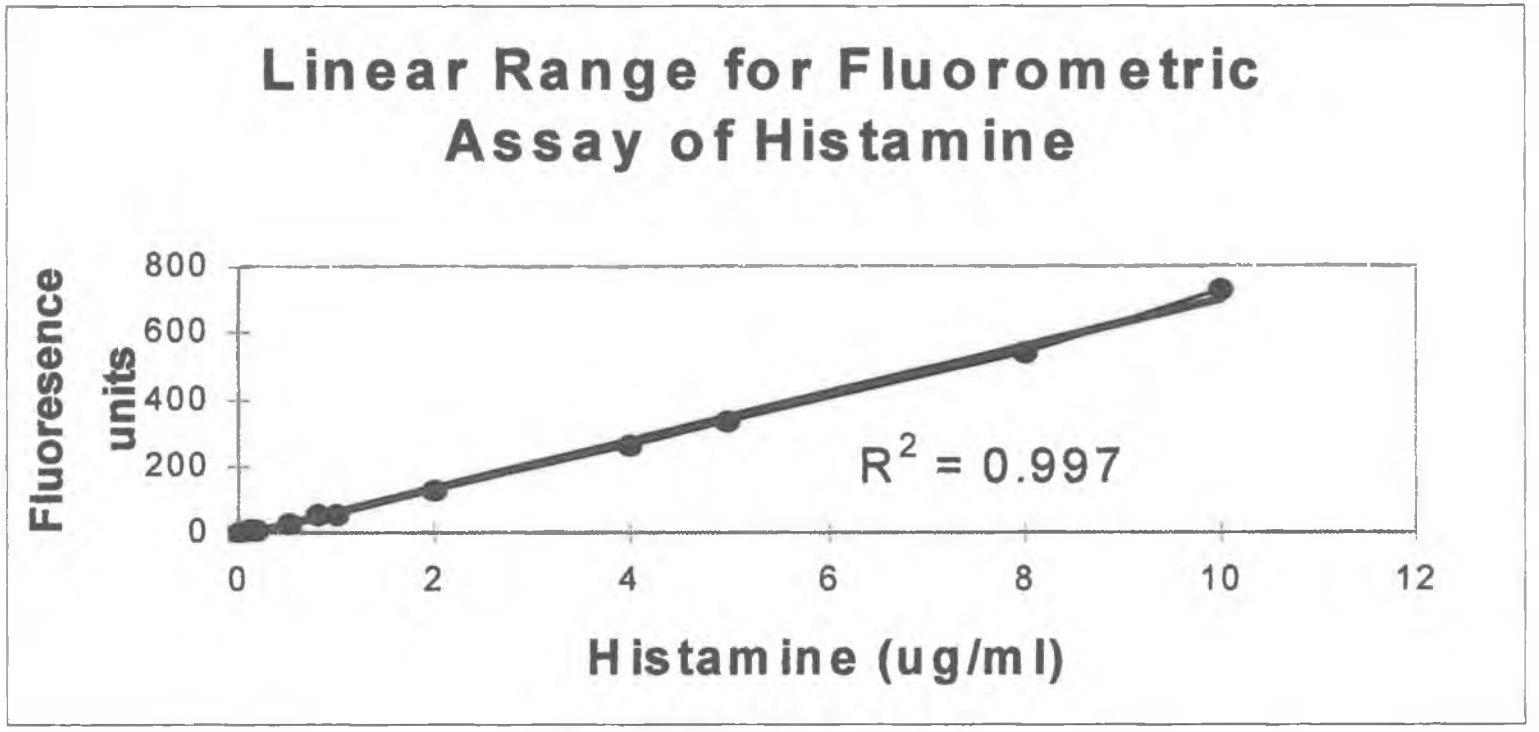


Table 3.3 Fluorometric Assay on Various Amines and Amino acids

	Fluoresence						
Conc. (ppm)	Histamine	Histidine (pre. col.)	Histidine (post col.)	Cadaverine	Tyramine	Putrescine	Amino acids*
0	0	0	0	0	0	0	0
0.005	0.906	0.182	0	0	0	0	0
0.025	4.693	0.6115	0	0.033	0	0	0
0.050	8.318	1.77	0	0	0	0	0
0.10	19.213	2.771	0	0	0	0	0
0.25	51.563	7.156	0	0.072	0	0	0
0.40	91.268	12.076	0.12	-	0	0	0
0.50	113.853	12.236	0	0.115	0.445	0	0
1.00	-	-	-	0.121	0	0	0
100.0	-	-	-	0.362	0	4.02	15.64

* Amino acid solution
  L-Lysine
  L-Leucine
  L-Proline
  L-Glutamine

3.3.1. Analysis of Irish Cheeses for Histamine Content

A detailed study was carried out into histamine levels in Irish produced cheeses. The five cheeses, (Cheddar, Cooleney, Brie, Edam and Emmental) were analysed over a three week period, before and after their expiry best before dates and were purchased from two retail outlets to examine batch to batch variation. Analysis was carried out in triplicate and standard deviations calculated. Samples were stored at 4°C during decomposition to emulate conditions in retail outlets and storage fridges. The results are displayed in tabular form (Table 3.4) and as a bar graph (Figure 3.2).

Table 3.4 Histamine Levels in Irish Cheeses with Standard Deviations

Cheese	Analysis 1 (mg/100g)	Analysis 2 (mg/100g)	Analysis 3 (mg/100g)
Cheddar 1*	0.452 ± 0.1	0.704 ± 0.11	0.539 ± 0.02
Cheddar 2	4.31 ± 0.22	2.95 ± 0.38	0.281 ± 0.04
Cooleney 1	0.355 ± 0.03	6.68 ± 0.58	8.522 ± 0.71
Cooleney 2	0.519 ± 0.127	3.15 ± 0.54	1.758 ± 0.12
Emmental 1	1.953 ± 0.32	2.28 ± 0.96	6.314 ± 0.67
Emmental 2	0.986 ± 0.19	1.71 ± 0.33	5.906 ± 1.31
Edam 1	0.219 ± 0.09	0.273 ± 0.023	3.071 ± 0.29
Edam 2	2.643 ± 0.17	1.892 ± 0.14	4.073 ± 0.62
Brie 1	2.912 ± 0.12	0.633 ± 0.03	0.375 ± 0.05
Brie 2	1.847 ± 0.22	0.453 ± 0.05	0.119 ± 0.04

*The numbers indicated after the cheeses represent which outlet they were obtained from.

Analysis 1: Carried out 7 days before the expiry date.

Analysis 2: Carried out on the expiry date.

Analysis 3: Carried out 7 days after the expiry date.

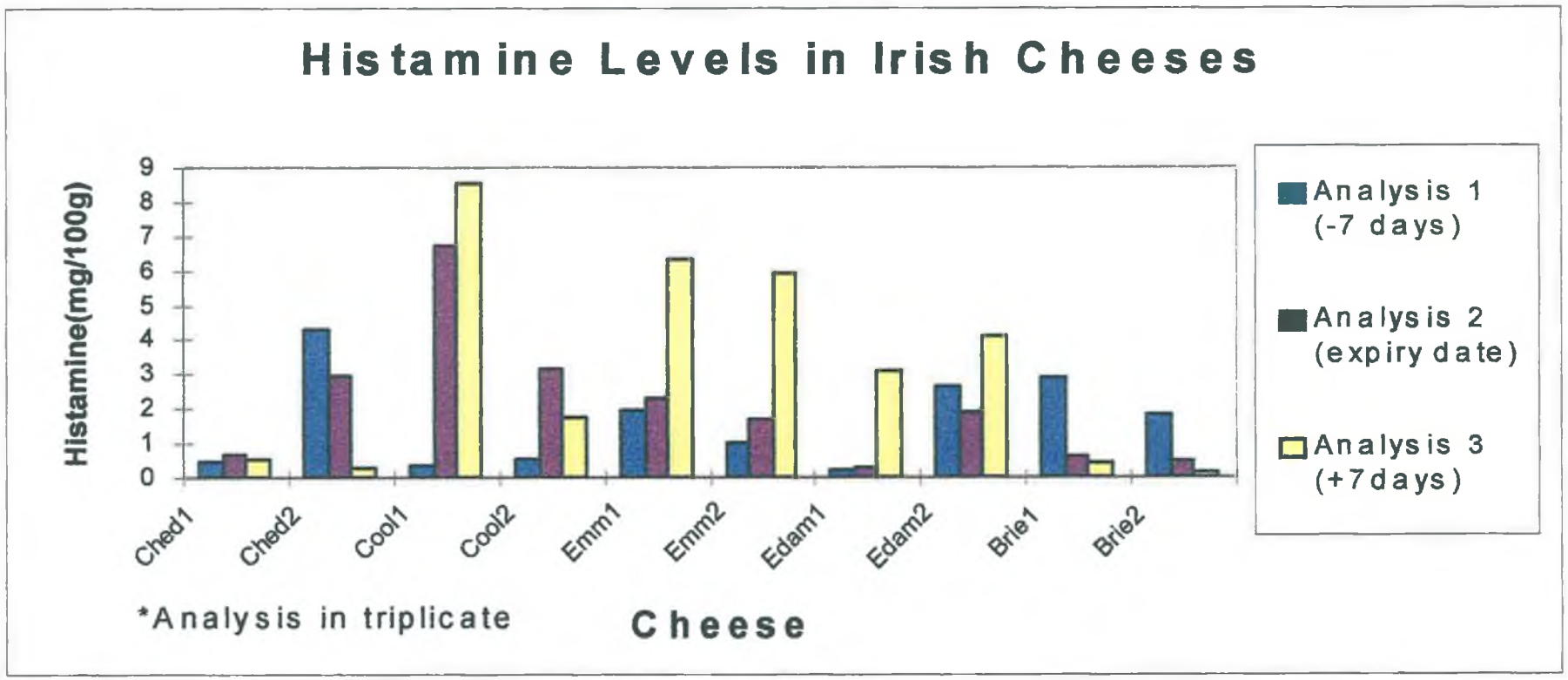
3.3.2 Spiking of Cheese Samples and Recoveries

A decomposed cheese sample (Emmental) was spiked with a 200 ppm histamine standard following homogenisation with methanol and brought through the extraction process as described in Chapter 2 (2.1). Recoveries were derived by calculating how much of the 200 ppm histamine spike was detectable in a cheese sample of known histamine concentration after the extraction procedure was carried out, ie. Adding 200 ppm histamine to a 47 ppm histamine cheese would yield a 100% recovery if 247 ppm histamine was detected. Table 3.5 shows the recovery of histamine spikes in the cheese samples.

Table 3.5 Recoveries of histamine standards from cheese

	Cheese	Spike 1	Spike 2	Spike 3
Level (ppm)	47	223.2	238.3	230.2
Recovery %	100	90.4	96.5	93.2

Figure 3.2 Bar graph of Histamine Levels in Irish Cheeses over three-week period.

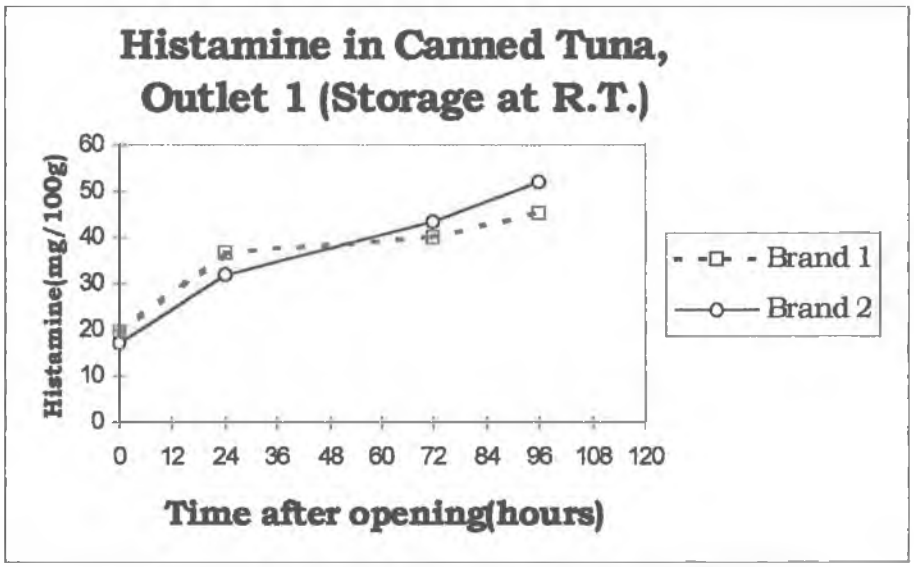


3.3.3 Histamine Analysis in canned tuna

Two brands of canned tuna were chosen for the survey. Samples were obtained as with the cheese survey from two different retail outlets to observe batch to batch variations and analysis was carried out in triplicate.

Canned tuna was tested on opening (Time =0) and then left in storage at room temperature and at refrigeration temperatures to decompose. Further analysis was conducted at daily intervals in triplicate.

Figure: 3.3 Analysis of Histamine levels over time on Canned Tuna at Room temperature storage from Outlet 1.



(R.T.: Room temperature)

Figure 3.4 Analysis of Histamine levels over time on Canned Tuna at Room temperature storage from Outlet 2.

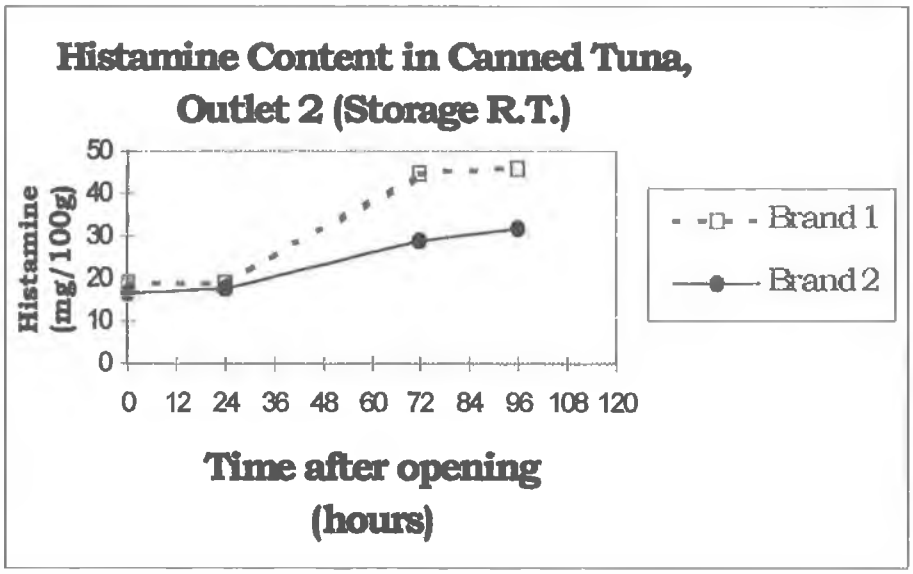


Figure 3.5: Histamine Analysis in Canned Tuna at 4°C Storage from Outlet 1.

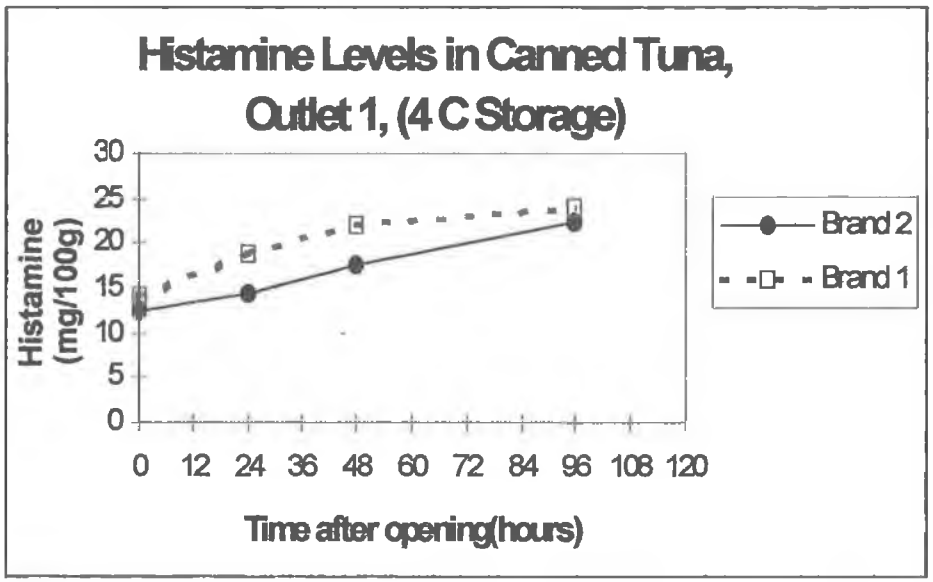


Figure 3.6 Histamine Analysis in Canned Tuna at 4°C storage from outlet 2

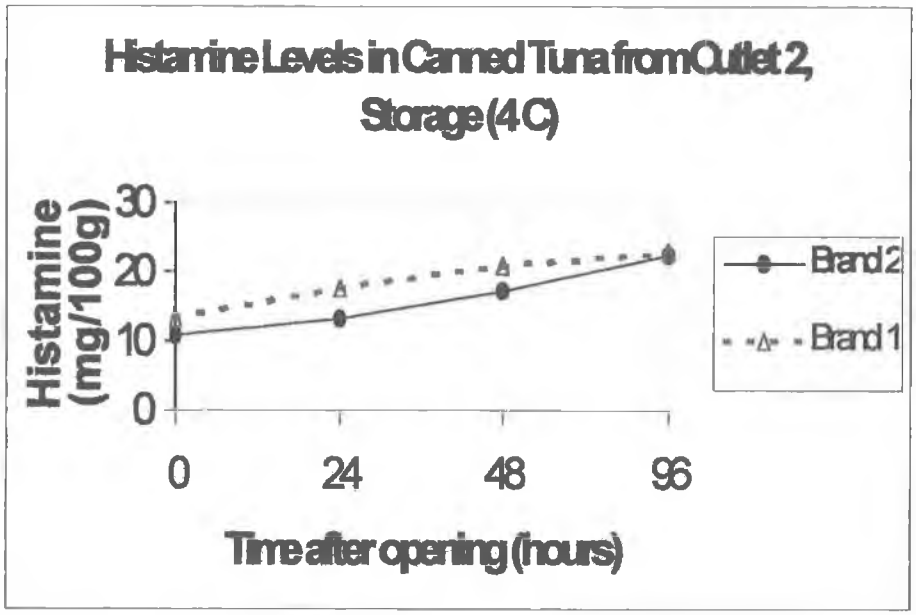


Table 3.6 Spiking and Recoveries of Histamine in Tuna

A 200ppm histamine spike was added to three tuna samples prior to homogenisation and extraction and the percentage of histamine spike recovered was determined by fluorimetry.

	Fresh tuna	Spike 1	Spike 2	Spike 3
Fluorescence	71.82	122.33	139.44	124.2
Level (ppm)	157	351.0	399.0	357.5
Recovery %	100	97.5	121	99.9

3.3 Histamine Analysis in other foods

Foods that have reported low histamine levels such as vegetables and fruits were analysed in order to show comparative figures to the high histamine levels in canned tuna. The vegetables were prepared and extracted in the same way as the cheese and tuna samples. Table 3.7 shows that there was no histamine present in the samples.

Table 3.7. Histamine levels in fruit and vegetables.

Food	Histamine (mg/100g)*
Potato (a)	0
Potato (b)	0
Carrot	0
Apple	0

*(Analysis in triplicate)

3.4 Identification of a Histamine producing micro- organism

A screening method based on the differential media developed by Niven *et al.* (1981) was employed to identify histamine-producing bacteria. The bacteria, following overnight growth were diluted to various concentrations and plated on the medium and incubated for 72 hours at 37°C. Three bacterial strains were grown on this media, with only one bacteria, *Providencia retgerri* 865 exhibiting positive purple colonies indicative of histamine producing bacteria. (Table 3.8). *P.retgerri* was used for further histamine studies, with its optimum growth rate established growing on LB media (Figure 3.8). Figure 3.9 demonstrates where the optimum production of histamine occurs in the lifetime of the species, which correlates to Figure 3.8 where exponential growth occurs between 2 and 8 hours.

Table 3.8 Micro-organisms showing histamine decarboxylase activity on differential plating medium.

Bacterial species	Differential media
Providencia retgerri 865	+
Proteus vulgaris	-
Escherichia coli	-

Figure 3.7

Effectiveness of the ion-chromatography column in removing histidine from culture fluid samples.

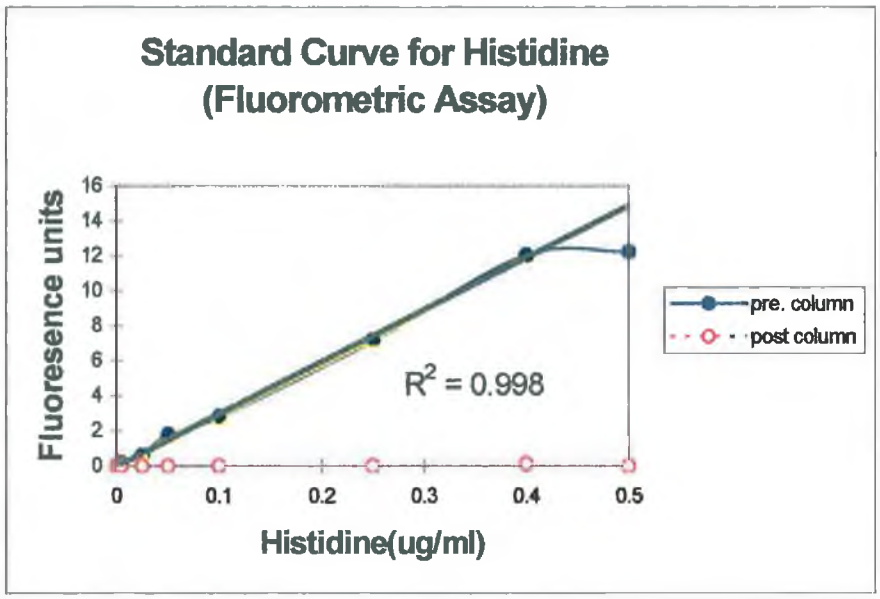


Figure 3.8: Growth Curve for *Providencia retgerri* 865 in unsupplemented and histidine supplemented LB broth.

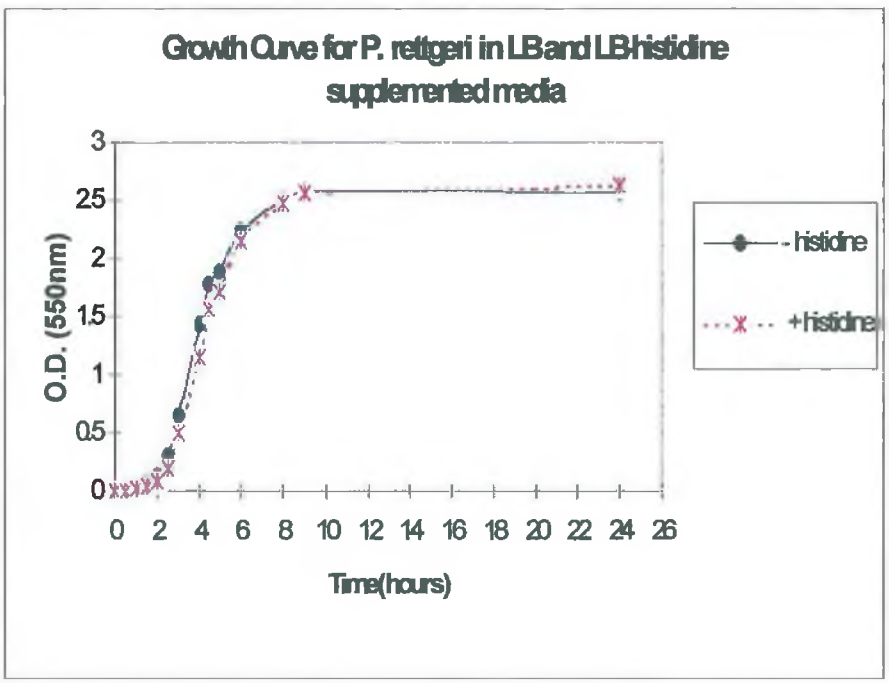
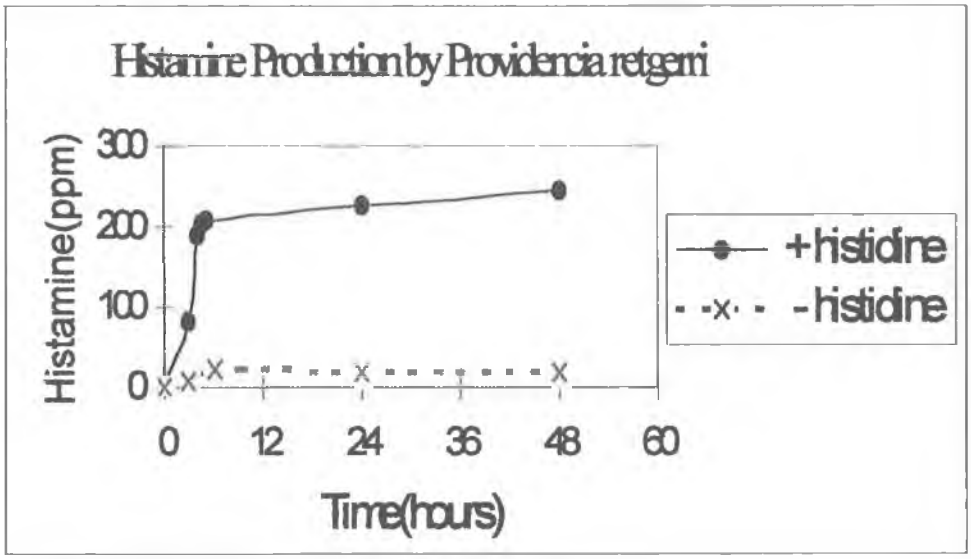


Fig 3.9: Production of Histamine by *Providencia retgerri* on unsupplemented and histidine supplemented LB media.



3.5 Diamine Oxidase Activity

Diamine oxidase was added to histamine samples extracted from tuna at various concentrations and at different incubation times to observe the effect of its activity.

Table 3.9 Effect of Diamine Oxidase (DAO) on Histamine in tuna.

Sample	Histamine (mg/100g)
Tuna (a sample)*	88.4
Tuna (b sample)*	68.8
+ DAO (5min)	19.1
+ DAO (15 min)	10.7
+ DAO (overnight)	0.0

*** decomposed canned tuna**

3.6 Discussion

Taylor et al. (1978b) showed water saturated n-butanol to be the best organic solvent for the extraction of histamine with a partition coefficient of 33. However Shore, (1971b) suggested that in samples where there are high histidine levels like in the case of canned tuna, n-butanol-chloroform (3:2) should be used. However these solvents only have a partition coefficient of 0.04 and may leave the majority of histamine in the aqueous phase and not the desired organic phase. Table 3.1 shows that some histidine was carried through into the organic phase (up to 4%) and displayed some residual fluorescence. The putrificative amines cadaverine and putrescine were analysed in the same way as they are associated with decomposition in fish and may be a source of interference. A solution of these amines along with glutamine and tryptophan gave an average recovery of 109%, again indicating that some of these substances were not totally excluded from the butanol phase. However these interferences were greatly decreased by the extraction steps and should not interfere to any great extent in the food assays.

The fluorometric assay is based on the reaction of o-phthaldiadehyde with amines at a high pH. Upon acidification all complexes are dissociated with the exception of the acid stable fluorophore OPT-histamine complex. Therefore it should be possible to observe the total amine levels by eliminating the acidification step and comparing levels to an acidified sample having only histamine fluorophores. This result would again demonstrate whether any other amines are being extracted along with histamine in the procedure. It is apparent from Table 3.2 that there was a difference in levels of upto 3 ppm (0.3mg/100g) between the acidified and unacidified samples which is the amount of interference coming through the butanol extraction and complexing with the OPT.

A broad linear range (0-10 μg/ml) was achieved for histamine with fluorescence detection (Figure 3.1) and both cheese and tuna samples fell within this range following dilutions in the sample preparation stages.

Table 3.3 lists a range of amines and amino acids subjected to fluorescence detection after butanol extraction in comparison to histamine. Tyramine and the putrificative amines putrescine and cadaverine showed little or no fluorescence even at very high concentrations. The amino acid solution showed some activity but only at high concentrations (100 ppm) where its fluorescence corresponded to that created by 0.1

ppm histamine. Histidine exhibited about 10% fluorescence compared to histamine but after subjecting it to an ion-exchange column all such fluorescence was eliminated.

The results indicate that the assay is specific enough for histamine determination in food samples without major interference from other amines and that an ion-exchange column is useful in the cases of high histidine levels that need removing.

Histamine levels in fresh Irish cheeses produced were low in comparison to levels in the reported literature, ranging from 0.2 to 4.3mg/100g and proved to be non-hazardous for consumption in relation to recommended safe levels (50mg/100g). Separate studies would be needed to analyse tyramine levels in case of a 'cheese reaction'. The extraction of histamine from the cheese matrix was deemed to be satisfactory with an average 93.3% recovery of spiked histamine standards.

Upon putrefaction at refrigeration temperatures a rise in histamine levels would be expected to some extent due to microbial decarboxylase activity and this was observed in most cases with the exception of Brie and Cheddar from outlet 2. An explanation for the drop in levels may be the presence of an oxidase-producing fungus. Brie is a soft cheese that contains a mould, which is added for flavour during the processing stage. The mould is usually a *Penicillium* species and may have the capacity to produce amine oxidase, which would deaminate any histamine formed in the cheese. As Cheddar contains no such mould the fungus would have had to come from an external source. There was evidence of batch to batch variation in the samples from each of the two outlets. Histamine levels in cheddar varied between 0.4 and 4.3 mg/100g and histamine levels in Edam varied between 0.2 and 2.6 mg/100g. An investigation of the microbial population by isolating decarboxylase positive colonies on differential media would act as a further method to confirm the increase in histamine levels and identify its source.

From the study it can be concluded that histamine levels in Irish-produced cheeses are well within safety limits although, like all other cheeses, are prone to spoilage and histamine production.

Histamine levels in canned tuna from the two outlets ranged from 10 to 20mg/100g, a figure that is notably high when reported results indicate that a level of 20mg/100g is considered evidence of decomposition. The FDA hazard action level for histamine in tuna is 50 mg/100g of fish (Food and Drug Administration, 1998). This level is achieved in canned tuna (both brands) 96 hours after being opened and let putrefy at

room temperature. From figures 3.3 and 3.4 an increase in histamine levels can be seen. Both brands purchased from outlet 1 show similar trends with histamine increasing at a steady rate over time. The brands from outlet 2, show histamine remaining constant up to 24 hours followed by an increase and further levelling off after 72 hours. It must be noted that during the course of the study the brine, which normally acts as a preserving agent, was removed from the tuna samples thus allowing spoilage to occur more rapidly. The effect of storage temperatures is very noticeable from the comparison of the trends in figures 3.5 and 3.6 to the previous figures. Refrigeration temperatures slow down the rate of decomposition and this can be seen by the small increases in histamine levels. Temperature is critical for the growth of micro-organisms and cold incubation conditions are usually good inhibitors of growth. A net increase of 10 mg/100g occurs at 4°C storage compared to an increase of 20-30 mg/100g at room temperature storage. These results verify the critical need for cold storage temperatures of fish upon harvesting on board fishing vessels and also in prolonging their safe shelf life.

Recoveries of histamine from the fish flesh were very efficient with an average recovery of 106%.

The number of microbial species with histidine decarboxylase activity within the microflora population of a food sample is relatively small in size. A screening process involving the utilisation of the differential plating medium (Niven et al, 1981), would be required to distinguish producing from non-producing strains. For the purpose of the experiment three bacterial strains were screened for decarboxylase activity, a known producer, *Providencia retgerri* 865 obtained from the National Collections of Industrial and Marine Bacteria Limited (NCIMB) and two unknowns *Proteus valgaris* and *Escherichia coli* DH5α from DCU stocks.

Positive growth of colonies on the differential media was represented by purple colonies growing on a yellow media background indicating that histamine has been produced from the histidine substrate. This was achieved for the *Providencia retgerri* 865 strain at a dilution of 10^{-7}. No growth appeared on plates from the other two strains even at lesser dilutions (Table 3.8), while decreased dilutions for the *Providencia* strain resulted in a complete colour change of yellow to purple for the whole plate.

Once the histamine producing strain had been identified it was decided to investigate the level of histamine production over a time period by *Providencia retgerri* 865. The bacterial strain was grown on two types of media, ordinary LB broth and LB broth supplemented with histidine to act as a substrate for decarboxylase activity. A growth curve was constructed in order to observe the different phases of growth especially the exponential phase where maximum activity takes place. The lag phase proceeded for about 2.5 hours, which is the time it takes for the micro-organism to adapt to its surroundings. This was followed by exponential growth until 8 hours. This is the period of maximum activity and growth where the rich supply of nutrients in the media is utilised. Eventually the nutrients begin to be exhausted and a balance is achieved between growth and cell death where a plateau in growth is obtained called the stationary phase.

The bacteria were grown overnight in 5 mls of LB broth and transferred to two culture flasks containing LB broth and histidine supplemented LB broth at a dilution of 1/100. At regular intervals optical density measurements were taken to observe growth and also culture extracts were subjected to the fluorometric assay according to the procedure by Behling and Taylor, (1982). This involved the use of an ion exchange column to remove histidine and figure 3.7 shows the extreme effectiveness of the technique. Figure 3.8 clearly shows the production of histamine by *Providencia retgerri* 865 by decarboxylase activity on the histidine substrate as compared to the lack of production when the substrate is absent. The availability of histidine supplement in the medium did not influence growth rate.

A rapid increase in histamine levels occurs in the early hours of growth (0-6 hours) corresponding to exponential phase on the growth curve where maximum activity is expected with increased growth. Histamine production levels off once stationary phase is reached with a level of just over 200 ppm produced after 24 hours. This level corresponds to 1.8 μmoles/ml. Each of these growth stages can be seen from figure 3.9. A similar level was produced by *Citrobacter freundii* T3 after 24 hours at similar incubation temperatures with levels continuing to rise on further incubation. Tuna fish infusion broth was used as the medium for growth in this case and based on the weight of tuna fish used to prepare the broth, it was calculated that a histamine level of 2.5 μmoles/ml was equivalent to 50 mg/100g, which is the FDA Hazard action limit for histamine in tuna. (Behling and Taylor, 1982).

Although a different medium was used in our studies and may not be a true representation of conditions in tuna fish flesh, the species *Providencia retgerri* 865 was observed to produce less histamine than some of the major producers like *Klebsiella pneumoniae* and *Proteus morganii*. However like these bacteria it was capable of producing histamine in a short period of time which is of concern as although some fish may look unspoiled in appearance, they may contain significant levels of histamine.

Table 3.9 shows the ability of the enzyme diamine oxidase to successfully break down histamine. In this study histamine was extracted from tuna samples according to the method described by Taylor et al. (1978b). Diamine oxidase (20mg/ml) was added to the extracted histamine and incubated at 37°C for various time periods (5 min, 15 min and overnight). A clear reduction in histamine levels resulted over time with the complete elimination of histamine after overnight incubation. Increasing the concentration of the commercial enzyme or using a more purified form of the enzyme would result in decreasing the time to totally eliminate the histamine levels. This has significant implications in the development of enzyme based sensors for histamine detection (Bouvrette *et al.* 1997). Obviously an enzyme that can breakdown histamine completely in the shortest time in order to detect one of the breakdown products is essential to the detection system.

Chapter 4
HPLC Analysis

4.1 Introduction

High performance liquid chromatography has been a very popular method for the analysis of biogenic amines (Gouygou *et al.* 1987, Vallé and Malle, 1996, Vallé and Gloria, 1997) and the method of detection has varied from UV to fluorescent detectors. Biogenic amines with the exception of the aromatic amines, do not possess any chromophore or fluorophore, which means that they have to undergo derivitisation to render them active.

Two methods of HPLC analysis were employed for the determination of histamine. The first method involves benzoylating the amino acid groups on the amines with benzoyl chloride followed by separation in a methanol:water gradient coupled with UV detection. (Yen and Hsieh, 1991). However due to the failure of this method to successfully detect histamine a second method according to Wei *et al.* (1995) was investigated. This method was based on derivitisation with dansyl chloride, separation in acetonitrile: methanol:water and UV detection. It is capable of analysing seven biogenic amines simultaneously with good sensitivity and selectivity.

Once the method had been successfully adopted and optimised the ability to identify and quantify histamine in both standards and food samples was examined. The extent of the correlation between the fluorimetric method and the HPLC method for analysis of histamine in food samples was examined.

4.2 Results

The results from derivitisation with benzoyl chloride were deemed to be inconclusive and shall only be discussed (section 4.4 below).

After derivitisation with dansyl chloride histamine standards were separated on a C_{18} column and analysed by UV detection. A number of peaks emerged and based on the linear increase in peak area corresponding to an increase in histamine concentration the peak at 11.5 minutes was selected as a possible candidate peak for histamine. The peak was also observed in decomposed tuna samples. The sensitivity of the detection method was established by repeated analysis of a lower standard range. Eventually a range of 0.066 to 0.33µg/200µl was determined (Figure 4.3) which corresponded to a range of 8 to 40 mg/100g in food samples after allowing for the dilutions in the extraction procedure (Taylor *et al.* 1978b).

Figure 4.1 Standard Curve for Benzoylated Histamine

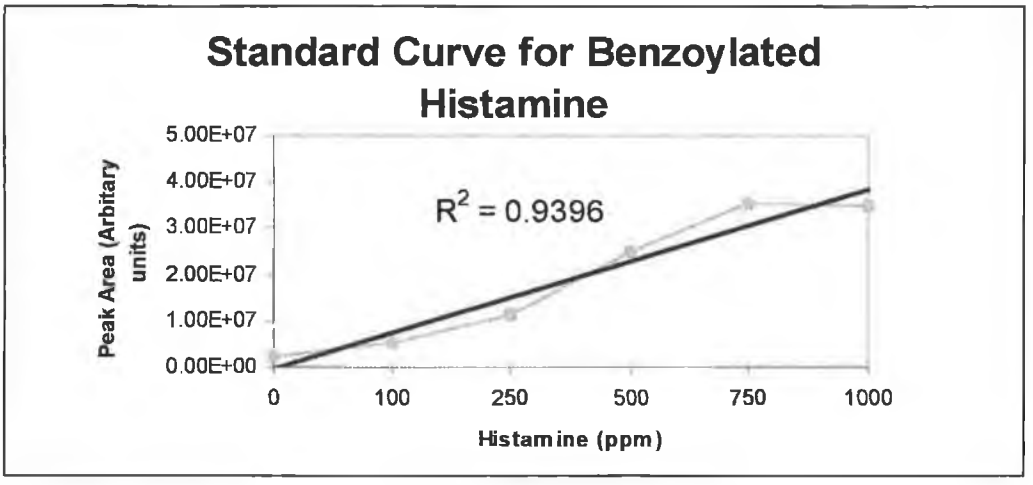


Figure 4.2 Chromatogram of Benzoylated Histamine Standard

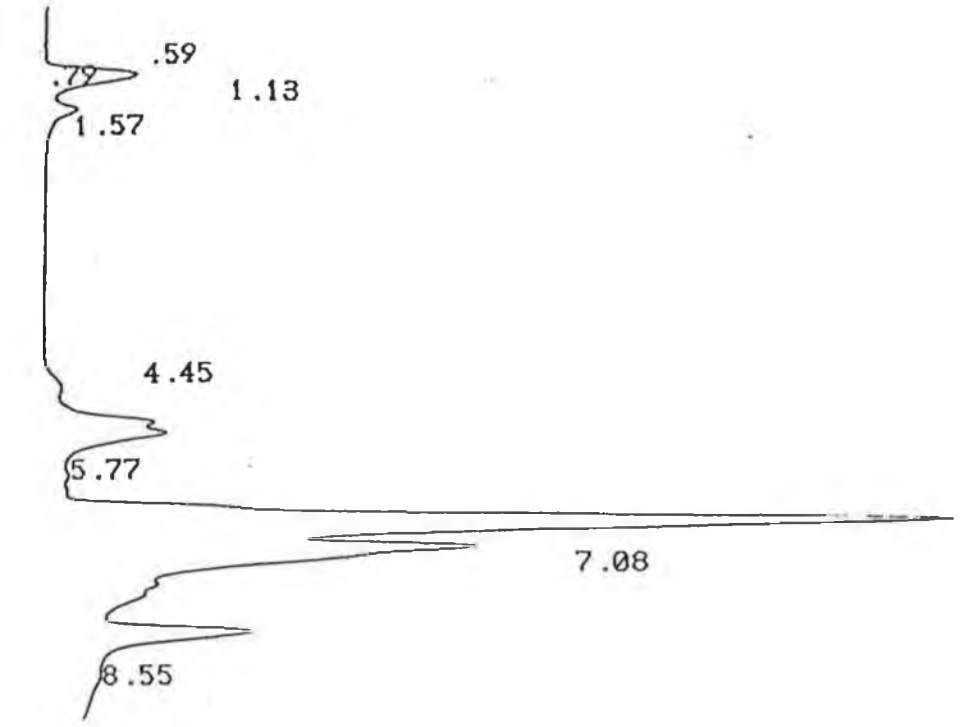


Figure 4.3 Standard Curve for Dansylated Histamine by HPLC determination

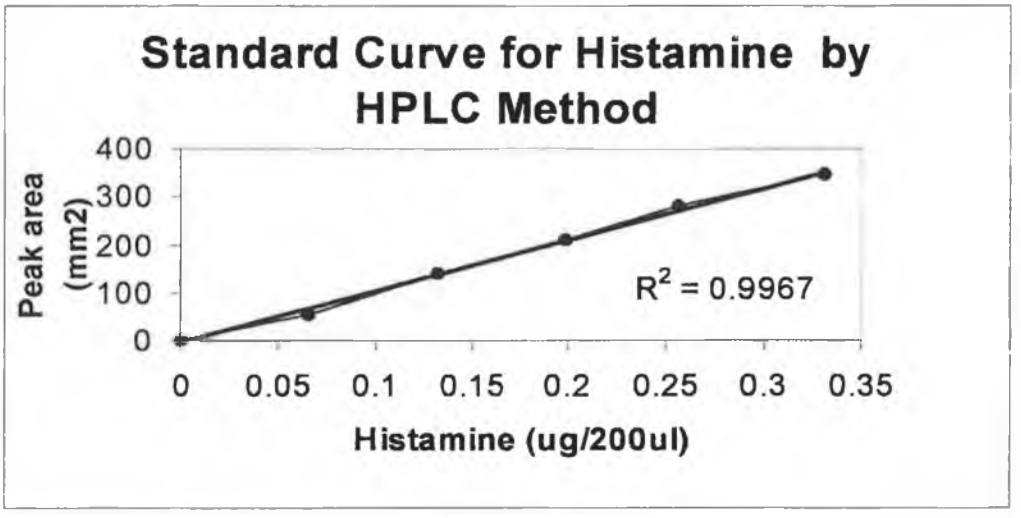
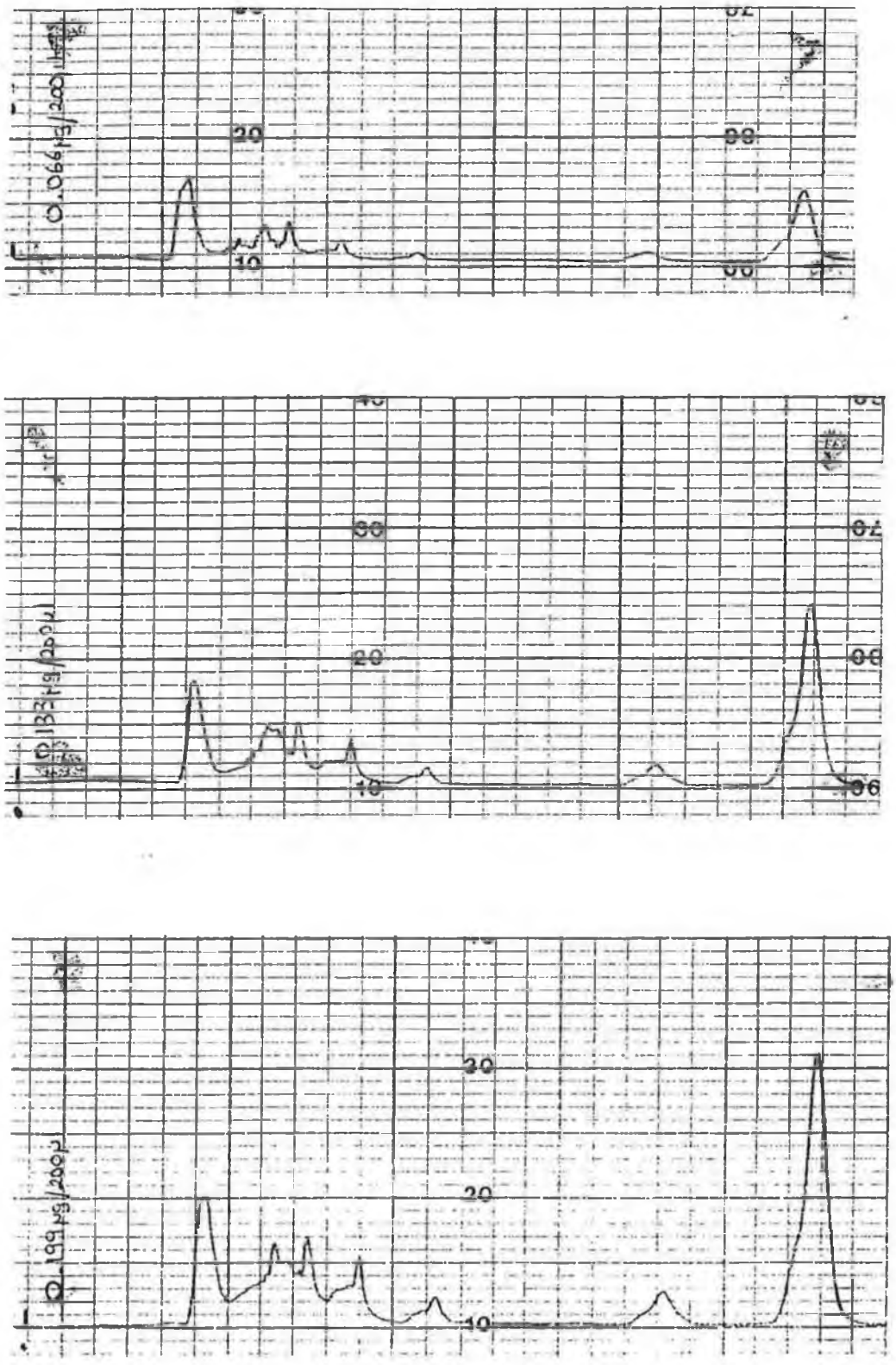
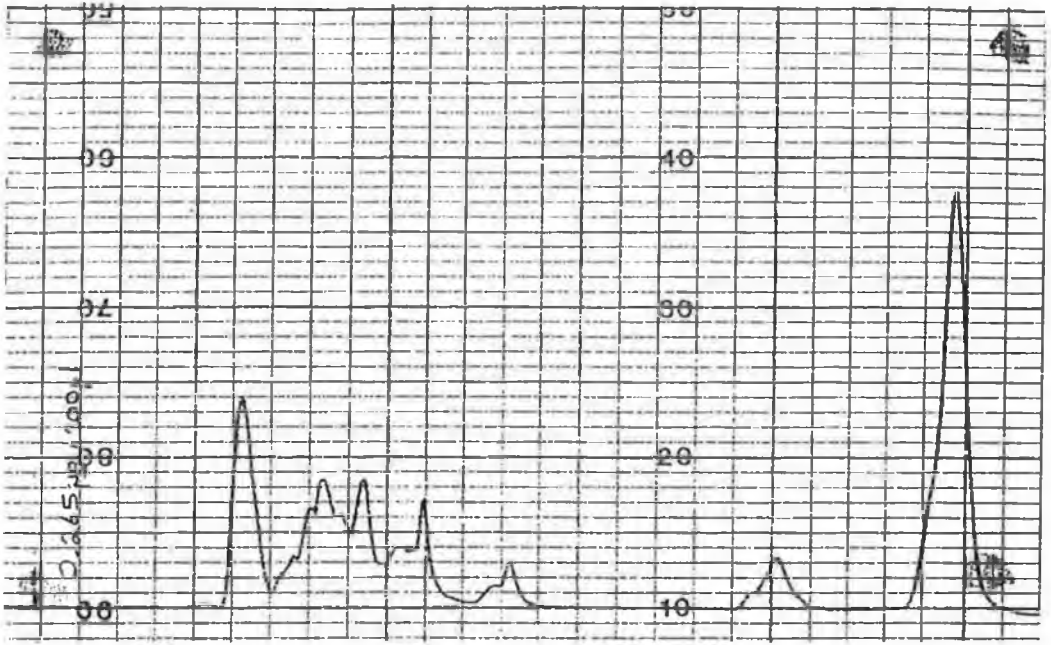
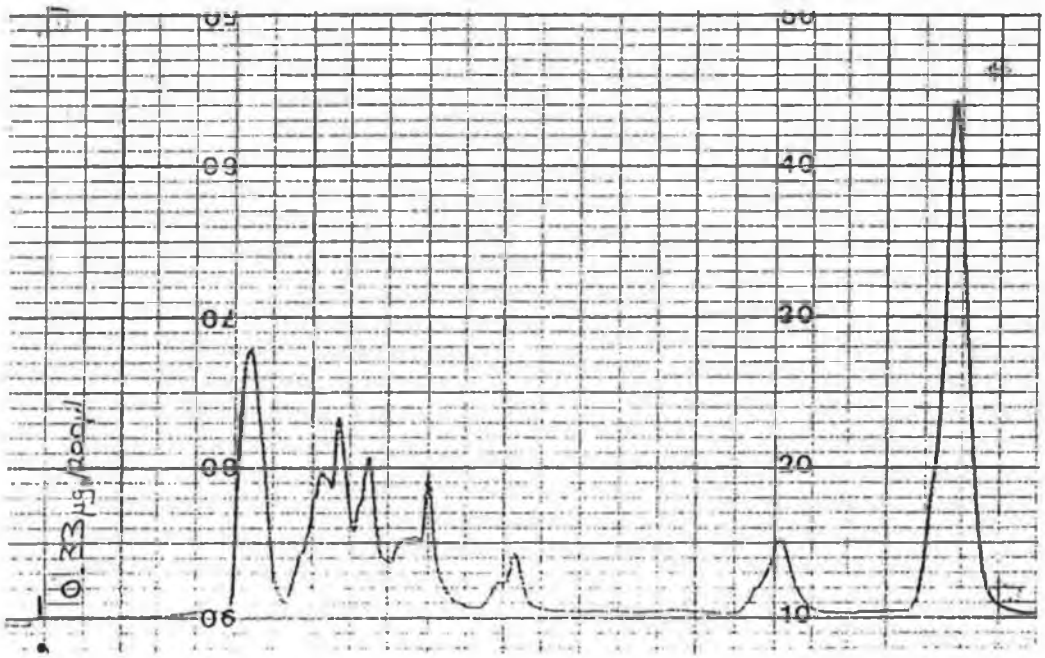


Figure 4.4 Chromatograms for HPLC Dansylated Histamine Standards.




40
30
20
10


50
40
30
20
10
0.23 µg/ml

4.2.1 Verification of Histamine Peak

In order to verify that the peak actually represents histamine a number of experiments were undertaken. A tuna sample was spiked with a histamine standard (200ppm), and brought through the extraction procedure and histamine was determined by HPLC analysis. In a similar way the effect of diamine oxidase (10mg) on this peak was investigated.

Figure 4.5 Histamine Spiked Tuna Sample

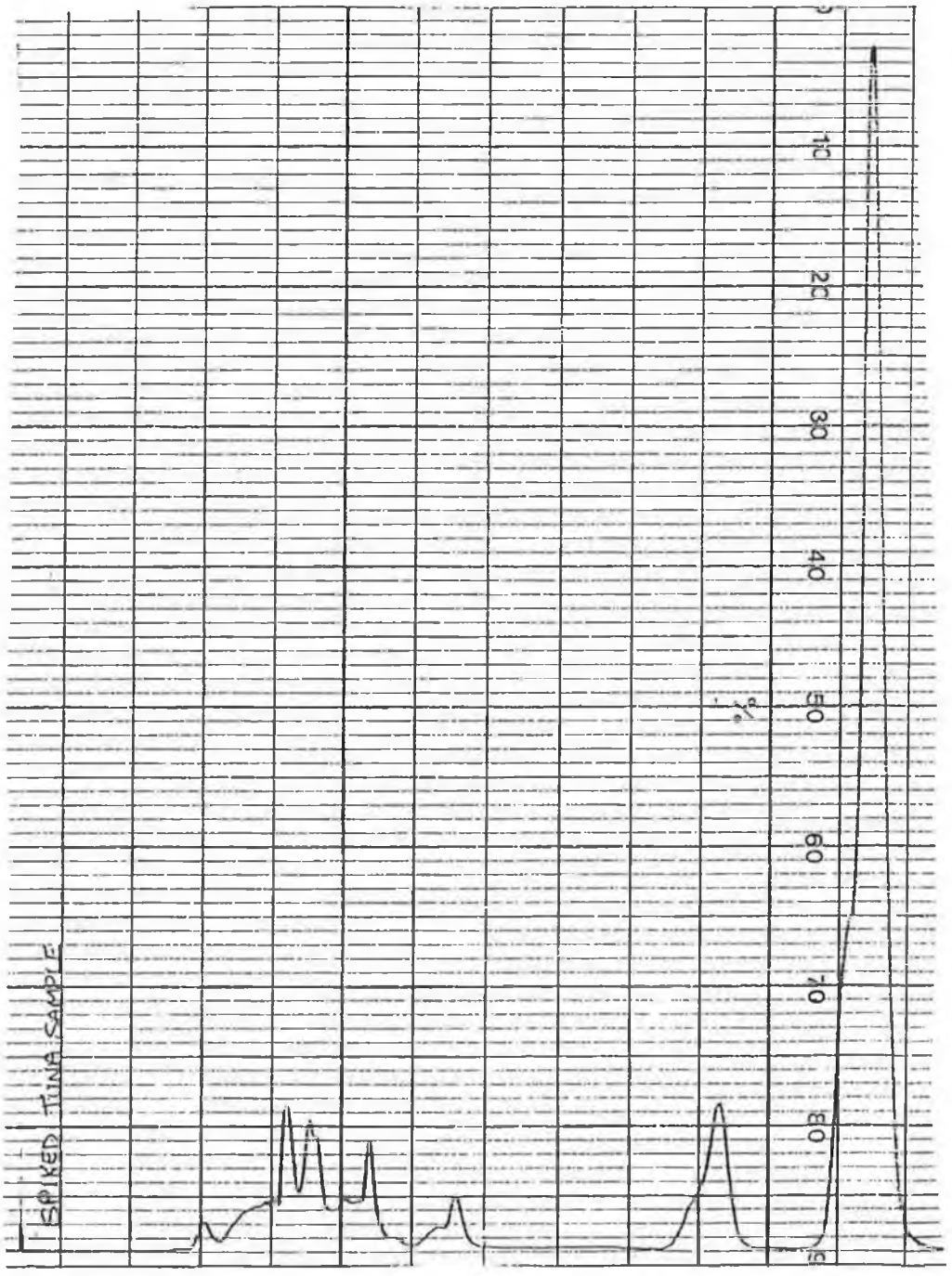
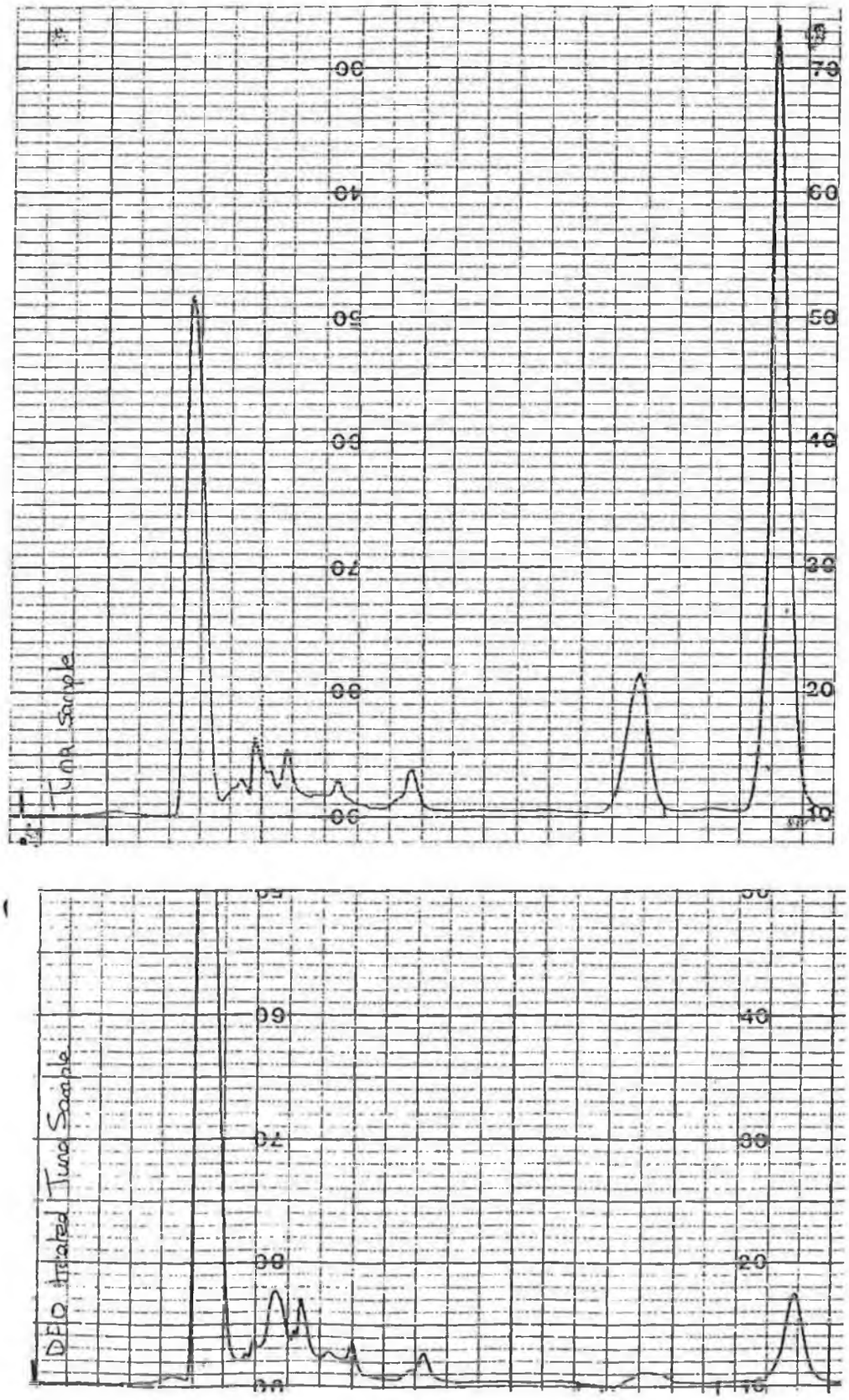


Figure 4.6 Chromatogram showing histamine peak in (a) tuna and (b) tuna treated with diamine oxidase.



4.2.2 Correlation of HPLC analysis with the Fluorometric Assay.

A tuna sample was divided into two portions, one part spiked with histamine (200mg) and the other left untouched. Analysis on the individual portions was to be carried out both by the fluorometric assay and by HPLC analysis. Analysis was carried out in duplicate. Table 4.1 shows the comparative results.

Table 4.1 Comparison of Histamine levels in Tuna by HPLC and Fluorometric analysis

Sample	Histamine (mg/100g) Fluorometric Assay	Histamine (mg/100g) HPLC Analysis
*Tuna 1	90.38	89.88
*Tuna 2	91.22	96.04
Spiked Tuna*	263.46	121.04

* Decomposed canned tuna

4.4 Discussion

The chromatogram from the analysis of histamine derivitised with benzoyl chloride showed far too many peaks so it was decided to try to improve the extraction technique in order to achieve a cleaner chromatogram. Derivitisation was allowed to proceed for 1 hour instead of twenty minutes and the aqueous phase was extracted 2 x 2 times with diethyl ether. Three main peaks were now observed, 6.08, 7.08 and 7.39 minutes. A blank was run and the only significant peak occurred at 7.4 minutes indicating that this is not an analyte peak but probably due to benzoyl chloride.

The most significant peak resulting from histamine standard injections occurred at 7.01 minutes and a standard curve (Figure 4.1) indicated that this peak was that of histamine with a R^2 value of 0.9396 which is indicative of linearity. It was thought that the linear response might have been improved by using an alternative to diethyl ether in the extraction, as volatile losses were thought to have been a potential source of error. Hexane was tried as an alternative but

proved to be a very poor extracting solvent with respect to the benzoylated histamine. Butanol was considered but owing to its high boiling point (117°C) it would not have been compatible with the evaporation step. As no substitute was found, diethyl ether was used for further investigations. It was found that many of the interfering peaks could not be successfully removed so it was decided that it would be more advantageous to achieve good separation in the presence of these interferences than to eliminate them from the analysis altogether. The poor separation of the histamine peak at 7.08 minutes was slightly improved by making the mobile phase more polar but two shoulders could still be observed on the analyte peak (Figure 4.2). Increasing the polarity of the mobile phase further did not improve the separation. The pH of the mobile phase was adjusted to pH 4 and the gradient run was changed such that the program proceeded linearly to 70:30, methanol: water. This improved separation but baseline resolution was not being observed. The mobile phase was increased to 65:30:5, methanol:water:acetonitrile and the pH dropped further but no improvement in resolution occurred and peak tailing was becoming evident. It was decided that baseline resolution would be difficult to achieve. The method could possibly be used if peak heights were used, but problems may be encountered whereby the interference peaks may swamp the signal at the retention time of histamine. This could result in a very high detection limit. Quantitation would be difficult based on peak areas owing to the poor separation achieved.

Analysis of dansylated amines proved to be less complicated and more accurate, but analysis time was prolonged due to the overnight derivitisation procedure. Chromatograms were clearer with relatively few peaks observed. Figure 4.4 shows the range of peaks obtained from the analysis of histamine standards and the peak at 11.5 minutes was postulated to be that of histamine. From the injection of a range of histamine concentrations a standard curve was constructed based on a retention time of 11.5 minutes and a linear response was found with a R^2 value of 0.9967. This was not conclusive evidence that the peak at 11.5 minutes was that of histamine, as an increase in peak area was observed with the peak at 9.5 minutes. Spiking a tuna sample with a known amount of histamine would lead to an increase in peak area in the identifying peak. From Figure 4.5 a notable increase in height/area is seen in the peak eluting at 11.5

minutes while the peak at 9.0 minutes remains unchanged. Further confirmation is achieved through treating a tuna sample with the enzyme diamine oxidase, which due to its activity on histamine results in a corresponding decrease in peak height at 11.5 minutes. (Figure 4.6). It is interesting to note that a decrease in peak height is also associated with the 9.0-minute peak, that aids in its identity. As it is subject to deamination by diamine oxidase, it therefore must be a diamine, which has passed into the organic layer during the extraction procedure. The figures in Tables 3.1 and 3.3 that identify the possibility of other amines or amino acids being selected to some degree by the extraction procedure suggest that the identity of the diamine may be cadaverine or putrescine. However the peak is also present in chromatograms of histamine standards which exclude the possibility of other diamines being present unless contamination of the stock histamine has occurred which is unlikely. A new stock of histamine was analysed with the same resulting chromatograms. The peak therefore is likely to be due to some component in the extraction or derivitisation procedure. A blank was derivitised according to procedure and the resulting chromatogram showed a large peak at 2.5 minutes, this is either the dansyl chloride itself or 5-dimethylaminonaphthalene-1-sulfonic acid, which is a hydrolysis product of dansyl chloride. (Hui and Taylor, 1983).

Table 4.1 compares histamine levels in tuna as determined by the two methods. The methods compare favourably with a 0.56% error between methods for sample 1 and a 5.02% error between methods for sample 2. However, although the fluorometric assay confirmed the spike with 86.5% of the 200mg detected the HPLC method failed to do so to such an extent. This may be as a result of the spike being much greater than the linear range which measures from 0-4.2 mg/10g i.e.: 0-4.2mg in the 100ml methanol extraction step where the spike is added. This overloading would account for the non-detection of the spike.

Both methods work well in this study of histamine but the HPLC method would prove to be more beneficial elsewhere as it has the ability to simultaneously detect several biogenic amines. In terms of analysis time the fluorometric assay has a distinct advantage over the HPLC method which requires overnight derivitisation. However the assay does not provide rapid analysis of analyte or high sample throughput needed in the food industry.

Chapter 5

Histamine Detection by Novel Methods

5.1 Introduction

Nitrophenylazophenol cailx[4]arene belongs to a group of compounds called chromogenic calixarenes that have the property of upon complexation with lithium and to a lesser extent sodium, in the presence of a base undergo a change in absorption. A colour change of yellow to red corresponding to a wavelength shift from 380 to 520nm. Colour generation arises from the deprotonation of the acidic chromophore (-COH) attached near the ligand polar cavity. A proton acceptor is required for the colour formation, which cannot proceed just by the formation of the metal ligand complex itself.

Equation 1: L-COH + M^+ ⇔ LM^+ + COH

Equation 2: L-COH + M^+ + B ⇔ LM^+ + CO^- + BH^+

No deprotonation occurs in the absence of a base (equation 1) while equation 2 shows deprotonation of the acidic chromophore. (McCarrick *et al.* 1994).

Figure 5.1 The structure of the chromogenic ionophore, nitrophenylazophenol calix[4]arene used in this study.

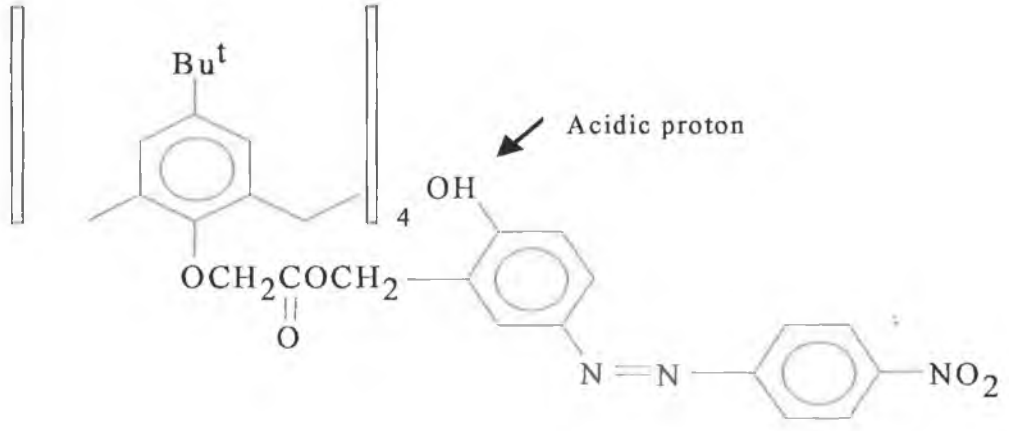
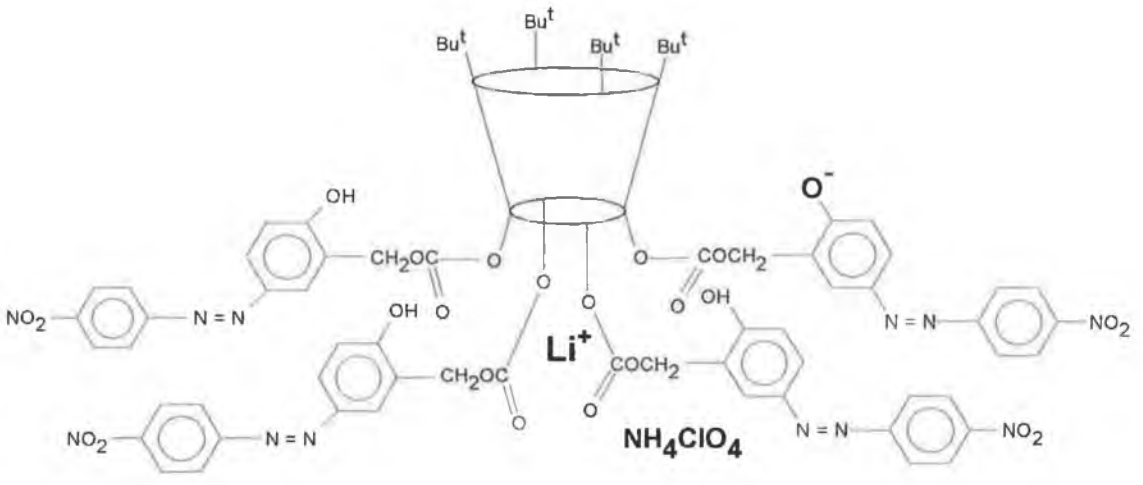


Figure 5.2 Representation of the deprotonation of lithium nitrophenylazophenol calix[4]arene complex as a result of the basic nature of ammonia gas.



By complexing the metal with the ligand it is now possible to determine the presence of a base by observing the generation of a colour change (equation 2), which is the procedure by which McCarrick *et al* used to detect trimethylamine (TMA).

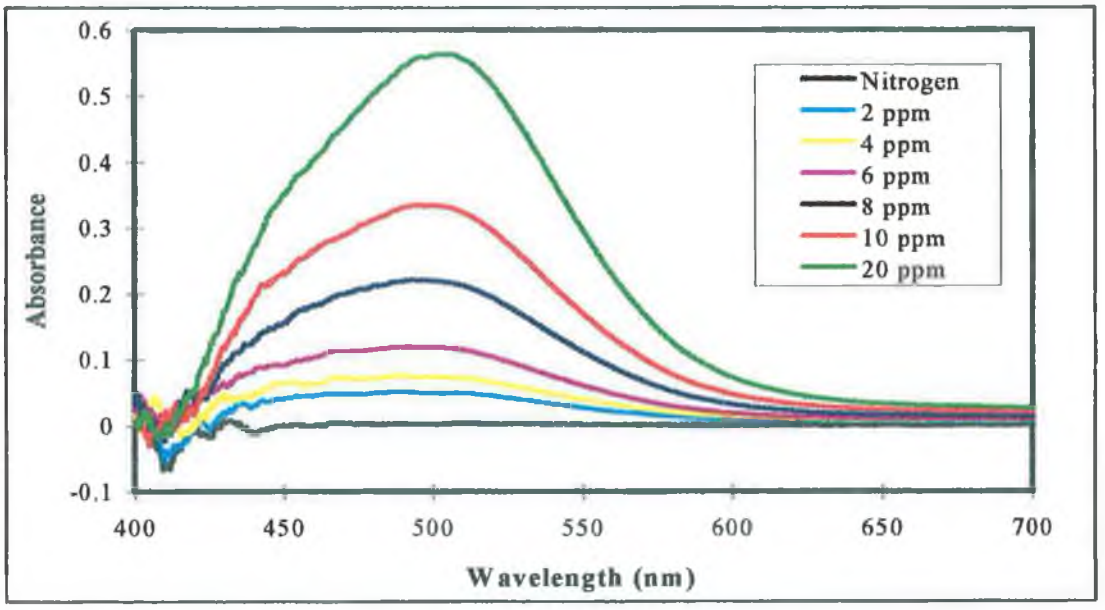
TMA is a degradation product of bacteria in marine fish after death and is often used as an indicator of spoilage in fish. Previous methods of detection ranged from colourimetric methods to gas chromatograpic analysis involving either elaborate handling or the need for instrumentation. The use of calixarenes as a non-instrumental indicator system that responds rapidly to volatile amines would benefit the food industry.

The calixarene complexes were immobilised onto filter discs and a colour change was generated on exposure to the gaseous amine. The detection limit ranged from 0.02 to 30 ppm and the reaction time was under 2 minutes. (McCarrick *et al*. 1994).

The ability of this compound to detect gaseous TMA led Grady *et al* to investigate the effect of ammonia gas (another volatile base) on a poly (vinyl chloride), PVC, membrane which incorporated the nitrophenylazophenol calix[4]arene ligand. The sensor configuration employed in this study was based on evanescent wave

interactions between guided radiation in an optical fibre and the calixarene immobilised on the core of the optical fibre. The characterisation system consisted of mass flow controllers which allowed known concentrations of ammonia to be passed over the optical fibre, a white light source and an Ocean Optics Spectrometer to collect the light from the fibre and disperse it to detectors.

Figure 5.3 The absorbance spectra of the PVC coated fibre with the calixarene for the addition of different ammonia concentrations.



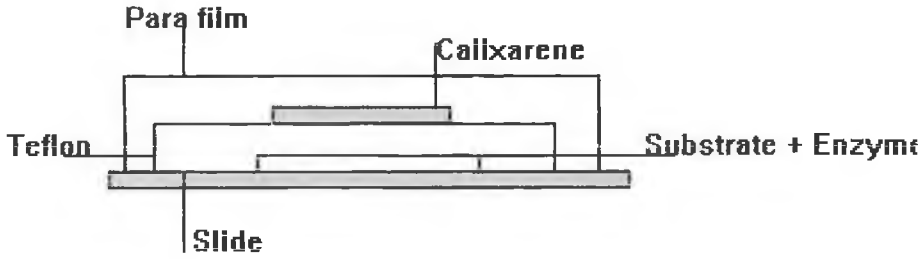
It was possible to alter the sensitivity of the calixarene membrane to ammonia by varying the lithium/ligand mole ration or by changing the metal ion from lithium to potassium. The sensor was able to detect the ammonia gas at very low levels (10 ppm). (Grady *et al.* 1997).

The ability of this system to detect ammonia gas at low levels prompted an investigation of its suitability in detecting endogenous diamines and monoamines in food and those produced by micro-organisms. An amine oxidase based system coupled to the ammonia-sensing sensor would provide a simple and efficient method for the detection of amines.

5.2 Results

A crude sensor based on the amine oxidase-calixarene detection system was designed. An ammonia source (the reaction of diamine oxidase and a substrate or pure ammonia) was enclosed by a gas permeable teflon seal and the calixarene complex was immobilised onto a filter disc and placed the other side of the teflon membrane. The whole system was enclosed with non-permeable parafilm.

Figure 5.4 Schematic diagram of the detection system.



A number of different conditions were investigated in order to achieve a colour change by the calixarene, these included pH, temperature, substrate and enzyme concentrations and the presence/absence of moisture.

Table 5.1 Conditions for colour generation in calixarene.

pH	Moisture	Heat	Time	Colour
8.4	Yes	No	-	yellow
8.4	No	No	-	yellow
10.3	No	No	-	yellow
10.3	Yes	No	15 mins	Red
10.3	No	Yes	45 mins	Orange
10.3	Yes	Yes	15 mins	Red

Substrate: 100µl of 1mM histamine dihydrochloride

Enzyme: 40µl 5mg diamine oxidase = 0.056 Units

Table 5.2 Controls for calixarene colour generation.

Control	Colour
Aqueous ammonia	Red
Histamine only	Yellow
Diamine oxidase only	Yellow

Table 5.3 Level of histamine detectable by calixarene.

Histamine	Time	Colour
0.5 mM	12 hours	Orange
0.25 mM	48 hours	Red/orange
0.1 mM	48 hours	Orange
0.05 mM	48 hours	Orange
0.01 mM	-	Yellow

Figure 5.5 Colour Generation on Calixarene doped filter discs.

A. Gaseous base absent

B. Gaseous base present

Table 5.4 Colour generation from a positive histamine-producing bacterial species with the calixarene-amine oxidase system.

Substrate and Enzyme	Colour
Providencia retgerri 865 + Diamine oxidase	Red
Providencia retgerri 865 - Diamine oxidase	Red

Histamine was extracted from cheese samples according to Taylor *et al.* (1978b) and treated with and without diamine oxidase. The samples were applied to the crude sensor as shown in figure 5.4. In most samples the red colour was generated regardless of diamine oxidase outlining the difficulties posed by background ammonia. However in one cheese sample colour was only seen after the addition of the enzyme which indicated that the system detected ammonia as a result of the oxidation of histamine in the food sample.

5.3 Discussion

The reaction of an amine with an oxidase enzyme yields a product of ammonia and in this case histamine is reacted with diamine oxidase. The chromogenic calixarene, which has been immobilised onto a filter paper disc, undergoes a colour change from yellow to red on exposure of ammonia gas. The reaction between enzyme and substrate produces ammonia but in the aqueous phase and so in order to cross the teflon barrier the ammonia must be encouraged to enter into the gaseous state.
Ammonia was encouraged to cross the membrane in a number of ways. Firstly, knowing that ammonia is a volatile base, heat was applied, then moisture was added to the calixarene disc on the opposite side of the reaction to force ammonia across and also by increasing the pH the NH_4 molecule is deprotonated forming NH_3 gas.

Firstly no colour change was observed at a pH of 8.4 even at 44°C and the presence of moisture. The pH was raised to 10.3 for the remainder of the experiment, as it proved

successful in producing a colour change. The substrate used was100ul of 1mM histamine and the enzyme quantity was (40ul) 5mg.

The trial was carried out at 44°C at pH 10.3 and using the above enzyme and substrate values but with no added moisture. A slight colour change was observed after 60 minutes.

The best reaction occurred at a pH of 10.3, with added moisture and no heat was needed. A red tinge was observed after about 15 minutes.

Table 5.2 lists the controls used in order to verify that the calixarene could detect ammonia and secondly that the substrate-enzyme reaction was the unique ammonia source.

Both the histamine and enzyme on their own could not produce a colour change.

The sensitivity of the system was investigated by finding the lower limit of detection for histamine. With 0.5 mM histamine as substrate a red/orange tinge was observed around the edges of the calixarene disc after 60 minutes. After 12 hours an orange tinge was seen over 90% of the disc and a fully developed red colour produced after 24 hours. At levels of 0.05 mM histamine an orange colour developed after 48 hours but very little colour change was observed at levels of 10μM.

Lower limits of detection can be obtained and hence increasing sensitivity by altering the lithium-ligand mole ratio. (Grady *et al.* 1997).

Provedencia retgerri 865, a positive histamine producing strain identified in Chapter 3 with a differential media was grown in histidine supplemented LB broth and a culture fluid extract was obtained according to Behling, A.R. *et al.* (1982).

This fluid was examined using the crude calixarene-amine oxidase sensor. A colour change occurred both in the presence and absence of diamine oxidase, which meant that a gaseous base was been generated from another source. The culture was examined for urease activity by streaking out a loopful of the culture on a urease slope. The slope, yellow in its uncontaminated form turned to red indicating that the culture was positive for urease activity. Two controls, *Proteus vulgaris* and *Escherichia coli* were also streaked onto the slopes and a negative result was observed with the slopes remaining yellow. Urease is an enzyme present in microorganisms which hydrolysis urea, producing ammonia. This would explain the change in colour of the calixarene in the absence of diamine oxidase in the culture fluid extract. Screening for a positive histamine producing bacterial species with negative urease

activity would yield an ideal microorganism to test with the sensor system. However the floral content in food is not of a homogenous nature and may include several microorganisms that possess this urease activity. This background level of ammonia present in the food matrix represents a difficulty in the detection of ammonia from amine oxidation.

The solution to the problem is to use a differential measurement of histamine in food, firstly without an enzyme added which would represent any background ammonia and secondly after addition of an oxidase, so only ammonia produced as a result of the enzyme activity on amines present would be detected. The experiment described here includes the lengthy extraction procedure in isolating histamine so this would not be a solution to a rapid and efficient method of analysis. For an ideal detection system the enzyme would have to be applied directly to a food sample and the evolution of ammonia monitored from a difference measurement.The system also would have to be in an enclosed environment as the calixarene is very sensitive and may be influenced by contamination, a problem experienced in one of the laboratories.

It was found that the specific activity of commercial enzyme used in the study was very low, 0.14 units/mg solid (1 μmol putrescine oxidised/hour at 37°C). Using the more conventional unit definition (μmol/min), the specific activity would be 0.0023 units/mg solid. Male *et al.* (1996) cited that for the development of an amperometric biosensor for diamines with a satisfactory response, sensitivity and detection limit, the enzyme must have a specific activity of about 1 unit/mg solid. This necessitates the need for purification of the enzyme from a source such as porcine kidney or the fungus *Aspergillus niger*. Already in existence are several biosensors capable of measuring histamine either by detecting the product of an amine oxidase reaction, hydrogen peroxide (Male *et al.* 1996), measuring the rate of oxygen consumption by the same enzyme reaction (Ohashi *et al.* 1994) and detecting gaseous trimethylamine directly using the nitrophenylazophenzl calix[4]arene (McCarrick *et al.* 1994).

The detection system used in this study is based on the same calixarene used by McCarrick *et al.* (1994) but is used to detect another product of the amine oxidase reaction with biogenic amines, ammonia. The sensitivity of the calixarene offers great potential in a detection system and with more investigations may be eventually used for histamine detection in the food industry.

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