A chemical method for the investigation of chemical changes in fish during smoking

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A CHEMICAL METHOD FOR THE INVESTIGATION
OF CHEMICAL CHANGES IN FISH DURING SMOKING

by

Suet Lan Tang B.Sc.

A Master's Thesis submitted in partial fulfilment of the requirements
for the award of a degree of Master of Science of the Loughborough
University of Technology.

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Abstract

This report surveys the literature on the effects of smoking on the nutritive value of flesh foods and the chemical changes associated with such processing methods. The development of a dye binding method for use in assessing protein damage in smoked Coley (Pollachius virens) fillets is described.

The use of blocking reagents with dye binding permitting estimation of lysine content is also described and the results so determined are compared with values for lysine obtained by the use of fluoro dinitrobenzene. The possibility of interference from smoke components in the dye binding and the fluoro dinitrobenzene methods was also examined.
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1.0.0 INTRODUCTION

It is now realised that the quality of a food protein depends on its amino acid availability and digestibility. An extensive range of methods is now available for amino acids analysis of proteins and total protein of food and feeding-stuffs. The choice of a suitable method depends on a number of factors, basically, on the protein to be determined, whether material is high or low in protein and the nature of the other substances present such as phenolic compounds in smoked products, cellulose and starch in feeding stuffs.

Procedure either determine the reaction of the intact protein or the ammonia into which the protein has been either wholly or partially converted. Another important factor is the reproducibility and precision of the method and the number of samples that could be analysed per day. Rapid and accurate determinations of protein quality is paramount while the food and feeding stuff are in the relatively fresh condition, if they are used as reference standards in a good quality control system.

Fish is an important source of protein particularly the essential amino acid lysine, in human and animal nutrition. While the application of heat and smoke is to preserve the fish flesh, heating, drying and reactions of smoke components with amino acids, either by "Maillard" type reaction in which the amino acid groups interact with the carbonyl groups of reducing aldehydes of sugars (Lea and Hannan 1950; Miller et al 1965); fats degradation products (Kwon et al 1965) and smoke phenolic, aldehydes and acidic components (Dvorak and Vognarova 1965; and Ruiter 1970) or the protein -
protein type damage (Bjarnason and Carpenter 1970) render the amino acids involved (particularly methionine and lysine) nutritionally unavailable. The concept of "available" as distinct from "total" amino acids present in a protein is used to differentiate between amino acids, modified or denatured during processing, with the consequent loss of nutritive value, and those amino acids which remain of nutritional use to the metabolic processes. The lysine which has had its $\varepsilon$-amino groups bound was said to have no nutritive value as these groups were not susceptible to enzymatic hydrolysis (Lea and Hannan 1950; Henry and Kon 1950; Buraczewski, Buraczewska and Ford 1967).

Extensive studies have been made on the chemical and nutritional changes in fish meal (Lea, Parr and Carpenter 1958, 1960, 1962) and dried fish products (Tarr 1954; Carpenter et al 1957; Jones 1962) but there is little information on the lysine levels of smoked-temperate fish in a Torry kiln, similar to that of a commercial products. Although there is abundant information on the dye binding of fish meals (Bunyan and Price 1960; Moran, Jensen and McGinnis 1963; Hurrell and Carpenter 1975; Jones 1974) and freeze-dried cod (Jones 1974), there is little or no information on the dye binding of smoked fish.

The objective here is to determine the dye binding capacity of smoked and the control unsmoked fillets of Coley and to find the Dye Binding Difference (DBD) or available lysine values by suitable blocking reagents and compare the available lysine obtained by DBD with those of 1-fluoro-2,4-dinitrobenzene (FDNB)-reactive lysine values.
LITERATURE REVIEW

Methods of smoking and relation to deposition of smoke on fish

Before the effects of smoke chemical reactions on fish proteins could be evaluated, a knowledge of the composition of smoke, reaction of smoke components on surface proteins, degree of smoke penetration and a review of the chemistry and technology of smoking and the methods of measuring lysine would be useful.

Traditional methods of smoking over woodfire usually have high wastage of droppers and there is no control on the combustion parameters, hence no control on the degree of smokiness (i.e. amount of smoke produced) or the temperature during the smoking process. Smoke is not uniformly distributed, resulting in some fish virtually unsmoked and undried in addition to the smuts and moulds deposited on the products.

The Torry kiln is now widely used by industries to produce good quality, uniformly smoked fish such as kippers, roes and smoked salmon. In the Torry mini-kiln, smoke is produced externally in three-tier fire boxes, it is drawn into the kiln and circulated by an electrically-operated fan. Fresh air can also be sucked into the kiln by opening the adjustable air inlet flap and the amount of wet smoky air displaced up the chimney is adjusted by a metal plate across the chimney. The temperature of the smoke is maintained by thermostatically controlled electric heaters of 1 to 3 Kw. The electric heaters are essential, because as the smoke passes over the fish, it is cooled by the process of evaporation of water from the fish.

The much larger 50 stones and 120 stones Torry kilns have
adjustable splitters or vertical partitions, aerofoils and diffuser walls for more uniform distribution of smoke and air throughout the large kiln with air velocity of 600-700 ft/min in the centre of the kiln. The large kilns also have a smoke by-pass damper adjustable in 2 positions, "open to chimney" position for smoke to be led out of kiln when the fans are stopped and dampers of the fire boxes are closed, when the positions of the trolleys of fish are interchanged during half-time. The "open to kiln" position is to recirculate the smoke. There is also a central heater battery controlled by a thermostat, which operates on 2 switches of 6 Kw and 12 Kw.

It is important to note that the more air allowed into the kiln, the more smoke is allowed out, the faster the drying process and the more dilute the smoke, hence less smoke deposition is obtained (Burgess and Bannerman 1963). Hence the proportion of smoke and air is important in the smoking process. The rate of deposition of smoke on fish have been found to increase proportionally with the optical density of the smoke (Tilgner 1958), temperature (Tilgner 1958) and velocity of smoke in the kiln (Linton and French 1945). At high humidity, the wet fish surface and interstitial water absorb smoke more rapidly than at low humidity or when the fish flesh dries. Foster and Simpson (1961) have suggested that most of the smoke is deposited or absorbed by the surface and interstitial water of the fish muscle and they (Foster, Simpson and Campbell 1961) have shown that the rate of deposition of smoke phenolic compounds on pre-dried fish is only 5% of that deposited on the wet fish surface; that vapour absorption seemed to be the main mode of smoke deposition and that the disperse particulate phase contribute a negligible amount of steam-non-volatile phenols.
Since the absorption of invisible smoke vapour components by surface and interstitial water of fish and meat is proportional to the mass concentration of that smoke component in the vapour phase, changes in the vapour phase, such as by raising the temperature, would result in changes in the composition of the smoke deposits.

At the very high temperature of combustion, the degradation products of wood are in the gaseous form, and as they mix with the air, they cool and the less volatile components condense to form the disperse liquid or particulate phase (i.e. the visible particles of smoke in which the more volatile compounds tend to dissolve.) The two phases thus form a gas-liquid partition system in which the different smoke components are partitioned between the vapour and particulate phases according to the Nersnt's law. Since the surface area of the particulate phase is large relative to its volume, equilibria are very rapidly attained.

Foster, Simpson and Campbell (1961) have also shown that when the wood smoke is diluted by air as in the kiln, the concentration of the more volatile phenols fall to a greater extent than that of the non-volatile phenols. Hence there must be a transfer of smoke components from the particulate or liquid phase to the vapour phase showing that a dynamic state of equilibrium exist. Within the temperature range of 30 to 80°C, absorption of smoke vapours is independent of temperature while deposition or absorption of the non-volatile phenols increase with increase in temperature, as the partition coefficients are also increased resulting in a transfer of smoke non-volatile components from the disperse liquid to the vapour phase.

Since the particulate phase contain small amounts of volatile
components and a large amount of non-volatile components, the composition of the vapour and particulate phase is continuously being changed as components are absorbed and reactions take place in the fish flesh during the smoking process. Since the moisture content of wood sawdust affects the smoke generation temperature, it could also affect the composition of the smoke (Draudt 1963).

Hence the disperse liquid phase acts as a reservoir of volatile and non-volatile smoke components and when the vapour-disperse liquid equilibrium is disturbed, the disperse liquid phase release part of its non-volatile components to the gas phase as when the smoke components are absorbed or when there is dilution of smoke by air or when the smoking temperature is raised.
2.2.0 Wood Smoke Composition

To understand the mechanism of flavour and colour development, epsilon-amino lysine inavailability and the auto oxidative and bacteriostatic properties of smoked food, a knowledge of the chemical composition of wood and the important reactions which takes place during pyrolysis is essential. The three major constituents of wood are cellulose, hemicellulose and lignin occurring in the ratio of 2:1:1 respectively. Pettet and Lane (1940) did some pioneering work on wood smoke composition. Since then there have been numerous technological advances by many workers. Substances produced during burning of wood depend on conditions employed such as the kind of wood used, moisture content and heating methods.

Cellulose heated under high vacuum yields levoglucosan (Pictet and Sarasin 1918) and burning in air yields carbon dioxide and water. Pyrolysis at atmospheric pressure yields, through hydrolysis of the cellulose components to glucose units, then dehydration to give 1,6-anhydro glucose (B-glucosan), followed by secondary pyrolysis to produce aliphatic acids such as acetic acid and its homologs, water and small amounts of furans and phenols (Goos 1952).

Pyrolysis of hemicellulose (least stable of the wood components) yield furan and its derivatives, together with a range of aliphatic carboxylic acids. Hard wood hemicellulose containing more pentosans yield larger amounts of aliphatic carboxylic acids than softwood. At lower combustion temperature hemicellulose would yield higher acid production in the smoke (Wasserman and Fiddler 1969).

Pyrolysis of both hard and softwood lignin generally yield the smoke flavouring components of phenol and phenolic ethers such
as guaiacol (2-methoxy phenol) and syringol (2,6-dimethoxy phenol) and their homologs and derivatives, having substituent groups of methyl-, ethyl-, propyl-, vinyl-, allyl- and propenyl-.

Hence these side chains are usually not more than 3 carbon atoms in length and paraposition to the phenolic hydroxyl group (Goos 1952). Hardwood lignin (polysaccharide polymers) contains 3,5-dimethoxy-4-hydroxy phenyl propane units and guaiacyl-propane units. When oxidised in alkaline nitrobenzene, vanillin and syringaldehyde are the main oxidation products. Softwood lignin contain in addition to the above, 4-hydroxy phenyl propane units. Hence when softwood lignin is oxidised, vanillin, syringaldehyde and p-hydroxy benzaldehyde are the main oxidation products, (Pearl I.A.)

Thermal degradation of lignin also yields ferulic acid as an intermediate and Fiddler et al. (1967) have shown that the pyrolysis of ferulic acid to phenolic compounds such as guaiacol and derivatives in nitrogen rich atmosphere and acetovanillone, vanillin and vanillic acid in normal air oxidation.

![Chemical structure diagram](attachment:image.png)

**Fig. 2.1** Thermal decomposition of ferulic acid (Fiddler et al. 1967)
Doerr, Wasserman and Fiddler (1966), Lustre and Issenberg (1969) and Fiddler et al (1966) have been able to obtain reproducible condensates from laboratory generated wood smoke under controlled conditions of combustion temperature, air-flow rate and they have identified a long list of phenolic compounds in wood smoke and liquid smoke. Volatile acetic, propionic and butyric acids in hickory sawdust smoke were identified by Hamid and Saffle (1965). Acids and carbonyl compounds in maple sawdust smoke have been obtained by fractionation, isolated as 2,4-dinitro phenyl hydrazone derivatives and identified by gas chromatography (gc) retention times (Porter, Bratzler and Pearson 1965; Love and Bratzler 1966).

Smoke phenolic compounds have been recognised as important components in the flavour of smoked food. Flavour components in the steam distillable fraction are mainly phenols; carbonyls and acids (Husaini et al 1957; Tilgner et al 1962). Using water filled casings as absorbents, Bratzler and Harper (1968) found that an increase in temperature led to less guaiacol (b.pt. 205°) and proportionately more syringol (b.pt. 261°) being absorbed. Taste and threshold level of guaiacol, syringol and 4-methyl syringol in oil and water systems have been determined by Bratzler et al (1969), Tilgner et al 1962 and Wasserman (1966).

Tucker (1942) and Kurko (1959) found decreasing amounts of phenols from outer to inner layers of smoked ham and sausage. Bratzler et al (1969) found a good correlation between degree of smokey flavour and depth of phenol penetration in Bologna sausage. Lactones have been identified as important aroma compounds (Reynolds 1970). Knowles, Gilbert and McWeeny (1975) have studied the phenolic composition of some commercial liquid smoke condensates and the
smoked bacon prepared with them. Approximately 50% of the phenolic fractions of all the condensates is made up of guaiacol, 4-methyl guaiacol, syringol, trans-eugenol and 4-methyl syringol. They found a selective uptake of guaiacol, phenol, 4-methyl guaiacol and m- and p- cresol, there was a negligible uptake of syringol and its derivatives or of eugenol and that there is relatively little diffusion of phenols in the smoked bacon.
2.3.0 **Effects of smoke components on protein surfaces**

Development of colour in smoked products have been correlated with a quantitative decrease in carbonyl groups in the smoke vapour condensate, especially more significant in the presence of amino compounds. Colour intensity has been found to increase with increase in pH value, and with increase in intensity and length of exposure to heat, light and oxygen (Ziemba 1969 a and b). Acidic compounds influence colour formation by surface protein hydrolysis to form the free bases which proceed to interact with the phenolic compounds. However brown pigments on surface tissues are said to inhibit penetration of carboxylic groups and other smoke components (Ziemba 1969 a and b). Colour formation does not directly involve the phenolic compounds (Chen and Issenberg 1972, Ziemba 1969b).

Both phenolic and neutral fractions of wood smoke condensate cause a significant loss in available lysine when applied to ground pork preparations (Chen and Issenberg 1972). Spectrophotometric studies on colour formation in food is mainly by reacting smoke absorbates with proteins and amino acids (Ziemba 1969 a) or using specific smoke components such as dicarboxylic with individual amino acid and proteins (Ruiter 1969; Kurko and Schmidt 1969; Chen and Issenberg 1972). Coniferaldehyde and sinapaldehyde of wood smoke have been found to react with protein casein to produce a characteristic orange and yellow colour while glyoxal, pyruvaldehyde and furfural produced yellowish brown to brown colour in contact with the protein of casein. (Chen and Issenberg 1972). It is assumed that the ε-amino group of lysine are irreversibly bound after smoking and forms the brown colouring. Loss of lysine can be correlated with increase in colour formation of smoked meat and model protein systems. Formaldehyde
has been reported to be the active smoke component in its reaction with the $\epsilon$-amino group of lysine (Dvorak and Vognarova 1965) but this reaction of formaldehyde with proteins does not contribute to colour development in smoked food (Chen and Issenberg 1972).

Ruiter (1970) used 25% fish paste in thin Cutisin sausage casing and found that brown colour developed during smoking and that the free $\epsilon$-amino groups of lysine are irreversibly bound (lysine was determined by Carpenter's FDNB method). The smoked water (petri dish of water placed in the smoke-kiln for 2.5 h) gave comparable browning reaction with 100 mg glycine as with model mixtures of glycolic-aldehydes, glyoxal, methyl glyoxal and formaldehyde. Hence he concluded that the $\epsilon$-amino group of lysine in fish provide the amino group for the browning reaction while the guanidino group remained unchanged during the smoking process.
2.4.0 Effects of heat and other components on protein that is, mechanisms of heat damage

(a) carbonyl groups (reducing aldehydes) of sugar + \( \varepsilon \)-amino group of lysine or "Maillard" reaction;

(b) carbonyl groups from fat oxidation products + \( \varepsilon \)-amino group of lysine;

(c) protein – protein interaction.

2.4.1 (a) "Maillard" type reaction

It is generally accepted that brown colour formation in foodstuff is due to carbonyl-amino reactions or "Maillard type" reactions between amines, amino acids and proteins with sugars, aldehydes or ketones at elevated temperature and low moisture content. The primary step in the carbonyl-amino reaction is a condensation reaction between the \( \varepsilon \)-amino groups of the amino acids and proteins and the carbonyl groups of the reducing sugars, forming the initial condensation product, a Schiff's base, which undergo cyclization to form N-substituted glycosylamine, the products being in equilibrium in aqueous solution. Partridge and Brimley (1952) have demonstrated the existence of condensation products using ion exchange chromatography. The carbonyl-amino reaction occur at above the iso electric point of the amino groups. (Lea and Hannan 1949; Underwood et al 1959), increase rate of reaction with rise in temperature over the range of 0°-90°C. (Lea and Hannan 1949) and at optimum moisture content which correspond to fairly low moisture levels (Lea and Hannan 1949 and 1950; Karel and Labuza 1968). Non-reducing sugars cannot react unless the glycosidic bond is cleaved while reducing
sugars of mono- and disaccharides provide the carbonyl group for interaction with the free $\alpha$-amino groups. Since the amino acids of proteins are joined covalently by peptide bonds, the amino groups involved are presumed to be unavailable for the condensation reaction hence the $\varepsilon$-amino group of lysine provide the majority of free groups for the reaction (Harris and Mattil 1940) while arginine, histidine, and tryptophan and N-terminal amino groups also participate.

The N-substituted glycosyl amine undergoes a series of rearrangement from the aldose to the ketose sugar, referred to as "Amadori Rearrangement" from Fig. IV to Fig. VII (Weygand 1940; Lea and Hannan 1950; Abram et al 1955). From the 1-amino-1-deoxy-2-ketose derivatives, three pathways are believed to occur, involving a series of condensations and polymerisations, leading to the formation of brown nitrogenous pigments or melanoidins.

Several workers including Anet (1960, 1964) and Kato (1962, 1963) have provided evidence of 3-deoxyhexosones as intermediates of pathway 2; while a second group of workers including Hodge (1953); Hodge et al (1963) and Simon and Henback (1965), postulated pathway 1.

The third or indirect pathway is believed to be the oxidative degradation or "Strekker degradation" at high temperature of amino acids by $\alpha$-dicarbonyls or other conjugated dicarbonyl compounds (provided from other reactions) to form amino compounds, corresponding aldehydes with one carbon less, which is lost as carbon dioxide, thus providing more reducing compounds for the carbonyl-amino reactions (see Fig. 3). Cole (1967) reported good correlation between carbon dioxide formation and formation of brown pigments and unsaturated dicarbonyl compounds.
Fig. 2.2 Pathways leading brown melanoidins or pigments formation

(adapted from Hodge 1967)
More recent theories regard Maillard reactions and browning process as independent processes occurring concurrently because the protein–sugar interactions occur under alkaline conditions, while browning due to sugar degradation occur at different pH conditions. Under alkaline conditions the non-enzymic browning due to degradation of sugar is more important than that of the "Maillard" reaction. (Hölttermann 1966).

Pyrazines have been identified in heated food e.g. potato chips. Dawes and Edwards (1968) have suggested that these volatile substituted pyrazines, which they determine from model systems of aldose-amino acid interaction, were from the Strekker degradation reactions. The carbon ring in the substituted pyrazine was derived from degradation of sugars while the nitrogen was from the Strekker degradation of the amino acids (Koehler et al 1969). Pyrazines and substituted pyrazines have been determined in wood smoke condensate (Kim et al 1974).

Formaldehyde (Baba 1960, Dvorak and Vognarova 1965, Chen and Issenberg 1972), acetal (Sasano et al 1968) and propional (Lea, Parr and Carpenter 1960) all readily reduced FDNB – reactive lysine in the fish protein.
Sugars which occur free in the fish muscle are glucose and ribose and their concentration in the muscle vary considerably, depending on the physiological condition of the fish (whether well rested and fed, or spawning, exhausted) at the time of death (Jones 1962). Phosphorylated hexoses are found in fish muscle and they are also reactive in the carbonyl amino reactions. Like the freely occurring sugars glucose and ribose, their concentration in the muscle also depend on physiological conditions before death. Fish muscle extractives contain the free amino compounds of which anserine and taurine are the major contributors to browning at the surface of dried fish, while B-alanine-1-methyl histidine and lysine become increasingly important as the fish muscle spoils (Jones 1962). Further 1-methyl histidine and ribose gave a dark brown colouration at high temperature and drying conditions (the ribose, derived from degradation of nucleotide and ribo nucleic acids, contributes much of the sugar - amino browning reactions in the fish muscle.)

2.4.1 (b) Carbonyl groups from fat oxidation products with \( \epsilon \)-amino of lysine

Carbonyl groups for the carbonyl-amino reactions are also readily provided from oxidative degradation of polyunsaturated fatty acids in lipids (Lea 1958). During the oxidation of these unsaturated fatty acids, many compounds such as aldehydes, ketones, peroxides, keto hydroxy and epoxy compounds are produced which are capable of reacting with the amino groups of fish amino acids or proteins.

Malonaldehyde (one of the products of autoxidised polyunsaturated fatty acids, Sinnhuber et al 1958) has been reported to react with
various food constituents including amino acids and proteins (Kwon et al. 1965). Buttkus (1967) has demonstrated that about 40% of the ε-amino groups of lysine in myosin have interacted, after only 8 h. at 20°C and approximately the same order occurred at -20°C due to the concentrating effect (due to the freezing effect) and due to catalytic effect of ice crystals. Lea et al. (1958) found that available lysine in herring meal was reduced by 4% when stored in air at 25°C for several months and no loss in lysine availability when the herring meal was stored under nitrogen. Lea et al. (1960) have shown that oxidised fat will bind with lysine. They found a 12% loss of lysine when oxidised herring meal was heated at 100°C for 30 h. and this was accompanied by an increase in bound lipids. No losses of lysine were found when fresh herring meal was processed under nitrogen. These results indicate that when oxidation of lipids is facilitated by heat, etc., carbonyl-amino type interaction occurs between the amino acids and the oxidation products of lipids, resulting in losses of amino acids availability. In autoxidation of unsaturated lipids in fish oil, hydrogen abstraction by peroxide radical (or other free radical) may be more effective in destroying the ε-amino group of lysine than the "active" carbonyls groups (which are the end-products of the autoxidation process). These carbonyls are usually easily detected as they have not further reacted. Free radicals are difficult to detect because they react very rapidly and probably therefore are more likely to cause the observed changes. The free radical decomposes after abstracting the hydrogen and may then give rise to the carbonyls, reported by other workers.
2.4.1 (c) Protein – protein interaction

Protein-rich foods that are free from carbohydrate and fat and isolated pure protein can still suffer heat damage during heat processing which can lower the nutritional quality but have little or no apparent effect on the amino acid composition.

Carbonyl-amino type reactions seem to occur to the same extent with pure protein preparations as in the binding of free amino acids of protein with reducing carbonyl groups from sugar and fat oxidation products. (Carpenter, Morgan Lea and Parr 1962) in an atmosphere of nitrogen or oxygen (Carpenter, Ellinger, Munro and Rolfe 1957). It is then suggested that the ε-amino group of lysine may be bound by cross-linkage formation with the carboxylic groups in the protein. There has been much speculation on the possible interactions between functional groups resulting in new linkages within and between the peptide chains. These new cross-linkages could be resistant to hydrolysis by protease enzymes of digestive tract, making digestion of adjacent bonds very difficult.

Mecham and Olcott (1947) observed that when a variety of proteins were subjected to dry heat, there were marked losses in amino nitrogen, decreased solubility and digestibility of proteins, this being attributed to the loss of polar groups through internal formation of amides or esters. They (Mecham and Olcott 1947) suggested that an increase in amide formation can take place and this to some extent is related to the breakdown of cystine, that the lysine losses during heating of proteins at 100°C or above may be due to Maillard-type reactions between the ε-amino group and the carbonyl compounds from cystine destruction.
Fig. 2.4 Possible degradation of cystine in heated proteins

(Patchornik A, Sokolovsky and Sadeh 1961).
Bjarnason and Carpenter (1970) however found a low degree of correlation between lysine destruction and cystine content in cystine-containing proteins and they suggested that this could be due to the different degrees of rearrangement of Schiff's base at that point in time. Hence they consider the binding of \( \epsilon \)-amino group of lysine with cystine destruction products to be partially responsible for the lysine binding in heated proteins. They suggested that the chemically-bound lysine (from heating carbohydrate free and oxidised fat-free proteins) can be liberated on hydrolysis, from the products of condensation reactions and is measured as residual lysine in the procedure of Roach et al (1967).

It has also been said that inactivation of lysine is caused by reaction between \( \epsilon \)-amino groups of lysine and carboxylic acids (as in Fig. 5d) but Bjarnason and Carpenter (1970) concluded that this reaction is not likely or very difficult on thermodynamic grounds as high temperature and removal of water is needed to bring about condensation between these groups. They, however, provided strong evidence that the reaction between the \( \epsilon \)-amino group of lysine and the amide group of asparagine \((n = 1)\) or glutamine \((n = 2)\) with the reacting units either in the same peptide chains or between neighbouring peptide chains. Asquith (1970) noted that the \( \epsilon \)-\((\gamma\text{-glutamyl})\) - lysine (which occurs naturally in wool keratin) content is increased on heating. Fig. 2.5 shows 4 possible intramolecular linkages.
(a) an ester link between the carboxyl of aspartic (n = 1) or glutamic acid (n = 2) and the hydroxyl group of a hydroxy amino acid.

(b) a thiol ester link between aspartic or glutamic acid and a thiol group of cysteine.

(c) an imide link between the carboxyl group of asparagine (n = 1) or glutamine (n = 2) and glutamine and aspartic or glutamic acid.
(d) Interaction between $\epsilon$-amino group of lysine and amide group of asparagine or glutamine

\[
\begin{align*}
\text{lysine} & \quad \text{asparagine or (n = 1)} & \text{glutamine (n = 2)} \\
\text{carboxyl group of} & \quad \text{amide group} & \\
\text{an imide link between lysine} & \quad \text{residue and asparagine} \\
& \quad (n = 2) \text{ or glutamine (n = 2)} & \\
& \quad \text{in the same peptide chain} & \\
& \quad \text{or in neighbouring ones.} & \\
\end{align*}
\]

Bjarnason and Carpenter (1970) further suggested these reactions could not be detected by total amino acid analysis. (The literature here concern mainly the lysine residues although there is destruction of cystine and \(-SH\) interactions in heated proteins.)
2.5.0 Nutritional aspects of heat-damaged proteins

The review here will concern mainly the amino acid lysine. Extensive studies have been made on the nutritional effects of heat processing of food and feedstuffs. The above reactions between carbonyl groups of sugar aldehydes and \( \varepsilon \)-amino groups of lysine residues form amide linkages, which have very poor gut protease enzymic digestion (Henry, Ken, Lea and White 1947-8, Henry and Ken 1950), rendering the lysine nutritionally unavailable.

Bissett and Tarr (1954) subjected normal and over-heated samples of herring meal to in vitro digestion with pancreatin plus "hog intestinal mucosa" and assayed the digests for individual amino acids with non-proteolytic bacteria, they found a relative decrease in the release of lysine, as with the other amino acids from the over-heated meal samples.

Laksesvela (1958) reported a low biological value and a 40% loss in available lysine in spontaneously heated herring meals. Miller (1956) has reported that fish proteins are readily damaged at high moisture content and their reduced nutritional value can be explained by formation of sugar-amino complexes which are resistant to digestion. He also found a decreasing Net Protein Utilisation with increasing relative humidity. Carpenter, Ellinger, Munro and Rolfe (1957) have shown that losses in available lysine, resulting

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Net Protein Utilisation (N.P.U.) is the percentage of the protein eaten that stays in the body (taking into consideration digestibility of protein) calculated from carcass and faecal analysis with allowance for metabolic and endogenous losses of nitrogen in proportion to food intake and body weight respectively.
from short periods of heating cod fillets (for 90 minutes at 38°C continued for a further 36 h at 105°C at 11% moisture level), were as much in nitrogen as in air and no significant loss when the samples were defatted. They also observed that there was no evidence of carbohydrate content high enough in fish (in white fish, the amount is generally less than 1%) to explain for the 28% reduction in Gross Protein Value and that lysine is not the only amino acid affected by heat processing of proteins.

Ousterhout, Gran and Lundholm (1959) reported that laboratory-dried tuna fish meal (for 3 h at 202°C) in which the availability of methionine and methionine plus cystine were judged, from the growth assay procedure with chicks, to be 41% and 33% respectively and that as a result of evaporation, the temperature and moisture content of the fish meal must have been changing during the course of heating. Miller, Carpenter and Milner (1965) have shown that with severely heated animal proteins (essentially free from reducing sugars or oxidised fats), the nutritional availabilities of lysine, methionine and tryptophan were all reduced to a similar degree, when cod fillets at 0% to 50% moisture content were heated for 9 to 729 h at 45°C to 116°C. The digestibility of the protein, as determined with rat tests, fell with increasing heat treatment (without sugar present.) However the fall in digestibility of the protein is not sufficient to explain the fall in Net Protein Utilisation, hence some of the digested methionine and lysine from

Gross Protein Value (G.P.V.) is the extra growth in g. obtained in 2 weeks, that is, the excess over the growth on basal ration, divided by the number of g. of supplementary protein eaten.
the heated materials may have become unavailable giving a reduced value for growth promotion. The same results were obtained with chicks feeding trials. Carpenter and Varnish (1975) obtained similar results with heat-damaged proteins.

Dvorak (1968) reported twice as much protein was digested in the albumen without glucose, heated at 121° than the albumin heated with glucose at 90° for the same period of time. Since only about 55.5% of the heated albumin without glucose was hydrolysed, the lowered digestibility cannot be explained solely by carbonyl-amino linkage between the reducing sugars and ε-amino group of lysine. Cross-links within or between protein molecules may be formed by heat treatment, as shown as Mecham and Olcott (1947) and these linkages probably caused resistance of heat-treated proteins against proteolytic enzymes.

Ford and Salter (1966) found that aspartic acid, glutamic acid, lysine and cystine in freeze-dried cod fillet heated at 135° were poorly released by successive pepsin-trypsin-erepsin enzymic digestion as compared to the other amino acids. They passed the enzymic digest of heat-damaged and unheated cod muscle samples through a sephadex gel G25 column. The eluted fractions were hydrolysed and the amino acid content was estimated by reaction with ninhydrin and expressed as "leucine equivalents". They found on digestion that the heated cod muscle gave less amount of free amino acids and more of the larger peptide units while the unheated sample gave mainly free amino acids and some small peptide units. Amino acid analysis showed lysine - and sulphur - containing amino acids to be relatively deficient in the free amino acid composition from the digest of heated fish meal.
Growth tests with rats indicated that biological availability of lysine was much lower than that of methionine and isoleucine and analysis of the small intestine contents showed an accumulation of undigested protein and peptides, which like the in vitro tests were rich in lysine, aspartic and glutamic acids (Buraczewski et al 1967, Buraczewska 1966). Erbersdobler et al (1969) found on digestion of heat-damaged casein in rats, that there were differences in the availability of different amino acids and lysine, asparagine and glutamine were the most reduced in availability. Since the peptide fractions, especially the larger peptides were strongly yellow-brown in colour they must have been the products of interaction between protein and reducing-sugars. Ford (1973) showed that freeze-dried cod muscle contain 335 mg ribose/100g before heat treatment. Peptide-fractions which are resistant to enzymic digestion could be due to this residual ribose and other sugar-containing substituents (possibly the products of nucleic acid breakdown). He also indicated that cysteic acid is produced as breakdown product of cystine and severe heat treatment had marked differential effect on the enzymic digestion and release of different amino acids from the heat-damaged cod muscle. The free amino acid components in the enzymic digest were deficient in several amino acids relative to the amino acid content in the original protein, especially deficient in lysine and the sulphur-containing amino acids. He has suggested that the damaging effect of heat treatment is probably indirect, resulting in the formation of new intramolecular linkages that are not easily hydrolysed by the gut enzymes and the linkages involved are mainly glutamine and asparagine and perhaps
also the corresponding glutamic and asparatic acids with lysine. The low biological value could be due to a poorer pattern of amino acid obtained on digestion or that some of the amino acids absorbed from the gut are still bound up in small undigestible peptide units.

Ford and Shorrock (1971) examined the effects of feeding heat-damaged cod muscle protein to rats and examined the peptides and free amino acids content in their urine. They found a increased urinary excretion of peptide-bound amino acids of approximately three-fold with cod fillets heated for 20 h at 135° and lysine, aspartic acid and glutamic acid together comprise nearly 70% of the total amino acid residues measured. There was also a higher total content of free amino acids excreted in the urine. They suggested that the presence of these "unavailable" peptides might lower the renal threshold for free amino acids hence hindering the reabsorption of amino acids by the renal tubules but they concluded that the urinary losses of undigested peptides were of marginal nutritional importance. This explanation was analogous to the findings of Buraczewski, Buraczewska and Ford (1967) who postulated that the accumulation of "unavailable" peptide material in the intestine might hinder the absorption of amino acids by saturating the absorption sites involved in their transport across the ileal-mucosal barrier.

Ford and Shorrock (1971) reported findings of lysine, aspartic and glutamic acid in the urinary residues and suggested that the biologically unavailable peptide residues could represent the amide-linked asparagine or glutamine with lysine (Bjarnason and Carpenter 1970). However this suggestion now seems less likely to be the case.
as Mauron (1970) and Waibel and Carpenter (1972) showed that lysine in $\varepsilon$-($\gamma$-L-glutamyl)-L-lysine is fully available to the rat but the compound is probably not completely hydrolysed in the lumen of the gut (Pisano et al. 1969, Asquith et al. 1970) or during its passage through the gut mucosal wall as it appears in the portal blood. Hence they suggested that its hydrolysis may occur in the kidney which contains enzymes that will hydrolyse a variety of $\varepsilon$-acyl derivatives of lysine. While the $\varepsilon$-($\gamma$-L-glutamyl)-lysine can be absorbed and utilised as a source of lysine, each new glutamyl-lysine linkage hinders the access of gut peptidases to several adjacent peptide bonds, so that little or no free glutamyl-lysine unit would actually be liberated into the lumen of the gut absorption. However more direct evidence of this is needed. Ford (1973) suggested that heat-damaged proteins are unavailable in that they are slow to digest, probably due to steric hinderance to access of proteases and that availability and unavailability are arbitrary and must relate to the conditions of the test, species of animal and many other factors. Rat growth assays indicated that the rate of digestion "in vivo" was inefficient compared to the "in vitro digestion" with the pepsin-trypsin-pancreatin enzyme digestion. Rats given heat-damaged fish meals contained a high concentration of undigested peptides in the intestine which could be due to an increased loss of endogenous protein such as digestive enzymes, mucus and desquamated gut epithelial cells into the gut lumen. These, however, are normally digested along with the other food proteins and reabsorbed. Buraczewski (1966) found that with rats given heat-damaged fish meal, the high concentration of
"unavailable" peptide units competes with other small peptides and free amino acids for absorption sites involved in the transport of amino acids across the mucosal barrier and in addition, there is increased proteolytic activity of the middle and distal portions of the rat small intestine. Shorrock (1972) showed that the presence of unavailable peptides depressed the absorption of leucine and small, nutritionally available peptides as the unavailable peptides compete for the absorption sites.

Summarising the existing literature, the reduction in nutritive value appears to be due to a combination of the following factors:

a) the resistance of Maillard-type compounds and protein-protein type cross-linked compounds to digestion as each new cross-linkage hinders the access of gut peptidases to the adjacent peptide bonds;

b) due to the above factor (a), large peptide units are released from the digestion of heat-damaged proteins instead of free amino acids and small peptide units;

c) lysine and sulphur-containing amino acids are poorly released and are deficient in the free amino acid composition of the digest of heated proteins;

d) an "apparent" accumulation of undigested proteins and peptides in the small intestine (rich in lysine, aspartic and glutamic acids.) This high concentration of unavailable peptide units competes with the small peptides for the absorption sites and saturate these
sites, depressing the absorption of the small peptides.

Hence in addition to the heat-damaging effect on fish proteins during hot smoking at high moisture content, one needs to consider the extent of damage caused by interaction between the protein constituents and smoke components, carbonyl groups of reducing sugars and lipid oxidation products, reactions which hopefully are limited to the top few centimetres of the smoked fish fillet surface.
2.6.0 Proteolytic activity in fish (post-mortem)

There is a possibility of proteolytic activity in fish from the time of death. The sarcoplasm in fish muscle fibres contain the cellular organelles called lysosomes which contain the hydrolytic proteolytic enzymes, including cathepsins, which are active at acid pH. These proteolytic enzymes are released by rupture of the lipoprotein membranes of the lysosomes when the pH dropped lower than the normal in vivo pH level.

Bate-Smith (1948) in a review article suggested that proteolysis by cathepsin was the most probable cause of tenderness developed during post-mortem ageing of meat. Sharp (1963) made extensive studies on aseptic autolysis in rabbit and bovine muscle at 37°C. He found that the main catheptic activity was confined to the sarcoplasmic proteins and none from the collagen friction. Cathepsin in fish muscle have not been studied in great detail. Siebert (1958) found that cathepsin activity in fish muscle was ten times greater than that of mammalian muscles. Cathepsins appear to have an optimum pH around 5.5 and are active at fairly high temperature of around 37°C for cold blooded animals. Makmodan and Ikeda (1969) reported proteolytic enzyme activity at slightly alkaline pH range, in addition to those active in the acid range. Wladyka and Dawson (1968) demonstrated that proteolysis occurred in poultry carcass held at -18°C for up to 90 days. McCain et al (1968) reported that there was no correlation between tenderization and proteolysis and that the free amino acid produced by catheptic activity are the precursors of meat flavour.

Cathepsin D have been purified with other lysozome enzymes in the white muscle of gold fish (Bird et al 1969). Reddi, Spiros,
Constantinides and Dymsza (1972) studied the intracellular localization of catheptic activity and the physical and chemical properties of cathepsin D from the skeletal muscle of the Winter flounder (*Pseudopleuronectes americanus*) in relation to the autolytic breakdown of haemoglobin. They found that the breakdown of haemoglobin increased with decreased pH with optimum range of 3 to 7, with the lysozomal fraction; it is stable at 5°C and still active at 66°C. They found that cathepsin D hydrolyses the protein in the fish tissue homogenate and the breakdown products were further hydrolysed by cathepsins A, B and C as suggested by Huang and Tappel (1971) and this autolytic spoilage facilitate subsequent spoilage by bacteria.

Makinodan and Ikeda (1969) reported a protease of Carp muscle which is active in the slightly alkaline range and retained 70% of its activity after heating at 60°C for 1 hour. This relative stability of catheptic enzymes to high temperature is an important feature in the heat processing of fish muscle as insufficient heat treatment would cause enzymic activity to be retained.

Advantage of catheptic activity is made by the process of ensilaging fish wastes by the addition of hydrochloric acid or formic acid to bring the pH down to pH 3 to 4, to obtain a product of high amount of soluble protein which is dried for mixing into animal feed and fish sauces.

Raa and Gildberg (1976) measured the rate of autolysis of cod viscera in the process of ensilaging and found that the initial rate of autolysis is the highest at pH 3.5 to 4.0 (measured as increase of dry weight of the soluble phases.) The protein sediment
left after autolysis is very similar to that of structural
glycoprotein extracted from connective tissue (Robert and Conte
1968) and that autolysis is the resultant of the action of proteolytic
enzymes and other enzymes splitting those B-glycosidic bonds which
interlink the glycoprotein with the other structural protein.

Hence from time of death of fish when it is landed in the
trawler, the pH of fish gradually decrease due to accumulation of
lactate from breakdown of muscle glycogen. The level of pH depends
on the physiological condition of the fish (whether well fed, rested
or exhausted or spawning) species of fish, type of fishing ground and
seasonal variation. The low pH ruptures the lysozome lipoprotein
membrane, releasing the proteolytic enzymes. Short chain peptides
and amino acids are produced as a result of catheptic activity and
one would expect the fish non-protein extractives to be high in some
free amino acids. Fish which not, quickly eviscerated on board
the trawler would have a higher level of free amino acids from the
protease action of the gut enzymes. Hence one must expect a
certain amount of lysine as free amino acid in the fish muscle
extractive before the smoking process and an increase during the initial
stages of smoking process, as the temperature of the fish fillet
gradually rose to the kiln temperature, before high temperature
eventually inactivated the enzymes. Of more importance than the
free lysine increase is the possible increase in N-terminal amino
groups.
2.7.0 Determination of 1-fluoro, 2,4-dinitro benzene (FDNB) reactive lysine

Chemical methods for the determination of available lysine have been developed because of the slow throughput and difficulties associated with the microbiological procedures and methods involving the use of experimental animals. An example of the chemical methods is the amino acid analysis by the method of Moore and Stein (1951). A disadvantage with this method is that the results give the total lysine content of the sample and do not indicate the level of available lysine. It is also difficult to obtain complete recovery of an amino acid and reproducibility of results have been reported to be poor. The method of 2,4,6- trinitro benzene sulphonic acid (TNBS) procedure developed by Kakade and Liener (1969) have been found to be useful in fish meal, soya bean oil meal and whole milk protein. Criticism of this method is that the estimation of available lysine is less specific than that of the FDNB procedure. Amino acid analysis after alkylation of epsilon-amino group of lysine, (the procedure by Finlay and Friedman 1973) is lengthy and complex.

The method of Carpenter (1960) is widely used for the determination of available lysine in proteins and protein-containing foods, but it is not suitable for samples containing high amounts of carbohydrates such as cereals. The reaction between the amino group of the amino acid and 1-fluoro-2,4-dinitro benzene was introduced by Sanger (1945). The FDNB method determines the number of lysine molecules which have free-amino groups, that is, groups not covalently bound by other amino acid residues. It is now generally accepted that only these lysine molecules are nutritionally available.
Fig. 2.6 Reaction between lysine residue and 1-fluoro-2,4-dinitro benzene

Baily (1957) found that $\varepsilon$-DNP lysine can be separated from possible interference of $\alpha$-DNP arginine (from N-terminal arginine) and imino-DNP-histidine by raising the pH of the filtered hydrolysate containing the dinitrophenylated amino groups and shaking this solution with methoxycarbonyl chloride (also called methyl chloroformate). The $\varepsilon$-DNP lysine reacts at its $\varepsilon$-amino group with the methoxycarbonyl chloride to form the methoxy carbonyl derivative, which is ether soluble when the solution is made acidic, while the DNP-arginine remains in the aqueous layer. Bruno and Carpenter (1957) retained the diethyl ether washings (after reaction with methoxy-carbonyl chloride and acidification) evaporate it and dissolve the
They use the colour in the ether layer as a measure of FDNB-reactive lysine and found useful correlation with the nutritional value of a range of vegetable materials (oil seed, cereals, grass and leaf protein concentrates).

\[ \text{DNP} - \text{NH}(\text{CH}_2)_4\text{CH(COOH)}\text{NHCOOCH}_3 \]

Fig. 2.7 C-DNP lysine-methoxy carbonyl complex

Carpenter, Jones and Mason (1959) observed that the colourless im-DNP-histidine react with methoxycarbonyl chloride to form an orange-yellow derivative which was also ether soluble. Carpenter (1960) solved this problem by measuring the absorbance of the ether-washed hydrolysate before and after treatment with methoxycarbonyl chloride (MCC) and a further twice extraction with ether before the residual colour (containing the interfering compounds that have not changed solubility) served as a blank. Hence the difference in the colour intensity of the hydrolysate before and after reaction with MCC and extraction with ether was taken as a measure of available lysine. This procedure is lengthy and laborious, requiring considerable analytical expertise and the errors involved can vary enormously (Jacobsen et al. 1972). It is also not suitable for food materials containing large amounts of carbohydrates such as cereal grains, cereal flours and vegetable protein, because reducing compounds are formed from the carbohydrate material during the hydrolysis stage, resulting in the destruction of a portion of
the \( \varepsilon \)-dinitrophenylated lysine and the content of the chemically available lysine is, hence, under-estimated. DNP-lysine is absorbed by dinitrophenols, carbonised starch and insoluble "humin" from vegetable materials. Booth (1971) recommended redigestion of the washed insoluble residues. He found yields of 0.7% of the initial yield for the second digestion of wheat, 1.6% for fish and meat meals and cereals average about 5%.

Polylysine is not easily hydrolysed and dinitrophenylation of polylysine makes digestion on hydrolysis even slower but in a sealed ampoule autoclaved at 120\(^{\circ}\)C, Booth (1971) obtained on second digestion, a yield of 4.3%.

A small weight of sample to volume of acid is recommended for hydrolysis to minimise the destruction of amino acid during acid digestion of protein (Shram, Dustin, Moore and Bigwood 1953). This was confirmed by Rao et al (1963) on cottonseed and Matheson (1968) on groundnut. Reflux of carbohydrate in HCl produced a yellow soluble compound called humin (in the case of sample with high carbohydrate content) which absorbs light at 435 nm. Booth (1971) reported that this problem can be overcome by hot filtration of the acid hydrolysate. He (Booth 1971) also showed that the loss of added DNP-lysine to fish meal in the absence of carbohydrate was 9% but loss was increased to 20% with the addition of starch, as carbohydrates especially pentoses when heated with HCl, produced furfural which may act as reducing agents for the DNP-lysine. Booth (1971) also showed that Carpenter's original correction factor of 1.09 may be too high for materials that contained no carbohydrate. He obtained a value of 1.04 for freeze-dried chicken meat, an average
of 1.05 for fish meals, 1.2 for wheat and some other cereals and
1.14 for beans, groundnut and maize.

2.7.1 FDNB-reactive lysine by difference

Rao, Carter and Frampton (1963) determined lysine before and
after dinitrophenylation of the sample, the difference represent
FDNB-available lysine by difference (Total lysine minus Inaccessible).
Rao et al. (1963) removed the ether-soluble breakdown products
before the acid digestion but in Carpenter's method, these products
are left to protect the \( \epsilon \)-DNP-lysine during the digestion. The
method also employ the use of an ion-exchange chromatographic column
containing a resin, size B Amberlite IR-120 in the sodium form in a
column of 6 cm. This requires considerable expertise in the
collection of various effluent fractions and regeneration of the
column with water-aqueous NaOH-water-HCl washings. Hence throughput
of samples is very slow and continuous attention is required when
collecting the effluent fractions. The method, however, is said to
be more specific as it includes the N-terminal lysine and free lysine
but excludes all the other amino acids.

Roach, Sanderson and Williams (1967) combined the methods of
Carpenter (1960) and Rao et al. (1963). The determination of
available lysine was calculated by measurement of the total lysine
in an acid hydrolysate of a sample and of lysine remaining in solution
after a separate hydrolysis of the sample treated with 1-fluoro-
2,4-dinitrobenzene according to the method of Carpenter (1960). The
lysine remaining in the solution, not reacted with FDNB, being the
inaccessible lysine. Hence the difference between these 2 values of
lysine (in the 2 separate hydrolysis is the lysine in the protein which have the free $\varepsilon$-amino group and is referred to as available lysine). The method of Roach et al (1967) is more rapid as it is semi-automated. The effluent fraction is drawn off the column at a rate of 0.42 ml/min. and is fed directly into the Technicon Autoanalyser analytical system and the colour developed with ninhydrin is read at 570 nm and recorded automatically. Standards of 0.1 mole/ml lysine solution is run together with the samples so that the peaks obtained with the standards are paired with that obtained for the sample. Like Booth (1971) they found that since the variability of recovery is high, determination of the recovery correction factor for individual runs is required.

Although many workers have found loss in lysine with heat damaged food samples, there is no literature on the possibility of $\varepsilon$-DNP lysine reacting with smoke-phenolic and -carbonyl compounds in smoked products. This possibility of this reaction has not been considered even by workers examining smoked meat (Dvorak and Vognarova 1965) and smoked fish (Hoffman et al 1977).
2.8.0 Dye binding of food and feedstuffs

The literature here will concentrate mainly on the azo acid dyes of Acid Orange 10 (AO.10) and Acid Orange 12 (AO.12).

Much work has been carried out in the employment of dye binding in the determination of protein quality in certain foodstuff but little have been published on the quantitative basis.

Fraenkel-Conrat and Cooper (1944) developed a method in which the dye acid orange G 10 (AO 10) was used to determine the number of basic groups in protein. They (Fraenkel-Conrat and Cooper 1944) found that the uptake of acid orange 10 by granules of gelatin in citrate-phosphate buffer at pH 2.2 was equivalent to the number of titratable basic groups. The dye binding procedures commonly used now for the estimation of protein and the evaluation of protein quality have generally adopted this basic method but it should be noted that the dye-binding procedures which are currently used for the estimation of basic groups in proteins are quite different from that originally employed by Fraenkel-Conrat and Cooper (1944), as different dye-stuffs are used and experimental parameters have changed considerably. Under the conditions currently employed, the reaction between the dye and the protein is no longer stoichiometric (Lakin 1973 a).

The basic technique of dye binding procedure involves:

1) the finely divided food sample is mixed with a reagent containing the dye stuff of known strength buffered at pH 2;

2) an equilibration period of violent shaking for intimate contact to allow the acidic or basic
amino groups of the protein react with the dye to form insoluble complexes;

3) insoluble complexes are removed by filtration through an inert material (such as glass micro-filter paper) or centrifugation;

4) the extinction of the excess dye in the supernatant solution is then measured after dilution or in a cuvette of short path length. This extinction value is inversely proportional to the amount of basic or acidic groups in the sample.

The mechanism of dye binding is an electrovalent attraction between the dye anion (in the case of azo acid dyes) and the basic amino acid residues of the protein, which are positively charged. In a buffer of low PH of 1 to 2, the cation imidazole, guanidine and ε-amino groups and the free amino end groups of the protein chains (small fraction compared to the basic amino acid residues in the protein) binds quantitatively with the anionic sulphonic sites of the basic dyes Acid Black, Acid Orange 12 and Acid Orange 10.
Fig. 2.8 Structures of 3 basic dyes commonly employed in dye binding procedures

Acid Black
molecular weight = 616

Acid Orange 10
molecular weight = 452

Acid Orange 12
molecular weight = 350

Fig. 2.9 Simulated protein chain showing the basic amino acid residues which are essentially protonated at pH 1 to 2 (Udy, 1971)

(Lys) +

(His) +

(Arg) +

Udy (1971)
Hence the dye binding of a lysine residue with Acid Orange 12 is as follows:

\[
\text{Acid Orange 12} \rightarrow \text{lysine residue} \rightarrow \text{insoluble complex}
\]

Lakin (1973) has given a list of possible secondary binding mechanisms between the dye molecules and the protein as being due to:

i) hydrogen bonding between the dye molecules and the proteins as the azo acid dyes contain the groupings (-OH; \(-N = N-\), etc.,) which can form hydrogen bond with the proteins instead of only with the basic amino groups;
ii) acid azo dyes have hydrophobic structures which are made water soluble by substitution of the polar groups hence the hydrophobic sites form hydrophobic bonds with the proteins;

iii) hydrogen and/or hydrophobic bonding between the dye molecules, already bound to proteins, and the free dye molecules giving dye-dye binding reactions, indicating more basic amino acid residues than there actually are.

To avoid this secondary binding mechanism, a correct ratio of dye-protein basic groups is essential. Ashworth (1971) have shown that the proteins of meat products do not react stoichiometrically with the dye acid orange 12 as an increased dye/protein ratio increased the dye binding capacity (DBC) and standard deviation also increased, possibly indicating secondary binding mechanisms. Milk protein have also been shown to react in a similar way. Ashworth and Chandry (1962) determined the dye binding capacity of milk proteins with Amido Black 10B and Orange G and stressed that the unbound dye or equilibrium dye concentration must be used to determine the proper value of the DBC before the DBC can be used to convert the dye bound to mg protein in the sample. Hurrell and Carpenter (1975) found that repeating the determinations with smaller quantities of test materials gave much higher values of DBC although the relative values of the different test material remained almost the same.

In addition to Dye/Protein ratio, (a) hydrogen ion concentration, (b) particle size of test sample, (c) reaction or equilibration time,
(d) fat content of sample, (e) starch content and (f) metal ions in buffer reagents have to be considered.

a) **hydrogen ion concentration**: Complete dissociation of the basic protein groups was necessary for stoichiometric binding by the dye and Fraenkel-Conrat and Cooper (1944) found that an optimum of pH 2.2 was necessary for binding gelatin granules with Orange G 10 and that the dye binding properties of proteins decrease with increasing pH. Vickerstaff (1954) have shown that the optical properties of dyes are affected by the hydrogen ion concentrations;

b) **Particle size of sample** is important as the finer the test sample the faster the dye binding occurs and the shorter the equilibration period (Moran et al 1963, Udy 1957). Most workers have used BS sieve 30 mesh or 0.5 mm (BS 410 1969 or ASTM E11 - 1970 sieve No. 35) to obtain a fairly uniform particle size. However Pomeranz (1965) found that the dye binding properties of soya flour were independent of particle size when equilibration time allowed for the reaction was only a few minutes;

c) Equilibration time is required for complete binding of all the basic sites of the protein. Fraenkel-Conrat and Cooper (1944) showed that an equilibrium time of 20 h is favourable with most proteins. While Moran et al 1963 found that a 1 h reaction time is sufficient to obtain a relatively accurate DBC, while Dolby (1961) found that soluble proteins, e.g. milk, have a very short equilibration time of only a few minutes;
d) **High fat content in test sample** Ashworth *et al* (1960) found that butterfat of milk had no effect on the dye binding of milk proteins and a similar report for the fat content of beef and pork proteins by Ashworth (1966). Moran *et al* (1963) suggested that since fat content of samples may interfere with the ability of the water soluble dye to bind the proteins, forming a physical barrier, thus preventing dye-protein reaction reaching equilibrium. Hence the amount of fat content present should be constant or it should be extracted. For fish meal, Moran *et al* (1963) found it necessary to extract the fat with diethyl ether in order to obtain reproducible results. Jones (1974) found that the high fat content in groundnut decreased the rate of dye penetration.

e) **Starch content of samples:** Ashworth (1971) reported that starch does not influence the dye binding measurements with AO.12. The effect of whole starch on dye binding in cereals was insignificant compared to gelatinised starch when it has been heat processed (Jones 1974). Udy (1973) found that partially gelatinised starch adsorb more dye than completely gelatinised starch. Hurrell and Carpenter (1975) found that under the conditions of their procedure, starch in groundnut meals account for less than 1% of the overall dye binding and sucrose and glucose do not interfere with dye binding.

Amounts of dye bound is said also to be affected by the composition of the buffer solution. Inert impurities also interfere in the measuring of the amount of dye bound by proteins (Lakin 1973). Udy (1971) maintained that different ions have pronounced effects on the DBC of proteins with AO.12. Acetate ions seemed to cause a
large decrease while phosphate and chloride ions increase the DBC. Heavy metal ions chelate the A0.12 dye irreversibly and the addition of oxalic acid helped to minimise or eliminate the gradual loss of colour and maintain the equilibrium dye concentration (EDC), especially with milk. Udy (1971) also reported that propionic acid effectively inhibited mould growth over 10 months and A0.12 dye is unstable to light.

Hydroxy lysine exists in the bone, tendon and skin collagen and may constitute about 12-21% of the total lysine + hydroxylysine. If bones, skins and tendons are carefully removed and excluded from the test samples, the small amount of hydroxy lysine in the muscle will be included in the measurement of lysine in both the dye binding and direct FDNB (1-fluoro 2,4-dinitro benzene)- reactive lysine methods.

In animals especially fish, fission of peptide bonds by proteolysis may lead to the production of significant amounts of free amino acids and terminal amino groups which are still of nutritional value (provided that they are not lost in the "drip loss" from fish flesh), but the free amino acids no longer cause precipitation of the dye in which case the DBC values would not correspond with the basic amino acids and would give misleading DBC values (Lakin 1975).

Dye binding has been used to indicate the protein quality on barleys (Mossberg 1968; Hagberg and Karlson 1968) to select strains of barley of high basic amino acid content especially lysine. Mossberg (1969) reported that the basic amino content of a range of cereals gave a better correlation with the amount of dye bound ($n = 0.94$) than with the protein content ($r = 0.767$). Moran et al
(1963) found a definite tendency of fish meal towards decreased ability to bind $A0.10$ with decreasing levels of lysine (as determined by method of Carpenter 1960). Udy (1971) found $A0.12$ useful in estimating the crude protein in milk. Jacobsen et al. (1972) reported a correlation coefficient of 0.883 with fish meal dye binding data and available lysine (as determined by fluoro-dinitro benzene procedure of Carpenter 1960). Good correlation have been reported between fish meal lysine and binding of $A0.10$ (Boyne et al. 1961; Baba et al. 1964). Lakin (1973 b) have obtained a linear relationship between absorbance of test filtrate and milk proteins provided that the calculated absorbance of the dye-protein mixture (EDC) is above 4. Roma et al. (1975) developed a dye binding procedure for the estimation of protein in seed extracts and solution of seed protein isolats.

As a result of such reactions as Maillard browning and protein-protein type interactions, the dye binding properties of the damaged protein would be expected to decrease. As the $\epsilon$-amino groups of lysine is already bound by the reactions mentioned above, this would result in fewer basic sites available for dye binding. These observations were reported by Udy (1954) with heated soluble wheat proteins; Mossberg (1965, 1966) with heated wheat flour and barley; Pomeranz (1965; Hymowitz, Collins and Gibbons (1969) with soya bean meal; Udy (1971) with whole and extracted soya flour and wheat flour; Ashworth (1961) with casein heated in the presence of lactose.

Dye binding procedure has also been tested as an index of the nutritive value of a protein. When the nutritive value of a food protein is limited by its content of lysine (or very rarely one of the other basic amino acid), dye binding data have been correlated
with the results of animal feeding trials. Acid Orange 10 has been found to correlate well with the nutritive value of fish meals (Thurston 1957); whale meat, fish meals and meat and seed meals (Bunyan and Price 1960, Boyne et al. 1961, Bunyan and Woodham 1974); meat and bone meals (Choppe and Kratzer 1963); soya bean meals (Moran et al. 1963) and a series of heated barley samples DBC values with mouse growth (Mossberg 1965; 1966). Jones (1974) reported that early Maillard-type reactions are not discriminated by dye binding as the sugar-protein complex in the early stages of the Maillard reaction are still basic in character. Hurrell and Carpenter (1975) used 3 dye binding procedures for the assessment of heat damaged protein and reported that mild heat treatment of groundnut meal (1h at 121°C) did not affect the DBC values of AO.12 or the nutritional value of a similar sample as reported by Anantharaman and Carpenter (1971). When the protein and glucose or sucrose system was more severely heated (4h at 121°C) the DBC values were lowered slightly but not to the same extent as the reduction in basic amino acids histidine and arginine as determined by amino acid analysis and reactive-lysine by FDNB method. The total of these 3 basic amino acids as determined by these methods is termed H A R-L values by Hurrell and Carpenter 1975). However, with severely heated meat products (autoclaved, oven-dried steak and ox gut) the AO.12 DBC values fall to the same extent as the H A R-L values, as the dye in these conditions, has not been able to penetrate to the reactive sites probably due to extensive cross-linkage formed in the protein (due to protein-protein type damage), in addition to "Maillard" type reactions. With bovine plasma albumin and fat-extracted, freeze-dried chicken muscle, unheated or severely heated in the absence of
sugar, the A0.12 dye binding values were close to the H A R L values. When animal feeding stuffs were treated to early, advanced Maillard and protein-protein type damage, they reacted with A0.12 in much the same way as with the model systems of lactalbumin acylated to form formyl lactalbumin and propionyl lactalbumin.

Hence fish hot-smoked over a period of several hours undergoes early to advanced "Maillard" type reactions and protein-protein type heat damage and would be expected to behave in a similar manner to that reported for A0.12 dye used under similar experimental conditions with other similarly heat-damaged materials.
2.9.0 A review of the different blocking reagents for ε-amino group of lysine

2.9.1 Acylation of ε-amino groups of lysine by propionic anhydride

Udy (1971) observed that when lysine was blocked (by an unspecified reagent) the dye binding value of the blocked protein was the same as that obtained by severely heated soya beans and wheat samples. He concluded that since the blocked samples and the heated samples behaved similarly, then all the lysine must have been made chemically unavailable for dye binding in the heated samples. The subsequent dye binding value must be proportional to the amount of available arginine and histidine in the protein sample.

If the dye binding value of the treated sample was subtracted from that obtained for the untreated sample, the difference would be proportional to the chemically reactive lysine content.

Hurrell and Carpenter (1975) used model systems of blocked lysine: formyl lactalbumin and propionyl lactalbumin in which 75% and 95% respectively of their ε-amino group of lysine was in a form that would not react with the FDNB. The DBC values gave a very good correlation with the HAR-L values (histidine and arginine determined by amino acid analysis and reactive lysine by the FDNB method). There was a decrease of 49.05% and 49.53% in the HAR-L and DBC (A0.12) values respectively for formyl lactalbumin and a decrease of 67.92% and 71.02% in the HAR-L and DBC value respectively for propionyl lactalbumin for the unacylated lactalbumin.

Hurrell and Carpenter (1976) described a method for the propionylation of lysine residues, determining the dye binding values before and after blocking the lysine, giving a specific measure of
lysine in whole wheat and wheat gluten, rice, sweet corn (zaa mays rugosa), groundnut flour, beans (soya, broad and wing), chick peas and bovine plasma albumin. The results showed good agreement but the meat meals and fish meals gave dye binding difference (DBD) values of only 0.7-0.9 of the corresponding FDNB values which they ascribed as due to incomplete propionylation under the conditions used so far.

A sample containing 15 mg lysine was shaken vigorously for 15 min with 0.2 ml propionic anhydride and 2 ml half saturated sodium acetate solution in a glass tube stoppered at each end. The suspension was then subjected to the "Foss" DBC procedure. 40 ml dye solution (3.89 mmol/litre oxalic acid-acetic acid-phosphate buffer pH 1.25) was added and shaken violently for 6 min with a steel disc in the reaction chamber. The dye sample mixture was filtered and the extinction of the filtrate was compared with that of the unreacted dye solution. The unacylated sample was subjected to the normal dye binding procedure with 2 ml acetate solution added to the 40 ml dye solution. The DBC obtained for the unacylated (unpropionylated) sample minus the DBC obtained for the propionylated sample gave the dye binding difference (DBD) values. Another reason that Hurrell and Carpenter (1976) gave for the low correlation for meat and fish meals was the difficulty of transferring the acylated sample from a small vessel to one suitable for dye binding reaction. This reason should surely apply also to the cereal, beans and bovine plasma albumin samples. It should, however, be noted that Hurrell and Carpenter (1976) used Acid Orange 12, ready mixed for use with a Pro Meter Mk II (A/S Foss Electric, Hillerød, Denmark). Both the dye and the reference standard were supplied by the manufacturers.
2.9.2 **Acylation of ε-amino group of lysine with 2,4,6-trinitro benzene sulphonic acid (TNBS)**

Kakade and Liener (1969) modified the method of Habeeb involving the use of 2,4,6-trinitro benzene sulphonic acid to determine specifically the lysine content of proteins and the available lysine of protein foodstuffs by the fact that subsequent to acid hydrolysis of the trinitro phenylated - (TNP)-proteins, the α-TNP amino acids may be extracted with diethyl ether while the ε-TNP lysine remains in the aqueous phase where it may then be determined spectrometrically. The method of TNBS can be applied with accuracy to low molecular weight protein where the total number of N-terminal amino groups is small compared with the number of free ε-amino groups. The N-terminal amino groups may also react with the TNBS but reaction is much slower and dependent on the pH of the system.

2.11 **Reaction between ε-amino group of lysine and 2,4,6-trinitro benzene sulphonic acid**

\[ \text{lysine residue} \rightarrow \text{trinitro phenylated lysine residue} \]
The TNBS reacts with the primary amino groups at pH 8.5 in 2% sodium bicarbonate at 40°C to form the trinitrophenylated lysine residues. The reaction between TNBS and the \( \text{\textalpha} \)- or \( \text{\epsilon} \)-amino groups of free amino acids and proteins depend on the pH of the system and pKa of the amino group. The \( \text{\epsilon} \)-amino group of lysine being the most reactive while the imidazole group of histidine (Freedman and Radda 1968) and guanidine group of arginine (Sataka et al. 1960) were found not to react with TNBS. Different proteins react at different rates with TNBS, requiring different concentration of TNBS and adsorption isotherms must be determined for the protein sample.

Jones (1974) found that the dye-protein equilibrium mixture may contain the residual TNBS reagent which could change the pH of the system or compete with the dye anions for basic sites on the proteins. Hence it was necessary to remove the excess TNBS from the sample reagent by filtration before proceeding with the dye binding reaction.

Jones's method is as follows:-

1) sample (100 ± 1 mg) was weighed into a 100 ml polyethene bottle and 10 ml 4% (w/v) sodium bicarbonate was added. The sample was dispersed by gentle aditation and incubated at 40°C for 30 min;

ii) TNBS solution (10 ml of 1% w/v) was added and the reaction was allowed to proceed at 40°C for 4 h with occasional agitation;
iii) the solution was then acidified by addition of 5 ml 1M HCl and the contents mixed;

iv) the contents of the bottle were filtered through glass micro fibre filter paper and the precipitate washed with 2 x 5 ml aliquots of 0.2M citric acid. The filter paper was allowed to drain;

v) the filter paper together with the treated sample was placed back in the polyethylene bottle, 3 glass beads were added and tube and contents were subjected to the normal dye binding procedure.

For the untreated sample the dye concentration was 3 meq l\(^{-1}\) and for the treated sample concentration was 2 meq l\(^{-1}\) of Acid Orange 10. It was necessary to use different concentrations of dye in the dye-buffer reagent for the treated and untreated samples. This ensured that the dye-binding properties were determined at similar equilibrium dye concentration (c 1.5 meq l\(^{-1}\)) so that the amount of dye bound to the sample by secondary associations were of the same order in each case.

For the dye binding procedure, 50 ml of dye buffer reagent citric acid monohydrate (42.03g) + Acid Orange 10 dissolved in 1 litre of distilled water was added from a Daffert pipette. The bottles were closed by leakproof polyethylene screw tops. Mixture were vigorously shaken overnight (c14-16 h). Portions of dye equilibrium mixture were poured into a 15 ml centrifuge tube and centrifuged at 2000 r.p.m. for 10 min. The absorbance of the supernatant was measured with reference to water at 482 nm.
Jones (1974) has shown that (a) lysine-sugar complexes in the early Maillard stages retained their basic character; (b) this basic character was lost as the browning reaction proceeded. This hypothesis has been proposed by Hurrell and Carpenter (1973). These early Maillard, basic lysine-sugar complexes and the uncharged lysine-sugar advanced Maillard do not react with the TNBS reagent. Hence the dye binding properties would be affected by these changes; and the dye would still bind with the positively-charged early Maillard lysine-sugar complexes and would not bind with the advanced Maillard sugar-lysine complexes which are not positively charged.

Jones (1974) presented the dye binding with TNBS as follows:

With control sample

\[
\begin{align*}
\text{histidine}^+ & \quad \text{dye}^- \\
\text{lysine}^+ & \quad \text{dye}^- \\
\text{lysine}^+ & \quad \text{dye}^- \\
\text{arginine}^+ & \quad \text{dye}^- \\
\end{align*}
\]

(b) dye binding after TNBS treatment

\[
\begin{align*}
\text{histidine}^+ & \quad \text{dye}^- \\
\text{lysine}^- & \quad \text{TNP} \\
\text{lysine}^- & \quad \text{TNP} \\
\text{arginine}^+ & \quad \text{dye}^- \\
\end{align*}
\]

Fig. 2.12 (a) dye binding before treatment with TNBS. (b) dye binding after TNBS treatment

Molecules of dye bound by sample prior to TNBS (a) = 5
Molecules of dye bound by TNBS treated sample (b) = 2
Hence dye binding difference (DBD) values (a-b) = 5-2 = 3
Fig. 2.13  Sample affected by early Maillard browning

(c) dye binding before TNBS treatment

P
R
O
T
E
I
N
histidine$^+$ dye$^-$
lysine-sugar$^+$ dye$^-$
lysine-sugar$^+$ dye$^-$
lysine$^+$ dye$^-$
arginine$^+$ dye$^-$

(d) dye binding after TNBS treatment

P
R
O
T
E
I
N
histidine$^+$ dye$^-$
lysine-sugar$^+$ dye$^-$
lysine-sugar$^+$ dye$^-$
lysine$^+$ dye$^-$
arginine$^+$ dye$^-$

Hence dye binding difference (DBD) values = c-d = 5-4 = 1

Fig. 2.14  Sample affected by advanced Maillard browning

(e) dye binding before TNBS treatment

P
R
O
T
E
I
N
histidine$^+$
lysine - x
lysine - x + n(dye)$^-$
lysine-sugar$^+$ (early)
arginine$^+$

(f) dye binding after TNBS treatment

P
R
O
T
E
I
N
histidine$^+$ dye$^-$
lysine - x
lysine - x
lysine-sugar$^+$ dye$^-$
arginine$^+$ dye$^-$

Molecules of dye bound by sample (with and without treatment with TNBS) = 3

Hence dye binding difference (DBD) values = 0

Jones (1974) concluded that the conventional dye binding procedures are not able to discriminate the early Maillard compounds until browning reaction has proceeded to an advanced stage and that the procedure is not satisfactory for materials which have undergone proteolysis i.e. dye binding occur only in the intact protein.
2.9.3 Acylation by ethyl chloroformate

Sandler and Warren (1974) used ethyl chloroformate as the blocking reagent. They argued that the blocking reagent should be of small molecular size so as to avoid steric hindrance to the reactions of the dye with other basic groups, react quantitatively at low temperatures with the ε-amino groups, and at the same time, do not modify the basic properties of the imidazole group of histidine or the guanidino group of arginine. Sandler and Warren's method is as follows:

i) about 120 mg of finely milled sample was moistened with 1 ml 0.5M NaOH. After 15 min, the reagent dye (40 ml) was added and mixture was stirred at 40°C at 20 min; cooled and hyflo super filter (120 mg) was added; and the solution was filtered through glass wool filter paper. An aliquot 15 ml of filtrate was diluted with phosphate buffer (potassium dihydrogen phosphate 3.4g, 85% phosphoric acid (1.7 ml), glacial acetic acid (60 ml), propionic acid (1 ml) and oxalic acid (2g) and the solution was made up to 1 litre).

ii) about 120 mg of the sample was moistened with carbonate buffer 1 ml (carbonate buffer is Na₂CO₃ in 8% NaHCO₃ to give pH 8.5.) The solution was cooled to 0° to 4°C and 0.5 ml ethyl chloroformate was added. The reaction mixture was maintained at 0° to 4°C and stirred occasionally for 20 min. Then 20 ml phosphate buffer + 20 ml dye reagent (AO 12 3.71 meq/l 0.05M phosphate buffer) and
mixture was allowed to react for 20 min at 40°C and equilibrium dye concentration determined as above.

Sandler and Warren (1974) found good agreement between DBD values and molar quantity of lysine by amino acid analysis and with lysine determined by Carpenter's method (Carpenter 1960).
3.1.0 Determination of FDNB - available lysine in the smoked and unsmoked freeze-dried coley fillets by method of Carpenter (1960) as modified by Booth (1971)

3.1.1 Materials: Mono-ε-N-dinitrophenyl lysine hydrochloride monohydrate (DNP-L) (Sigma London Chemical Co. Ltd.)

104 mg in 20 ml distilled water. Take 2 ml which then contained the equivalent of 10.2 mg or 30 μmol DNP-L FDNB solution: disposable polythene gloves were used as 1-fluoro-2,4-dinitro benzene (FDNB) is a vesicant and carcinogenic compound. Approximately 0.3 ml of FDNB in 12 ml ethanol, hence FDNB is in considerable excess and exact measurement of the FDNB solution is not essential.

Enough fresh FDNB solution was made in a conical flask for all the samples to be determined for that day. If the FDNB was solid, it was melted by standing the bottle in a beaker of hot water.

Methoxy carbonyl chloride (methyl chloroformate, MCC manufactured by Aldrich Chemicals Co., Gillingham, Dorset.) The bulk was kept in the refrigerator and a small amount kept in a service sample bottle. It does not need to be accurately measured. About 5 drops from a pasteur or dropping pipette. It is handled carefully under the fume hood as it is lachrymatorous.

NaHCO₃ – Na₂CO₃ buffer solution: 19.5g NaHCO₃ + 1g Na₂CO₃ in 250 ml distilled water and pH adjusted to pH 8.5 and stored in a well stoppered bottle.

NaHCO₃ solution of 80g in 1 litre distilled water. Phenolphthalein solution 400mg/l of 60% ethanol. NaOH solution (120g/l) for raising the pH of the ether extracted filtrate to pH 8.5 for the MCC reaction.
Peroxide free diethyl ether
Defatted (diethyl ether extracted) and finely ground
(to pass through sieve BS410 0.25 mm aperture)
smoked and unsmoked freeze-dried coley fillets.

3.1.2 Procedure:

Stage 1

i) defatted and finely ground sample (200 mg) was weighed into an ampoule (10-11 ml capacity) by standing the ampoule in a 10 ml beaker in the weighing balance. A small plastic funnel was used to introduce the sample. The funnel was tapped gently, after each weighing to make sure that all the powder went into the ampoule;

ii) sodium bicarbonate solution (1.6 ml of 8% w/v) was added to each ampoule. The ampoule was shaken gently by hand and left to stand for 10 min. to allow hydration of the sample;

iii) about 2.7 ml of ethanolic FDNB solution was added to each ampoule and the ampoules were mechanically shaken for 2 h;

iv) the ampoules were placed in a boiling water-bath until there was no effervescence on shaking. A weight loss of approximately 2.5g corresponds to complete removal of the ethanol added;

v) hydrochloric acid (6ml 8.0 M) was added to each ampoule from the burette;
vi) the space above each acid mixture in the ampoule was gently heated to drive out the air before the ampoules were carefully sealed;

For the DNP-L recoveries, the procedure was as above, with 2 ml DNP-L added before sealing the ampoule.

vii) the ampoules were placed in cans lined with cotton wool. The cans were loosely covered with a lid and placed in an oven set at 110°, for 24 h;

viii) the cans were removed from the oven, cooled and the neck of the ampoule was sawed off. The contents were filtered through a Whatman No. 541 filter paper, into a 100 ml volumetric flask. The ampoule was washed several times with small aliquots of distilled water and the filtrate was made up to volume.

**Stage 2**

Aliquots of 2 ml of the diluted filtrate was pipetted into stoppered test tubes A, B and a small sample bottle C. The tubes were twice extracted with 5 ml peroxide-free diethyl ether. The ether layer was removed by means of a pasteur pipette. The tubes were placed in a hot water-bath operating in a fume cupboard, at about 70-80° until the last traces of ether was removed (effervescence ceased) and the tubes were cooled. Tube A was made up to 10 ml with M HCl.
Stage 3

A drop of phenolphthalein was added to the 2 ml filtrate in the sample bottle C and the contents were titrated with the NaOH solution until the first pink colour appeared. The same amount of NaOH was added to tube B followed by 2ml carbonate buffer (pH 8.5).

Under the fume hood, 5 drops (about 0.05 ml) of methoxy carbonyl chloride (MCC) was added. The tube was stoppered and vigorously shaken. Pressure was released cautiously.

After about 10 min, 0.75 ml concentrated HCl was added dropwise with agitation to minimise frothing. The tube was stoppered and shaken, the solution was extracted 4 times with diethyl ether as before and the residual ether was boiled off in a hot water-bath. The tube was cooled and contents were made up to 10 ml with distilled water.

Absorbance of tubes A and B was read at 435 n.m. against a water blank in a SP500, using a 1 cm cuvette.

Reading A minus reading B (the blank) is the nett absorbance or extinction due to ε-DNP-L.

3.1.3 Absorbance of Standard ε-DNP-Lysine

The standard solution ε-DNP-L (2ml of 30 µmol in M HCl) was carried through the above procedure from stage 2 onwards, omitting the first diethyl ether extractions. Nett extinction of this standard was used as reference for calculating the unknowns.
3.1.4 Calculations

The results were calculated as in Booth (1971) in the following manner.

\[
\text{mg lysine/g sample} = \frac{w_s \times A_s \times V \times 100 \times 1000}{w_u \times A_u \times a \times (\text{CP})}
\]

- \(w_s\) = weight of standard expressed as mg lysine in 2 ml (10.2 mg used).
- \(w_u\) = weight of sample in mg.
- \(A_s\) = nett absorbance of standard.
- \(A_u\) = nett absorbance of unknown.
- \(V\) = volume of filtered hydrolysate (100 ml).
- \(a\) = aliquot of filtrate (2 ml).
- \(\text{CP}\) = crude protein, \(N \times 6.25/100\text{g sample}\).

Crude protein was determined by method of Kjeldhal digestion and the Technicon Autoanalyser for Nitrogen determination. Ammonium sulphate was used as standard for the calibration curve.

3.1.5 Recoveries of added DNP-L with samples - results and discussions

After a series of determinations on different occasions, recoveries of DNP-L with the unsmoked freeze-dried Coley fillets gave a mean correction factor of 1.043 with \(n = 11\) and 10 degrees of freedom and a range of 87-101% (Standard deviation = 0.047).

The corresponding smoked freeze-dried fillets gave a mean correction
factor of 1.096 with \( n = 11 \) (10 degrees of freedom) and a recovery range of 87-94% (standard deviation = 0.031; \( t \) test obtained was \( t = 2.93 \), significant at \( p = 0.01 \), \( F \) value = 2.29, that is, the 2 sets of correction factors were significantly different).

From these results, it is observed that the recoveries for the smoke- and heat-damaged proteins were significantly smaller, hence required a larger compensation factor of 1.096. Correction factors obtained by Booth (1971) are shown in Table 3.1.

Table 3.1 Correction factors as determined by Booth (1971)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Correction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-dried chicken meat</td>
<td>1.04</td>
</tr>
<tr>
<td>Fish meal x 836</td>
<td>1.04</td>
</tr>
<tr>
<td>Fish meal x 835</td>
<td>1.06</td>
</tr>
<tr>
<td>Meat meal x 804</td>
<td>1.07</td>
</tr>
<tr>
<td>Bovine plasma albumin</td>
<td>1.08</td>
</tr>
<tr>
<td>Heat damaged lactalbumin</td>
<td>1.16</td>
</tr>
<tr>
<td>Partly propylated lactalbumin</td>
<td>1.21</td>
</tr>
</tbody>
</table>

The results of Booth (1971) suggested that a higher correction factor is required for heat damaged protein such as heat-damaged lactalbumin and acylated lactalbumin. Booth (1971) suggested that for materials that contained no carbohydrate, a recovery correction factor of 1.05 is more suitable than the Carpenter's original of 1.09. It is suggested that either a series of recoveries should be
carried out along with the sample determinations and the mean of the recoveries should be used for the actual correction factor (as in this experiment) or individual correction factor could be used to compensate for the recovery of added DNP-L in that batch of samples.

As heat-damaged proteins seemed to yield smaller DNP-L recoveries, it is only appropriate to use a mean correction factor of 1.043 for the unsmoked and a correction factor of 1.096 for the smoke- and heat-damaged fillets. The smaller recoveries for the smoked fillets suggest that "Maillard" type compounds and residual smoke components in the smoked samples react with the added DNP-L (and with the DNP-L formed by FDNB and inherent lysine) resulting in a larger loss of DNP-L than from the unsmoked samples. There is no previous report on such interactions. The results of 1, 3 and 5 h smoked and the control unsmoked fillets are shown along with the DBD values in Tables 4.8 and 4.10. The conclusion here is that the recovery of DNP-L is influenced by the reaction conditions and the recovery factor must be modified accordingly.
3.2.0 Investigation into the effects of smoke components on DNP-L during the acid hydrolysis

Equimolar amounts of smoke component (see Table 3.2 and DNP-L were hydrolysed together in a sealed ampoule with 8 M HCl in order to assess the effects of residual smoke component or smoke-component-protein complex on the DNP-L recovery.

3.2.1 Materials: 10-11 ml ampoule; DNP-L (2 ml = 10.2 mg = 30 μmol); vanillin; vanillic acid, etc., 8 M HCl, smoke deposited from the Torry mini-kiln door.

3.2.2 Procedure:

i) equimolar quantities of ε-DNP-lysine (2 ml) and a smoke component was weighed into an ampoule. 5 ml 8M HCl was added;

ii) smoke deposit (10 mg) was weighed into an ampoule and 2 ml DNP-L + 5 ml 8.0M HCl were added;

iii) the tubes were sealed and placed in the cottonwool-lined cans in the oven at 110°C for 24 h;

iv) the tubes were cooled and the neck of the ampoule was sawn off;

v) the contents of each ampoule was filtered through a Whatman No. 541 filter paper into a 100 ml flask. The filter paper and residues were washed several times with distilled water and filtrate was made up to the 100 ml mark with distilled water;

vi) the filtrate (2 ml) was used to determine the absorbance of the recovered DNP-L as in the procedure in section 3.1.2 on the SP500 at 435 n.m. using a 1 cm cuvette;
vii) the control was DNP-L + 5 ml 8M HCl placed in an ampoule, sealed and placed in the oven, together with the other ampoules. At the end of the acid digestion time, the ampoule was opened and contents diluted to 100 ml with distilled water using a 100 ml volumetric flask. 2 ml of this filtrate was carried through the procedure as with the other samples and the mean duplicate absorbance reading for the DNP-L control was determined.

The % loss in extinction of DNP-L with the smoke component was calculated from the extinction of the control DNP-L carried through the procedure.

3.2.3 Results and discussions

The results presented in table 3.2 showed that smoke deposit from the smoking kiln and a range of smoke components reduced DNP-L recovery. From the few data available, it is not possible to suggest a mechanism for DNP-L destruction, but these results justify the use of different recovery factors for smoked and unsmoked fish samples.
Table 3.2  Effect of smoke components on DNP-L recovery after acid hydrolysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>mean A*</th>
<th>mean B</th>
<th>A-B</th>
<th>% loss in Recovery</th>
<th>% loss in Extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanillin</td>
<td>.3775</td>
<td>.035</td>
<td>.3425</td>
<td>6.73</td>
<td>6.5</td>
</tr>
<tr>
<td>vanillic acid</td>
<td>.3825</td>
<td>.0325</td>
<td>.350</td>
<td>4.21</td>
<td>3.4</td>
</tr>
<tr>
<td>acetovanillone</td>
<td>.3825</td>
<td>.0395</td>
<td>.343</td>
<td>6.57</td>
<td>5.3</td>
</tr>
<tr>
<td>guaiacol</td>
<td>.395</td>
<td>.039</td>
<td>.356</td>
<td>2.19</td>
<td>1.8</td>
</tr>
<tr>
<td>syringic acid</td>
<td>.3775</td>
<td>.0405</td>
<td>.337</td>
<td>8.59</td>
<td>7.0</td>
</tr>
<tr>
<td>syringol</td>
<td>.390</td>
<td>.0385</td>
<td>.3515</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>.395</td>
<td>.045</td>
<td>.350</td>
<td>4.21</td>
<td>3.5</td>
</tr>
<tr>
<td>smoke deposit from kiln wall</td>
<td>.338</td>
<td>.040</td>
<td>.298</td>
<td>21.72</td>
<td>17.79</td>
</tr>
<tr>
<td>DNP-L</td>
<td>.400</td>
<td>.0375</td>
<td>.3625</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Where A and B are the extinction of DNP-L of the samples measured at 435 nm on a SP500, using a 1 cm cuvette and the value of DNP-L above is taken as 100%.

Chen and Issenberg (1972) treated bovine serum albumin with selected smoke components and then observed the effects of these compounds on the availability of ε-amino groups of lysine by the addition of alcoholic 1-fluoro-2,4-dinitrobenzene (FDNB) solution. The mixture was shaken for 2 h, 8.1M HCl was added and the ampoule was sealed and hydrolysed at 110° for 24 h. DNP-lysine was extracted and determined by the method of Carpenter (1960). Chen and Issenberg
(1972) found that of the components tested, formaldehyde exhibited the maximum reaction rate in reducing the number of ε-amino groups available for reaction with FDNB, while coniferaldehyde and sinapaldehyde reacted initially at a slow rate and appeared to stop at the end of 24 h. They obtained in decreasing order of reaction, formaldehyde, glyoxal, pyruvaldehyde, furfural, sinapaldehyde, coniferaldehyde and furfuryl alcohol, while phenol, cyclotene, eugenol and syringol were the least reactive. The results in table 3.2 also show syringol and guaiacol to be less effective in destroying DNP-lysine.
The destruction of DNP-L can be illustrated in the following Fig. 3.1

**situation 1**
- formaldehyde, glyoxal, pyruvaldehyde
- added to
- Bovine serum albumin-lysine
- get X% lysine loss
- FDNB
- DNP-L
- get Y₁% DNP-L loss
- HCl

**situation 2**
- phenol, cyclotene, euginal
- no reaction with lysine
- FDNB
- DNP-L
- get Y₂% DNP-L loss
- HCl

**DNP-lysine loss in**

1) lysine loss of X%
2) DNP-L loss of Y₁%

1) DNP-L loss of Y₂%

It is difficult to determine whether DNP-L recovery was low because of:-

a) lysine destruction during the smoking process;

b) DNP-L destruction by the hydrolysis process and by the residual smoke components;

c) both (a) and (b) contribute simultaneously.
4.1.0 **Dye-binding procedures for the estimation of basic groups in smoked and unsmoked freeze-dried defatted Coley fillets**

A large number of parameters have been studied and established in the binding of Acid Orange 12 (AO.12) dye with fish proteins. \cite{Bunyan1960, Jones1974, Sandler1974, Hurrell1975} and the literature contains reviews of some of the problems associated with dye binding. From the literature, it is essential:

1) to determine the calibration curve of AO.12 dye;
2) to determine the equilibrium dye concentration (EDC) or optimum dye concentration of AO.12 dye;
   
   In the absence of previously published data, it is also essential
3) to test for smoke components interaction with the AO.12 dye;
4) to determine how the dye AO.12 reacts with short chain peptides produced by proteolysis during smoking;
5) to determine the dye binding values for both smoked and the unsmoked fillets of Coley fish;
6) to determine the dye binding difference (DBD) values of the smoked and unsmoked fillets of Coley fish.

The materials were prepared as follows:

i) one fillet of the Coley was smoked while the other fillet was the control and was not smoked;

ii) both fillets were freeze-dried, ground finely, defatted and stored as described in 4.1.1 until required.
4.1.1 Smoking and treatment of fillets before dye binding

F.A.O. (1970) recommends a low-high-low temperature cycle of 80°C-100°C-80°C during the hot smoking of fish. However, for simplicity, a constant temperature was used in this investigation, hence attempts were made to keep the production of smoke and temperature constant, rather than maintain the F.A.O. cycle.

Three fresh Coley fish purchased (from the Grimsby Fish Docks) were filleted and the fillets were placed in 5% NaCl for 15 min. The top 0.5 to 1 cm layer of one fillet (see fig. 4.1 below) was placed in the blast freezer (the control) while the other fillet of the same Coley fish was drained and placed horizontal on a wire mesh tray, in the middle shelf of a Torry Mini kiln, where smoke production from one fire box was in progress and temperature in the kiln was about 50°C. The fillets were smoked for 3 h and maximum temperature reached and maintained at 115°C. Air velocity in the middle of the kiln varied between 0.7-0.8 m/sec. After 3 h, the fillets were removed from the oven, cooled and the upper layer to a depth of approximately 0.5-1 cm was removed. Similarly, the top layer was removed from the unsmoked control before the freezing process, taking care to exclude any bones, scales and skin (as calcium ions are said to affect dye binding, Lakin 1973).

Fig. 4.1
The top layers of both unsmoked and smoked fillets were frozen before freeze drying and the freeze-dried products were ground in a hammer mill using a fine mesh. Samples were passed through a sieve of 0.25 mm aperture BS410, patent No. 667924 Serial No. 209918. Both finely divided samples of smoked and control fillets were soxhlet extracted with 100 ml diethylether for 8 hours. The extracted samples were stored in vacuum sealed high density polyethene bags at 4°C until required.

4.1.2 Determination of calibration curve of AO.12 dye

4.1.3 Procedure

1) Dye concentration of 1.42 meq AO.12 (A/SN Foss Electric, Hillerød, Denmark) in 1 litre 0.2M citric acid (0.5g acid orange 1Z + 43.02g citric acid monohydrate (AR) in 1 litre distilled H₂O) pH measured was 1.25;

ii) Dilution of this dye solution was made to obtain a range of concentrations of dye from 0.01 mg/ml to 0.034 mg/ml. Using a cuvette of 1 cm path length, the optical density was measured at 482 nm wavelength on a SP500 spectrophotometer.

It was thought wise to use the very low dye concentration range, as at the high dye concentration, De Beer's Law is not obeyed.
4.1.4 Results are as shown below:

Table 4.1 Calibration of AO.12 dye

<table>
<thead>
<tr>
<th>Concentration in y mg/ml</th>
<th>Optical density (O.D.) readings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$x_1$</td>
</tr>
<tr>
<td>0.034</td>
<td>1.52</td>
</tr>
<tr>
<td>0.0272</td>
<td>1.25</td>
</tr>
<tr>
<td>0.025</td>
<td>1.18</td>
</tr>
<tr>
<td>0.02</td>
<td>0.94</td>
</tr>
<tr>
<td>0.0167</td>
<td>0.785</td>
</tr>
<tr>
<td>0.0125</td>
<td>0.592</td>
</tr>
<tr>
<td>0.01</td>
<td>0.463</td>
</tr>
</tbody>
</table>

Correlation coefficient $r = 0.9988$

where $N$ numbers = 14, degrees of freedom $v = 12$.

Regression line obtained was $y = -0.000776 + 0.022x$.

Since the term $-0.000776$ was very small, it was ignored.

Hence $y = 0.022x$ where $y =$ amount of dye in mg/ml

$x =$ optical density reading at 482 nm.
4.2.0 Investigation into the Adsorption Isotherms of skimmed milk powder

Since extensive studies have been made about the dye binding of milk with acid azo dyes (Udy 1956; Ashworth 1961; Ashworth and Chandry 1962; Dolby 1961) and milk isotherms with acid azo dye are well established, hence it was thought appropriate initially to investigate the AO.12 with skimmed milk powder.

4.2.1 Experimental Materials

Skimmed milk powder (Marvel Brand); 100 ml polythene centrifuge tubes with fitted caps; glass beads 0.5 mm diameter; glass microfibre paper Whatman GF/A (as recommended by Sandler, 1972); acid orange 12 dye (AO.12), 0.2M citric acid (42.04g in 1 litre distilled water).

4.2.2 Procedure

i) skimmed milk powder (0.15g ± 59 mg protein) was weighed accurately into a 100 ml polythene centrifuge tube;

ii) glass beads (3 to 4) were added to each centrifuge tube;

iii) AO.12 dye (40 ml) of increasing concentrations (1.14 meq to 3.14 meq/litre 0.2M citric acid) was added. The determinations were carried out in duplicates;

iv) the centrifuge tubes were stoppered and the dye-sample mixtures were mechanically shaken for 1 h;

v) the mixtures were centrifuged at 2500 r.p.m. for
10 min. As the supernatant (after centrifuging) was still opaque, the samples were filtered through the glass micro fibre paper Whatman GF/A;

vi) the filtrate, where necessary, was diluted with 0.2M citric acid and read on a SP500 at 482 nm using a 1 cm cuvette.

4.2.3 Results and discussions

From the results shown in graphs 4.1 and 4.2 (Table 4.2 in the Appendix) of dye binding capacity (meq dye bound per g. sample) against mg of unbound dye in 40 ml, the A0.12 dye adsorption isotherm for skimmed milk powder was typical and this indicates that the experimental procedure was correct. It is essential that secondary dye binding is avoided in dye binding determinations (see literature review section 2.8.0) that is, the dye binding conditions corresponding to the horizontal portion of graph 4.1 (the saturation plateau before the secondary dye binding). The ratio (mg dye used per mg protein in sample) corresponding to this is shown in Table 4.2. Accordingly a ratio of between 0.34 to 0.61 could be adopted in future investigation if skimmed milk powder was the material to be examined. In order to examine smoked fish, the corresponding data must be similarly determined and interpreted.

The A0.12 dye worked well with the skimmed milk powder and could perhaps also work with the fish sample.
Graph 4.1  Dye binding isotherms of skimmed milk powder with acid orange 12 dye.
Graph 4.2  Graph of mg Acid Orange 12 dye bound by skimmed milk powder with increasing dye concentrations

mg bound dye g⁻¹ sample

mg dye added in 40 ml
4.3.0 Investigation of dye adsorption isotherms with smoked and unsmoked defatted fish samples

The same dye concentrations and procedure could be used to access the dye binding properties of unsmoked and smoked Coley fillets.

4.3.1 Experimental materials

As previously described in 4.2.1 except for skimmed milk powder. Finely divided, diethyl ether extracted smoked and control fillets as prepared in section 4.1.1 (the upper layers of 3 smoked fillets were mixed in a polythene bag to give a homogenous mixture. The unsmoked layers were similarly treated, to provide a larger amount of homogenous material for the initial investigations).

4.3.2 Experimental procedure

i) unsmoked and smoked samples (0.15g c. 140 mg protein) were weighed into 100 ml centrifuge tubes;

ii) glass beads (3 to 4) 0.5 mm diameter were placed in each centrifuge tube;

iii) 0.12 dye (40 ml) of increasing strength (0.86 meq/l to 3.42 /l in 1 litre 0.2M citric acid) was added. The determinations were carried out in duplicates;

iv) the tubes were tightly stoppered with plastic caps and mechanically shaken for 1 hour;

v) the tubes were then centrifuged at 2500 r.p.m. for 10 min;

vi) the supernatant was pipetted off using a dropping pipette and 1 ml diluted where necessary. The absorbance was read against 0.2M citric acid on a SP500 spectrophotometer at 482 nm using a 1 cm cuvette.
4.3.3 Results and discussion

The dye binding isotherms for unsmoked and smoked samples are shown in Table 4.3 in the Appendix. The curves of dye binding capacity (DBC, meq dye bound per g sample) with equilibrium dye concentration are similar to the dye binding isotherms of skimmed milk powder. (Graphs 4.1 and 4.2). The milliequivalents of dye bound per unit weight of sample (DBC) increased with increasing dye concentrations. However, the DBC of the smoked samples were higher at the lower dye concentration of the isotherm than the DBC of the unsmoked samples. This suggested that the smoked and heat-processed proteins contained more basic amino acids than the unsmoked controls. It is reasonable to expect hot smoking to reduce the number of basic groups and thus the DBC of the smoked fillets, compared to the unsmoked controls.

Assuming that at these low dye concentrations, there was no dye-dye or other secondary binding mechanisms operating, possible explanations for the anomalous results include:-

i) effect of the 5% NaCl solution used for both the smoked and unsmoked fillets before the smoking and freeze-drying process;

ii) reactions of residual smoke basic phenolic components with the dye anions;

iii) proteolysis during the smoking process, resulting in short chain peptides and N-terminal groups which could bind with the AO.12 dye.
Graph 4.3 Dye binding isotherms of freeze-dried smoked and unsmoked Coley fillets in the presence of chloride ions.
Graph 4.4 Mg dye bound g⁻¹ sample of freeze-dried smoked and unsmoked Coley fillets with increasing dye concentration in the presence of chloride ions.
4.4.0 Investigation of dye binding properties of smoked and unsmoked Coley fillets without the 5% NaCl

In the typical commercial smoking process, fish fillets are soaked in brine containing up to 10% NaCl before the smoking process. The salt reacts with the surface free amino acids and proteins to give the smoked fish a glossy appearance which appeals to the customer.

4.4.1 Experimental materials

The fillets were prepared as in 4.1.1. Since the salt might be responsible for the anomalous results in section 4.3.2, the brining operation was omitted.

4.4.2 Experimental procedure

The dye binding properties of this batch of samples (without sodium chloride) were determined as in the procedure in 4.3.0.

4.4.3 Results and discussion

Dye binding isotherms of smoked and unsmoked samples are shown in Table 4.4 in the Appendix. At the high dye concentrations, the amount of dye bound by the unsalted smoked samples was slightly more compared to the salted smoked fillets. Similarly dye binding was less for the unsalted unsmoked samples (see graphs 4.5 and 4.6). The difference between the salted and unsalted samples was small as the amount of sodium chloride, used in the previous batch of fish samples, was very small. Udy (1971) found that when chloride ions were incorporated in buffers, high dye binding occurred. Jones (1974) reported the reverse effect with chloride ions. Consistent with the report of Jones (1974) omission of the salt increased the dye binding. However, at the low-dye concentrations (16 mg to 30 mg per 40 ml), the
Graph 4.5  Dye binding isotherms of freeze-dried smoked and unsmoked Coley fillets without chloride ions

dye binding capacity (DRC) meq dye g⁻¹ sample

equilibrium dye concentration meq l⁻¹
Graph 4.6  \( \text{Mg dye bound g}^{-1} \) sample of freeze-dried smoked and unsmoked Coley fillets without chloride ions.
smoked samples still bound more dye than the unsmoked samples indicating that the salt content was not responsible for the anomalous results presented in section 4.3.2. It was observed that the dye supernatant from the unsmoked samples were turbid. It was assumed that this turbidity might account for a larger than normal replicate variation. Of more importance, however, is the possibility that light scattering was causing false high "light absorption" readings that in turn implied high dye concentration in the supernatant and low dye binding by the unsmoked samples. It was postulated that the turbidity might be caused by:-

i) residual lipids in the unsmoked fish samples; or

ii) effects of low dye concentration on dye binding by proteins.

Fraenkel-Conrat and Cooper (1944) have stated that

"when less than equivalent amounts of dyes were added to dissolved proteins, the resulting protein-dye complexes were not completely precipitated. This resulted, paradoxically, in higher colourimetric readings in the soluble phase, than when sufficient dye for the saturation of the protein was added."

Fraenkel-Conrat and Cooper (1944) used gelatin granules. Similar observations were reported by Rawlins and Schmidt (1930).

The data here suggest possible additional cause for the anomalous dye binding behaviour in sections 4.3.0 and 4.4.0, and
the following factors must be investigated:

a) interaction between residual smoke components and AO.12 dye;

b) interaction between AO.12 dye and basic ends of short chain peptides and N-terminal of free amino acids produced by proteolysis during the smoking process.
4.5.0 Investigation of possible interactions between smoke components and A0.12 dye

It is possible that the diethyl ether extractions have not completely removed all the smoke components from the smoked fillet samples and that these residues may modify the optical properties of the dye supernatant.

To observe how the smoke components may react with the A0.12 dye, the following experiment was made.

4.5.1 Materials

Selected smoke components 0.2M citric acid; 1.42 meq A0.12 dye/litre

4.5.2 Procedure

i) smoke component (10 mg) was dissolved in 1 ml propan-2-ol in a test tube (determinations were in duplicates);

ii) to this was added 5 mg dye in 10 ml 0.2M citric acid (i.e. 0.5 g/l dye solution);

iii) the absorbance of each mixture was read against 0.2M citric acid on the SP500 at 482 nm and the wavelength maximum absorbance was recorded;

iv) a steel plate 10.2 cm x 15.4 cm was hung in the kiln and smoked for 3 hours, 40 ml methanol was used to wash the plate. 1 ml of the wash solution was added to 10 ml of above dye solution and absorbance recorded as above.
The same experiment was repeated using the same smoke components and a higher dye concentration of 12mg dye in 10 ml 0.2M citric acid (3.42 meq A0.12 dye/litre 0.2M citric acid).

Suitable dilutions with 0.2M citric acid were made where necessary. The "control" or unreacted dye was 10 ml dye of the appropriate concentrations + 1 ml propan-2-ol (70% w/v).

4.5.3 (a) Results and discussions

The results for the selected smoke components with dilute dye are shown in Table 4.5 and show a reduction in extinction of the A0.12 dye with each smoke component tested and with the smoked plate deposit. In all cases \( \lambda_{\text{max}} \) fell in the range 482 ± 2 nm. These results suggest that any residual smoke components in the diethyl ether extracted smoked samples will reduce the supernatant extinction and thus imply a false high dye binding ability for that smoked sample.

It would appear that simple phenols or phenol ethers have only a small effect but the presence of a carbonyl group, a carboxylic acid group or an unsaturated aliphatic side chain para-positioned to the hydroxyl group, markedly increased the effect. The phenolic fraction of wood smoke alone is reported to be complex and some of the components is said to have carbonyl as well as phenolic functional groups (Issenberg, Korneich and Lustre, 1971) who also showed the difficulty in completely removing all the phenols in the form of smoke condensates added to pork belly. When 10 ml of the low dye concentration of 1.42 meq A0.12 dye/litre 0.2M citric acid was added to 10 mg of these compounds, the extinction was reduced. When converted to reduction per mole of compound; of the syringol and
Table 4.5 Reduction in extinction of AO.12 dye (5 mg in 10 ml or 1.42 meq/litre) per mole of compound used, at 482 nm on SP500

<table>
<thead>
<tr>
<th>Substitution Groups R</th>
<th>O</th>
<th>C-CH₃</th>
<th>COOH</th>
<th>H-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>p hydroxy benzaldehyde</td>
<td>103</td>
<td>408</td>
<td>350</td>
</tr>
<tr>
<td>-CHO</td>
<td>p anisaldehyde</td>
<td>286</td>
<td>396</td>
<td>450</td>
</tr>
<tr>
<td>-COOH</td>
<td>benzoic acid</td>
<td>199</td>
<td>729</td>
<td>785</td>
</tr>
<tr>
<td>-CH₂ = CH= CH₂</td>
<td>guaiacol</td>
<td>87</td>
<td>55</td>
<td>77</td>
</tr>
<tr>
<td>-CH=CH-CHO</td>
<td>cinnamaldehyde</td>
<td>396</td>
<td>449</td>
<td>449</td>
</tr>
<tr>
<td>-CH=CH-COOH</td>
<td>4(OH)-3(CH₂O) cinnamic acid</td>
<td>1010</td>
<td>1081</td>
<td>450</td>
</tr>
</tbody>
</table>

Reduction R = CH₂CHO acetate aldehyde 44.

Formaldehyde

Smoke solution 0.0356/square cm/min smoking time.
guaiacol group, acetylodyringone, syringaldehyde and syringic acid gave
the greatest reduction then came acetovanillone, vanillin and
vanillic acid in decreasing order. Syringol and guaiacol, not
surprisingly, did not give a strong reduction but the derivatives
of syringol greatly reduced the extinction coefficient showing
functional activity of the aldehyde, ketone and carboxyl groups.
The methoxy and hydroxy groups are electron-donating, contributing
to electron delocalisation and hydrogen bonding between the smoke
components and the $-N = N-$ bond of the A0.12 dye, hence causing the
reduction in the extinction coefficient of the dye. If the ortho-
hydroxy benzoic acid (which is very likely to have intramolecular
hydrogen bonding) is compared with $p$-hydroxy benzoic acid, the
reduction effect by the $o$-hydroxy benzoic acid is less due to its
intramolecular hydrogen bonding effect, hence there is less binding
to the $-N = N-$ bond of the A0.12 dye. Hence the largest reductions
per mole were obtained with ferulic acid, caffeic acid, 4-allyl-
2,6-dimethoxy phenol, 3,4,5-trimethoxy benzoic acid, 2-allyl phenol
3,5-dimethoxy-4-hydroxy cinnamic acid, 3,4-dimethoxy benzaldehyde and
cinnamaldehyde in decreasing order. During the hot smoking of meat
products at high temperature and high relative humidity, the phenols
such as syringaldehyde, acetylodyringone and acetovanillone are said
to be better adsorbed in the meat product because of the greater
polarity of these components. Potthast (1971) showed that there is
great loss of phenols with reactive carbonyl groups caused by
condensation reactions of the carbonyl groups with the free amino
groups of the protein when he compared the distillate of the water-
smoked solution and distillate of the smoked-meat homogenate, and he
also found phenol, guaiacol, vanillin, acetylodyringone and syringaldehyde.
in high amounts in the distillate of the smoked meat homogenate containing the phenol fraction. Hence not surprisingly, when all these components were present in the smoke solution (washings of the smoked steel plate), the combination of these smoke compounds together gave the greatest reduction in the optical density of the dye.

4.5.3 (b) At the high dye concentration of 3.42 meq A0.12 dye/litre 0.2M citric acid (i.e. 12 mg in 10 ml), there was no change in the extinction of the dye solution. It could be that interactions between the dye and the smoke components (at that dye concentration used) had reached saturation.

Although the amount of smoke removed from the smoked fillets after the ether extraction is not known, it would appear that the effect at high dye concentration is reduced as the molar ratio of "smoke" to dye in the supernatant is reduced. Therefore it is desirable to use a suitably high dye concentration (see dye isotherms with smoked samples section 4.7.0), consistent with other constraints such as no secondary dye binding prevailing.
4.6.0 Investigation of the effects of proteolysis during smoking - dye binding of short chain peptides, amino acids and amines

Hurrell and Carpenter (1975) reported that proteolysis leads to increase in terminal-amino groups by fission of peptide chains. The terminal-NH₂ groups will also bind with the dye. Lakin (1975) has also mentioned it in his report on dye binding of proteins at the Food Group Symposium. If proteolysis could have taken place while the fillets were hung in the smoking kiln during the smoking process, this would result in more terminal-NH₂ groups which in turn would bind more dye. This increase in DBC might account for the smoked fillets binding more dye than the unsmoked controls.

4.6.1 Experimental materials

3.71 meq AO.12 dye/litre 0.2M citric acid (1.3g AO.12+42.03g citric acid monohydrate (AR); 0.2M citric acid; amino acids, short chain peptides and polypeptides of different molecular weights as shown in Table 4.6.

4.6.2 Procedure

i) approximately 8 mg of short chain peptides and polypeptides were weighed accurately into test tubes in duplicates;

ii) AO.12 dye (10 ml of 3.71 meq/litre 0.2M citric acid) was added to each tube;

iii) the tubes were stoppered and vigorously shaken for 2 minutes to mix and the tubes were centrifuged;

iv) each supernatant (1 ml) was suitably diluted with
0.2M citric acid and the extinction read against 0.2M citric acid on the SP500 at 482 nm using a 1 cm cuvette. 1 ml of the unreacted dye was diluted (x400) and the extinction was recorded. Hence absorbance of the unreacted dye minus the absorbance of the dye + polypeptides mixture would give the reduction in extinction. The calibration curve in section 4.1.1 was used to calculate the amount of dye bound by 8 mg of sample. Hence mole of dye bound per mole of sample per basic residue (histidine, arginine, reactive lysine and terminal-amino groups), was determined.

4.6.3 Results and discussion

Proteolysis is said to be most active during the initial hours of the smoking process, as the temperature of the fillets rose, before the proteolytic enzymes were denatured by heat and smoke components.

The results presented in Table 4.6 indicate that the low molecular mass compounds bound a barely detectable amount of dye (a range of 0 to 0.05 moles of dye per basic group per mole of compound).

In contrast several of the naturally occurring higher molecular mass compounds bound substantially more dye (a range 0.59 to 0.72 moles per basic (histidine, arginine, reactive-lysine and terminal amino) group per mole of compound) indicating a tendency for the binding to rise with increased molecular mass. This mass-binding relationship however requires confirmation by using a larger range of compounds. From the results of Hurrell and Carpenter (1975), the
Table 4.6  Dye binding of AO.12 dye by short chain peptides, N-terminal-amine groups and amino acids

<table>
<thead>
<tr>
<th>Sample</th>
<th>molecular weight</th>
<th>O.D.*</th>
<th>No. of basic * groups*</th>
<th>mole dye/mole compound per basic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine monoHCl</td>
<td>209.63</td>
<td>3.4</td>
<td>2</td>
<td>0.030</td>
</tr>
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<td>Lysine mono HCl</td>
<td>182.65</td>
<td>3.0</td>
<td>2</td>
<td>0.020</td>
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<tr>
<td>Arginine mono HCl</td>
<td>174.20</td>
<td>11.0</td>
<td>4</td>
<td>0.040</td>
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<tr>
<td>Glycine</td>
<td>75.07</td>
<td>7.4</td>
<td>1</td>
<td>0</td>
</tr>
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<td>Glyglycine</td>
<td>132.12</td>
<td>0</td>
<td>1</td>
<td>0.043</td>
</tr>
<tr>
<td>Valine</td>
<td>117.15</td>
<td>1.0</td>
<td>1</td>
<td>0.001</td>
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<td>Butylamine</td>
<td>73.14</td>
<td>9.4</td>
<td>1</td>
<td>0.050</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>95.57</td>
<td>6.2</td>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>69,000</td>
<td>11.0</td>
<td>98</td>
<td>0.59 (1.05)*‡</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,500</td>
<td>19.0</td>
<td>62</td>
<td>0.600</td>
</tr>
<tr>
<td>Poly-L-leucine</td>
<td>15,000</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Poly-L-leucine</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Poly-L-lysine</td>
<td>13,000</td>
<td>43.0</td>
<td>62</td>
<td>0.720</td>
</tr>
<tr>
<td>Insulin</td>
<td>5,733</td>
<td>11.0</td>
<td>14</td>
<td>0.620</td>
</tr>
</tbody>
</table>

* O.D. = reduction in optical density.

* histidine, arginine, lysine and terminal-NH₂ groups in 1 mole of compound.

‡ as determined by Hurrell and Carpenter (1975) with bovine plasma albumin.
moles of dye bound per mole of bovine plasma albumin per basic residue was calculated to be 1.05 as they (Hurrell and Carpenter 1975) used optimum conditions in which one would expect a 1:1 molar binding. However, for a short investigation of the naturally defined proteins such as bovine serum albumin, myoglobin, poly-L-lysine and insulin, the conditions used here were not ideal or optimum conditions and would perhaps need a greater amount of dye for a 1:1 molar binding ratio.

Since the fish proteins are treated as optimum dye binding conditions, that is, 1 mole dye per basic group, these proteins might bind 30 times as much dye as the basic group in a free amino acid. If 30 amino acids or short chain peptides are split off from a high molecular mass protein during the early stages of smoking, it follows that the released small residues would bind as much dye as the remaining large molecule.

Logically this implies:—

1) that proteolysis during the early stages of smoking is very slight; or

2) the released fragments are rapidly lost by extensive "Maillard" type reactions; or

3) combination of factors (a) and (b).

otherwise dye binding would rise substantially for smoked products. It is possible, therefore, that the slightly higher DBC at the low dye isotherm for the smoked samples could in part be accounted for in this way. Alternatively these anomalous results in section 4.3 and 4.4 could arise because of incomplete protein precipitation (Fraenkel-Conrat and Cooper 1944).
Hurrell and Carpenter (1975) reported that proteolysis may lead to the production of free amino acids which are still of nutritional value but no longer cause dye binding as measured by the precipitation of the dye. However, the results here show that the small molecular mass amino acids hardly bind dye while small peptides bind to a very small extent, that could only be significant if proteolysis is extensive, thus essentially confirming their (Hurrell and Carpenter, 1975) report.
4.7.0 Investigation of dye binding properties of AO12 dye with the smoked and unsmoked freeze dried Coley fillets

Dye adsorption isotherms of AO12 dye with the smoked and unsmoked freeze dried fillets

The dye binding of the smoked and unsmoked defatted and freeze-dried samples were tested with increasing concentrations of AO12 dye as in previous experiment.

4.7.1 Materials

As in 4.4.1

4.7.2 Procedure

was as in 4.4.2.

4.7.3 Results and discussions

The adsorption isotherms were constructed (from table 4.7 in the appendix) by plotting the dye binding capacity (DBC meq dye g\(^{-1}\) sample) against the equilibrium dye concentrations (EDC). The curves obtained were similar to the adsorption isotherm curves obtained by other workers (Ashworth and Chandry 1962; Jones 1974). At the low AO12 dye concentrations of 0.86 meq to 1.42 meq/1 litre 0.2M citric acid, the effect of insufficient dye concentration on the unsmoked fillet protein was again observed as in section 4.3 and 4.4. (Fraenkal-Conrat and Cooper 1944 and Rawlins and Schmidt 1930), but the effect was to a much smaller extent. The smoked samples attained dye binding saturation earlier (that is, at lower dye concentration than the unsmoked samples). This behaviour is consistent with a reduction in the number of basic groups by the smoking process, because advanced "Maillard" type compounds and "protein-protein" type
Graph 4.7  Adsorption isotherm on smoked and unsmoked Coley fillets using acid orange 12 dye
Mg free dye against mg of acid orange 12 added to freeze-dried smoked and unsmoked Coley fillets.

Graph 4.8

mg free or unbound dye supernatant

mg acid orange 12 added in 40 ml
Graph 4.9  Mg acid orange 12 bound g⁻¹ sample of freeze-dried smoked and unsmoked Coley fillets
compounds (see section 2.9.2) are not able to bind with the dye anions.

The DBC of both the unsmoked and smoked samples increased with increase in equilibrium dye concentration (EDC) until the dye binding saturation plateau was reached (about 3.85 meq dye/litre 0.2M citric acid cf the dye isotherms in section 4.4.) From the data on the dye binding saturation plateau, a tentative procedure for the dye binding of smoked and unsmoked samples can be devised.
4.8.0 Investigation of dye binding properties of fillets smoked over a period and blocking or acylation of the \( \varepsilon \)-amino groups of the smoked and unsmoked fillets

The results in section 4.7.0 indicate that the smoked and unsmoked samples reached the dye binding saturation plateau by 4.0 meq/litre dye solution. Accordingly this concentration was used in all subsequent work to determine the dye binding properties of the unsmoked controls and fillets smoked over 1, 3 and 5 h.

In order to follow the changes in the \( \varepsilon \)-amino groups of lysine during the smoking, in addition to the total basic residues (histidine, arginine and reactive-lysine), blocking of the lysine side chains was by methoxy carbonyl chloride acylation. These results obtained for lysine can be compared with the results for lysine obtained by the FDNB method in section 3.1.0.

4.8.1 Materials

Eleven Coley fish were filleted and the fillets marked with small coloured beads so that pairings could be maintained. One set of 11 fillets were kept as unsmoked controls. From each, 2 successive approximately 0.5 to 1 cm layers were removed and freeze-dried. Bones, scales and skin were carefully removed. The other set of 11 fillets were smoked in the Tarry mini-kiln as in section 4.4.1. After 1 h, 3 fillets were removed from the top tray; after 3 h 4 fillets from the middle tray and after 5 h, the last 4 fillets on the bottom tray which was shifted up to middle position of the kiln at 3 h. Temperature of smoking kiln was maintained at 115\(^{\circ}\). After the smoked fillets were cooled, the layers were removed as described for the unsmoked fillets (see fig. 4.2 overleaf).
layers were placed in the blast freezer and the frozen layers were freeze-dried. The samples were then ground in a hammer-mill to pass through a sieve BS410 of 0.25 mm aperture, defatted by diethyl ether extraction in a soxhlet apparatus (twice with 100 ml diethyl ether) and stored vacuum sealed in polythene bags at 4°C (as they were to be used fairly rapidly over a short period of time, there was no essential need to store them in the frozen state).

4.8.2 Investigation of dye binding values of unsmoked and smoked Coley fillets (1, 3 and 5 h smoking time)

4.8.3 Materials

4.0 meq AO.12 dye/l 0.2M citric acid (1.40g
AO.12 dye + 42.03g citric acid monohydrate (AR) in
1 litre distilled water; glass beads 0.5 mm diameter;
100 ml polythene centrifuge tubes with plastic caps.

4.8.4 Experimental procedure

1) approximately 0.15g of the sample (c. 140 mg protein)
prepared in section 4.8.1 was weighed accurately into
a 100 ml polythene centrifuge tube;
ii) Glass beads (3 to 4) were added to each sample in the centrifuge tubes. A glass microfibre filter paper Whatman GF/A was wetted with 0.2M citric acid, drained and placed in (this is to account for the c.2 ml citric acid in the GF/A paper used in the blocking process);

iii) A0.12 dye (40 ml of 4.0 meq. A0.12 dye/litre 0.2M citric acid, i.e. 1.4g dye/litre or 56 mg in 40 ml; compared to Hurrell and Carpenter 1975, 54.5 mg in 40 ml and Jones 1974, 54 mg in 50 ml but they used different sample size, Hurrell and Carpenter, 1975, c.5 mg lysine sample and Jones, 1974, c.100 mg protein sample);

iv) the tubes were stoppered with the plastic caps and mechanically shaken for 1 h;

v) the sample dye mixture was centrifuged at 2500 r.p.m. (1 ml of the supernatant was diluted to 10 ml with 0.2M citric acid, where necessary) and the absorbance was read against 0.2M citric acid on the SP500 at 482 nm using a 1 cm cuvette.
4.8.5 Results and discussions

The results are presented in Table 4.8.

The DBC (dye binding capacity meq g\(^{-1}\) sample) was calculated and percentage loss in basic amino acid of the smoked layers compared with the unsmoked controls were determined from the O.D. readings, using the calibration curve in section 4.1.2.

There was an increasing percentage loss of basic amino acids with increased time of smoking. The upper layers of the smoked fillets showed a greater loss in basic amino groups than the lower layers. This was observed for all the smoked fillets of 1, 3 and 5 h smoking time.

There was a small loss of 2.49% (mean of the 2 layers) in basic amino groups in the upper and lower layers of the 1 h smoked fillets. As reported by Jones (1974), the early "Maillard" type compounds are still basic and will bind with the dye (see section 2.9.2). The percentage loss in basic amino groups from the upper layers of the 3 h smoked fillets were significant from the unsmoked controls (student 't' test = 4.88, F = 1.40, \(p = 0.01\)) with a mean of 9.16% loss in DBC, but the average % loss of both the upper and lower layers showed a mean of 6.01%.

Greater loss in basic amino acids of both upper and lower layers of the 5 h smoked fillets were observed with a mean of 17.85% for the upper layers and a mean of 8.26% for the lower layers (3 samples as the lower layers of the fourth sample was lost), hence the average of both upper and lower layers showed a loss of 13.05%.

The final 2 hours of smoking produced a smaller loss of lysine in the upper layers than the upper layers of the first 3 hours of
the smoking process. This could be because of:-

a) the smoke-protein interaction approaching saturation towards the end of 5 h smoking;

b) the declining relative humidity and moisture content of the surface layer, limiting adsorption and damage towards the end of the process as reported by Foster and Simpson (1961) for smoked fish;

c) combination of factors (a) and (b) above.

Caurie (1975) has shown that the amount of smoke adsorbed increased with increasing temperature from 45° to 72° and depended on the speed of smoke flow. When the oven was held smoke tight, the amount of smoke adsorbed was limited and the drying process was slow. When smoke was allowed to escape rapidly with a high air velocity, adsorption was rapid initially and gradually slowed down as the fish flesh dried. Therefore, with high air velocity, the drying process was rapid.
4.9.0 Blocking or acylation of the $\varepsilon$-NH$_2$ groups of lysine by methoxy carbonyl chloride (methyl chloroformate)

4.9.1 Materials

Unsmoked control and smoked (1, 3 and 5 h) fillets of section 4.8.1., 3.2 meq AO.12 dye/litre 0.2M citric acid (1.12g dye/litre 0.2M citric acid); buffer 8% ($w/v$) NaHCO$_3$ – 8% ($w/v$) Na$_2$CO$_3$ solution (19.5g NaHCO$_3$ + 1g NaCO$_3$ in 250 ml distilled water), pH adjusted to pH 8.5; methoxy carbonyl chloride (MCC).

4.9.2 Experimental procedure

i) freeze-dried defatted sample (0.15g) was weighed into a 100 ml polythene centrifuge tube;

ii) sodium bicarbonate-sodium carbonate (prepared in section 3.1.0) buffer pH 8.5 (2 ml) was added and the tube was shaken by hand to mix and left to stand for 15 min to allow hydration of the samples to take place;

iii) methoxy carbonyl chloride (0.5 to 1 ml) was added by a graduated pipette attached to a pumpette transfer was made under the fume hood as MCC is lachrymatus);

iv) four glass beads were added, the tube was stoppered and mixture was vigorously shaken occasionally over 20 min and filtered through a glass micro fibre filter paper Whatman GF/A. The filtrate was washed with 5 ml citric acid and allowed to drain. The filter paper and its contents was carefully wrapped and placed back into the 100 ml polythene centrifuge tube;
v) the dye (40 ml of 3.2 meq/1) was added, the tubes were re-stoppered and samples were shaken mechanically for 1 h;

vi) the samples were centrifuged at 2500 r.p.m. for 10 min, supernatant was removed by a dropper pipette and 1 ml was diluted to 20 ml with 0.2M citric acid. The absorbance was read against 0.2M citric acid on the SP500 at 482 nm using a 1 cm cuvette.

It is essential to reduce the amount of dye in 40 ml for the acylated sample so that the dye binding properties of the blocked and untreated samples were determined at (3.2 meq l⁻¹) and 4.0 meq l⁻¹ for blocked and untreated samples respectively) similar equilibrium dye concentration (c. 2 meq l⁻¹). Hence the amount of dye bound by secondary binding associations were of the same order (Jones 1974).

4.9.3 Results and discussions

The amount of dye bound per g sample was calculated from the O.D. readings, using the calibration curve in section 4.1.2. Hence the dye binding capacity (milliequivalents of dye bound per gram of MCC - treated sample) values were calculated as in section 4.8.0.

The dye binding difference values were calculated as in Jones (1974).

\[
\frac{\text{meq dye bound to untreated sample}}{\text{sample weight}} - \frac{\text{meq dye bound to treated sample}}{\text{sample weight}} = \text{dye binding difference (DBD)}
\]
Table 4.8  Dye binding values of unacylated samples and DBD-lysine the samples treated with MCC compared with FDNB-reactive lysine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Untreated samples</th>
<th>MCC Treated samples</th>
<th>FDNB-available lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBC-1% loss DBC</td>
<td>mg lysine % loss lysine</td>
<td>Average % loss of 2 layers</td>
</tr>
<tr>
<td></td>
<td>meq g in sample</td>
<td>g sample</td>
<td>meq g in sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UU 1 1</td>
<td>.995 .616 .902  c</td>
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Table 4.8 cont.

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<th>Sample</th>
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<th>MCC Treated samples</th>
<th>FDNB-available lysine</th>
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<td>mg lysine multiplied % loss average of 2</td>
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<td>meq g⁻¹ in DBC sample</td>
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DBD x 146.2 = mg lysine g⁻¹ sample.
UU = upper 0.5 to 1 cm layer of unsmoked fillet.
UL = lower 0.5 to 1 cm layer of unsmoked fillet.
SU = upper 0.5 to 1 cm layer of smoked fillet.
SL = lower 0.5 to 1 cm layer of smoked fillet.
1.1 = sample 1, 1 hour smoking.
3.1 = sample 1, 3 hours smoking
5.1 = sample 1, 5 hours smoking.
The unsmoked, freeze-dried control fillets showed a DBD (MCC)-lysine content average of 92.25 mg/g sample while FDNB-reactive lysine average was 95.06 mg/g sample. Tookey and Lawrie (1974) showed calculated values of 103.68 mg/g sample and obtained 94.55 mg/g sample FDNB-lysine (average for fresh and frozen raw Coley fillets, converted to dry matter basis, assuming 80% water). This difference might reflect some loss of available lysine during the acid hydrolysis of the protein or natural variations.

With the 1 h smoked fillet layers, there is little loss of FDNB-available lysine or lysine by dye binding difference (DBD), giving an average (of the 2 layers of the 3 fillets) of 2.71% and 2.97% respectively. Dames and Johnson (1968) reported that provided the temperature does not exceed 100°C and the period of heating is not more than 1 h., there is little damage to protein. The upper layer of the smoked 1 h Coley fillets showed a greater loss than the lower layer reflecting their relative exposure to heat and smoke.

After 3 h smoking at 115°C and at high relative humidity (wetted mixture of hardwood and softwood sawdust was used), there was significant average loss of 13.95% FDNB-lysine and of 13.98% DBD-lysine from the upper smoked layers, while the loss from the lower layers was relatively small, with losses of 6.97% FDNB-lysine and 4.61% DBD-lysine. The grand average losses for both layers of 4 fillets were 10.46% FDNB-lysine and 9.29% DBD-lysine.

There were large losses of lysine by both methods with the fillets
smoked for 5 h. The top layer showed average losses of 24.95% FDNB-lysine and 25.27% DBD lysine, while the lower layers showed average losses of 11.32% FDNB-lysine and 10.91% DBD lysine.

Taking all values for smoked and unsmoked samples the DBD (MCC)-lysine values correlated well with FDNB-lysine values ($r = 0.97$, $p = 0.01$) and regression equation obtained is $Y = 0.998X - 2.120$ where $Y =$ mg FDNB lysine and $X =$ mg DBD (MCC) lysine.
4.9.4 Effects of smoke components

Dvorak and Vognarova (1965) suggested that short-time smoking has no effect on the lysine availability but loss was evident after exposure to smoke for several hours or days. They reported that with sausages hot smoked for a short time (2-3.5 h maximum), the content of available lysine was reduced by approximately and not more than 20% and that formaldehyde might be the most active smoke component in the reactions with the ε-amino group of lysine.

Chen and Issenberg (1972) obtained a loss of 44.5% FDNB-available lysine in 2-3 mm thick lean beef sirloin slices smoked for 10 h at 65°C and in the unsmoked controls, heat-treated for 10 h at 65°C samples, a loss of 15.2% FDNB-lysine was obtained, clearly showing that loss of available lysine in smoked samples is not by heat alone and that there is inter-reaction between the ε-amino groups of the protein and the smoke components. Chen and Issenberg (1972) also showed by the effects of treating meat homogenate with smoke fractions, that the phenolic and neutral fractions of the smoke components react with the ε-amino groups of the protein and obtained 45% destruction of FDNB-available lysine by the neutral fractions, 38% loss by the phenolic fractions and 14% by the acidic fractions.

In the 5 h smoked samples (section 4.9.3) the greater loss of available lysine in the surface layer compared with the lower layer must reflect the interactions between the ε-amino groups of lysine with the smoke components in the surface layer (see literature review, section 2.2), while the deeper layers beneath the surface could have suffered loss of available lysine by heat and drying.
process with "Maillard" type reactions and protein-protein interactions (see section 2.2).

Caurie (1975) smoked butterfish for 24 h at 45° and obtained 42% loss of lysine (for whole fish), and for 24 h at 120°, obtained 55.5% loss of lysine (for the whole fish). The African fish Tilapia lidole, reported by Hoffman et al. (1977) showed approximately 6% loss of FDNB-lysine in the whole fish tilapia smoked at 75°, and 9% loss in FDNB-lysine in the whole fish tilapia smoked at 100°, both stored under nitrogen, while a sample of I. lidole traditionally smoked for 4 h with 95° temperature (recorded in the centre of fish), showed a loss of 6%, Sardinella aurita, traditionally smoked for 36 h at temperature between 100° to 112° (in wood fireclay-kilns) showed 29% loss of FDNB-lysine.

4.9.5 Effect of protein-protein interaction

Carpenter et al. (1962) have shown that the small amount of free and combined sugar in herring meal made from press cakes, contribute only a small fraction of the major loss of available lysine resulting from severe over-heating of fish meal. It is known that carbonyl-amino browning reactions are not stoichiometric, as the first formed condensation products, after rearrangement or degradation, can react with further molecules containing either carbonyl or amine groups but the discrepancy between the amount of active carbonyl groups available and potential amino acids is too large for this mechanism to account solely for the loss in available lysine. Hence other amino acids in side chains must be involved but lysine is usually the first to be affected (Lea and Hannan 1950).
Ford and Shorrock (1971) found 33.7% fall in FDNB-available lysine with cod heated for 20 h at 135°C in air. It must be remembered that the amount of pentose (ribose), hexose phosphate and fat in cod is very small and therefore could only amount for a small part of the loss of lysine. Most of the loss must be from amino acid cross-linkages in the denatured protein.

Hurrell and Carpenter (1975) obtained in bovine serum albumin, heated for 8 h at 121°C, a loss of 22.0% FDNB-lysine and in defatted chicken muscle heated for 2 h at 121°C, a loss of 6.04% FDNB-lysine and for the same defatted chicken muscle heated for 4 h at 121°C a loss of 11.76% FDNB-lysine. The destructive effect of heating carbonyl or aldehydric groups from glucose or ribose is illustrated by Hurrell and Carpenter (1975) when they heated albumin mix (ovalbumin-lactalbumin, 3:2 w/w) with glucose for 15 min at 121°C and results showed a large loss of 88.00%.

4.9.6 Effect of fat and protein-protein interaction

Lea, Parr and Carpenter (1960) showed the effect of fat oxidation products on lysine with cod muscle heated for 48 h at 37°C with propanal.

In the presence of propanal, lysine loss (FDNB-lysine) was about 33% higher than in the control sample to which no propanal was added. Propanal seemed to react rapidly with dry cod muscle but the effect was retarded by high moisture content. Fresh herring meal and pre-oxidised herring meal, heated in the laboratory in the absence of oxygen for 30 h at 105°C suffered available lysine losses of 19% and 27% respectively, and for 30 h at 130°C, losses were 67% and 69% respectively. Hence at high temperatures, rancid fat contributes
significantly to loss of available lysine. One would also expect the unsaturated lipids in fresh herring to become oxidised rapidly, thus accounting for similar losses from fresh and rancid herring samples after a long period of heating.

Tooley and Lawrie (1974) obtained mean loss of 17.9% FDNB lysine in fish samples (including Coley fillets) fried for 4 min at 180° in fresh corn oil, ground nut oil and olive oil and a greater loss of 28.9% FDNB-lysine when fried for 4 min at 180° in thermally oxidised oil.

4.9.7 Effect of heating protein at high relative humidity

Miller (1956) reported that fish protein are more readily damaged at high moisture content. Carpenter, Ellinger, Munro and Rolfe (1957) obtained approximately 30% loss of available lysine in vacuum-dried cod fillets brought up to 11% moisture, heated for 24 h at 105° in the air, while a similar set of vacuum-dried cod fillets at 1.7 to 2.3% moisture level, heated for 31 h at 100°, suffered approximately 12% loss of lysine.

Carpenter, Morgan, Lea and Parr (1962) observed approximately 20% loss of lysine in freeze-dried herring press-cake (fat-free basis) heated at 115° for 27 h at 11.8% moisture level and that for herring press-cake (fat-free basis) heated at 130° for 2 h, amino acid binding was found to be a maximum in the region of 4-12% moisture content, while loss of lysine was appreciately less for the very moist as well as for the driest samples.

Miller, Carpenter and Milner (1965) obtained 69% loss of available methionine and a similar loss for FDNB-available lysine when they heated cod fillets at 14% moisture content at 116° for 27 h.
4.9.8 Effect of heating glucose or ribose (aldehyde groups) with protein

Miller, Carpenter and Milner (1965) observed loss of about 15% and 20% FDNB-lysine when cod-glucose mixtures were heated at 45° and 55° respectively for 9 h and when cod was heated with 10% glucose for 27 h at 85° and 14% moisture level, a loss of 46.5% FDNB-lysine was obtained. With low content of ribose as in a vacuum-dried cod fillet 1.7 to 2.3% moisture, heated at 100°C for 31 h, loss of lysine was about 12% (Carpenter, Ellinger, Munro and Rolfe 1957).

Carpenter, Morgan, Lea and Parr (1962) obtained about 0.8 mole of lysine bound per mole of ribose added, with bovine plasma albumin heated with ribose at 85° for 1 h, but when heated alone at 85° for 27 h, no detectable loss of lysine was obtained.

4.9.9 Some observations on the colour of the smoked fillets

The fillets smoked for 1 h were very moist and showed a range of colour from lightly yellowish-brown to light orange-brown. The top approximately 0.5 to 1 cm surface of fillet flesh was still very "raw" and approximately 1 cm of the underlying layer was very like the initial unsmoked fish fillet. The top approximately 0.5 to 1 cm surface layer of the fillets of 3 h smoking time showed a darker or deeper range of the colours above, from dark yellow and orange to light brown and brown colour. The flesh showed signs of "firming of muscles or having a slightly cooked appearance." The top surface layer was slightly dry and lower layers still "raw" and moist with a milky fluid exudate. After 5 h smoking at 115°, the top
approximately 0.5 to 1 cm surface was dark brown and the muscles were dry and hard. The underlying layers were fairly dry and showed a light brown or opaque cream colour. The whole fillet had the "cooked and edible" appearance. (Moisture content was not determined here as the main concern here was with dye binding of the proteins). In addition there was a graduation of colour in the (1, 3 and 5 h) trays of fillets, with the strongest brown coloured fillets nearest the fire boxes and the palest colour farthest away from the fire boxes.

Bratzler et al. (1969) found that phenol content decreased towards the centre of the bologna sausage, there was no change in the carbonyl content and the acid content increased towards the centre.

To correlate this with colour, Chen and Issenberg (1972, see literature review section 2.2) found that casein treated with formaldehyde and dried at 65°C for 24 h showed no pigment formation, while glyoxal produced yellowish-brown; pyruvaldehyde-light brown; furfural-brown; coniferaldehyde-yellow and sinapaldehyde - an orange pigment, indicating the colour produced by phenolic interaction with proteins. Kurko and Shmidt (1969) suggested that the colour developed was from non-enzymic interaction between polyhydric phenolic and carbonyl compounds of smoke vapour with the collagen of the connective tissue. Caurie (1975) found that:

a) the hydroxy proline (degradation product of collagen and connective tissue) content was proportional to both the phenol content and colour of the smoked butterfish;
b) the farther the butterfish was from the source of
smoke, the lower the phenolic content adsorbed on
the fish surface; and
c) there was increased deposition of phenols on the fish
skin with increased time of smoking.

During smoking, we would expect "Maillard" type reaction
involving smoke phenolic and carbonyl groups, polyunsaturated
lipid oxidation products, such as aldehydes, ketones, peroxides
and epoxides, small amounts of reducing sugar (ribose) and sugar
phosphates to be mainly responsible for the browning of the
fish flesh (see section 2.3 and 2.4). Degradation products of
1-methyl histidine are also said to make some small contribution
(Jones 1956) and possibly complex compounds formed from interaction
among these components themselves in addition to reactions with the
$\varepsilon$-amino groups of lysine and other amino acids (products of
protein degradation to give free $\varepsilon$-amino groups of the N-terminal
residues and sulphydryl groups from the breakage of disulphide cross-
linkages which are important in maintaining the tertiary structure
of the protein).
4.10.0 Investigation of dye binding difference (DBD) of \( \varepsilon \)-amino groups of lysine acylated by:

a) methoxy carbonyl chloride (MCC);

b) 2,4,6-trinitrobenzene sulphonic acid method of Jones (1974, section 2.9.2).

Since the use of methoxy carbonyl chloride (MCC) permitted lysine to be estimated by the method of dye binding difference, such that the results correlated well with the DNP-L values \((r = 0.97, p = 0.01)\) it was thought worthwhile to try the 2,4,6- trinitrobenzene sulphonic acid as the blocking reagent (Jones 1974) and compare MCC and TNBS as reagents for analysis of lysine.

4.10.1 Materials

A fresh batch of 12 Coley fish were filleted and the fillets from one side of each fish were smoked as in section 4.8.1 while the fillet from the other side of the fish were layered and placed in the blast freezer. The fillets for smoking were divided into 2 groups: first group of 6 fillets were smoked 3 h and second group of 6 fillets were smoked for 5 h, layered, ground, defatted and stored as in section 4.1.1. FDNB-available lysine of the fillets were determined as in section 3.1.0.

4.10.2 Procedure to determine dye binding and dye binding difference values (DBD) of the samples by methoxy carbonyl chloride was as in the method of section 4.8.2 and 4.9.0

The dye binding values (DBC) of the untreated (unacylated) samples minus the dye binding values (DBC) of the acylated samples gave the dye binding difference values (DBD) as in section 4.9.0.
4.10.3 Procedure to determine the dye binding difference values of the samples by the method (modified) of Jones (1974) using 2,4,6-trinitro benzene sulphonylic acid (TNBS)

4.10.4 Materials

1% (w/v) tribitro benzene sulphonylic acid in distilled water; 4% (w/v) NaHCO₃ solution in distilled water; 1M HCl, 0.2M citric acid, Whatman glasswool microfibre filter paper GF/A.

4.10.5 Procedure

i) sample (0.15g) was weighed into 100ml polythene centrifuge tube and 10% (w/v) NaHCO₃ solution was added. Mixture was gently shaken and incubated at 40°C for 30 min;

ii) TNBS solution (10 ml of 1% w/v) was added and the reaction was allowed to proceed at 40°C for 4 h with occasional shaking;

iii) the solution was stoppered by addition of 5 ml of 1M HCl and the contents were shaken to mix;

iv) contents of the centrifuge tube were filtered through the glass wool microfibre filter paper GF/A and precipitate was washed twice with 2 x 5 ml aliquots of 0.2M citric acid. The filter paper was allowed to drain;

v) the filter paper together with the treated sample was placed back into the 100 ml centrifuge tube and 3-4 glass beads were added;

vi) AO.12 dye (40 ml of 3.2 meq dye/litre) was added and the tubes were stoppered and mixture were
mechanically shaken for 1 h;

vii) the mixture was centrifuged at 2500 r.p.m. for 10 min and 1 ml of the supernatant was suitably diluted (x20) and extinction or absorbance was read on the SP500 at 482 nm against 0.2M citric acid using a 1 ml cuvette.

The DBC of the acylated samples were calculated from the O.D. readings using the calibration curve in section 4.1.0. The DBC values of the untreated samples were determined as in section 4.8.2.

Hence the DBD (dye binding difference) values of the samples were calculated as in Jones (1974)

\[
\text{meg dye bound to untreated sample} - \frac{\text{meg dye bound to blocked sample}}{\text{sample weight}} = \frac{\text{sample weight}}{\text{dye binding difference (DBD)}}
\]

As was observed in section 4.9.9, there was a graduation in the intensity of smoke colour along each tray of 6 fillets, when they were removed from the Torry mini-kiln at the end of 3 h and 5 h smoking time. The darkest brown or smoked fillet was nearest the fire boxes while the palest was farthest away. The results presented in table 4.9 showed a graduation in loss of DBC and in loss of DBD-lysine in the 6 fillets corresponding to the fillets positioned along the wire tray.
4.10.6 Results and discussions

Results of this batch of smoked fillets were similar to those obtained in section 4.8.0, with a loss of 4.90% DBC or basic groups in the unacylated 3 h smoked samples and 12.32% decrease in DBC in the unacylated 5 h smoked samples (total mean of both layers of 6 fillets).

The DBD(MCC)lysine and FDNB-lysine values showed a similar degree of loss with the 3 h smoked fillets as in section 4.8.0 with 7.31% DBD lysine, 8.72% FDNB-lysine and 6.52% DBD (TNBS) lysine (total mean of both layers of 6 fillets). The 5 h smoked samples showed loss of 18.0% DBD (MCC) lysine, 18.01% DBD (TNBS)-lysine and 17.55% FDNB-lysine values (total mean) with the upper layers of both 3 h and 5 h smoking showing greater reduction in available lysine as shown in Table 4.10.

The values of the DBD (MCC)-lysine were much closer to the FDNB-lysine on this occasion, probably because the second analysis was performed shortly after the first ($r_{xy} = 0.986, p = 0.01$; regression line is $Y = 1.0285X - 2.9210$, where $Y$ = mg DBD (MCC) lysine and $X$ = mg FDNB-lysine; with student 't' test = 1.42, F value = 1.38).

The TNBS-lysine results were slightly lower, perhaps due to any excess TNBS left in the precipitate, that is, the 2 x 5 ml citric acid may not be sufficient to wash off any excess TNBS not reacted with the sample, as TNBS also binds with the AO.12 dye (Jones 1974) Regression line obtained $Y = 1.0201 - 3.3794$, where $Y$ = mg FDNB lysine and $X$ = mg DBD (TNBS) lysine; $r = 0.97, p = 0.01$, student 't' test = 3.9, F value = 1.99.

As shown on the graph 4.10, the overall rate of loss in lysine in the upper layers relative to the lower layers in this batch of
Table 4.10  Dye binding values of unacylated samples and DBD-lysine the samples treated with MCC and TNBS compared with FDNB-reactive lysine.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Untreated samples</th>
<th>MCC treated</th>
<th>TNBS treated</th>
<th>FDNB-reactive lysine</th>
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<td>% loss in DBC</td>
<td>DBD meq g lysine</td>
<td>% loss in lysine</td>
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<td>meq g⁻¹ sample</td>
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DBD $\times$ 146.2 $=$ mg lysine g⁻¹ sample.

UU = upper 0.5 to 1 cm layer of unsmoked fillet.
UL = lower 0.5 to 1 cm layer of unsmoked fillet.
SU = upper 0.5 to 1 cm layer of unsmoked fillet.
SL = lower 0.5 to 1 cm layer of smoked fillet.

1.1 = sample 1, 1 hour smoking.
3.1 = sample 1, 3 hour smoking.
5.1 = sample 1, 5 hour smoking.
smoked fillets were similar to the first batch of smoked fillets (section 4.8.0) with very high initial rate of lysine loss in the first 3-4 h as the surface muscle ε-amino groups of lysine bind with the smoke components etc (see section 4.8.0).

Gradually the rate decreased as the level of available lysine fell (Bjarnason and Carpenter 1970). The rate of loss of lysine in the deeper layers initially rose slowly and showed an increase in rate when heated beyond 3 hours, that is, when the internal temperature of the fillet rose.

As was observed in section 4.8.0, the smoke and heat distribution in the Torry mini-kiln was not uniform. The fillets nearest the 3 fire boxes suffered more smoke, heat and drying damage than the fillats farthest away from the fire boxes.
Graph 4.10  Percentage loss of DBD (MCC) lysine in the upper layers, lower layers and average of both layers with time of smoking

- upper layers
- average results of section 4.8.0
- lower layers
- upper layers
- average results of section 4.10.0
- lower layers

Percentage loss of DBD (MCC) lysine

time of smoking in hours

- 128 -
4.11.0 General discussions

The investigations here have been concerned primarily with the feasibility of dye binding procedures for the evaluation of lysine content or protein quality in smoked fish. These investigations have shown that dye binding can be applied to smoked fish.

From the results obtained, it has been found that acid azo orange 12 dye should be used at 4.0 meq/litre and a dye:protein ratio of 0.38 for the untreated samples and at 3.2 meq/litre for the acylated samples. Comparison of the results in section 4.7.0 and 4.4.0 indicate that it is essential to have sufficient dye to saturate the basic groups. The effects of smoked components and of proteolysis during smoking on dye binding properties have been investigated and it can be concluded that under the conditions employed, neither caused significant interference. However very high levels of smoke components in relation to dye (hence diethyl ether extraction of smoked fish is necessary) or severe proteolysis could interfere.

The results obtained for DBC and DBD (both by methoxy carbonyl chloride and 2,4,6 trinitro benzene sulphonic acid) have been compared with the established Carpenter's (1960) method, and it was observed that smoke components interfere with the recovery of the dinitrophenylated lysine. However provided an allowance was made for this, it was observed that a change in DBC (an estimation of histidine, lysine and arginine) was paralleled by a change in the available lysine by Carpenter's Method. ($r_{xy} \geq 0.968$, $p = 0.01$). Lysine estimated by DBD using methoxy carbonyl chloride (MCC) as blocking agent was statistically indistinguishable from lysine estimated by Carpenter's Method ($F$ value = 1.37; Student 't' test = 1.42; $r_{xy} = 0.974$ and $p = 0.01$). The use of 2,4,6-trinitro benzene sulphonic acid
as blocking agent gave a slightly lower lysine content than either MCC or Carpenter's Method but the cause of this discrepancy was not investigated.

Analysis of smoked fish showed that the lysine loss and the total basic amino acid loss was progressive and concentrated in the outer layer. Losses of up to 25.27% were observed in the most exposed sections of the fillet, whereas the layer immediately below did not exceed 14.44%. It follows, therefore, that the overall reduction in N.P.U. will decrease as the fish fillet (or steak or whole fish) thickness increases. Provided that the thicker material can be adequately preserved, it follows that there are nutritional advantages in using thicker raw material for smoking.

Dye binding procedure used to estimate the basic amino acids and lysine content in a sample of animal or plant origin have the advantages of being low cost, rapid throughput of samples to be analysed and good reproducibility of results, compared to the more laborious method of determining available lysine by the 1-fluoro-2,4-dinitro benzene or the amino-analysis. The major problem in dye binding with AO.12 dye (as with other methods is the variation in the raw materials, particularly fish (which is affected by seasonal variations, physiological conditions, species variation etc.).
4.11.1 Conclusion

1) lysine in smoked and unsmoked fish can be estimated using acid orange 12 dye and methoxy carbonyl chloride as the blocking reagent;

2) smoke damage in the fish fillets is substantially greater at the surface than the centre and to minimise destruction of lysine, thick fillets or whole fish should be used;

3) recovery of dinitro phenylated lysine in Carpenter's method (1960) is affected by smoke components.

4.12.0 Suggestions for Future Work

The dye binding procedures developed in this investigation should be used to investigate more fully the effects of smoking upon the available lysine content of smoked foods. There is a need for more information about the behaviour of smoke components and their reactivity with lysine and their generation and deposition under different conditions of smoking. Such information might permit lysine loss to be minimised while producing an acceptable and stable product.
### Table 4.2 Dye binding isotherms of skimmed milk powder with acid orange 12 dye

<table>
<thead>
<tr>
<th>mg dye in 40ml</th>
<th>EDC (meq (l^{-1}))</th>
<th>mg dye bound (meq g(^{-1}) sample)</th>
<th>DBC (meq g(^{-1}) sample)</th>
<th>D/Protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.049</td>
<td>104.27</td>
<td>0.299</td>
<td>0.27</td>
</tr>
<tr>
<td>20</td>
<td>0.149</td>
<td>119.40</td>
<td>0.341</td>
<td>0.34</td>
</tr>
<tr>
<td>24</td>
<td>0.354</td>
<td>126.92</td>
<td>0.363</td>
<td>0.40</td>
</tr>
<tr>
<td>28</td>
<td>0.594</td>
<td>131.27</td>
<td>0.375</td>
<td>0.47</td>
</tr>
<tr>
<td>32</td>
<td>0.836</td>
<td>135.33</td>
<td>0.387</td>
<td>0.54</td>
</tr>
<tr>
<td>36</td>
<td>0.052</td>
<td>141.73</td>
<td>0.405</td>
<td>0.61</td>
</tr>
</tbody>
</table>

**EDC** = equilibrium dye concentration or unbound dye in milliequivalent per litre.

**DBC** = dye binding capacity or milliequivalent dye bound per gramme sample.

**Dye/protein ratio** = mg dye used per mg protein in the sample.

**Crude protein of skimmed milk powder** = 39.66% (dry matter basis by Kjeldahl method).
APPENDIX 2

Table 4.3 Dye binding isotherms of smoked and unsmoked freeze-dried Coley fillets with acid orange 12 dye with chloride ions

<table>
<thead>
<tr>
<th>mg dye in 40 ml.</th>
<th>Unsmoked fillets</th>
<th></th>
<th>Smoked fillets</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDC meq l^-1</td>
<td>mg dye bound g^-1 sample</td>
<td>DBC meq g^-1 sample</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>.068</td>
<td>73.53</td>
<td>.210</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>.113</td>
<td>109.40</td>
<td>.313</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>.125</td>
<td>121.60</td>
<td>.347</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>.125</td>
<td>148.26</td>
<td>.424</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>.106</td>
<td>176.66</td>
<td>.504</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>.085</td>
<td>185.33</td>
<td>.530</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>.062</td>
<td>205.13</td>
<td>.586</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>.040</td>
<td>233.33</td>
<td>.675</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>.044</td>
<td>262.60</td>
<td>.750</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>.047</td>
<td>275.66</td>
<td>.788</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>.062</td>
<td>314.2</td>
<td>.899</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations see Table 4.2.

Dye/protein ratio refer to Table 4.7.
Table 4.4 Dye binding isotherms of smoked and unsmoked freeze-dried Coley fillets with acid orange 12 without chloride ions

<table>
<thead>
<tr>
<th>mg dye in 40 ml.</th>
<th>EDC meq 1^-1</th>
<th>mg dye bound g^-1 sample</th>
<th>EDC meq g^-1 sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsmoked fillets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>.106</td>
<td>70.0</td>
<td>.200</td>
</tr>
<tr>
<td>18</td>
<td>.141</td>
<td>106.73</td>
<td>.305</td>
</tr>
<tr>
<td>20</td>
<td>.175</td>
<td>116.86</td>
<td>.334</td>
</tr>
<tr>
<td>24</td>
<td>.188</td>
<td>142.40</td>
<td>.406</td>
</tr>
<tr>
<td>28</td>
<td>.194</td>
<td>168.53</td>
<td>.482</td>
</tr>
<tr>
<td>32</td>
<td>.181</td>
<td>196.46</td>
<td>.561</td>
</tr>
<tr>
<td>36</td>
<td>.084</td>
<td>232.26</td>
<td>.664</td>
</tr>
<tr>
<td>40</td>
<td>.088</td>
<td>258.46</td>
<td>.739</td>
</tr>
<tr>
<td>44</td>
<td>.090</td>
<td>284.93</td>
<td>.814</td>
</tr>
<tr>
<td>48</td>
<td>.143</td>
<td>306.66</td>
<td>.876</td>
</tr>
<tr>
<td>Smoked fillets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>.025</td>
<td>77.6</td>
<td>.221</td>
</tr>
<tr>
<td>18</td>
<td>.031</td>
<td>117.13</td>
<td>.335</td>
</tr>
<tr>
<td>20</td>
<td>.026</td>
<td>130.93</td>
<td>.374</td>
</tr>
<tr>
<td>24</td>
<td>.028</td>
<td>157.46</td>
<td>.450</td>
</tr>
<tr>
<td>28</td>
<td>.027</td>
<td>184.20</td>
<td>.526</td>
</tr>
<tr>
<td>32</td>
<td>.034</td>
<td>210.20</td>
<td>.600</td>
</tr>
<tr>
<td>36</td>
<td>.039</td>
<td>236.40</td>
<td>.675</td>
</tr>
<tr>
<td>40</td>
<td>.066</td>
<td>260.46</td>
<td>.744</td>
</tr>
<tr>
<td>44</td>
<td>.084</td>
<td>285.33</td>
<td>.815</td>
</tr>
<tr>
<td>48</td>
<td>.279</td>
<td>294.00</td>
<td>.840</td>
</tr>
</tbody>
</table>

Abbreviations: refer to Table 4.2.
Dye/protein: refer to Table 4.7.
**APPENDIX 4**

Table 4.7 Dye binding isotherms of smoked and unsmoked freeze-dried Coley fillets with acid orange 12 dye

<table>
<thead>
<tr>
<th>mg dye in 40 ml</th>
<th>mg unbound dye</th>
<th>EDC meq l⁻¹</th>
<th>mg dye bound g⁻¹ sample</th>
<th>DBC meq g⁻¹ sample</th>
<th>Dye/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsmoked fillets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>.425</td>
<td>.03</td>
<td>77.20</td>
<td>.22</td>
<td>.081</td>
</tr>
<tr>
<td>16</td>
<td>.369</td>
<td>.03</td>
<td>104.20</td>
<td>.30</td>
<td>.108</td>
</tr>
<tr>
<td>20</td>
<td>.120</td>
<td>.01</td>
<td>132.52</td>
<td>.38</td>
<td>.135</td>
</tr>
<tr>
<td>24</td>
<td>.120</td>
<td>.01</td>
<td>159.20</td>
<td>.45</td>
<td>.162</td>
</tr>
<tr>
<td>32</td>
<td>.185</td>
<td>.013</td>
<td>212.13</td>
<td>.61</td>
<td>.216</td>
</tr>
<tr>
<td>36</td>
<td>.323</td>
<td>.023</td>
<td>237.86</td>
<td>.68</td>
<td>.243</td>
</tr>
<tr>
<td>48</td>
<td>1.375</td>
<td>.098</td>
<td>310.86</td>
<td>.89</td>
<td>.325</td>
</tr>
<tr>
<td>52</td>
<td>1.640</td>
<td>.117</td>
<td>335.73</td>
<td>.94</td>
<td>.352</td>
</tr>
<tr>
<td>54</td>
<td>2.723</td>
<td>.195</td>
<td>341.86</td>
<td>.98</td>
<td>.365</td>
</tr>
<tr>
<td>56</td>
<td>3.12</td>
<td>.223</td>
<td>352.53</td>
<td>1.01</td>
<td>.379</td>
</tr>
<tr>
<td>58</td>
<td>3.65</td>
<td>.260</td>
<td>362.26</td>
<td>1.03</td>
<td>.392</td>
</tr>
</tbody>
</table>

| Smoked fillets |
|----------------|----------------|-------------|------------------------|-------------------|-------------|
| 12             | .074           | .005        | 79.53                  | .23               | .082        |
| 16             | .102           | .007        | 106.00                 | .30               | .109        |
| 20             | .102           | .007        | 132.66                 | .38               | .136        |
| 24             | .120           | .009        | 159.20                 | .45               | .163        |
| 32             | .268           | .019        | 211.53                 | .60               | .218        |
| 36             | .590           | .042        | 236.06                 | .67               | .245        |
| 48             | 2.390          | .171        | 304.06                 | .87               | .327        |
| 52             | 3.28           | .234        | 324.80                 | .93               | .354        |
| 54             | 3.83           | .274        | 334.46                 | .96               | .368        |
| 56             | 4.83           | .345        | 341.06                 | .97               | .381        |
| 58             | 5.52           | .394        | 349.86                 | .99               | .395        |

Abbreviations: please refer to Table 4.2
Crude protein of freeze-dried, defatted, unsmoked fillets = 98.60%.
(dry matter basis, by Kjeldahl Method).
Crude protein of freeze-dried, defatted, smoked fillets = 97.95%.
(dry matter basis by Kjeldahl Method).
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