DEVELOPMENT OF NITRITE-FREE MEAT CURING SYSTEMS

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BY

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ABSTRACT

The pigment responsible for the colour of cooked cured-meats has been synthesized from bovine red blood cells directly, or indirectly through a haemin intermediate using sodium nitrite and nitric oxide, respectively. The preformed cooked cured-meat pigment (CCMP) so obtained exhibited absorption characteristics similar to those of pigments extracted from a nitrite-cured sample of ham. Since the CCMP is sensitive to light and oxygen and ultimately decomposes in their presence over a short period of time, the pigment was encapsulated in carbohydrate-based wall materials in an effort to extend its shelf-life and for its easy handling. The resultant powdered cooked cured-meat pigment (PCCMP) remained stable during 18 months of refrigerated storage in some preparations. Application of CCMP or PCCMP to comminuted meat systems produced upon thermal processing the typical pink colour of nitrite-cured products. The colour characteristics of pigment-treated meats depended on both the myoglobin content of muscles from the various species used as well as the level of pigment added. Presence of some myoglobin was deemed necessary in order for the pigment to impart a cured colour to meats. No detrimental effects on the colour or oxidative stability of CCMP-treated pork systems were noted after radiation processing at levels of 5 and 10 kGy. The absence of N-nitrosamines in cooked nitrite-free meat and fish systems containing CCMP was confirmed using a gas chromatography-thermal energy analyzer (GC-TEA) methodology.

Pilot-scale preparation of CCMP-treated frankfurter and salami products was successful, and the flavour characteristics were indistinguishable from their nitrite-cured counterparts even after 30 days of refrigerated storage. Application of nitrite-free curing pickle containing CCMP to solid cuts of pork conferred the characteristic cured-meat colour throughout the muscles after thermal processing. The concentration of CCMP in pickle had a more pronounced effect on the extent and rate of the pigment's penetration throughout the meat than did the effect of temperature of the pickle.

The oxidative stability of cured-pork meat and the methodology of the modified 2-thiobarbituric acid (TBA) test were examined. Addition of sulphanilamide played a beneficial role in evaluating the oxidative state of cured meats prepared with the addition of $\geq$ 100 ppm of sodium nitrite. In the absence of nitrite, sulphanilamide reacted with malonaldehyde forming a 1-amino-3-iminopropene complex. Multiple interactions between malonaldehyde and sulphanilamide, TBA or their combinations were examined. The structures of the above complexes were elucidated using ultraviolet-visible (UV-VIS), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopic (MS) techniques.

Pentanal and hexanal were the dominant volatile aldehydes generated from cooked pork during storage as determined by a rapid headspace-gas chromatographic (HS-GC) methodology. The concentration of hexanal increased faster than any other aldehyde and it has been suggested to serve as an index of meat flavour deterioration (MFD). Hexanal levels of cooked pork increased during the first 6 days of storage and then declined quite

markedly. Caution should be exercised when using hexanal as an indicator of lipid oxidation because a given hexanal level may correspond with two points during the storage period of cooked meats. The hexanal and pentanal concentrations of CCMP-treated and nitrite-cured pork systems were depressed even after 4 weeks of refrigerated storage.

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LIST OF ABBREVIATIONS

AA	- Ascorbic acid
ACS	- American Chemical Society
AP	- Ascorbyl palmitate
APT	- Attached proton test
BHA	- Butylated hydroxyanisole
BHT	- Butylated hydroxytoluene
BRBC	- Bovine red blood cells
^{13}C	- Carbon thirteen atoms
$^{13}C\{^{1}H\}$	- Carbon thirteen spectra which is broadband decoupled
CCMP	- Cooked cured-meat pigment
DMA	- Dimethylamine
DMSO	- Dimethyl sulphoxide
DMSO-d_6	- Deuterated dimethyl sulphoxide
EA	- Erythorbic acid
EDTA	- Ethylenediaminetetraacetic acid
ESR	- Electron spin resonance
FDA	- Food and Drug Administration
FSIS	- Food Safety and Inspection Service
FTIR	- Fourier Transform Infrared

GC	- Gas chromatography
GC-MS	- Gas chromatography-Mass spectrometry
GC-TEA	- Gas chromatography-thermal energy analyzer
GRAS	- Generally recognized as safe
^1H	- Hydrogen atoms
Hb	- Haemoglobin
HETCOR	- Heteronuclear correlation
HPB	- Health Protection Branch
HPLC	- High pressure liquid chromatography
HS	- Headspace
IHP	- Inositol hexaphosphate
IR	- Infrared
LAPB	- Lactic acid-producing bacteria
Mb	- Myoglobin
MbO_2	- Oxymyoglobin
metMb	- Metmyoglobin
MFD	- Meat flavour deterioration
MS	- Mass spectrometry
MSCM	- Mechanically separated chicken meat
MSSM	- Mechanically separated seal meat
NADH	- Nicotinamide adenine dinucleotide

NDMA	- N-Nitrosodimethylamine
NDPA	- N-Nitrosodi-*n*-propylamine
NMR	- Nuclear magnetic resonance
NOHb	- Nitrosylhaemoglobin
NOmetMb	- Nitrosylmetmyoglobin
NOMb	- Nitrosylmyoglobin
NPYR	- N-Nitrosopyrrolidine
NTHZ	- N-Nitrosothiazolidine
NTHZC	- N-Nitrosothiazolidine-4-carboxylic acid
PCCMP	- Powdered cooked cured-meat pigment
PG	- Propyl gallate
PP-IX	- Protoporphyrin-IX
ppb	- Parts per billion
ppm	- Parts per million
PUFA	- Polyunsaturated fatty acids
SAPP	- Sodium acid pyrophosphate
SHMP	- Sodium hexametaphosphate
SHP	- Sodium hypophosphite
SMS	- Complex of sulphanilamide and 2-thiobarbituric acid in the ratio of 2:1, respectively

SMT	- Complex of sulphanilamide, malonaldehyde and 2-thiobarbituric acid in the ratio of 1:1:1, respectively
SNC	- S-Nitrosocysteine
STPP	- Sodium tripolyphosphate
TBA	- 2-Thiobarbituric acid
TBARS	- 2-Thiobarbituric acid reactive substances
TBHQ	- Tertiary-Butylhydroxyquinone
TEA	- Thermal energy analyzer
TMAO	- Trimethylamine N-oxide
TMP	- 1,1,3,3-Tetramethoxypropane
TMS	- Tetramethylsilane
TMT	- Complex of 2-thiobarbituric acid and malonaldehyde in the ratio of 2:1, respectively
TPP	- Tetraphenylprotoporphyriniron
USA	- United States of America
USDA	- United States Department of Agriculture
UV-VIS	- Ultraviolet-Visible
WOF	- Warmed-over flavour

CHAPTER 1. INTRODUCTION

Cured meats represent a large portion of the processed meat products consumed in North America. These processed meats are attractive in their colour, texture and flavour and are popular with consumers because they combine this variety with the convenience of high storage stability. The origin of meat curing is lost in antiquity, but it was not until the turn of this century that nitrite was ascertained to be the fundamental ingredient of the curing process. This ubiquitous compound is responsible for the development of the characteristic pink colour and pleasant flavour of cooked cured-meats. It has an antioxidative property delaying the onset of the deterioration of meat flavour, thereby providing an extended shelf-life to processed meat products. Most importantly, nitrite, in combination with sodium chloride, has bacteriostatic action and inhibits production of the neurotoxin by *Clostridium botulinum*.

Even with all of the benefits conferred by this multifunctional food additive, addition of nitrite to meat and meat products is a source of concern, due to its role in the formation of carcinogenic N-nitrosamines. These carcinogens may be formed by the reaction of nitrite and its dissociation products present in the muscle tissue with secondary amines during processing, cooking or after the ingestion of nitrite-cured meats in the stomach. Despite this concern, the meat industry is committed to the use of nitrite in cured products since there is no suitable alternative available. The necessity for studying the formation and occurrence of N-nitrosamines in cured meats and other food systems stems from the absolute nature of the Food and Drug Regulations in the USA and other

regulatory bodies in Canada and Europe which deny the use of any food additive which in itself is carcinogenic or produces carcinogens in food. Therefore, it is only reasonable that usage of nitrite in cured meats be reduced or phased-out as soon as effective and safe substitutes are found.

1.1 Thesis Objectives

The basic objectives of this thesis involved the development of composite nitrite-free curing systems which bestow the characteristic and desirable attributes of cooked cured-meat products without the fear of N-nitrosamine formation and which may be employed at the industrial level. The emphasis of this study was on the development and efficacy of compounds to be used in nitrite-free formulations to reproduce the colour and flavour characteristics of nitrite-cured meats. The key component of these systems, with regard to colour fixation, was the cooked cured-meat pigment (CCMP). This pigment was made from bovine red blood cells (BRBC) in either a direct, one-step process or through a haemin intermediate, and then applied to meat systems. The colouring efficacy of this pigment as part of a composite package was examined in comminuted and solid cuts of pork, other red meat species, poultry, seal, fish and specific retail products. The effect of low-dose, γ-irradiation on the colour and flavour characteristics of nitrite-free cured pork systems was examined as was the occurrence of N-nitrosamines in meat model systems. Preparation of an encapsulated CCMP, to stabilize the pigment, thereby extending its shelf-life and making it easier to incorporate into nitrite-free composite systems, was accomplished. The colour characteristics of this powdered cooked cured-

meat pigment (PCCMP) were examined.

To assess the flavour attributes of nitrite-cured and pigment-treated systems, analyses of dominant aldehydes, with particular emphasis on hexanal, as opposed to the classical TBA test, was carried out. The limitations of the TBA test for assaying nitrite-cured meats were revisited and some surprising implications as well as novel interactions involved in the test were uncovered.

CHAPTER 2. LITERATURE REVIEW

2.1 History of the Curing Process

The curing of meat is based in part upon the art as practised through aeons of time and perhaps to a far greater extent upon sound scientific principles developed since the turn of this century (Binkerd and Kolari, 1975). The origin of nitrate usage, as saltpetre, in meat curing is lost in antiquity, but preservation of meat with salt preceded the intentional use of nitrate by many centuries. Rock salt was an important commodity in ancient times. It was reported to be in common use for muscle food preservation in ancient China, the Jewish Kingdom, Babylonia and Sumeria, long before the Christian era (Jensen, 1953). In ancient Greece, salt obtained from "salt gardens" was used to preserve fish. The Romans learned the use of salt from the Greeks and used it themselves extensively to cure fish. The Romans also learnt how to preserve various kinds of meat such as pork with pickle containing salt and other ingredients, thereby, establishing a trade for these products in the Roman empire (Jensen, 1954). It was nitrate impurities in the rock salt, which upon incorporation into the meat matrix and after reduction to nitrite by the post-mortem reducing activity of the muscle tissue, that were truly responsible for the curing effect.

By medieval times, addition of salt, saltpetre and smoke to meats was commonplace, and the effect of saltpetre on colour impartation to meats was recognized. Gradually, sweet pickle and sugar cures evolved as sucrose became available as a commodity of trade. Sugar added flavour to the meat and helped to mask some of the

harshness of salt. As the art progressed, the term "meat curing" eventually was understood as the addition of salt, sugar, saltpetre (nitrate) or nitrite to meat for its preservation and flavour enhancement (Townsend and Olson, 1987).

Toward the end of the nineteenth century, significant changes in meat curing had occurred. Various methods of curing, namely dry, wet or pickle cures and combinations of the two, were commonplace. Dry curing involves using uniform and quantitated mixtures of salt, sugar, spices and sodium nitrate and/or nitrite over solid pieces of meat such as hams. The cure is massaged over the surface of the meat and time is required for its penetration into the interior. More than one application of the salt mixture is generally necessary to effect a cure. This process requires a considerably longer period than is the case for curing of comminuted meats. Pickle curing involves the immersion of whole cuts of meat into brine solutions which also generally contain sodium nitrate or nitrite. The meats are then held in vats for long periods of time at 2-4°C to allow penetration of the curing salts. If sugar is included in the brine, it is referred to as a sweet pickle. The practice of pumping/injecting meat with a perforated needle originated in the late nineteenth century. Stitch pumping involves addition of pickle to the interior of the meat at several locations *via* insertion of a needle having a series of small openings near the pointed end. The cure is rapidly distributed through channels in the muscle tissue. Tumbling further accelerates the curing process which occurs by diffusion. In the case of bone-in hams, meat pieces may be placed in vats and immersed in pickle for 5 to 7 days to allow even distribution of the cure throughout the meat.

When nitrite *per se* was first used to cure meat is unknown, but classical studies by Polenske (1891), Kisskalt (1899) and Lehmann (1899) demonstrated the importance of nitrite rather than nitrate in the curing process. Polenske (1891) provided the first technological advance in curing by concluding that the nitrite found in cured meats and curing pickle arose from bacterial reduction of nitrate. Shortly afterwards, Kisskalt (1899) and Lehmann (1899) demonstrated that the typical colour of cured meats was due to nitrite and not to nitrate. By 1901, Haldane had investigated the pigment responsible for the redness of cooked cured meats. He prepared nitrosylhaemoglobin (NOHb) by adding nitrite to haemoglobin (Hb) and showed that its conversion to nitrosyl-haemochromogen upon thermal processing was the pigment responsible for the red colour of cooked cured meat. Haldane (1901) also stated that the colour change during cooking was a consequence of NOHb decomposition into two constituents, namely haemin, the colouring group, and a denatured protein. Hoagland (1908) confirmed Haldane's findings and suggested that reduction of nitrate to nitrite, nitrous acid and nitric oxide by either bacterial or enzymatic action, or a combination of the two, was essential for NOHb formation.

By 1917, proprietary curing mixtures containing nitrite were marketed in Europe. At the same time, a US patent was issued to Doran (1917) for nitrite usage in meat curing. Because data indicated that the nitrite content of meat cured by processes solely containing nitrate yielded extremely variable and, at times, high levels of nitrite in the product, the USDA permitted direct addition of nitrite to meat in early 1923. Studies by

Kerr *et al.* (1926) revealed that the flavour and keeping quality of nitrite-cured meats were equal to those cured by traditional processes; judges were unable to distinguish meats cured by either method. A limit of a 200 ppm nitrite content in all finished meat products was established at this time. The products so cured included pork shoulders, loins, tongues, hams, bacon as well as corned and dried beef. On the basis of the results obtained in these experiments, the use of sodium nitrite to cure meats in federally inspected establishments was formally authorized by the USDA in 1925 (United States Department of Agriculture, 1926).

During the 1930s, progress continued as meat processors adopted the use of nitrite to accelerate their cures. Surveys showed average nitrite levels of 100 ppm or less in finished products (Mighton, 1936; Lewis, 1937), but nitrate levels remained quite high. Stitch pumping was formally introduced in the 1930s (Fox, 1974). This decade also saw the next technological advance, namely, the discovery that ascorbic acid would effectively reduce nitrite to nitric oxide (Karrer and Bendas, 1934). It was not until the 1950s that ascorbic acid, ascorbate, or its isomer, erythorbate, were formally authorized for use in cures by the USDA (Hollenbeck, 1956). These ingredients provide reducing conditions in meat and meat products which are necessary for a rapid reaction between nitrite and myoglobin. These adjuncts accelerate and stabilize the finished colour of cured meats.

The need to decrease curing time to meet increased demands for finished products led to the use of various acidulating agents during the 1960s (Karmas, 1977). Glucono-δ-

lactone, acid phosphates and citric acid were most common. Direct usage of nitric oxide gas for curing of meat was proposed during this period (Shank, 1965), but this was not commercially feasible. Emulsification and mixing under vacuum of various comminuted meat formulations were also considered to speed up the process and to decrease the curing time.

Up to the early 1970s, the primary technological emphasis of nitrite usage had been to reduce the time required for curing as much as possible, in order to increase production capacity. Modern technology and scientific understanding had made it possible to utilize smaller quantities of nitrite while exercising vastly improved control over the curing of meat and meat products. Suddenly, the technological emphasis shifted to problem-solving with particular regard to N-nitrosamine production (Sebranek, 1979).

Although meat curing processes, including smoking of meats, were designed for preservation without refrigeration, cured meats continue to have an important place in our diet. Current curing mixtures used in North America contain salt, nitrite, certain colour fixing ingredients, and frequently, seasonings, phosphates, and reductants. Salt still remains the bulk of curing mixtures even though the 1990s health-conscious consumer searches for low sodium-containing foodstuffs. In addition to its preserving effect by inhibiting the growth of microorganisms due to an increase in the osmotic pressure of the medium, salt also helps to solubilize proteins which are important for the emulsion stability of comminuted meat products. Addition of phosphates aid solubilizing proteins and therefore improve binding of comminuted and restructured-type meat products.

Phosphate and polyphosphate usage in cures has also been reported to perform other functions such as increase the retention of moisture and improve the colour and texture of finished products (Savich and Jansen, 1957; Mahon *et al.*, 1971; Smith *et al.*, 1984). Of all these ingredients, nitrite is the most important when used in sufficient quantities, but it is harmful if used too freely. Nitrite is responsible for the typical colour and flavour associated with cooked cured meat. It also acts as an antioxidant and retards the formation of *Clostridium botulinum* toxin. The characteristic attributes which nitrite imparts to meat and their ramifications are discussed below.

2.2 Chemistry of Meat Colour

An important property of meat, whether fresh or cured is its colour. It has a major influence on the consumer's decision to purchase (Hood and Riordan, 1973) because it is usually associated with the quality of the product (Cassens *et al.*, 1988). The colour of meat may range from the deep purplish-red of freshly cut beef to the light pink of cured chicken breast. Deterioration of meat colour has long been used as an early warning of meat "going-off", and FDA regulations prohibit the use of chemical substances such as ascorbic acid or nicotinic acid to artificially prolong fresh meat colour. In the case of preserved meats this is not so, and the bright pink colour of cured bacon and ham has long been used as a selling point, particularly since the development of transparent plastic vacuum packaging. The fading of cooked cured-ham colour under fluorescent lighting in supermarkets has long been recognized as a problem (Pate *et al.*, 1971). Fortunately, the colour of meat can be controlled if the many factors that influence its

colour are understood.

2.2.1 Fresh Meat Colour

The term "meat" signifies the edible flesh or muscle of animals which are acceptable for consumption by man. Edible and acceptable have different interpretations depending on one's cultural background and religion. Meat is a complex biological system. The approximate composition of lean meat is 75% water, 19% protein, 2.5% lipid, 1.2% carbohydrate, 2.3% non-protein compounds containing nitrogen and inorganics and trace amounts of vitamins (Lawrie, 1979).

The native pigment in muscle tissue is a haemoprotein called myoglobin (Mb). In living tissue, Mb is the storehouse of oxygen that is used in the normal biochemical processes of the living muscle. Because the activity of muscles differs greatly and their oxygen demands vary, different Mb concentrations are found in various muscles of the animal. For example, the back muscles of hogs (*i.e.* loin) are used primarily for support and posture and, therefore, have a much lower oxygen requirement than a leg muscle (*i.e.* ham or shoulder) which is used for movement. Besides muscle type, Mb levels are influenced by a large number of intrinsic factors related to function. The most important factors include species, breed, age, sex, anatomical location of the muscle, training or exercise and nutrition (Lawrie, 1979). Species is perhaps the most easily appreciated factor affecting the Mb content of muscles. Typical Mb concentrations of *longissimus dorsi* muscle in mature meat animals are 0.02% in rabbit, 0.25% in sheep, 0.06% in pig, 0.50% in ox, and 0.91% in blue whale (Lawrie, 1979). The overall redness of fresh meat

is largely governed by its Mb content (molecular weight *ca.* 16,700) and to a smaller extent by its Hb content (molecular weight *ca.* 67,000), as well as the forms in which they exist. The greater the Mb concentration, the more intense the colour.

Myoglobin is a globular protein. It is made up of a protein, a globin consisting of 153 amino acids and a prosthetic haem group, an iron (II) protoporphyrin-IX complex. The haem moiety is held in a cleft of the globin by a coordinate bond between the imidazole nitrogen of the proximal histidine residue and the ferrous iron atom, and by a large number of nonpolar and H-bonding interactions at the porphyrin periphery. It is this haem group which gives Mb and its derivatives their distinctive colour as well as being the principal site for meat curing as it relates to colour development.

The haem molecule is an organometallic compound. The organic portion consists of four pyrrole groups linked by methine bridges forming a tetrapyrrole ring. Four methyl, two vinyl, and two propionate side chains are attached to the ring to yield the molecule, protoporphyrin-IX. The iron atom is bonded to the four nitrogens in the centre of a near planar ring. Two additional bonding sites normal to the plane of the ring are occupied by an imidazole group of a histidine residue of globin and an atom possessing a free electron pair. These bonding sites are called the fifth and sixth coordination positions, respectively. The haem iron atom may exist in the ferrous (+2) or the ferric (+3) state, depending on the presence of reductants and oxidants (See Appendix for electronic configurations). In the absence of a covalent complex, either state may coordinate with water. Besides Mb, the remaining tissue pigments include Hb,

cytochromes, vitamin B_{12} and the flavins, but these contribute little to meat colour.

In the purple-red deoxy-form, the pentacoordinate haem Fe(II) compound is high spin (t_{2g}^4 e_g^2, S=2) with an ionic radius of 78 pm which is too large to fit into the porphyrin plane. Consequently, the ferrous ion projects greater than 25 pm above the porphyrin ring plane towards the proximal histidine giving the molecule a square pyramidal configuration (Kendrew, 1963; Thompson, 1988). A vacant binding site for ligands lies on the side of the porphyrin away from the proximal histidine. Cherry-red oxymyoglobin (MbO_2) is formed upon oxygenation in the sixth coordination position. This hexacoordinate low spin (t_{2g}^6, S=0) Fe(II) complex is diamagnetic and is believed to lie in the plane of the porphyrin ring giving the molecule an octahedral configuration (Thompson, 1988). Besides oxygen, Mb can complex with other ligands such as nitric oxide in its vacant sixth coordinate position.

The bright red colour of fresh meat is due to MbO_2 and is present only on the meat's surface. This is a consequence of an adequate supply of molecular oxygen and reducing substances such as cytochrome c and to the nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase system in the mitochondria. In contrast, the interior tissue is purple-red in colour. This is the colour of Mb in the ferrous state as long as reductants generated within the cells by enzyme activity are available. When these substances are depleted, the haem iron is oxidized to the ferric state. The brown pigment formed, which is characteristic of the colour of meat left standing for a period of time, is called metmyoglobin (metMb). When metMb is denatured by heat, meat remains

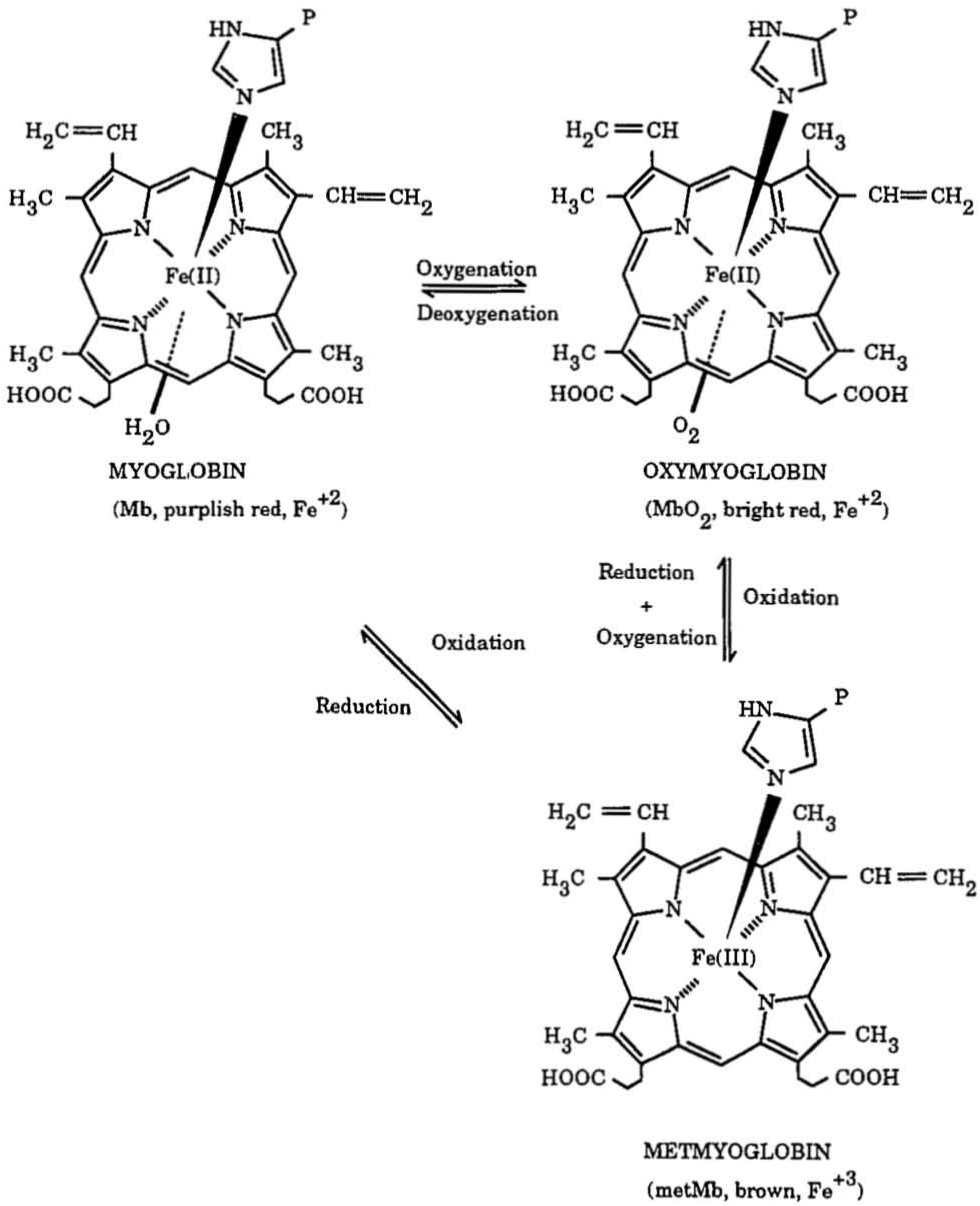
brown in colour, but this denatured pigment may be oxidized further to yellow, green or colourless porphyrin substances by bacterial action or photochemical oxidation. The interrelationship between fresh meat pigments is illustrated in Figure 2.1.

2.2.2 Cured Meat Colour

The chemistry of nitrite curing is complex and a number of possible routes of interaction of nitrite with the various forms of Mb exist. When nitrite is added to comminuted meat, the meat turns brown since nitrite acts as a strong haem oxidant. Myoglobin or MbO_2 is oxidized to metMb by the nitrite ion which itself may be reduced to NO. These products can combine with one another to from an intermediate pigment, nitrosylmetmyoglobin (NOmetMb). Nitrosylmetmyoglobin is unstable and autoreduces on standing, due to the presence of endogenous and exogenous reductants in the post-mortem muscle tissue, to the corresponding relatively stable Fe(II) form, nitrosylmyoglobin (NOMb) (Yonetani *et al.*, 1972).

The characteristic red colour of fresh cured meats (*i.e.* before thermal processing) is due to NOMb. Nitrosylmyoglobin is a ferrous mononitrosylhaem complex in which the reduced iron atom is coordinated to four nitrogen atoms of the protoporphyrin-IX plane, one nitrogen atom of the proximal histidine residue of globin (fifth coordinate position) and a NO group (sixth coordinate position). The NOMb pigment can be produced by the direct action of NO on a deoxygenated solution of Mb, but in conventional curing it arises from the action of nitrite as stated above. Upon thermal processing, globin denatures, detaches itself from the iron atom and surrounds the haem

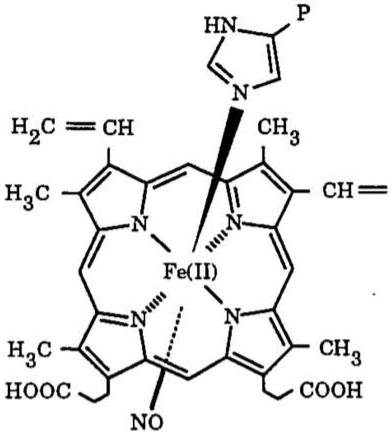
Figure 2.1 Interrelationship between pigments of fresh meat. Adapted from Bard and Townsend (1971).


HN
P
N
H_2C═CH
CH_3
H_3C
CH═CH_2
N
N
Fe(II)
N
N
H_3C
CH_3
HOOC
COOH
H_2O
MYOGLOBIN
(Mb, purplish red, Fe^{+2})
Oxygenation
Deoxygenation
O_2
OXYMYOGLOBIN
(MbO_2, bright red, Fe^{+2})
Reduction
+
Oxygenation
Oxidation
Oxidation
Reduction
Fe(III)
METMYOGLOBIN
(metMb, brown, Fe^{+3})

moiety. Nitrosylmyochromogen is the pigment formed upon cooking and it confers the characteristic pink colour to cooked cured meats. Haldane in 1901 was first to recognize the cooked cured-meat pigment as a nitrosylhaem complex. The pigment contains at least one NO moiety in either the fifth or sixth coordinate position and a molecule such as water in the other, but the possibility of a dinitrosylhaem complex in which NO groups are bound in both axial positions has been suggested (Tarladgis, 1962; Lee and Cassens, 1976; Rennerre and Rougie, 1979). This will be discussed in some detail below. The formation of the cooked cured-meat pigment from NOMb and its possible side reactions are illustrated in Figure 2.2. This pigment is susceptible to photooxidation and decomposes upon standing. A two-step process involving light-accelerated dissociation of NO from the haem followed by oxidation of both the NO moiety and the ferrous haem iron has been suggested as the probable mechanism (Fox, 1966).

Specific biochemical reducing systems which may be important in the development of cured meat colour have been the subject of intensive investigation (Walters and Taylor, 1965; Walters *et al.*, 1967; Möhler, 1974; Walters *et al.*, 1975). Endogenous reductants capable of reducing nitrite to NO in meat include cysteine, reduced NADH, cytochromes and quinones (Fox, 1987). A number of workers have investigated the effects of endogenous muscle metabolites including peptides, amino acids, and carbohydrates on the formation of NOMb. Tinbergen (1974) concluded that low-molecular-weight peptides such as glutathione and amino acids with free sulphydryl groups were responsible for the reduction of nitrite to NO which is subsequently

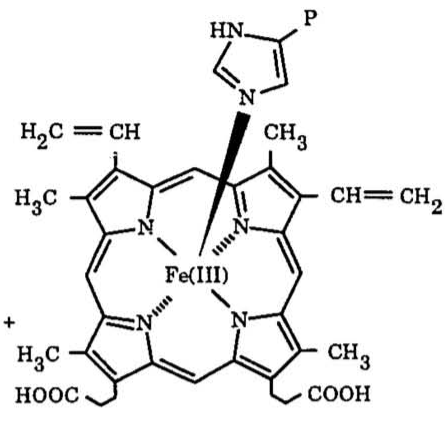
Figure 2.2 Some of the possible curing reactions that result from the addition of nitrite to meat. Adapted from Bard and Townsend (1971).



NITRIC OXIDE MYOGLOBIN

(NOMb, red, Fe^{+2})

Oxidation ⇌ Nitric Oxide + Reduction

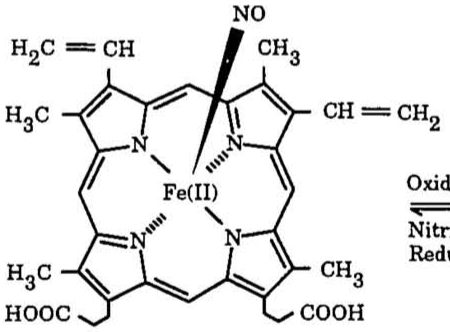


METMYOGLOBIN

(metMb, brown, Fe^{+3})

Protein Denaturation and Detachment (Heat)

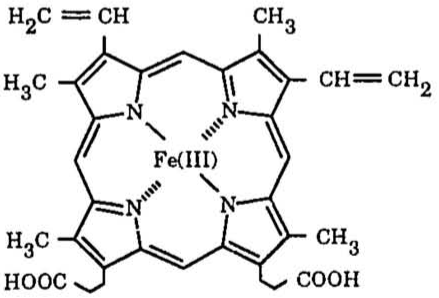
Protein Denaturation and Detachment (Heat)



COOKED CURED-MEAT PIGMENT

(CCMP, pink, Fe^{+2})

Oxidation ⇌ Nitric Oxide + Reduction



DENATURED METMYOGLOBIN

(den. metMb, brown, Fe^{+3})

OXIDIZED PORPHYRINS

(green, yellow, colourless)

complexed with Mb to produce NOMb. Similar work by Ando (1974) also suggested that glutathione and glutamate are involved in cured colour formation. Depletion of these compounds in meat through oxidation occurs with time, but reductants such as sodium ascorbate or erythorbate are added to nitrite-cured meats prior to processing to ensure good colour development (Alley *et al.*, 1992). The role of reductants in haem-pigment chemistry is ambiguous, however, they can promote oxidation and even porphyrin ring rupture under certain conditions.

2.2.3 Characterization of Nitrosylhaem Pigments

Hornsey (1956) described a simple and rapid method for extracting and measuring the content of NO-haem pigments present in cooked cured meat. The author stated that selective extraction of NO-haem pigments was achieved using a 4:1 (v/v) acetone:water mixture. No other meat pigments were extracted under these conditions. The absorbance of the extract was measured spectrophotometrically at a wavelength of 540 nm and based on a known molar extinction coefficient, the concentration of the NO-haem pigment determined. Since the extract was sensitive to light and oxygen reductants were added to improve its stability. Hornsey did not characterize the number of NO ligands or sites of their attachment to the denatured haemoprotein.

2.2.3.1 Evidence for a Dinitrosylhaem Complex

Based on spectral studies using acetone extracts made by Hornsey's method (1956), Tarladgis (1962) concluded that the pigment of cooked cured meats was a low-

spin ferrous-porphyrin coordination complex. The author observed that the α band at 563 nm was more intense than the β band at 535 nm indicating a strong donation of electrons from the ferrous ion to the unsaturated NO ligand for the formation of a π-d covalent bond. Nitrosylhaem can be selectively extracted from NOMb and NOHb by aqueous acetone. Complexation of the metalloporphyrin with the strongly *trans*-directing nitrosyl ligand is believed to weaken the coordinate bond linking the haem and the globin. The protein is denatured upon acetone addition and the nitrosylhaem enters solution. Fresh cured meat pigments (*i.e.* NOMb and NOHb) are released from the protein and dissolved in acetone, while other haemoprotein derivatives such as MbO_2 and metMb are denatured intact. Hornsey's method is not specific for the cooked cured meat pigment, as Tarladgis (1962) has implied, even though extraction is more efficient when the iron-imidazole bond has been cleaved after cooking. Although the pigment was easily extracted into acetone, no infrared spectra for these extracts were presented. The identity of the ligand in the vacant coordination position of CCMP is uncertain. Tarladgis (1962), using optical and electron spin resonance (ESR) spectroscopies, has suggested that both axial coordinate positions of the iron in CCMP are occupied by NO groups. The author proposed that the two unpaired electrons from the NO moieties should have their spins coupled, thus rendering the molecule diamagnetic. Because no ESR signals were observed, Tarladgis concluded that the pigment was a dinitrosylferrohaemochrome indicating the presence of no unpaired electrons. Lack of an ESR spectrum may only establish the diamagnetic nature of the pigment extract and is not a proof of dinitrosyl ligation.

Further evidence for this dinitrosyl form of the cooked cured meat pigment is available from studies by Lee and Cassens (1976) and Rennerre and Rougie (1979) in which $Na^{15}NO_2$ was used to determine the amount of NO bound to unheated as compared to heated solutions of Mb. They found that heated samples contained twice as much ^{15}N as unheated counterparts when analyzed by a modified Kjeldahl method. It was considered likely that the globin portion was detached from Mb during heating, making available two sites for NO binding. These authors failed to consider, however, that NO may bind with other constituents of the haemoprotein, not just the ferrous iron atom (Bonnett and Nicolaidou, 1979; Bonnett *et al.*, 1980*b*).

The presence of a dinitrosylhaem complex has also received support from studies of Wayland and Olson (1974). They have shown that tetraphenylporphyriniron (III) chloride dissolved in toluene in the presence of excess NO reacts with methanol to produce a ferrous nitrosyl derivative, $Fe^{II}TPP(NO)$. This compound was characterized as a low spin (S=½) ferrous porphyrin complex. The IR spectrum of $Fe^{II}TPP(NO)$ in Nujol mull had a strong ν_{NO} at 1700 cm^{-1} while the ESR spectrum showed 3 g values with NO ^{14}N hyperfine splitting characteristic of a pentacoordinate haem complex as reported by Bonnett *et al.* (1980*a*). The $Fe^{II}TPP(NO)$ formed one-to-one adducts with nitrogenous donors such as pyridine and piperidine showing a rhombic g tensor with ^{14}N hyperfine splitting from both NO and the ring N donor in the g_z region characteristic of the hexacoordinate species such as those of NO complexes of $Fe^{II}Mb$ and $Fe^{II}Hb$. This ^{14}N hyperfine coupling provides evidence for placing the odd electron in a molecular orbital

with substantial iron d_{z^2} character. The odd electron which originates on NO becomes highly delocalized to iron in the complex. Wayland and Olson (1974) also reported that the ESR spectrum of $Fe^{II}TPP(NO)$ decreased in intensity as the pressure of NO was increased or the temperature decreased. No ESR transitions were detected suggesting the formation of an even-electron species and magnetic susceptibility measurements in solution, as a function of temperature, indicated that $Fe^{II}TPP(NO)_2$ was diamagnetic. These authors reported that a new electronic spectrum similar to $Fe^{III}TPP(Cl)(NO)$ and low spin $Fe^{II}TPP(C_5H_5N)_2$ was observed. Two NO stretching frequencies in the mull were found at 1870 cm^{-1} and 1690 cm^{-1}. The band at 1870 cm^{-1} is in the range expected for a linear $Fe^{II}NO^+$ moiety and the 1690 cm^{-1} band is consistent with a bent $Fe^{II}NO^-$ fragment. Upon evacuation of excess NO, the intensity of the 1870 cm^{-1} band decreased and eventually disappeared. While the cooked cured meat pigment could possibly have such a structure, there is no evidence for the formation of a dinitrosylprotohaem complex in the meat matrix.

Burge and Smith (1992) attempted to characterize the structure of the organic-soluble pigment from cooked cured ham by synthesizing a pigment as described by Shahidi *et al.* (1985*b*) to model the CCMP and then analyzing its structure with ^{15}N NMR and IR spectroscopies. Although IR spectroscopy for identification of functional groups in molecules is commonplace, overlapping of symmetric stretch ranges for bent (1725-1525 cm^{-1}) and linear (2000-1600 cm^{-1}) nitrosyl moieties limits the usefulness of this technique in identifying nitrosyl coordinate geometry (Bell *et al.*, 1983). ^{15}N NMR is a

more precise technique for studying nitrosyl ligands of nitrosyl-metal complexes because the number of NO ligands and their coordinate geometry can be determined. Burge and Smith (1992) assumed that the synthesized nitrosylhaem pigment was a dinitrosylhaem complex, but this had not been satisfactorily proven by Shahidi *et al.* (1985*b*). By comparing acetone extracts of nitrite-cured ham, preformed CCMP, and pigment whose volume was reduced under a stream of nitrogen, Burge and Smith (1992) concluded that the disappearance of the 557-nm band was attributed to loss of the second NO moiety attached to haem. These authors experienced difficulties in recording an NMR spectrum of $Fe^{II}PP(^{15}NO)_2$.

2.2.3.2 Evidence for a Mononitrosylhaem Complex

Bonnett *et al.* (1978) attempted to characterize the pigment of cooked cured meat, nitrosylprotohaem, as its dimethyl ester, which was obtained by the reaction of NO with protohaem dimethyl ester and with methoxyiron (III)-protoporphyrin dimethyl ester. Presence of a strong IR band at 1660 cm^{-1} was diagnostic of the stretching mode of a bent Fe-NO moiety and a pentacoordinate complex. Although the visible spectra of these compounds were similar to one another and resembled that of cured meat, Bonnett *et al.* (1978) suggested that use of visible spectroscopy may be complicated by aerial oxidation of nitrosylhaem which becomes especially important at the dilution required to observe the Soret band. Hence, ESR spectroscopy using more concentrated samples in closed, oxygen-free systems at a low temperature was considered (Bonnett *et al.*, 1980*a*). The ESR spectrum of the nitrosylhaem in acetone showed a triplet signal due to hyperfine

splitting by a single axial nitrogenous ligand of NO indicating a pentacoordinate nitrosylhaem system. When this sample was kept in the sealed, oxygen-free ESR tube at room temperature and in the dark, the signal remained virtually unchanged over a two-year period reflecting the considerable thermodynamic stability of this compound (g_1 = 2.102, g_2 = 2.064, g_3 = 2.010, α_3 = 1.63 mT). When the ESR spectrum of nitrosylhaem was monitored in piperidine, a solvent providing a second nitrogenous ligand, the g_1, g_2 and the hyperfine structure g_3 were no longer resolved (g_1 = 2.08, g_2 = 2.04, g_3 = 2.003). The resulting spectrum with a minimum of the broad high-field feature located at an effective g value of 1.98 was characteristic of a hexacoordinate system. Nitrosylmyoglobin showed this type of ESR spectrum, indicating that it was a hexacoordinate complex where the fifth coordinate position was occupied by an imidazole group of the globin. Identical ESR characteristics of nitrosylprotohaem can be obtained from solutions of NOHb by treatment with acetone. The protohaem groups of Hb cannot be extracted with acetone, thereby suggesting that there is a structural *trans*-effect created by the nitrosyl ligand which results in the weakening and lengthening of the bond between iron and the coordinated N atom of the imidazole group. Similarly, nitrosylprotohaem extracted with acetone from cured meats showed an ESR spectrum expected for a pentacoordinate nitrosylhaem. While these extraction experiments provided confirmation of the general chemical nature of the chromophore of cured meat, they did not reveal the coordination sphere *in situ*.

Bonnett *et al.* (1980*a*) examined various cured meat samples directly by ESR

spectroscopy as opposed to pigment extracts. Spectra of cured meats subjected to thermal processing showed an ESR signal with the hyperfine splitting characteristic of the pentacoordinate nitrosylhaem indicating that the iron-imidazole bond was effectively broken. These authors suggested that the colour of cooked cured meat was due to the pentacoordinate nitrosylprotohaem which was physically trapped in a matrix of denatured globin offering some protection to the pigment against aerial oxidation. Further support for this view comes from the ESR spectrum of uncooked bacon which indicated the presence of both penta- and hexa-coordinate nitrosylhaems. After gentle heating of the sample in the ESR tube, the broad high-field feature located at an effective g value of 1.990, characteristic of a hexacoordinate species, disappeared and the features characteristic of a pentacoordinate species became sharper and more intense.

Killday *et al.* (1988) isolated and characterized an acetone extract of the CCMP from cooked corned beef by IR and VIS spectroscopies and thin-layer chromatography. They also identified the pigment as a mononitrosyl ferrous protoporphyrin complex which was confirmed by fast atom bombardment mass spectrometry.

Maxwell and Caughey (1976) reported the preparation of a solid pentacoordinate nitrosylhaem ester from pyridine using protoporphyrin-IX dimethyl ester iron (II). The haem pigment was heated at 80°C under vacuum until all the liganded pyridine, which was detected quantitatively, had been removed. Upon exposure of the solid to NO, uptake of 1.0 mol of NO/mole of Fe was observed, consistent with formation of a nitrosylhaem with one NO ligand. Infrared spectra of nitrosylhaem complexes prepared

in solution revealed stretching of a single ν_{NO} at 1670 cm^{-1}, indicating a pentacoordinate complex in which non-nitrogenous solvents were used. Hexacoordinated species with one NO moiety were observed in solutions in which either the solid pentacoordinate NO compound had been added to solvent containing a nitrogenous base or the haem had been exposed to NO gas in a solution with excess nitrogenous base present. A single ν_{NO} value at 1620 cm^{-1} was observed which is consistent with bent-end-on bonding (*i.e.* Fe-N-O) with iron (II) serving as π donor and the N of NO as σ donor with an overall shift of electron density from iron to NO upon bonding. This interpretation is not consistent with conclusions drawn from ESR studies by Wayland and Olson (1974), to the effect that the electron density shift was in the opposite direction, namely from NO to iron (II) to give a partially positive NO ligand (Yonetani *et al.*, 1972). Because ESR data gave evidence of spin density but did not indicate the charge distribution, the ESR data need not be considered inconsistent with the conclusions drawn from IR data. Maxwell and Caughey (1976) also showed that the ν_{NO} in the IR spectrum of NOHb exhibited the hexacoordinate configuration similar to a 1-methyl-imidazole protohaem nitrosyl compound. Upon addition of inositol hexaphosphate (IHP) to the system, a 50 cm^{-1} shift in the ν_{NO} to approximately 1670 cm^{-1} was characteristic of the pentacoordinate structure of nitrosylprotohaem. These authors suggested that the IHP-induced frequency shift provides a strong evidence for loss of the *trans*-histidine ligand since this shift is precisely of the same magnitude as that measured upon loss of imidazole in protein-free haems. Electron spin resonance spectra of NOHb in which $^{14}N^{16}O$ and $^{15}N^{16}O$ were used with and without

IHP were quite striking.

The ESR spectra of frozen solutions of native bovine NOMb (buffer pH 5.5) with strongly resolved hyperfine splitting were recorded by Kamerei and Karel (1983) and they resembled the ESR spectrum of NOHb in the presence of IHP. These authors stated that IHP converts NOHb from a relaxed to a tense quaternary state. In the tense state, the bond between the proximal histidine and iron is ruptured in the α-chains of NOHb. The observed hyperfine splitting was consistent with coupling of the ^{14}N nucleus of the proximal histidine. This was explained by assuming that the *trans*-effect of the NO ligand results in such a dramatic stretching of the Fe-N_{His} bond that no spin transfer from iron to N_{His} occurs. Thus, native bovine NOMb (uncooked) behaves as a pentacoordinate complex, but Dickinson and Chien (1971), who measured the ESR spectra of single crystals of sperm whale NOMb, observed for the first time clear splitting of resonance lines due to the imidazole nitrogen of the proximal histidine, thereby providing definite proof of hexacoordination. Clearly pH and other factors are important in determining whether native NOMb or NOHb exist as penta- or hexa-coordinate species.

Trittelvitz *et al.* (1972) stated that analysis of NOHb offers interesting aspects because NO is both a spin label for ESR studies and a strong ligand at the sixth coordination site of the iron. Lang and Marshall (1966) noted that the unpaired electron of the paramagnetic NOHb occupies a *d*-orbital of the iron atom. Trittelvitz *et al.* (1972) stated that the hyperfine structure resulting from the interaction of the unpaired electron with the N-nucleus of NO should bring evidence of a conformationally induced change

of the binding properties of the sixth ligand. These authors indicated that the g_1 value from the ESR spectrum of ^{14}NOHb showed a hyperfine structure of three lines similar to that observed by Maxwell and Caughey (1976) and Bonnett *et al.* (1980*a*). Using ^{15}NO, however, the three line spectrum became a two line spectrum and by comparing these spectra with theoretical splitting energy, the hyperfine structure at g_1 was ascribed to the sixth ligand of the haem iron. The ESR spectrum of ^{14}NO-Mb did not show any hyperfine structure similar to that of Bonnett *et al.* (1980*a*).

2.3 Oxidative Stability of Meat Lipids

Lipids are an integral part of foodstuffs. Two main classes of lipids in meat are adipose and intramuscular tissue. Adipose tissue consists primarily of triacylglycerols while intramuscular tissue is composed of both triacylglycerols and membrane-bound fats, such as phospholipids and lipoproteins. The fatty acids associated with these tissues are either saturated or unsaturated. Oxidation of unsaturated lipids has been extensively studied since it relates to deterioration of muscle foods, production of both desirable and undesirable breakdown products and numerous reactions associated with other food constituents (Wong, 1989).

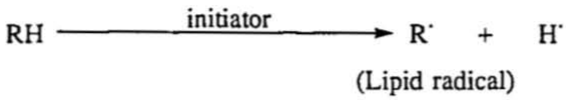
Autoxidation is the main pathway of oxidative deterioration of meat lipids. The process proceeds *via* a free-radical mechanism involving initiation, propagation, and termination steps as illustrated in Figure 2.3. An initiator causes homolytic cleavage of the lipid-hydrogen covalent bond adjacent, or α, to the site of unsaturation in fatty acid

molecules. It has been postulated that singlet oxygen is the active species involved in free radical formation in the initiation step, with tissue pigments such as Mb acting as sensitizers. A lipid radical reacts with molecular oxygen forming a peroxy radical. This radical in turn abstracts a hydrogen atom from a second lipid molecule producing a hydroperoxide and a new lipid radical which may also react with oxygen. This chain reaction appears to be self-sustaining as long as oxygen and unoxidized lipids are present. Due to resonance stabilization of lipid radical species (R'), a shift in the position of double bonds results in the formation of hydroperoxide positional- and geometric-isomers, but intermolecular reactions of radicals may result in the formation of non-radical species (*i.e.* termination products) such as dimers, polymers, cyclic peroxides, and hydroperoxy compounds.

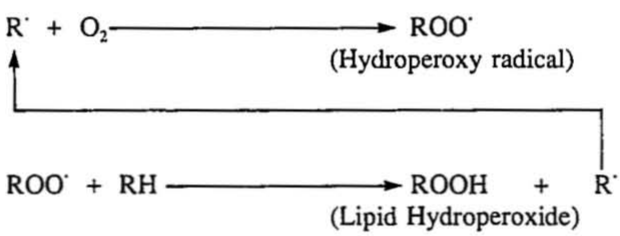
The hydroperoxides themselves do not contribute to off-flavours of oxidized fats. They are colourless, odourless and unstable and tend to breakdown to smaller compounds such as aldehydes, ketones, alcohols, hydrocarbons and organic acids which have characteristic pungent odours associated with rancidity of meats. The extent of autoxidation depends on many factors including oxygen partial pressure, the degree of unsaturation of lipids, the presence and concentration of antioxidants, packaging materials, exposure to light, and temperature of storage. The nature of fatty acids in meat and their concentrations have a very pronounced effect on the rate of autoxidation.

Figure 2.3 Mechanism of autoxidation. Adapted from Wong (1989).

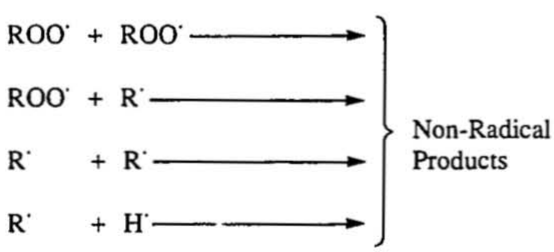
Initiation



Propagation



Termination



2.3.1 Lipid Oxidation of Uncured Meats

Tims and Watts (1958) observed that lipid oxidation in refrigerated cooked meats was more pronounced than that in raw or frozen uncooked meat. To describe this rapid development of lipid-derived oxidized flavour, they coined the term "warmed-over flavour" (WOF). The rancid or stale flavour becomes readily apparent within 48 hours in cooked meats as opposed to the more slowly developing rancidity encountered in raw meats which becomes evident only after prolonged freezer storage (Pearson *et al.*, 1977; Spanier *et al.*, 1992*a*). Warmed-over flavour has been reported to develop rapidly in raw meat that has been comminuted and exposed to air (Greene, 1969; Sato and Hegarty, 1971). It is now generally accepted that any process involving disruption of the integrity of muscles, such as cooking, grinding or restructuring, enhances the development of WOF (Spanier *et al.*, 1992*b*). In recent years, demand has grown for pre-cooked, ready-to-eat meat products in the marketplace and in fast food franchises, thereby providing expanding potential for consumer exposure to WOF (Stoick *et al.*, 1991). Because WOF development is a dynamic process of flavour change, due principally to a cascade of oxidative events (Asghar *et al.*, 1988), an understanding of the mechanism and prevention of its occurrence in meat and meat products is important to the food scientist.

In the late 1980s, various researchers showed that WOF was not solely a consequence of lipid oxidation (Vercellotti *et al.* 1987*a,b*; St. Angelo *et al.* 1988; Spanier *et al.* 1988). These researchers suggested that there was strong evidence that protein degradation reactions were also involved and that heteroatomic compounds formed from

these reactions may be implicated with the phenomenon of WOF, particularly with the deterioration of desirable meaty flavour notes. It was therefore proposed that meat flavour deterioration (MFD) was a more accurate term to use.

Of the lipids in meat, phospholipids are most susceptible to oxidation. Their tendency to undergo rapid oxidation is largely due to their high unsaturated fatty acid content which is accelerated upon thermal processing (Igene *et al.*, 1979). Oxidation of the unsaturated C_{18} fatty acids of meat, namely oleate, linoleate and linolenate, has been reported to produce low-molecular-weight aldehydes (C_3-C_{12}) such as pentanal, hexanal and 2,4-decadienal which are believed to be partially responsible for WOF and rancidity development of cooked meats during storage.

The catalytic effect of iron porphyrins and metal ions during oxidation has been the subject of a great deal of study over the years (Tichivangana and Morrissey, 1984). Haemoproteins have been implicated as major pro-oxidants of lipid peroxidation in meats (Watts, 1954; Greene *et al.*, 1971; Love and Pearson, 1974; Igene *et al.*, 1979). Wills (1966) and Liu and Watts (1970) assessed the role of haem and non-haem iron as catalysts of lipid oxidation in various animal tissues and concluded that both haem and non-haem iron had catalytic activity in raw and cooked systems. Catalysis of lipid oxidation by haem pigments was an accepted mechanism until the work of Sato and Hegarty (1971). These authors removed haem pigments from muscle tissue by dialysis, added Mb or Hb back to dialysed samples, cooked them and then stored the meats at refrigeration conditions. The TBA test revealed that haem pigments had no significant

effect on the extent of lipid oxidation (Fox and Benedict, 1987). Love and Pearson (1974) and Igene *et al.* (1979) extended and confirmed this basic finding. They reported that intact haemoproteins had little effect on the rate of oxidation in cooked meats, while non-haem Fe^{+2} at concentrations as low as 1 ppm resulted in enhanced oxidation in samples of water-extracted cooked meats. Resolution of the roles played by haem and non-haem iron as catalysts of lipid oxidation in meat products is very important in understanding the factors responsible for the development of off-flavours. Igene *et al.* (1979) proposed that thermal processing releases a significant amount of non-haem iron from the native muscle pigments which then accelerates lipid oxidation in cooked meats. Studies by Schrinker *et al.* (1982), Schrinker and Miller (1983), Chen *et al.* (1984) and Tichivangana and Morrissey (1984) have concurred with this finding.

2.3.2 Lipid Oxidation of Nitrite-Cured Meats

In 1954, Watts noted that development of oxidative rancidity was delayed in nitrite-cured meats. Younathan and Watts (1959) investigated the extent of lipid oxidation in cured and uncured cooked pork stored at refrigeration temperatures over a two week period using the TBA test to assess the degree of oxidative rancidity. Uncured samples yielded significantly ($P<0.05$) higher TBA values than their cured counterparts at all storage periods indicating that nitrite addition to meat suppresses oxidative deterioration of meat lipids. Zipser *et al.* (1964), Cho and Bratzler (1970) and Hadden *et al.* (1975) have all shown the inhibitory effect of nitrite against oxidation in cooked cured-meat products.

Sato and Hegarty (1971) reported that nitrite inhibits WOF development even at levels as low as 50 ppm, and it could completely retard lipid oxidation in ground beef when used at a concentration of 2000 ppm. Bailey and Swain (1973) confirmed the antioxidant role of nitrite in refrigerated cooked hams by correlating subjective taste-panel flavour scores with TBA values. MacDonald *et al.* (1980*a*,*b*) went a step further and studied the effects of various levels of nitrite (0, 50, 200, and 500 ppm) on the oxidative stability of cooked hams. A comparison of the data revealed a significant reduction ($P<0.05$) in TBA values of nitrite-cured ham, but no significant difference ($P>0.05$) in mean TBA values between hams cured with sodium nitrite at 200 and 500 ppm level was noted.

Fooladi *et al.* (1979) investigated the role and function of nitrite in preventing development of WOF in cooked beef, pork and chicken. Samples treated with nitrite at a fixed level of 156 ppm were evaluated against controls with no additives by the TBA test and by sensory panel scores before and after cooking at day 0 and again after 2 days of storage at 4°C. For all three species, a significant difference ($P<0.01$) in TBA values between cured and uncured meats was observed. Added nitrite inhibited WOF development in cooked meat, resulting in a 2-fold reduction in TBA values for beef and chicken and a 5-fold reduction in pork. Sensory panel data were in agreement with findings of the TBA test. Differences in taste panel scores between cured and uncured samples were significant ($P<0.05$) for chicken and highly significant ($P<0.01$) for pork and beef. The effect of sodium nitrite on lipid oxidation in cooked, minced muscles from

various species stored at 4°C for 24 h as monitored by TBA values is presented in Figure 2.4 (Morrissey and Tichivangana, 1985). At a level of 200 ppm, nitrite brought about a 17-fold reduction in TBA values of fish and a 12-fold reduction in TBA values of chicken, pork and beef compared to those of their uncured counterparts.

2.3.3 Mechanism of Nitrite's Antioxidative Action

The mechanism(s) by which nitrite prevents or retards the peroxidation of meat lipids is not fully understood. The literature suggests that mechanisms involved may include the following:

(i) formation of a stable complex between haem pigments and nitrite, thereby preventing the release of iron from the porphyrin molecule.

(ii) interaction of nitrite as a metal chelator which ties up trace metals in meat as well as any liberated non-haem iron from denatured haem pigments.

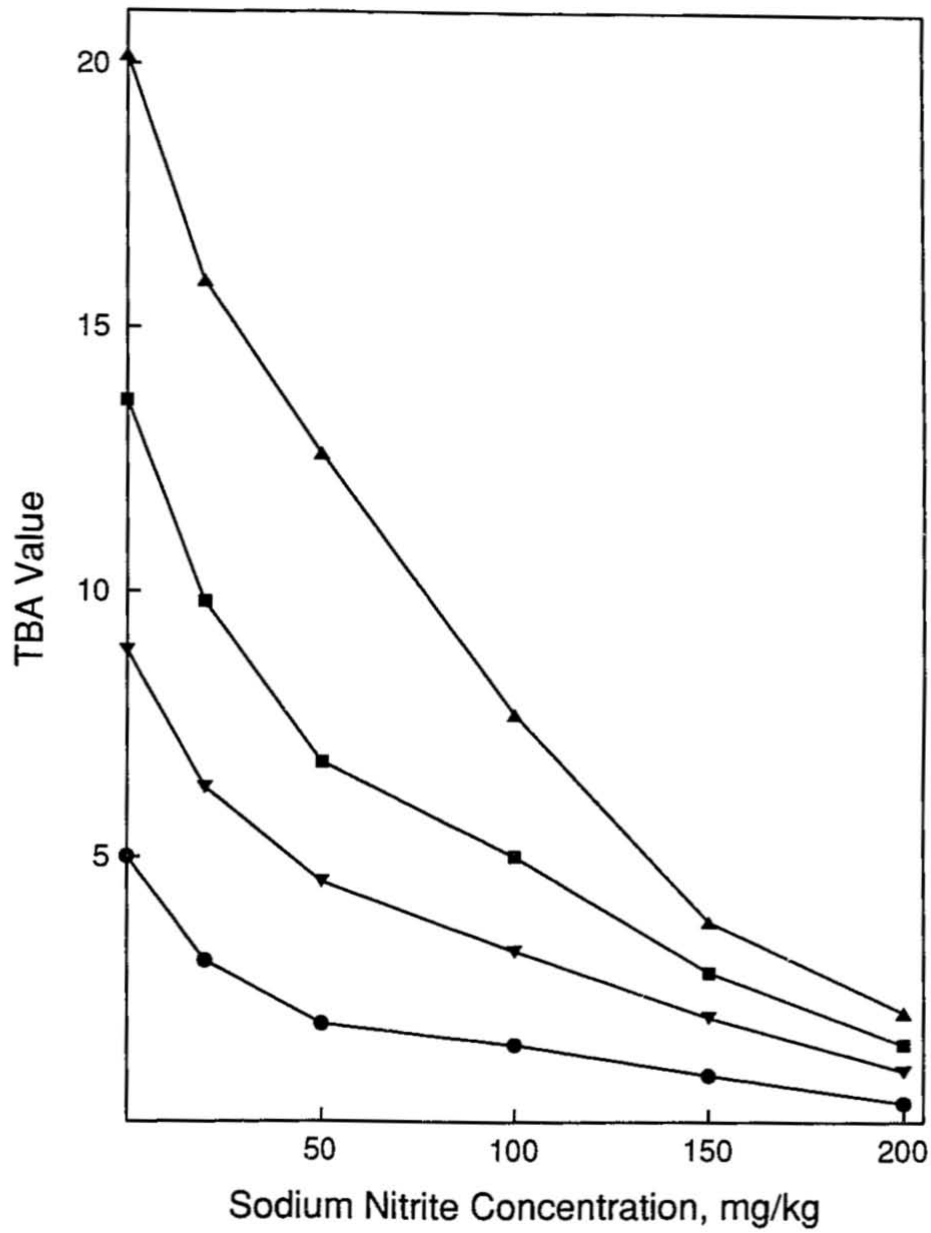
(iii) stabilization of unsaturated lipids within the membranes against oxidation.

(iv) formation of nitroso compounds in meat which possess antioxidative properties.

According to Gray and Pearson (1987), preventing the release of Fe^{+2} during thermal processing by stabilizing the porphyrin ring appears to be the most important mechanism. Igene *et al.* (1985) reported that cooking significantly ($P<0.05$) increased the proportion of non-haem iron in beef from 6.6 to 10.8 µg Fe/g muscle tissue whereas the levels of non-haem iron remained unchanged in the nitrite-cured sample (*ca.* 6.8 µg Fe/g muscle tissue).

Sato and Hegarty (1971) and Goutefongea *et al.* (1977) support the view that

Figure 2.4 Effect of sodium nitrite on lipid oxidation in cooked, minced muscles from various species stored at 4°C for 48 h: ▲ , fish; ■ , chicken; ▼ , pork; ● , beef. Adapted from Morrissey and Tichivangana (1985).


20
15
10
5
TBA Value
50
100
150
200
Sodium Nitrite Concentration, mg/kg

nitrite reacts with lipids in tissue membranes, leading to a stabilization and retardation of lipid oxidation. Walters *et al.* (1979) found evidence that nitrite added to the double bonds of unsaturated fatty acids forming pseudonitrosites. In a more recent study, Freybler *et al.* (1993) confirmed this finding and showed by IR analyses that nitrite or dinitrogen trioxide reacts with unsaturated lipids to form other nitro-nitroso derivatives, thereby stabilizing the lipids toward peroxidative changes. Igene and Pearson (1979) studied the reaction of nitrite with purified unsaturated phospholipids and demonstrated that nitrite significantly reduced TBA values while improving sensory scores. They also suggested that nitrite functions as an antioxidant by forming a complex with the phospholipid components, thereby stabilizing the membranes, as well as by forming a chromogen with haem pigments. By analyzing the difference in the TBA values of cooked nitrite-cured beef surimi with its uncured counterpart, Igene *et al.* (1985) attributed the effective inhibition of lipid oxidation to nitrite by its stabilization of membrane lipids. They also demonstrated that nitrite was an effective antioxidant against the degradation of phosphatidylethanolamine, the major phospholipid responsible for the development of MFD in cooked meat. Zubillaga *et al.* (1984) reported that the polar-lipid fraction of raw nitrite-cured beef and pork had sufficient activity in inhibiting the oxidation of linoleate as determined by β-carotene bleaching. While the reactive compounds were not identified, they concluded that an addition product of nitrogen oxides to olefinic double bonds of unsaturated lipid moieties did not account for the observed antioxidant activity.

Kanner *et al.* (1984) proposed that the antioxidant effects of nitrite in cured meat resulted from the formation of NO which reacts with metals, haem pigments and other biomolecules in the meat matrix. Kanner (1979), Kanner *et al.* (1980), Morrissey and Tichivangana (1985) and Shahidi *et al.* (1988) have clearly demonstrated that some nitrosylhaem compounds possess antioxidant effects. The preformed CCMP was found to act as a weak antioxidant in meat model systems (Shahidi *et al.*, 1987*a*) and in β-carotene/linoleate model systems (Shahidi, results not published). Suggestions have been made that nitrosylated iron porphyrin compounds act in the early stages of lipid autoxidation to quench substrate-free radicals and thereby inhibit their propagation (Kanner *et al.*, 1980). S-Nitrosocysteine (SNC), a possible reaction product in the meat curing process, has been shown to be a potent antioxidant (Kanner, 1979). The inhibitory effect of SNC on lipid oxidation in a cooked turkey meat product was reported by Kanner and Juven (1980). Equimolar concentrations of SNC and nitrite imparted a similar inhibitory effect. It was also demonstrated that at room temperature, or upon cooking, SNC dissociates to form haem-NO complexes in meat. Since only 1-2 ppm of nitrite is sufficient for cured-meat colour production (MacDougall and Hetherington, 1992), the concentration of SNC, on a molar basis, may arguably be much smaller than that of the added nitrite. Consequently, comparison of the antioxidant activity of nitrite with SNC at equimolar concentrations may not be realistic (Shahidi, 1992).

2.3.4 Assessment of Lipid Oxidation in Meats by the TBA Test

A relatively minor product of autoxidation of polyunsaturated fatty acids in meat is malonaldehyde. It has been extensively studied due to its reactivity with biological molecules such as amino moieties of amino acids, proteins, nucleic acids as well as with sulphydryl groups (Chio and Tappel, 1969*a*,*b*; Draper *et al.*, 1986). Malonaldehyde is generally bound to biological materials and therefore, prior to determination, it must be released from muscle tissues by acid treatment. Its presence and concentration in foodstuffs is commonly monitored as a marker of lipid peroxidation by the TBA test (Shahidi and Hong, 1991*a*). This spectrophotometric determination, first reported by Kohn and Liversedge (1944) and then described in detail by Tarladgis *et al.* (1960), involves the reaction of malonaldehyde in oxidized foods with the TBA reagent forming a pink adduct with a distinctive absorption maximum at 532 nm.

Various procedures have been employed for performing the TBA test. They generally involve heating the food product with an acid to liberate malonaldehyde from its precursors as well as to hasten condensation of malonaldehyde with TBA. The TBA reagent and an acid may be added to food directly followed by heating for a sufficient period to obtain maximum colour development. The pink pigment formed may be extracted into butanol or a butanol-pyridine mixture and then quantified (Placer *et al.*, 1966; Uchiyama and Mihara, 1978; Ohkawa *et al.*, 1979). The TBA test may also be carried out on a trichloroacetic acid extract of a foodstuff (Witte *et al.*, 1970; Siu and Draper, 1978; Caldironi and Bazan, 1982). The concentration of the chromogen formed

is then determined spectrophotometrically using a precursor of malonaldehyde such as 1,1,3,3-tetramethoxypropane or its tetraethoxy analogue as a standard. The latter procedure reported by Siu and Draper (1978) often affords more realistic results than other methodologies. Since malonaldehyde is extracted from the foodstuff into the trichloroacetic acid solution before heating with the TBA reagent, the possibility of artifact formation as a result of further oxidation during thermal processing is lessened and therefore prevents the overestimation of TBA values.

The TBA test was once believed to be specific for malonaldehyde (Tarladgis *et al.*, 1960; 1964), but this is not so. A variety of lipid oxidation products, such as aldehydes other than malonaldehyde and dienals (alka-2,4-dienals), may react with the TBA reagent to form a pink-chromogenic adduct with an identical absorption maximum as the TBA-malonaldehyde complex (Marcuse and Johansson, 1973; Kosugi and Kikugawa, 1986; Witz *et al.*, 1986; Kosugi *et al.*, 1987; 1988). Therefore, the term "thiobarbituric acid-reactive substances" (TBARS) is now commonly used in place of TBA number or value (Ke *et al.*, 1984; Gray and Pearson, 1987). Because the TBA reagent is not specific for malonaldehyde alone, certain limitations exist when performing the test for evaluation of the oxidative state of foods and biological systems due to the chemical complexity of these systems. For example, Dugan (1955) reported that sucrose and some constituents of woodsmoke react with the TBA reagent to give a red colour. Baumgartner *et al.* (1975) found that a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532-nm absorbing pigment identical to that

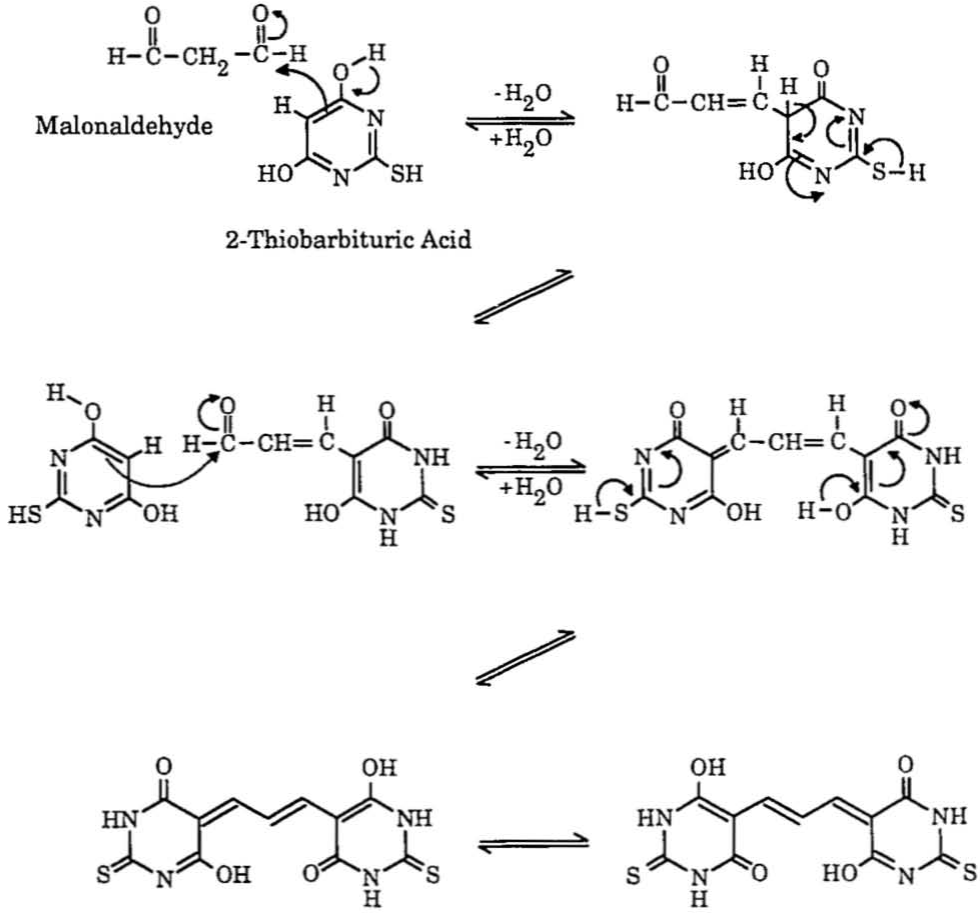
produced by malonaldehyde and TBA. To compensate for these factors, numerous modifications of the original TBA test have been reported in the literature (Marcuse and Johansson, 1973; Ke and Woyewoda, 1979; Robles-Martinez *et al.*, 1982; Pokorný *et al.*, 1985; Tomás and Funes, 1987; and Schmedes and Hølmer, 1989), but this raises a host of new problems. Sample preparation, types of acidulants and their concentration in the reaction mixture, pH of the reaction mixture, composition of the TBA reagent, length of the TBA reaction, and possible use of antioxidants and chelators in the systems are amongst the factors which may influence results reported by researchers from various laboratories. For example, Moerck and Ball (1974) suggested that Tenox II be added to the distillation mixture prior to heating in order to retard further oxidation and subsequently artifact formation during this step, whereas Ke *et al.* (1977) reported the use of propyl gallate (PG) and ethylenediaminetetraacetic acid (EDTA) during distillation for this purpose. Rhee (1978) pointed out that some phenolic antioxidants such as butylated hydroxyanisole (BHA), used to retard further oxidation of samples, may in fact enhance the decomposition of lipid peroxides during distillation. It is always preferable to quantitate the extent of lipid oxidation by a complementary analytical procedure to verify the results.

In addition to chemical reactivity of substances other than malonaldehyde with the TBA reagent, physical properties of the system may interfere with the test. The presence of coloured additives such as the CCMP as well as turbidity of extracts due to solubilized proteins or fat droplets may interfere with accurate determination of the coloured

chromogen(s) of TBARS-TBA by the spectrophotometer. Conversely, a steam-distillation methodology may be used to recover malonaldehyde from the acidified food product. An aliquot of the distillate is reacted with the TBA reagent, and the intensity of the chromophore is again determined spectrophotometrically. Unfortunately, the distillation method generally affords higher values of TBARS due to artifact formation resulting from further breakdown of labile hydroperoxides. Ward (1986) suggested that without knowledge of the exact nature of the TBARS, what TBARS-adduct(s) are formed, the fatty acid profile of the lipids in question, the oxidative pathways taken by components of the lipid system leading to the formation of TBARS, and the relationship of the TBARS to flavour producing molecules, the TBA assay is only of limited value for assessing the extent of oxidation or relating it to the sensory response. Shahidi and Hong (1991*a*,*b*) suggested that the relative, rather than the absolute, values of TBARS should be compared against one another in such determinations.

The pink pigment of the TBA-malonaldehyde reaction was first isolated and characterized by Sinnhuber *et al.* (1958), who showed it to be a condensation product of one molecule of malonaldehyde with two molecules of TBA. Nair and Turner (1984) elucidated the structure of this complex, purified by crystallization, by IR, UV-VIS and NMR methodologies, and showed that it existed in two prominent tautomeric forms. The proposed mechanism for the formation of the complex is illustrated in Figure 2.5.

Figure 2.5 Possible mechanism between malonaldehyde and TBA in the classical TBA test for lipid oxidation. Adapted from Nair and Turner (1984) and Pegg and Shahidi (1991).


Malonaldehyde
2-Thiobarbituric Acid
-H2O
+H2O

2.3.5 Complications Raised by Nitrite in the TBA Test

Nitrite curing inhibits MFD and rancidity development in cooked meats, but determining analytically nitrite's effectiveness as an antioxidant is difficult. Residual nitrite present in cured products interferes with the TBA test (Zipser and Watts, 1962). This interference is believed to be due to the nitrosation of malonaldehyde, which renders all or a portion of it unreactive in the TBA test, thereby resulting in an underestimation of TBARS (Zipser and Watts, 1962; Shahidi *et al.*, 1985*a*; Kolodziejska *et al.*, 1990). For nitrite-cured products, Zipser and Watts (1962) modified the TBA test by adding sulphanilamide prior to the distillation step to scavenge the residual nitrite and to hinder the nitrosation of malonaldehyde. Sulphanilamide reacts with residual nitrite to yield a diazonium salt, and permits malonaldehyde to react quantitatively with the TBA reagent. These authors concluded that sulphanilamide addition allows accurate quantification of malonaldehyde in nitrite-cured meat products within the limits of precision of the TBA test. Shahidi *et al.* (1985*a*) suggested that sulphanilamide itself may give rise to the formation of condensation products with malonaldehyde in the form of a 1-amino-3-iminopropene derivative. Multiple interactions between malonaldehyde with sulphanilamide and TBA have been suggested (Shahidi and Pegg, 1990*b*).

2.3.6 Hexanal Analysis as an Alternative to the TBA Test for the Oxidative Stability of Meat Lipids

An alternative approach for assessing lipid oxidation in meat products is to measure the carbonyl compounds formed upon degradation of fatty acid hydroperoxides.

Carbonyl compounds have been identified as significant contributors to the flavour of uncured meats (Shahidi *et al.*, 1986*b*; Shahidi, 1989*b*, Ramarathnam *et al.*, 1991*a*,*b*). Some have exceptionally strong aromas and can be detected during autoxidation of fatty acids, even if they are present at low concentrations. The concentration of some of these aldehydes has been shown to correlate with MFD. In particular, the concentration of hexanal has been suggested to be a useful primary marker of MFD (Bailey *et al.*, 1980; Dupuy *et al.*, 1987, Shahidi *et al.*, 1987*c*, Shahidi, 1989*b*).

Hexanal is a seemingly ubiquitous component of food, both fresh and stored. This stems from the fact that practically all foods have some linoleate (18:2ω6), the fatty acid from which hexanal is derived. A profile of the fatty acids found in muscle tissue of various animals is presented in Table 2.1.

Linoleate plays a significant role in the oxidized flavour of all meats, especially pork. Initial products of autoxidized linoleate consist predominately of the 9- and 13-hydroperoxides (46.5 and 49.5%, respectively) because the reactivity of the diallylic system favours attack of oxygen at carbon positions 9 and 13 (Belitz and Grosch, 1987). The 9-, 10-, 12- and 13-hydroperoxides at 32, 17, 17 and 34%, respectively, are products of photosensitized oxidation of linoleate (Belitz and Grosch, 1987). These hydroperoxides are unstable, and fragmentation occurs by homolytic and heterolytic cleavage mechanisms (Frankel *et al.*, 1984). Homolytic β-scission of 13-hydroperoxyoctadeca-9,11-dienoic acid produces an alkoxy radical intermediate. This undergoes carbon-carbon splitting forming either pentane and 13-oxo-9,11-tridecadienoic acid, or hexanal and an unsaturated C_{12}

Table 2.1
Unsaturated fatty acid content of lipids in various muscle foods.

Fatty Acid	Content (%)				
	Beef[a]	Chicken[b]	Fish[c]	Lamb[d]	Pork[e]
18:1ω9	33.44	46.02	19.59	19.51	12.78
18:2ω6	10.52	12.55	5.88	18.79	35.08
18:3ω3	1.66	1.86	8.07	0.44	0.33
20:2ω6	0.69	0.34	0.20	0.35	---
20:3ω6	2.77	0.16	0.36	0.62	1.31
20:4ω6	8.51	0.84	3.75	13.01	9.51
20:5ω3	0.76	tr	7.16	---	1.31
22:4ω6	0.88	---	0.65	---	0.98
22:5ω3	0.92	tr	2.39	---	2.30
22:6ω3	---	tr	2.39	---	2.30
Total	60.15	61.77	50.44	52.72	65.90

[a] Adapted from Igene *et al.*, 1980.
[b] Adapted from Onodenalore, 1993. tr -- trace.
[c] Adapted from Mai and Kinsella, 1979.
[d] Adapted from Lazarus *et al.*, 1977.
[e] Adapted from Yamauchi *et al.*, 1980.

fatty acid (Frankel, 1991). Products of the homolytic β-scission of 9-hydroperoxyoctadeca-10,12-dienoic acid include octanoic acid and 2,4-decadienal, or 9-oxo-nonanoic acid and a C_9 unsaturated hydrocarbon (Figure 2.6). Autoxidation of methyl linoleate in model systems has been reported to produce many aldehydes as shown in Table 2.2.

By far, hexanal predominates among these volatile aldehydes, but this is not surprising. Hexanal is the only aldehyde that arises from both the 9- and 13-hydroperoxides of linoleate, and from other unsaturated aldehydes formed during oxidation of linoleate (Schieberle and Grosch, 1981). The production of 2,4-decadienal is always less than that of hexanal because this dienal can only arise through β-scission of 9-hydroperoxyoctadeca-10,12-dienoic acid. In the autoxidized linoleate model system containing both saturated and unsaturated aldehydes, 2,4-decadienal oxidized faster forming hexanal than the saturated aldehydes. Schieberle and Grosch (1981) suggested that attack of free peroxy radicals ($RO_2^{\bullet}$) on the unsaturated moieties of 2,4-decadienal produces peroxyl peroxides which are more labile than the primary hydroperoxides themselves. They decompose readily to hexanal, 2-butene-1,4-dial and other organic compounds. Matthews *et al.* (1971) identified pentane, furan, ethanal, hexanal, acrolein, butenal, 2-heptenal, 2-octenal, benzaldehyde, glyoxal, *trans*-2-butene-1,4-dial, acetic acid, hexanoic acid, 2-octenoic acid, 2,4-decadienoic acid and benzene as the oxidation products of 2,4-decadienal in model systems.

In the late 1970s and early 80s, reports appeared which noted the presence of

Figure 2.6 Autoxidation of linoleic acid and the production of hexanal. Adapted from Frankel *et al.* (1984).

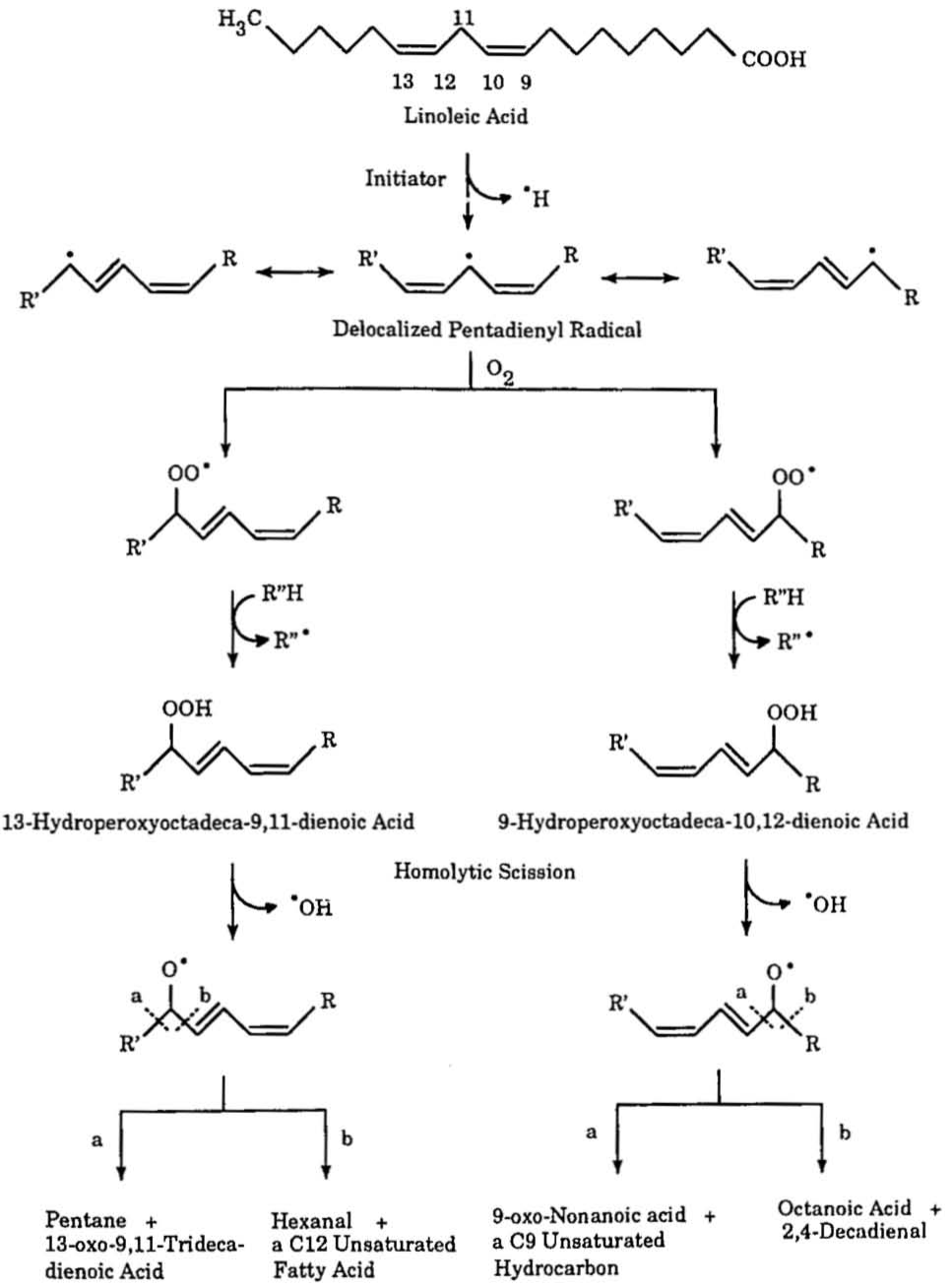

H3C
11
COOH
13 12 10 9
Linoleic Acid
Initiator
•H
R
R'
Delocalized Pentadienyl Radical
O2
OO•
R"H
R"•
OOH
13-Hydroperoxyoctadeca-9,11-dienoic Acid
9-Hydroperoxyoctadeca-10,12-dienoic Acid
Homolytic Scission
•OH
O•
a
b
Pentane + 13-oxo-9,11-Trideca-dienoic Acid
Hexanal + a C12 Unsaturated Fatty Acid
9-oxo-Nonanoic acid + a C9 Unsaturated Hydrocarbon
Octanoic Acid + 2,4-Decadienal

Table 2.2
Volatile dominant aldehydes derived by autoxidation of linoleate[a]

Aldehyde	Quantity[b] $\mu g \cdot g^{-1}$	Odour Threshold Value (ppb)	
		in Water	in Oil
Pentanal	55	10	100
Hexanal	5100	4.5	150
Heptanal	50	30	45
trans-2-Heptenal	450	50	14000
Octanal	45	40	50
cis-2-Octenal	990	--	--
trans-2-Octenal	420	4	7000
cis-3-Nonenal	30	--	--
trans-3-Nonenal	30	--	--
cis-2-Decenal	20	--	--
trans-2,*trans*-4-Nonadienal	30	90	460
trans-2,*cis*-4-Decadienal	250	--	20
trans-2,*trans*-4-Decadienal	150	0.1	200

[a] Adapted from Belitz and Grosch (1987).
[b] One gram of linoleate was autoxidized at 20°C by an uptake of 0.5 mole oxygen per mole of methyl linoleate.

hexanal in cooked muscle foods and its possible role as an indicator of lipid oxidation. Occurrence of hexanal and other aldehydic degradation products from autoxidation of edible oils had already been known for some time (Matthews *et al.*, 1971; Warner *et al.*, 1978; Henderson *et al.*, 1980). Bailey *et al.* (1980) reported the formation of low-molecular-weight aldehydes in cooked roast beef upon storage, and commented that hexanal and 2-pentylfuran were good indicators of lipid autoxidation. They also found that there were little, if any, qualitative differences in the volatiles produced during storage of meat at 4°C over 3 days, but there were quantitative differences.

Dupuy *et al.* (1987) noted that in cooked ground roast beef, pentanal, hexanal, 2,3-octanedione, nonanal and the total volatiles increased appreciably during the storage period at 4°C as did the sensory scores and TBA numbers. Of all of these compounds, hexanal content increased most, from 0.05 to 35 ppm, after 5 days of storage. A similar trend was observed in cooked chicken and turkey meats. In the white and dark muscles of chicken, hexanal levels increased from 0.1 to 15 ppm and from 0.9 to 11 ppm, respectively, during the same period. Similar data were acquired for the white and dark muscles of turkey. The level of hexanal and total volatiles was approximately 3 times greater for cooked beef compared to chicken or turkey after 5 days of storage. It was concluded that since the concentration of hexanal increased more rapidly than any other aldehyde, it should be a useful primary marker of WOF development.

Dupuy *et al.* (1987) also noted that addition of sodium chloride to meats, prior to thermal processing, stimulated the formation of carbonyl compounds during storage,

whereas, the addition of sodium tripolyphosphate (STPP) in the presence of sodium chloride inhibited its formation at the levels tested. Love and Pearson (1976) had previously reported that the addition of STPP, which retards oxidation in meats by its chelating ability, caused a 50% decrease in hexanal production in a model system. Stoick *et al.* (1991) who examined the hexanal levels of cooked, restructured beef steaks reported that STPP reduced hexanal levels to 50% of a sodium chloride control, whereas, addition of the antioxidant, *t*-butylhydroquinone (TBHQ), provided more complete protection by keeping hexanal levels at 3% of the salt-containing control.

The addition of antioxidants to meat systems retards autoxidation and limits production of overtone carbonyl compounds. Barbut *et al.* (1985) showed that addition of rosemary oleoresin or a butylated hydroxyanisole/butylated hydroxytoluene (BHA/BHT) antioxidant mixture to a cooked turkey sausage substantially reduced measurable TBARS as well as the content of oxidatively-derived carbonyls such as pentanal, hexanal, heptanal and 2,3-octanedione. Their results were in agreement with those of Shahidi *et al.* (1987*c*) who demonstrated that hexanal levels in cooked ground pork could be controlled by the addition of various antioxidants and chelating agents. Shahidi *et al.* (1987*c*) further showed that the hexanal content of meats treated with different antioxidants and chelating agents were linearly interrelated with their corresponding TBA values and sensory scores. These authors noted that after storing the cooked pork control sample for 2 days, the TBA numbers were practically identical. It was suggested that the hexanal content would be a better indicator of the oxidative state

of cooked meats than TBA values in the early stages of storage.

Morrissey and Apte (1988) examined the volatile constituents of cooked beef, pork and fish after 2 days of refrigerated storage and the role of haem and non-haem iron in hexanal production. The carbonyl compounds isolated were derivatized with 2,4-dinitrophenylhydrazine, and the resulting hydrazones were separated using reversed-phase high performance liquid chromatography and identified at 360 nm by a UV-VIS detector. Because preliminary studies had indicated that hexanal production continually increased during the early stages of storage of muscle foods, while other volatiles did not show a consistent pattern of increase during the same period, Morrissey and Apte (1988) focused their attention solely on hexanal generation. They ascribed the inconsistencies in the other volatiles to further oxidation or degradation resulting in new compounds. The hexanal concentration in fish muscle after 2 days of storage at 4°C was more than 2 times that of beef and 3 times that of pork. Noteworthy is the fact that these values correlated highly with TBA values. The influence of haem and non-haem iron in the systems showed that their stimulating effect on hexanal production was in the order of Fe^{+2} > Hb > ferritin. Hexanal formation is obviously a function of the lipid profile and the presence of pro-oxidants, antioxidants and chelators in the system.

Ang and Young (1989) investigated the flavour volatiles of cooked chicken during storage by a static headspace-gas chromatography (HS-GC) methodology. They reported that TBA values and hexanal levels increased in cooked chicken patties during a 5-day storage period at 4°C (correlation coefficient 0.95). These authors also observed that

addition of STPP depressed TBARS and hexanal values accordingly. Su *et al.* (1991) showed that in cooked chicken breast patties during 3 days of refrigerated storage significant correlations existed between values of TBARS, hexanal, and other HS volatiles, namely pentanal, heptanal and the total volatiles. These studies suggested that the rapid HS-GC technique may substitute for the TBA test.

Spanier *et al.* (1992*b*) went a step further and reported relationships among GC volatiles, TBARS markers, and descriptive sensory attributes of cooked beef patties. They showed that during a 4 day storage period significant correlations (correlation coefficients > 0.7) existed between pentanal, hexanal and TBARS values and desirable sensory descriptors (such as cooked beefy/brothy) and WOF descriptors (such as painty and card-boardy). Similar correlations have been reported (St. Angelo *et al.*, 1987; 1988; 1990).

2.4 Flavour of Meat

Meat flavour is a complex stimulus involving many sensory properties such as taste, odour, and temperature (Gray *et al.*, 1981). Flavour is an important characteristic which contributes to the acceptability of meat. Although significant advances in understanding the nature of meat aroma have been made in the last 30 years, no single class of compounds or group of factors has been identified as responsible for the flavour perception of meat.

2.4.1 Flavour of Uncured Meat

Raw meat has a slight odour and a blood-like taste, whereas thermal processing

results in creation of the pleasant aroma of cooked, roasted and fried products (Crocker, 1948). The method of cooking employed contributes significantly to the volatile compounds which are formed, and thereby relates to differences in the overall meat flavour sensation (MacLeod and Seyyedian-Ardebili, 1981). Volatile compounds produced during the cooking of meat are believed to be derived from non-volatile precursors, most of which are water-soluble. Flavour appears to be a combination of thermal degradation products of low-molecular-weight precursors which include reducing sugars, vitamins, amino acids, peptides, nucleotides as well as products of browning (Maillard) reaction and fat oxidation (Batzer *et al.*, 1962; Wasserman and Gray, 1965). Upon heat processing, free amino acids in meat such as cysteine, produced from the action of proteolytic enzymes during the post-mortem period, react with reducing sugars, products of glycolysis, and vitamins such as thiamine (Shahidi, 1989*b*). Often products of one reaction become precursors for others. Interaction of these volatiles with lipid-derived products may produce desirable flavours, but the progress of oxidation may mask the natural flavour of heat-processed meats, and it eventually leads to MFD.

Hornstein and Crowe (1960) suggested that meat aroma, derived from water-soluble precursors, was similar in all meat and that the characteristic species differences were due to the contribution of volatiles derived from the lipid fraction. Elimination of the lipid-derived flavours should reveal the natural flavour of meat itself. Fat influences flavour by formation of organoleptically significant amounts of carbonyl compounds (aldehydes and ketones) resulting from the oxidation of unsaturated fatty acids, and by

acting as a depot of fat-soluble compounds that volatilize upon heating. The spectrum of secondary products of lipid oxidation will of course depend on the fatty acid composition of the lipids which also varies from one species to another. One of the main functions of thermal processing is to produce flavour and flavour precursors from lipids, and to allow intimate mixing of fat-soluble and water-soluble compounds (Herz and Chang, 1970).

Volatile flavour compounds have been isolated and identified using gas chromatographic-mass spectrometric techniques. Nearly 1000 compounds have been identified in the volatile constituents of cooked red meats and poultry (reviewed by Shahidi *et al.*, 1986*b*). Chang and Peterson (1977) concluded that aliphatic and aromatic hydrocarbons, saturated alcohols, carboxylic acids, esters, ethers, and carbonyl compounds (aldehydes and ketones) were probably not the main contributors to meat flavour. Rather, lactones, acyclic sulphur-containing compounds (mercaptan and sulphides), non-aromatic heterocyclic compounds containing either sulphur, nitrogen or oxygen (*e.g.* hydrofuranoids) and aromatic heterocyclic compounds containing either sulphur, nitrogen or oxygen (pyrazines and thiophenes) possessed characteristic meaty flavour notes.

2.4.2 Flavour of Nitrite-Cured Meat

Nitrite is responsible for the production of the characteristic flavour of cured meat (*i.e.* a flavour that distinguishes cooked ham from pork). The role of nitrite in cured meat flavour is complex and the chemical changes that are responsible for this unique flavour brought about in meat are not entirely understood (Shahidi, 1989*b*). Cured meat flavour

is probably a composite sensation derived from contributions of many odoriferous compounds (National Academy of Sciences, 1982). Research into cured meat flavour has been divided into two main areas, namely the sensory evaluation of flavour imparted to meat by nitrite, and the qualitative and quantitative identification of volatile and non-volatile components responsible for it, but caution must be exercised. A compound-by-compound search of meat flavour volatiles may miss-identifying the true nature of cured meat flavour since a mixture of two or more odours can produce an aroma that is perceived as qualitatively distinct from the odours of their components.

The relationship of nitrite to cured meat flavour was first described by Brooks *et al.* (1940) who concluded that the characteristic flavour of bacon was primarily due to the action of nitrite. They further suggested that a satisfactory bacon product could be produced using only sodium chloride and sodium nitrite and that an adequate cured flavour could be obtained with a nitrite concentration as low as 10 ppm. Mottram and Rhodes (1974) studied the effect of varying nitrite concentrations in bacon by sensory analyses. Brines containing 20% (w/v) sodium chloride and sodium nitrite at concentrations ranging between 0 and 2000 $mg \cdot L^{-1}$ were used to cure pork middles. Sensory data showed that a significant difference ($P<0.01$) existed between the flavour of uncured and cured pork. Sensory studies with similar findings have been reported (Cho and Bratzler, 1970; Herring, 1973; Simon *et al.*, 1973). MacDougall *et al.* (1975) showed that taste panel scores of bacon flavour were linearly related to the logarithm of the nitrite concentration in the brines, but the level of nitrite required for a satisfactory

flavour varied between products depending on the nature of the meat. MacDonald *et al.* (1980*c*) demonstrated through sensory evaluation studies that 50 ppm of nitrite were required to develop a significant ($P<0.05$) cured-meat flavour as opposed to 10 ppm proposed by Brooks *et al.* (1940). In addition to nitrite, the effect and concentration of other curing ingredients, namely salt, sugar, polyphosphates and smoke, plays an over-riding role in the appreciation of cured meat flavour (MacDougall *et al.*, 1975) as does the holding time, temperature, and storage conditions employed (Kemp *et al.*, 1975).

Nitrite's role in the development of cured meat flavour involves its antioxidative activity which, as described previously, retards the breakdown of unsaturated fatty acids and the formation of secondary oxidation products. Numerous researchers have attempted to identify the volatile compounds produced during the thermal processing of cured meat (Ockerman *et al.*, 1964; Cross and Ziegler, 1965; Lillard and Ayres, 1969; Mottram and Rhodes, 1974; Ho *et al.*, 1983). Ockerman *et al.* (1964) extracted volatile compounds from dry-cured hams by vacuum distillation and cold trap collection. The main components identified by GC retention times and verified by IR spectroscopy included 6 aldehydes, 3 ketones, 5 acids, the bases of ammonia and methylamine, and hydrogen sulphide, but all compounds identified are contributors to the aroma of uncured cooked meat. Ho *et al.* (1983) isolated volatile aroma compounds from fried bacon and subjected them to extensive gas-chromatographic fractionation, thus, enabling the pure fractions obtained to be identified by IR spectroscopy and mass spectrometry. In all, 135 compounds were identified, and included hydrocarbons, alcohols, ketones, furans,

pyrazines, and sulphur- and nitrogen-containing heterocycles. These authors postulated that some compounds identified in their study which had not been detected in other investigations of cooked cured-meat volatiles were possibly due to the smoking and frying of bacon. An extensive listing of volatile compounds in cured pork has been reported (Shahidi *et al.*, 1986*b*; Ramarathnam *et al.*, 1991*a*).

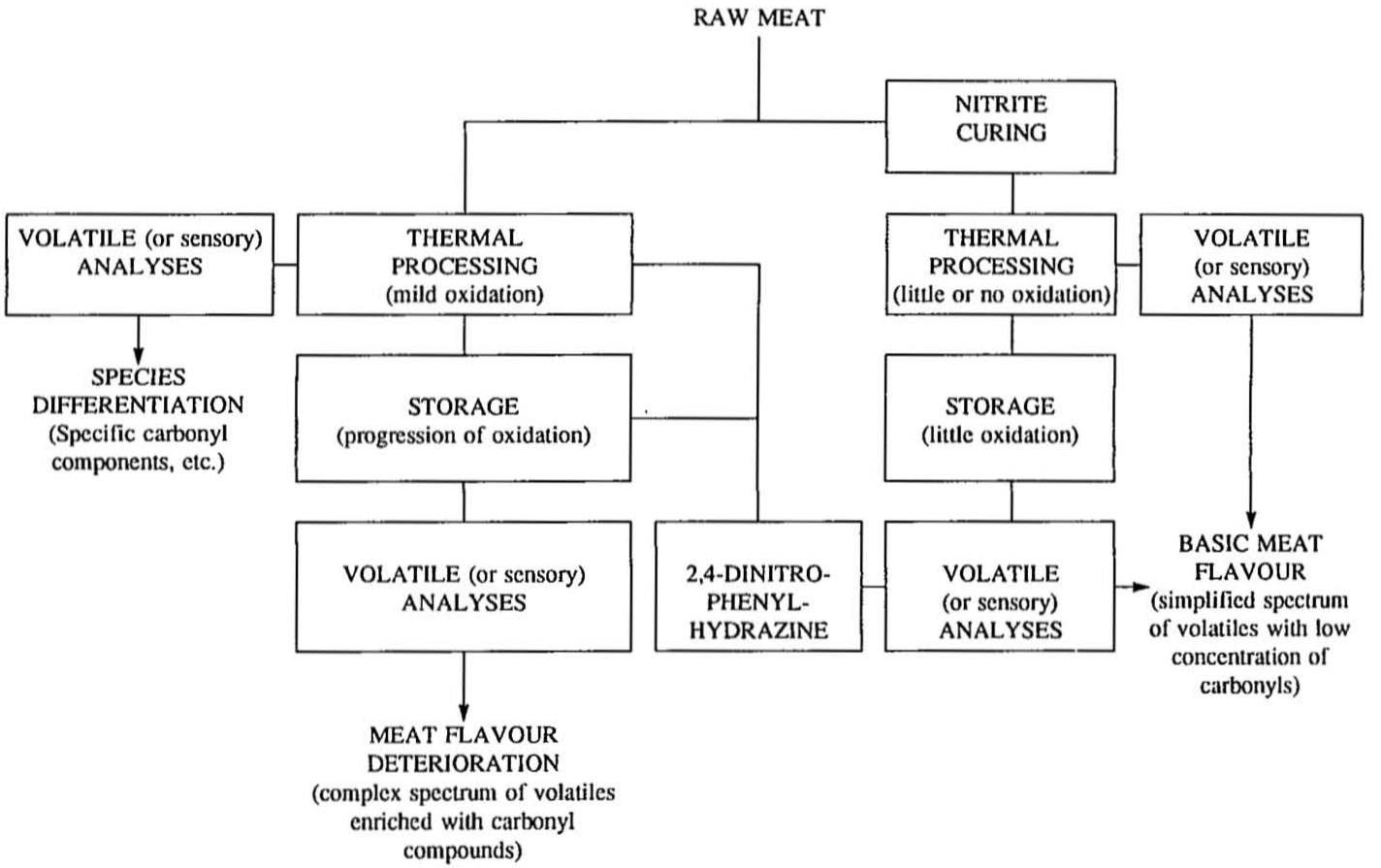
Cross and Ziegler (1965) examined the volatile constituents isolated from uncured and cured hams by a GC methodology. Qualitatively, the volatile compounds of cured ham were similar to uncured samples, but were quantitatively different. They reported that hexanal and pentanal were present in appreciable amounts in the volatiles of uncured, but were barely detectable in the volatiles of cured ham. Swain (1972) concurred with this finding and reported that nitrite appeared to retard the formation of higher molecular-weight aldehydes (*i.e.* > C_5). Cross and Ziegler (1965) also noted that the volatiles, after passage through a solution of 2,4-dinitrophenylhydrazine, had the characteristic cured-ham aroma, regardless of whether cured or uncured hams were used. Cured and uncured chicken and beef volatiles, after stripping their carbonyl compounds by passage through 2,4-dinitrophenylhydrazine solutions, also possessed an aroma similar to that of cured ham. Cross and Ziegler (1965) concluded that treating meat with nitrite does not seem to contribute any new volatile compounds to the flavour of cooked meats, with the exception of nitrogen oxides that are not present in cooked uncured meat. Therefore, they postulated that cured-ham aroma represents the basic flavour of meat derived from precursors other than triacylglycerols, and that the aromas of various types of cooked

meat depend on the spectrum of carbonyl compounds derived by lipid oxidation.

Shahidi (1989*b*) reported that the elimination of lipid oxidation, either by curing or by stripping of carbonyl compounds from volatiles of untreated cooked meats, caused a major effect on the flavour perception of meats, but this author noted that qualitative differences due to the possible presence of less active flavour components can not be ruled out. Nonetheless, GC analyses of the volatiles of cured meat revealed a much simpler spectrum than their uncured counterparts, with drastic suppression in the content of major aldehydes, such as hexanal and pentanal, which are known to be responsible for MFD. Shahidi (1989*b*) proposed that any agent, or combination of agents that prevents lipid oxidation, with the exception of nitrite precursors, would in principal, duplicate the antioxidant role of nitrite in the curing process, thereby preventing hexanal generation and MFD. According to Shahidi (1992), this is in line with findings of other researchers and its validity was confirmed by preliminary sensory evaluations, but mutton was not included in these studies.

A simplistic view, attempting to present a unifying theory of the origin of the basic flavour of meat, species differentiation, and MFD is provided in Figure 2.7. It postulates that meat when cooked acquires its characteristic species flavour which is caused by volatile carbonyl compounds, such as hexanal and pentanal, formed by oxidation of its lipid components (*i.e.* primarily phospholipids). Further oxidation during storage of cooked meat results in the deterioration of its flavour. Curing with nitrite suppresses the formation of oxidation products. It may be assumed that the flavour of

Figure 2.7 Consequence of cooking, curing, and storage on flavour of cooked meats and development of meat flavour deterioration. Adapted from Shahidi (1992).


RAW MEAT
NITRITE CURING
VOLATILE (or sensory) ANALYSES
THERMAL PROCESSING (mild oxidation)
THERMAL PROCESSING (little or no oxidation)
VOLATILE (or sensory) ANALYSES
SPECIES DIFFERENTIATION (Specific carbonyl components, etc.)
STORAGE (progression of oxidation)
STORAGE (little oxidation)
VOLATILE (or sensory) ANALYSES
2,4-DINITRO-PHENYL-HYDRAZINE
VOLATILE (or sensory) ANALYSES
BASIC MEAT FLAVOUR (simplified spectrum of volatiles with low concentration of carbonyls)
MEAT FLAVOUR DETERIORATION (complex spectrum of volatiles enriched with carbonyl compounds)

nitrite-cured meats is actually the basic natural flavour of meat from different species without being influenced by overtone carbonyls derived from oxidation of their lipid components. Further support for this view has recently been provided by Ramarathnam *et al.* (1991*a*,*b*; 1993*a*,*b*), but the postule does not easily explain the fact that the intensity of cured meat flavour is proportional to the logarithm of nitrite concentrations as reported by MacDougall *et al.* (1975), or the apparent persistence of the characteristic "mutton" flavour after nitrite curing of sheep meat (Reid *et al.*, 1993).

2.5 Microbial Protection of Cooked Meat

2.5.1 *Clostridium botulinum* in Meats

A wide variety of cooked meat products is available in the market, but care must be exercised during their preparation to avoid botulism. Because *Clostridium botulinum* spores are widely distributed, they may find their way into processed meats through raw food materials or by contamination of the meats after processing. Unless processors and consumers take preventive measures to eliminate *C. botulinum* or to inhibit growth and toxin production by this organism, botulism outbreaks will occur.

The causative organism was first isolated in 1896 by van Ermengen from a salted ham which had caused several human fatalities (Pierson and Reddy, 1988). The isolated organism was a gram-positive, spore-forming, anaerobic bacillus which produced a heat-labile toxin that was lethal to a variety of animals. van Ermengen named the organism *Bacillus botulinus* but in 1923 it was renamed to *Clostridium botulinum*. To date, there

are seven recognized serotypes of *C. botulinum* (A, B, C, D, E, F and G), but only serotypes A, B, E and F are involved in human botulism (Pierson and Reddy, 1988). There are marked differences among the strains in their tolerance to sodium chloride and water activity, minimum growth temperature, proteolytic activity, and in the heat resistance of their spores. Types A and B are of most concern to the food processor since both form extremely heat-resistant spores.

Foodborne botulism results from consumption of food in which *C. botulinum* has grown and produced toxin. The botulinal neurotoxins are proteins which are produced intracellularly as protoxins. They are liberated when the botulinum vegetative cell lyses, and they are activated to the maximum toxic state by proteolytic enzymes. Serotype A toxin is more lethal than strains B and E. The toxin is absorbed and bound irreversibly to peripheral nerve endings. Signs and symptoms of botulism poisoning, which include nausea, vomiting, fatigue, dizziness, headache, dryness of skin, mouth and throat, constipation, paralysis of muscles, double vision, and difficulty in breathing, develop within 12-72 hours after consumption of the toxin-containing food. Treatment of the poisoning includes administration of botulinal antitoxin and appropriate supportive care, particularly respiratory assistance. Recovery may take several weeks to a month if death does not occur, but today the mortality rate is less than 10% (Pierson and Reddy, 1988).

Conditions that favour growth and toxin production by *C. botulinum* include a relatively high-moisture, low-salt, low-acid (*i.e.* pH > 4.6) food that is devoid of oxygen and stored at temperatures in excess of 3.3°C. Meat provides an adequate medium with

nutrients for the growth of *C. botulinum* and toxin production. The excellent safety record of cured meats has been largely attributed to the use of nitrite as a curing ingredient. Many studies have been published on the efficacy of sodium nitrite in inhibiting *C. botulinum* growth and toxin production in perishable cured meats such as wieners, bacon, canned ham, luncheon meat, and canned comminuted meats. Safety cannot be totally attributed to nitrite alone, but rather to a variety of factors, such as heat treatment, acidity (pH), salt and bacterial spore levels. Other curing adjuncts, such as ascorbic acid and sodium erythorbate, have been reported to influence the efficacy of nitrite.

2.5.2 Bacteriostatic Properties of Nitrite

It was during the 1920s that investigation of the antibacterial properties of nitrite commenced. It was first observed by MacNeal and Kerr (1929) that nitrite was more inhibitory under acid conditions, and he hypothesized that this may be due to the presence of nitrous acid. Tanner and Evans (1933; 1934) studied the effect of meat curing solutions on anaerobic bacteria. Tarr (1941; 1942) reported the bacteriostatic action of sodium nitrite at a concentration of 200 ppm in fish muscle against *Achromobacter*, *Aerobacter*, *Escherichia*, *Micrococcus* and *Pseudomanas* in bacteriological media at a pH of 6.0. Many investigations indicate that not all bacteria are affected in the same way by nitrite, and that some may be more resistant to nitrite than clostridia. The initial contamination level or incidence of *C. botulinum* spores in raw meats to be cured can greatly affect the control of *C. botulinum* growth and toxin formation in the finished

product. In general, it is considered that the incidence of *C. botulinum* spores is low in raw meats, but some concern has been shown with respect to spore incidence in bacon products following studies such as those by Roberts and Smart (1976).

Perigo *et al.* (1967) reported that nitrite heated in bacteriological media was more inhibitory towards vegetative cells of P.A. 3679 than nitrite added aseptically after the medium had been autoclaved. This effect was found to occur in the temperature range of 95 to 125°C at pH values greater than 6.0, but at pH 6.0, heating in the range to 100 to 110°C enhanced this inhibitory effect tenfold or greater. This inhibitory substance has since become known as the Perigo factor or Perigo-like factor. Roberts (1975) confirmed these findings and showed the enhanced inhibitory effect of nitrite against the vegetative cells of 30 clostridia strains including 14 strains of *C. botulinum* serotypes A, B, E and F in laboratory media after heat treatment. Roberts' studies also indicated that a reducing agent and protein source were necessary components of the laboratory media in order to observe the Perigo effect. Present evidence strongly suggests that while there is an inhibitor formed in meats following the addition of nitrite, this inhibitor, which is not nitrite itself, is significantly different from the Perigo inhibitor formed in laboratory media (Holley, 1981). Meat does not reach 105°C during thermal processing which is the minimum temperature reported for Perigo inhibitor formation. Lee *et al.* (1978) reported that while it is evident that the classic Perigo inhibitor is absent from meat systems, a Perigo-type inhibitor not requiring sulphydryl groups for activity was present in meats.

Although the inhibition of *C. botulinum* spores and those of other clostridia species

by nitrite has been extensively studied, the exact mechanism by which nitrite exerts this action remains elusive. Johnston *et al.* (1969) have suggested that nitrite's mode of action in cured meats might be an enhanced destruction of spores by heat, an increased germination of spores during the heat treatment followed by thermal destruction of the germinated spores, an inhibited germination and outgrowth of spores surviving the heat process, or a reaction with some components in the meat system to produce a more inhibitory compound. In 1980, Yarbrough *et al.* proposed that nitrite has several sites of attack in the bacterial cell. Evidence suggests that nitrite delays, but does not entirely prevent, clostridial outgrowth. Although nitrite does not inhibit spore germination, its inhibitory effect can be seen upon emergence of the vegetative cell from the spore during cell division (Tompkin, 1978; Genigeorgis and Riemann, 1979; Sofos *et al.*, 1979*b*). Inhibition of energy dependent transport systems within the cell results from the presence of the nitrite anion, but growth inhibition is believed to be caused by undissociated nitrous acid (Freese *et al.*, 1973). It has also been proposed that nitrite inhibits *C. botulinum* outgrowth through a reaction with an iron containing compound, such as ferredoxin, thereby interfering with energy metabolism within the germinating spore (Tompkin, 1978). Tompkin *et al.* (1978) showed that erythorbate, ascorbate and cysteine enhanced the anticlostridial efficacy of nitrite in cured meats by sequestering metal ions in the meat rather than by an antioxidative or reductive mechanism. It was suggested that an essential metabolic step involving a cation is blocked by the reaction of nitric oxide within the vegetative cell. Eventual outgrowth could be dependent upon depletion of nitrite or nitric

oxide to nontoxic levels and repair of nitric oxide damaged material within the vegetative cell. Benedict (1980) in his review of the biochemical basis for nitrite's inhibition of *C. botulinum* reported that in cured meats it was most likely due to several interacting factors, namely (1) reaction and oxidation of cellular biochemicals within the spores and vegetative cells; (2) restriction of the use of iron, or other essential metal ions, through inhibition of solubilization, transport or assimilation, thereby interfering with metabolism and repair mechanisms; and (3) cell surface membrane activity limiting substrate transport by the outgrowing cell.

It is recognized that the safety of cured meats such as wieners, bacon, canned ham and luncheon meats cannot be totally attributed to nitrite alone. A variety of factors such as heat treatment, acidity, reductants, salt concentration, storage temperature, bacterial spore level and their interactions with nitrite provide the safety from botulism afforded cured meats. The thermal processes used on cured meats are sufficient to inactive vegetative bacterial cells with the occasional exception of the relatively heat resistant enterococci (Roberts, 1975). Processors are most concerned, however, with residual *C. botulinum* spores in cured meats after thermal processing and the effect of nitrite on outgrowth of the bacteria when the meats are subjected to temperature abuse. If refrigerated storage could be assured there would be no potential botulinal hazard in bacon, sausage, wieners and luncheon meat products.

It has been observed and generally accepted that the effect of nitrite on *C. botulinum* growth and toxin production increases as the concentration of nitrite does.

Numerous studies utilizing several inoculum levels of *C. botulinum* spores in a variety of cured meat products have demonstrated that as the spore concentration is increased, the inhibitory effects of nitrite and other curing adjuncts can eventually be overcome, thus allowing *C. botulinum* growth and toxin production. Greenberg (1972) studied the relationship between nitrite concentrations and spore levels added to hams which were canned and then thermal processed. The author reported that clostridial outgrowth occurred in hams treated with 150 ppm of nitrite when 100 clostridial spores/g were present, but not when a 200 ppm nitrite concentration was used for the same microbial load. As the microbial contamination increased to 10 000 spores/g, botulinal toxin was detected in all products containing 400 ppm of nitrite or less. Christiansen (1980) postulated that the nitrite level present at the time of temperature abuse is an important factor in determining outgrowth of *C. botulinum*. Christiansen (1980) also stated that clostridial spores readily germinate in the presence of nitrite, but nitrite inhibits by preventing the outgrowth of germinated spores. Because nitrite levels decrease during storage of meats, bacterial growth occurs when there is an insufficient concentration of nitrite to check outgrowth. In other words, the extent of nitrite inhibition of *C. botulinum* can be explained as being a race between nitrite depletion and death of germinated clostridial spores (Christiansen, 1980). Factors such as pH and ascorbate concentration affect inhibition because they influence the rate of nitrite depletion. Chelating agents such as ascorbate, EDTA and cysteine can also enhance the efficacy of nitrite by binding ferrous and ferric ions.

2.6 Physical Application of Cures to Meat and Meat Products

Addition of curing ingredients to comminuted meat products is very simple and generally involves standard mechanical devices to achieve uniform distribution of the cure. In frankfurter manufacturing, the cure is further homogenized in the product by the emulsifiers used. The introduction and distribution of curing ingredients to solid cuts of meat is more complex. Treatment of these meats involves either dry curing, brine/pickle curing or stitch pumping/multiple injections followed by tumbling or massaging, as has been previously outlined. All these methods utilize the same ingredients, but, unlike the rest, dry curing does not use aqueous solutions. The extent and rate of penetration of the curing ingredients into muscle tissue therefore depend on the technique employed, and are important factors in the preservation process.

Penetration of the cure in meats is a mass transfer. A component in a mixture migrates in the same phase, or from one phase to another, due to a difference in concentration. There are two types of mass transfer, namely molecular diffusion and eddy diffusion. Molecular diffusion involves the transfer of mass in stagnant fluids or fluids in laminar flow due to a gradient from an area of high concentration to one of low. On the other hand, eddy diffusion involves the transfer of mass by the motion or mixing of finite parcels of fluid (Bennett and Myers, 1982). The rate of this turbulent diffusion is quite fast in comparison to that of molecular diffusion, but for this discussion, only molecular diffusion will be considered.

The driving force of molecular diffusion is the difference between the

concentration of a species at the phase boundary (e.g. a solid surface or a fluid interface) and the concentration at some arbitrarily defined point in the fluid medium. The rate of mass transfer for molecular diffusion is governed by Fick's first law,

$$J_z = -D_z \, dC_z/dz$$

where J_z is the molar flux of component A in the z direction due to molecular diffusion (kg mol $A \cdot s^{-1} \cdot m^{-2}$), D_z is the molecular diffusivity of molecule A in phase B ($m^2 \cdot s^{-1}$), C_z is the concentration of A (kg mol $\cdot m^{-3}$) in the z direction and z is the direction of diffusion (m).

Wilson (1960) observed that the rate of curing solid cuts of meat depended on the rate of diffusion of curing ingredients into the tissue. In turn, this rate depended upon several factors including the manner in which the cure was applied, the size of the meat cut, and the amount of fat covering it. The author stated that penetration of the cure could be greatly facilitated by an increase in temperature during processing, but also mentioned the danger of bacterial proliferation and spoilage as a consequence of using higher temperatures.

Wood (1966) concluded that the preservation of meat by curing depended on the amount of salt reaching all parts of the meat, including the fatty tissues and the bones. The author stated that the rate of diffusion in a given tissue was largely governed by the concentration of the solution ingredients and curing temperature. Wistreich *et al.* (1959; 1960) had previously investigated the effect of temperature on the accumulation value of sodium chloride in pork muscles, and noted that Fick's law could not be rigorously

applied to diffusion of sodium chloride into muscle tissue because the tissue did not behave in the same manner as a simple solvent. They defined accumulation value as the amount of sodium chloride that diffused from the solution into the muscle through one square centimetre of contact area. The results of their experiment showed that accumulation of salt in pork muscle varied linearly with solution concentration, but the accumulation value-time relationship was logarithmic.

Fox (1980) undertook a study to investigate the rate of diffusion of sodium chloride, nitrite and nitrate in both beef and pork using a porous disc technique to determine the effect of salt-salt and salt-tissue component interactions. The author realized that if more than one solute was present in the cure, interactions may occur whose effects would be difficult or impossible to separate. To further complicate the issue, chemical reactions in the tissue would take place such as nitrite's reaction with components of the meat (Fox and Nicholas, 1974). The effects of curing ingredients were studied by a steady-state diffusion system in which a concentration gradient was established across a membrane and the diffusion of one solute was observed by varying its concentration in a series of experiments while keeping the concentration of other solutes constant. Fox (1980) reported that diffusion constants for chloride and nitrite in pork (0.21×10^{-5} and 0.13×10^{-5} $cm^2 \cdot s^{-1}$, respectively) were lower than corresponding values for beef (0.26×10^{-5} and 0.20×10^{-5} $cm^2 \cdot s^{-1}$, respectively), and that no difference in the rate of chloride diffusion was observed in the different muscles of pork.

The diffusion characteristics of small solute molecules (*i.e.* curing salts and CCMP

in the pickle) and macromolecules (*i.e.* proteins of meat) in multicomponent aqueous solutions are complex and difficult to model. Interactions of large molecules with small solvent or solute molecules affect the diffusion of the macromolecules themselves as well as small solute molecules, but the rates of diffusion of curing salts in meats are important because they ultimately determine the length of time required for processing and the uniformity of cure distribution (Fox, 1980).

O'Boyle *et al.* (1992) examined the distribution of the preformed CCMP as part of a composite nitrite-free curing system in hams. They reported that unlike nitrite, the CCMP is a relatively large molecule with a molecular weight approximately 10 times that of nitrite, and is sparingly soluble in water or curing pickle. These authors suggested that CCMP does not readily diffuse through an intact matrix of muscle fibres, but this was never verified. They also postulated that the collagenous coating around bundles of muscle fibres offered resistance to pigment transport, and suggested that injection of a curing pickle containing CCMP at uniform distances, using a 4-hole radial injection needle followed by tumbling, relaxation and a refrigeration period, would afford a visually appealing colour to meats. These authors reported that the interior colour of treated inside muscle (*Semimembranosus*, including the adductor), outside muscle (*Biceps femoris*) and knuckle (*Quadriceps femoris*) muscles from hogs, which had been manually deboned, was a strong, bright pink, with no uncoloured regions after cooking. There were however some very fine, barely noticeable lines of higher pigment concentration parallel to muscle fibre bundles.

2.7 The Fate of Nitrite

Nitrite is a very reactive anion and when added to meat well-recognized changes in the colour, flavour and shelf-life of meat occur as discussed above. Added nitrite reacts with proteins, lipids, pigments and other constituents of meat, but a portion of it remains unreacted in the free form (*i.e.* NO_2^- and HNO_2). This unreacted nitrite is often referred to as residual nitrite, whereas nitrite which has reacted with constituents of the meat matrix is referred to as bound nitrite. There is general agreement that most of the nitrite in meat exists in a form other than the nitrite anion (Cassens *et al.*, 1977). Nitric oxide bound to Mb to form the CCMP is one such example. The concentration of residual nitrite in meat depends on factors such as the type of muscle, pH and temperature of the system (Olsman and Krol, 1972). During storage, a decrease in its concentration takes place and by the time meat and meat products reach market, they contain only 5-30 ppm residual nitrite (Cassens *et al.*, 1979). A great deal of work on the fate of nitrite in meat has been carried out. For example, studies have shown that only a small fraction of the nitrite added to meat is, on occasion, detected as volatile N-nitrosamines.

Fox and Nicholas (1974) examined the fate of nitrite in meat, and found that the disappearance of nitrite was related to the production of NO. Fujimaki *et al.* (1975) investigated the fate of nitrite in model systems containing various ratios of Mb, nitrite and sodium ascorbate. These systems were stored at 4°C to mimic the curing and then heated at 70-80°C to mimic the thermal processing. Fujimaki *et al.* (1975) found that they could account for all the added nitrite as the sum of residual nitrite, nitrate, nitrosyl

groups, NOMb or gaseous nitrogen compounds. In meat systems, however, the fate of all added nitrite was more difficult to account. Emi-Miwa *et al.* (1976) reported that when ascorbate was added to a nitrite-cured meat system, a larger proportion of the added nitrite could not be accounted for as compared to a counterpart system devoid of ascorbate. By comparing the meat system to a Mb/ascorbate/nitrite model system, Emi-Miwa *et al.* (1976) found that larger quantities of gaseous nitrogen compounds were evolved. The meat was then divided into four fractions and the reaction of nitrite with each fraction was examined. Most of the unidentifiable nitrogen compounds were produced by reaction with a fraction containing small molecular-weight compounds. Miwa *et al.* (1980) found that a subfraction obtained by acid extraction could bring about conversion of some 30% of the added nitrite to compounds they could not identify. The authors proposed that these were produced by the action of a number of different components in meat.

Cassens *et al.* (1977) used labelled ^{15}N-nitrite to identify several components of cured meats that reacted with nitrite. They were able to recover 70-80% of the added nitrite nitrogen, and found that it was distributed as follows: 5-15% in Mb, 1-10% in nitrate, 5-20% in nitrite, 1-5% in volatiles, 5-15% bound to sulphydryl groups, 1-5% bound to lipid and 20-30% bound to protein. Their results showed that the amount of nitrite nitrogen bound to sulphydryl groups was low and that the majority of nitrite nitrogen was bound to non-haem proteins. Reaction with adipose tissue, connective tissue and unsaturated fatty acids also occurred. An excellent review on this topic is provided

by Woods *et al.* (1989).

2.8 Potential Health Hazards of Nitrite

In the late 1960s and early 70s, nitrite usage in cured meats became the source of some very serious concerns. Despite all of its desirable effects, nitrite is the culprit in the formation of N-nitrosamines in some cooked cured products. N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR), examples of such reaction products, were found to be carcinogenic, mutagenic and teratogenic in experimental animals (Magee and Barnes, 1967; Gray and Randall, 1979; Newberne, 1979; Preussmann and Stewart, 1984). N-Nitrosamines are formed by the reaction of naturally-occurring secondary amines and some amino acids in meat with added nitrite. Early work by Mirvish (1970) revealed that rate of N-nitrosamine formation in meat was first order with respect to amine concentration and second order with respect to residual nitrite concentration. The concern over N-nitrosamines has led to technological changes in the meat processing industry. These include the elimination of nitrate from most curing applications to allow more complete control of curing reactions, the reduction of nitrite addition levels, particulary for bacon, and the incorporation of N-nitrosamine-blocking agents such as sodium ascorbate or its isomer, erythorbate, in cures. An excellent review on the history of the N-nitrosamine question and industry's response is presented by Cassens (1990).

Various studies have confirmed the presence of volatile N-nitrosamines in cured meats, but there appear to be discrepancies as to both the qualitative and quantitative

nature of findings reported in the literature. Many factors such as mode of cooking, cooking temperature and time, nitrite concentration, salt concentration, pH and the presence and concentration of ascorbate affect the potential for N-nitrosamine formation (Sen *et al.*, 1979; 1985). Bacon has received the most attention with regard to N-nitrosamine formation. Trace levels (*ca.* 1 ppb) of NDMA are occasionally detected in cured meat products whereas NPYR is consistently detected in fried bacon at levels up to 20 ppb (Gough *et al.*, 1976). Although the origin of NPYR has not been conclusively established, its formation is dependent on frying temperature. Decarboxylation of N-nitrosoproline formed by the reaction of proline with nitrite has been proposed as the major route of NPYR formation.

Unlike volatile N-nitrosamines, presence of non-volatile N-nitroso compounds has not been widely reported, perhaps because their non-volatile character does not facilitate their isolation from foodstuffs. An assessment of the overall concentration of all N-nitroso compounds (*i.e.* volatile and non-volatile) in cured meats was obtained using a chemical denitrosation/chemiluminescence detection procedure described by Walters *et al.* (1978). This procedure provides no information on the levels of individual N-nitrosamines and may be subject to interference from some non-nitroso compounds, but the technique showed that the concentration of total N-nitroso compounds in cured meats is in the range of 0.5-5.0 ppm (Massey *et al.*, 1986). Comparison of these levels with those of volatile N-nitrosamines and N-nitrosoacids suggests that a large majority of N-nitroso compounds in cured meats are of unknown identity. Over 90% of the more than

300 nitroso compounds which have been tested in laboratory animals caused cancer (Preussmann and Steward, 1984), but no known case of human cancer has been shown to result from exposure to N-nitroso compounds. Much indirect evidence suggests that humans would be susceptible, and Doll and Peto (1981) have estimated that 35% of all cancer in humans is of dietary origin.

Nitrite is not permitted as an additive in the curing of fish in Canada because fish generally contains more dimethylamine (DMA) than meat and concerns regarding the formation of NDMA are therefore warranted (Canadian Food and Drug Regulations, 1981). Of particular concern is the enzymatic breakdown of trimethylamine N-oxide (TMAO) to formaldehyde and DMA in commercially important gadoid fish, such as cod, haddock, hake, pollock, and whiting (Pensabene and Fiddler, 1988). In the USA, the USDA Food Safety and Inspection Service (FSIS) has been petitioned to amend the standard of identity for cooked sausage to permit inclusion of up to 15% fish protein, in the form of surimi or unwashed or washed minced fish, with red meat or poultry in a variety of processed products such as frankfurters, bolognas, and salamis. This would not only make use of underutilized fish protein, but it also has the potential to increase the nutritional and sensory quality of formulated products (Pensabene *et al.*, 1991). The FSIS raised concerns over the possible presence of N-nitrosamines, particulary NDMA, in cured fish-meat products. Brooker (1985) reported that higher levels of NDMA were found in fish-meat mixtures compared to all-meat (control) frankfurters, but Pensabene and Fiddler (1988) questioned the possibility of artifactual NDMA formation in this study as a result

of the method of analysis employed. Pensabene *et al.* (1991) reported that the content of N-nitrosothiazolidine-4-carboxylic acid (NTHZC) and N-nitrosothiazolidine (NTHZ) in Alaska pollock surimi-meat frankfurters was similar to or lower than those found in an all-meat control, even at 50% substitution. N-Nitrosothiazolidine-4-carboxylic acid and NTHZ are reaction products between formaldehyde and nitrosylated cysteine or its decarboxylated derivative, nitrosylated cystamine respectively. Occurrence of NTHZC and NTHZ in smoked, cured, all-meat products has also been reported (Sen *et al.*, 1986; Fiddler *et al.*, 1989). Cuppet *et al.* (1989) added nitrite to smoked Great Lakes whitefish as a means of preservation. Although nitrite curing of fish is a cause for concern, the formation of N-nitrosamines in fish products has been shown to occur primarily in salt-water species which contain higher levels of trimethylamine and TMAO which are readily degraded to formaldehyde and DMA (Sikorski and Kostuch, 1982). Nitrite-cured whitefish samples were analyzed for volatile N-nitrosamines and for NTHZC and NTHZ because these compounds have been associated with smoked foods. No detectable levels of N-nitrosamines were found in any of the samples tested (Cuppet *et al.*, 1989).

Due to the importance of cured products in our diet, without nitrite a large class of well-loved muscle foods would be eliminated. It is therefore prudent to develop alternatives to nitrite in the curing of meat and fish products.

2.8.1 Meat Industry and Regulations

Despite the concern regarding N-nitrosamine formation, the meat industry is committed to the use of nitrite in cured meat products as there is no suitable alternative.

The long term goal of the Canadian government is to phase out the use of nitrite if safe and effective alternatives become available (Pim, 1978), and in the meantime the increased awareness of the dangers of nitrite has prompted changes in the Canadian Food and Drug regulations for nitrite and nitrate usage in preserved meats. This had not been totally unexpected since N-nitrosamine formation in meat is directly proportional to the square of the residual nitrite concentration (Mirvish, 1970). In 1975, the Health Protection Branch (HPB) of Health Canada reduced the permissable levels of sodium nitrite in bacon, before processing, from 200 ppm to 150 ppm, but many processors opted to use an even lower addition level of 120 ppm. The HPB also eliminated the addition of nitrate salts to curing formulations from bacon at this time (Sen *et al.*, 1977). Similar regulatory changes for nitrite were adopted in the USA by the USDA. These regulatory changes have resulted in a considerable decrease in the NPYR levels in bacon. Reported mean values of NPYR in fried bacon were 63 ppb in 1973 (Fazio *et al.*, 1973) and 21 ppb in 1982 (Sen and Seaman, 1982).

2.8.2 N-Nitrosamine Inhibitors

Total elimination of N-nitrosamine formation is impossible because the precursors (*i.e.* nitrite and amines) occur naturally in the environment, but there are agents which suppress N-nitrosamine formation. Sodium ascorbate and erythorbate have been the preferred compounds to date. Their effectiveness is limited, however, due to a lack of solubility in adipose tissue (Sebranek, 1979). Attention was focused on the use of lipophilic derivatives of ascorbic acid. L-ascorbyl palmitate (AP) and PG were found to

more effective than sodium ascorbate in reducing NPYR formation during the cooking of bacon (Sen *et al.*, 1976). Other potential anti-N-nitrosamine agents investigated include long chain acetals of ascorbic acid, the combination of α-tocopherol and ascorbate, and the use of lactic acid. Bharucha *et al.* (1980) reported that long-chain acetals of ascorbic and erythorbic acid (*i.e.* C_{16} and C_{18}) were excellent blocking agents of N-nitrosamine formation in bacon (approximately 96%) when used at a 1000 ppm level. They also reported that the acetals retained their efficacy in bacon even after 35 days at 3°C unlike AP which tends to lose its activity during storage. The use of α-tocopherol together with ascorbate has been reported to serve as effective N-nitrosamine blocking agents. In some bacon products, addition of microbes to convert sugar to lactic acid has been tested. Lactic acid build up results in a lowering of the pH level of bacon and accelerates the breakdown of nitrite. Despite these results, there has been a major effort to discover substitutes for nitrite.

2.8.3 Impact of a Nitrite Ban

If the use of nitrite in meat processing was to be discontinued, a large number of traditional meat products would be eliminated, and the economic implications would far outweigh the loss of these foodstuffs. The retail value of cured meat products sold annually in the USA was calculated to exceed $12 billion (Binkert, 1978). The costs of elimination of nitrite in meat products are numerous (Madsen, 1976):

1. Possible botulism
2. Reduced income/loss of cash and future markets

3. Reduced employment in farming, meat packing, distribution, and retailing
4. Loss of export markets for pork
5. Depressed trimmings market
6. Losses from closing of facilities
7. Fewer choices for consumers at meat counters
8. Fewer convenience foods

The costs listed justify the need of a safe nitrite substitute which preserve the characteristic properties of cured meat products.

2.9 Possible Substitutes for Nitrite

Kemp (1974) posed the question, "are we looking for substitutes that will do all the things that nitrite does, or should we be satisfied with materials that are specific for only one of the effects? If one substitute will affect colour and another has bacteriostatic activity, should we use a mixture?" Kemp (1974) further stated that from a sales point of view, colour development is the most important function of nitrite. From a health standpoint, bacteriostasis is of paramount importance. For the food connoisseur, flavour might be uppermost, while the antioxidant effect probably is the least important attribute. The possibility of finding a single compound to mimic all functions of nitrite is remote. Sweet (1975) in a US patent was first to propose the use of composite non-nitrite curing mixtures for duplicating the cumulative action of nitrite. His multicomponent system consisted of a colorant, an antioxidant/sequestrant, an antimicrobial agent and all other

typical curing adjuncts with the exception of nitrite. Shahidi and co-workers continued with this theme but used different composite systems (Wood *et al.*, 1986; Shahidi *et al.*, 1987*a*,*b*; 1988; 1990; Shahidi and Pegg, 1990*a*; 1991*b*; 1992). A summary of substitutes which reproduce the characteristic properties of nitrite-cured meats is provided below.

2.9.1 Colour Characteristics

Visual appearance is one of the main factors influencing consumers when assessing the quality and palatability of meat products. Many experiments have been performed which support the idea that certain colours do, in fact, influence food acceptance (Kostyla and Clydesdale, 1978). Although it is important to find a substitute for nitrite that reproduces the characteristic cured-meat colour, the National Academy of Sciences (1982) noted that there are relatively few reports of attempts to find compounds or processes that mimic the selective colour fixing effect of nitrite in the muscle tissue of cured meats. Some of these attempts are presented below.

Various nitrogenous ligands have been studied which preserve or stabilize the red colour of meat. These include the use of nicotinic acid (Coleman and Steffen, 1949), pyridine derivatives (Dekker, 1958; Hopkins and Sato, 1971), tetrazole (van den Oord and DeVries, 1971) and heterocyclic compounds such as purines, pyrimidines, imidazoles, pyrazines and triazines as well as derivatives of these ring systems (Tarladgis, 1967). Brown (1973) suggested that a compound which would react with Mb in a manner similar to that of nitric oxide might produce a colour similar to NOMb. The author tested many nitrogen-containing heterocycles and found that methyl and hexyl nicotinate and N,N-

diethylnicotinamide were able to react with Mb and form an acceptable red colour, but these pigments were less stable than the pigment of nitrite-cured meat after thermal processing. Howard *et al.* (1973) investigated a variety of nitrogenous ligands, including derivatives of pyridine, amino acids and amino acid esters for their ability to form stable pink pigments in model and cured meat systems. Methyl and hexyl nicotinate and N,N-diethylnicotinamide were found to be the most promising and produced stable pink ferrohaemochromes in cooked ground meat mixtures. Addition of ascorbic acid or glucono-δ-lactone at 0.05% (w/w) improved the colour as well as the stability of the pigment formed. Methyl nicotinate, trigonelline and N,N-diethylnicotinamide were also effective in combination with 10 or 20 ppm of sodium nitrite in forming a stable pink colour in meat systems. The colour of these meat systems was more acceptable than that of the nitrite-cured control after storage for 10 weeks at 5°C and upon exposure to air (Howard *et al.*, 1973). Unfortunately, N,N-diethylnicotinamide and some derivatives of nicotinic acid and nicotinamide are known to have vasodilatory properties.

Dymicky *et al.* (1975) tested more than 300 compounds from various classes of chemicals for their ability to form haemochromes in meat slurries and emulsions at 70°C. Most of the compounds tested were nitrogenous heterocycles and imparted colours to cooked meat from beige to pink to purple. The most effective colour-forming compounds were substituted pyridines and isoquinolines. Colour fixation was believed to be related to the nature of the substituent and its position on the ring. The best colour was produced by pyridine derivatives containing carbonyl moieties at the 3-position. These studies were

undertaken solely for the purpose of establishing the structure of compounds that would react with meat components to provide a satisfactory colour, and the effect on flavour, and the toxicity of any of the substitutes tested, were not considered.

Since the number of artificial colorants is limited, and the safety of some has been questioned, von Elbe and Maing (1973) and von Elbe *et al.* (1974*a*,*b*) investigated the use of natural pigments obtained from the red beet root (*Beta vulgaris*). Because beet powder is permitted as a food colorant, these authors used betalain pigments from beets to simulate cured meat colour in cooked, smoked and semi-dry, fermented sausage products. The colour of formulated products was measured using Hunter **L**, **a**, **b** values. Similar **a** and **b** values were noted in sausages containing nitrite/nitrate and those containing beet pigments. Lower Hunter **L** values were observed for pigment-treated meats denoting darker products. The colour of betalain-containing sausages was more stable to light exposure during storage than the colour of their nitrite/nitrate-treated counterparts, but expert tasters were able to detect subtle flavour and colour differences in betalain and nitrite-containing samples.

Sweet (1975), in his composite nitrite-free curing systems, used erythrosine as the colorant. In 1982, the National Academy of Sciences reported that although a stable uniform cured-meat colour can be achieved with sodium nitrite addition levels as low as 50 ppm, no suitable means of fixing colour in cured meat, other than reduced nitrite levels, have been demonstrated to be effective in products made under commercial conditions. Shahidi and co-workers attempted to solve this problem by using the

approach of Sweet (1975), but their colorant of choice was the actual CCMP. The CCMP was preformed outside of the meat matrix and then applied to meat systems. The colour characteristics of such nitrite-free systems have successfully duplicated those of nitrite (Shahidi *et al.*, 1984; 1985*b*; Shahidi and Pegg, 1991*b*). Smith and Burge (1987) tried to mimic cured meat colour using protoporphyrin-IX, but Hunter **L**, **a**, **b** values and spectra of pigments extracted from protoporphyrin-IX-treated systems were markedly different from those of nitrite-cured samples.

2.9.2 Antioxidant Properties

Agents other than nitrite have antioxidant activity in meat products. Sodium tripolyphosphate was shown to offer protection to precooked frozen pork products against lipid oxidation (Tims and Watts, 1958). An enhanced antioxidant action was observed when it was used in combination with ascorbic acid (Lehmann and Watts, 1951). Chang and Watts (1949) suggested that ascorbic acid and phosphates would act synergistically to prevent lipid oxidation in cooked meats. High levels (1000 ppm) of ascorbic acid also inhibited rancidity in a meat model system (Sato and Hegarty, 1971). The same researchers also observed that the addition of either STPP, sodium hexametaphosphate (SHMP) or tetrasodium pyrophosphate retarded rancidity development in ground beef. Haymon *et al.* (1976) reported that increased antioxidant properties were observed in frozen meat products which had been treated with STPP and a lemon juice concentrate. The mechanism by which phosphates retard lipid oxidation appears to be related to their ability to sequester metal ions, particularly Fe^{+2} ions, which are the major pro-oxidants

in meat systems (Love and Pearson, 1974).

Sato and Hegarty (1971) tested a variety of compounds for their ability to inhibit lipid oxidation, as measured by TBA values. The most active compounds were the disodium salt of EDTA, STPP, SHMP, sodium citrate, sodium ascorbate, BHA and BHT, but only the last two compounds were effective at concentrations as low as 100 ppm. MacDonald *et al.* (1980*a*) tested citric acid and BHT for their antioxidant activity and compared the results with those of nitrite at various concentrations. They reported that citric acid at 1000 ppm and BHT at 200 ppm were less active than sodium nitrite at its lowest concentration of 50 ppm.

Shahidi and Hong (1991*b*) reported that the addition of polyphosphates such as STPP at 3000 ppm or the disodium salt of EDTA to meat systems containing pro-oxidants such as iron and copper ions or haem pigments resulted in a substantial decrease in their content of TBARS. The coordination properties of nitrite may potentially be duplicated by the action of other ingredients (Shahidi *et al.*, 1986*a*). Shahidi (1992) reported on various curing adjuncts which inhibited lipid oxidation in cooked ground pork systems. Ascorbate (500 ppm) retarded lipid oxidation, possibly by upsetting the balance between Fe^{+2} and Fe^{+3} ions or by an oxygen scavenging mechanism (Decker and Hultin, 1990), but it has been reported to have pro-oxidant activity in some instances (Igene *et al.*, 1985). Presence of non-haem iron, tocopherols, citric acid and amino acids which are naturally present in meat may change the role of ascorbate from an antioxidant to a pro-oxidant. Ascorbyl palmitate and the C_{16} acetal of ascorbic acid at a 2000 ppm level gave rise to

strong antioxidant effects in cooked pork systems. Shahidi *et al.* (1987*a*,*b*) proposed that this activity may be due to their enhanced solubility in the fat portion of meat as compared to ascorbic acid itself.

A strong synergism has also been noted between polyphosphates and ascorbates (Chang and Watts, 1949). The TBA values of meats treated with combinations of polyphosphates and sodium ascorbate, or with either ascorbyl acetal or AP, were similar to those of nitrite-cured meat in model systems. At 0.4%, SAPP effectively retarded lipid oxidation in chicken nuggets. Long-chain polyphosphates are better sequestering agents for light metal ions such as calcium and magnesium compared to short-chain polyphosphates for iron and copper ions. As pH increases, the chelating ability of long-chain polyphosphates also increases, while the opposite is true for short-chain polyphosphates (Barbut *et al.*, 1989).

In some cured products, herbs and spices may be added in conjunction with the cure to impart a desired taste and aroma to the products. Rosemary, sage, thyme, marjoram and oregano are among the herbs, and clove, ginger and mace are among the spices which possess strong antioxidant effects (Shahidi and Wanasundara, 1992). The antioxidant activity of herbs and spices is due to the presence of natural inhibitors of lipid oxidation. It is generally derived from a diverse group of phenolic-based compounds. For example, cloves contain 1.26% gallic acid and 3.03% eugenol, both of which are known to be strong antioxidants at relatively low concentrations (Kramer, 1985; Al-Jalay *et al.*, 1987).

In emulsified meat products, protein extenders such as soy protein isolates or concentrates and other plant proteins are often incorporated. Many of these protein extenders are known to contain a relatively high concentration of phenolic compounds. Rhee *et al.* (1983) and Ziprin *et al.* (1981) reported that addition of protein to meats from glandless cotton seed or their aqueous or methanolic extracts retarded lipid oxidation. Shahidi (1992) reported that low-pungency mustard flour when added to comminuted meat systems possessed strong antioxidant effects at levels of addition between 1.5 and 2%, but its aqueous and methanolic extracts were less effective.

2.9.3 Flavour Characteristics

Flavour is a complex stimulus involving characteristics such as taste, odour, texture and temperature (Gray *et al.*, 1981). The National Academy of Sciences (1982) reported that the generation of cured meat flavour is probably a composite sensation derived from the contribution of many odoriferous compounds. A positive contribution by nitrite to flavour cannot be specified in chemical terms, but the committee suggested that nitrite probably influences the flavour of cured meat by virtue of its antioxidative effects. Because the mechanism involved in the production of the characteristic cured-meat flavour is uncertain, there is no known nitrite substitute which can duplicate this flavour.

Some meat products cured without nitrite have been found to be acceptable by panellists. Taste tests conducted on bacon treated with salt, sugar, STPP, sodium ascorbate, and varying levels of nitrite showed that an acceptable bacon product could be prepared without the use of nitrite (Wasserman and Kimoto, 1977). Further studies by

Wasserman *et al.* (1977) and Huhtanen *et al.* (1981) revealed that no difference between the preference for nitrite-free and nitrite-cured bacon could be discerned. These results were also supported by Williams and Greene (1979). Kimoto *et al.* (1976) reported that sodium chloride was more important than nitrite to the flavour of bacon while MacDougall *et al.* (1975) stressed the importance of sodium chloride for cured meat flavour. These authors reported that sodium chloride-free samples had almost no bacon flavour, whereas, salted, nitrite-free bacon did. On the other hand, Paquette *et al.* (1980), who varied sodium nitrite levels in bacon samples, found that samples containing nitrite had a significantly more desirable flavour than did nitrite-free analogs. No significant differences in the desirability among samples containing nitrite at different concentrations were noted. Although nitrite-free cured bacon had a less desirable flavour than its nitrite-cured counterpart, it was still acceptable.

Paquette *et al.* (1980) also reported that bacon containing potassium sorbate at 2600 ppm and sodium nitrite at 40 or 80 ppm was judged to be as desirable as that containing sodium nitrite and no potassium sorbate. No undesirable flavours were introduced by addition of the antimicrobial agent to the bacon products. Similar reports on the effect of antimicrobial agents in nitrite-free or nitrite-reduced cured meat products were complied by the National Academy of Sciences (1982). Data suggested that bacon processed with sodium chloride and sodium hypophosphite at 3000 ppm or bacon prepared with sodium chloride, sodium hypophosphite at 1000 ppm, and sodium nitrite at 40 ppm were judged to have a flavour as desirable as that of the conventionally

prepared nitrite-cured product. Bacon processed with sodium chloride alone was included as a control and was judged to have a flavour as desirable as that of the other products. Sensory data suggested that bacon treated with methyl fumarate at 1,250 ppm could not be distinguished from the nitrite-cured control. Hedonic scores for methyl fumarate-treated bacon and a conventional counterpart were equivalent.

For frankfurters, Simon *et al.* (1973) found that all-beef nitrite-free wieners had an equivalent flavour to nitrite-cured samples, but the flavour quality of 50% pork/50 % beef wieners varied directly with the nitrite concentration. On the other hand, Greene and Price (1975) reported that salt was the major contributor to cured meat flavour in samples of ground pork, whereas sodium nitrite alone at a level of 200 ppm produced very little cured-meat flavour. Yun (1984) and Yun *et al.* (1986) evaluated combinations of ingredients which would effectively prevent lipid oxidation in cooked ground pork systems to be used in the nitrite-free curing of meat products such as frankfurters. The authors reported that sensory evaluation scores of pork systems treated with 3000 ppm STPP, 550 ppm sodium ascorbate and 30 ppm of BHA or TBHQ were not significantly different ($P>0.05$) from their nitrite-cured (156 ppm) counterpart. Yun (1984) also observed that the concentration of volatiles identified in the distillate of cooked pork samples, notably hexanal, was significantly reduced ($P<0.05$) when samples had been pretreated with the above antioxidant/chelating agent combinations. The concentration of volatiles in these systems was depressed almost to the level of the nitrite-cured control.

The studies cited above suggest, for the most part, that it is possible to prepare

nitrite-free cured-meat products without seriously compromising their flavour. If one accepts the views of Cross and Ziegler (1965) that cured-ham flavour represents the basic flavour of meat derived from precursors other than triacylglycerols and that the different aromas of the various types of cooked meat depend on the spectrum of carbonyl compounds derived by lipid oxidation, then any agent or combination that suppresses lipid oxidation, would, in principal, duplicate the flavour of nitrite-cured meat.

2.9.4 Antimicrobial Properties

Nitrite exerts a concentration-dependent antimicrobial effect in cured meat products, including, but not limited to, inhibition of the outgrowth of spores of putrefactive and pathogenic bacteria such as *C. botulinum* (National Academy of Sciences, 1982). The degree of protection provided to cooked meats against microbial contamination depends on many factors including the concentration of residual nitrite, duration of temperature abuse, and extent of contamination. Irrespective of future decisions on the fate of nitrite, its removal or reduction must be counter-balanced by alternatives that will assure the safety from botulinal hazards in abused products (Shahidi and Pegg, 1991*a*). The traditional identity of cured meat products, however, must be preserved. According to Sofos and Busta (1980), any substance to be considered as an alternative to nitrite should be suitable for use in all cured meat products and should control other microorganisms of public health significance, delay product spoilage, and not interfere with beneficial microorganisms such as lactic acid-producing cultures, necessary for the manufacture of fermented meat products. The compound of choice must

also be (a) at least as effective as nitrite itself (b) safe, (c) heat stable, (d) flavourless, and (e) preferably effective at low concentrations.

The propyl ester of *p*-hydroxybenzoic acid (*i.e.* propylparaben) is approved for use in the casings of dry sausages to retard mold growth (Sofos and Busta, 1980). Use of parabens as antimicrobial agents in nitrite-free cured meats has been suggested. Sweet (1975) used methyl- and propyl-parabens as antimicrobial agents in his composite nitrite-free curing system. Robach and Pierson (1978) found that these parabens were good candidates as inhibitors of toxin production by *C. botulinum* strain 10755A in microbiological media, but their effectiveness in meat against *C. botulinum* was questionable. Tanaka *et al.* (1978) showed bacteriostasis against *C. botulinum* serotypes A and B spores to be only slightly effective in frankfurters, while Deibel (1979) reported that they were ineffective in a commercial wiener system. Dymicky and Huhtanen (1979) found an increasing effectiveness of the paraben as the ester chain length increased. The undecyl ester was 3000 times more inhibitory than its methyl counterpart, but on the whole the outlook of parabens as potential alternatives to nitrite in meat products is not very promising.

Sorbic acid and its potassium salt are known inhibitors of yeasts and molds, but knowledge on their action against bacteria is not as comprehensive (Sofos *et al.*, 1979*d*). Potassium sorbate is a white crystalline compound with GRAS (generally recognized as safe) status. It is approved for use in dry sausages to retard the growth of molds, and the casings are dipped in a 2.5% (w/v) solution. Tompkin *et al.* (1974) reported that sorbate

also delayed toxin production by *C. botulinum* in a nitrite-free sausage product. The efficacy of potassium sorbate or sorbic acid for controlling growth of *C. botulinum* in meat products, when used either individually or in combination with reduced levels of nitrite, has been evaluated by many investigators (Ivey and Robach, 1978; Tanaka *et al.*, 1978; Ivey *et al.*, 1978; Sofos *et al.*, 1979*a*,*b*; 1980*b*).

Tanaka *et al.* (1978) demonstrated that potassium sorbate addition to wieners at 2700 ppm provided an anticlostridial action similar to that of 100 ppm nitrite. Sofos *et al.* (1979*a*,*b*,*c*; 1980*a*) reported that sorbic acid when used at a level of 2000 ppm delayed *C. botulinum* toxin production in wieners to an extent similar to that of 156 ppm nitrite and longer than that of 80 ppm nitrite. These effects were pH dependent and only developed at pH values of less than 6.0. The protonated acid is more active than its anion, consequently, lowering the pH greatly enhances its anticlostridial effect. When nitrite was incorporated into the formulations at either 40 or 80 ppm, the bacteriostasis increased and the effective pH level was raised to 6.2 (Sofos *et al.*, 1980*b*). These investigators also demonstrated that sorbic acid in wiener emulsions, with or without nitrite, inhibited clostridial spore germination.

Several researchers have proposed the use of sorbate-polyphosphate combinations as antimicrobial agents. Ivey *et al.* (1978) presented data demonstrating that a mixture of potassium sorbate, STPP and SAPP was more effective against *C. botulinum* outgrowth in bacon than 120 ppm of added nitrite. Synergistic sorbate-polyphosphate effects were also observed in wieners by Tanaka *et al.* (1978).

In four bacon studies, combinations of 40 ppm nitrite and 2600 ppm potassium sorbate showed an anticlostridial effect similar to or greater than that of treatments with 120 ppm nitrite (Ivey *et al.*, 1978; Pierson *et al.*, 1979*a*,*b*; Price and Stevenson, 1979). In a commercial bacon trial, Sofos *et al.* (1980*b*) found that 40 ppm nitrite and 2600 ppm sorbate were effective against clostridial outgrowth but not to the same extent as that of 120 ppm nitrite addition. This combination was also found to reduce N-nitrosamine formation in cured products from nearly 100 ppb to less than 5 ppb (Shaver, 1979). Sensory data of these cured meats revealed no differences in colour and flavour scores.

Sodium hypophosphite (SHP), another GRAS substance, has been proposed for use as an antimicrobial agent in foods (Pierson *et al.*, 1981; Rhodehamel and Pierson, 1990). Microbiological studies indicated that a total or partial replacement of nitrite with this compound effectively inhibits production of *C. botulinum* toxins (Banner, 1981). Rhodehamel (1983) found SHP to be effective in inhibiting the growth of *C. perfringens* and *C. botulinum* strains 62A, 52A and other gram positive bacteria. Increasing sodium chloride concentrations in the media enhanced SHP's inhibition of both *Clostridia* strains. Rhodehamel and Pierson (1990) found SHP to suppress growth of certain gram negative spoilage bacteria near neutral pH. In their study, the efficacy of SHP increased slightly with decreasing pH, but they reported in all other studies that SHP's effectiveness as an antimicrobial agent seemed to be independent of the pH of the media. Rhodehamel and Pierson (1990) postulated that since the pK_a (1.1) of SHP's conjugate acid, hypophosphorous acid, was much lower than those of traditionally used food acidulants

such as benzoic, sorbic and propionic acids (pK_a 4-5), it is the dissociated acid anion of SHP which exhibits antimicrobial activity. Because SHP exists primarily in the dissociated form over the pH range of 5-7, this may explain why its inhibitory effect is not enhanced by decreasing pH.

At 3000 ppm alone or at 1000 ppm in combination with 40 ppm of nitrite, SHP imparted anticlostridial protection to meat products equivalent to that provided by 120 ppm of nitrite. Wood *et al.* (1986) demonstrated that SHP, added at 2600 ppm, was an effective antimicrobial agent in nitrite-free treated meat systems. This preservative is bland in taste and as such nitrite-free bacon containing 3000 ppm of SHP had a flavour as desirable as that of its conventionally cured counterpart.

Huhtanen (1984) found that methyl and ethyl esters of fumaric acid to be inhibitory to *C. botulinum* in a bacon model system. Monomethyl and monoethyl fumarates at 1250 ppm exhibited more anticlostridial activity than bacon cured with 120 ppm nitrite, while dimethyl and diethyl fumarates had activity only equal to that of the nitrite-cured control. These fumarate-treated meat samples were sensorially indistinguishable from that of their nitrite-cured counterpart. Wood *et al.* (1986) reported a similar finding on the efficacy of these fumarates in nitrite-free cured comminuted meats, but SHP was reported to be a superior anticlostridial agent.

Lactic acid, its sodium or potassium salts, or lactic acid-producing bacteria (LAPB) lower the pH of cured meat products and may provide microbial stability to muscle foods (Andres, 1985). Some adventitious LAPB that grow on meat cause flavour

and odour defects, but conditions that favour the growth of these bacteria generally result in a dramatic extension of the storage life of chilled meats (Farber *et al.*, 1990). While lactic acid, as such, may be used for surface treatment against microbial activity, use of lactate salts as a component of muscle foods may prove to be more beneficial. Incorporation of lactic acid, preferably in an encapsulated form, or LAPB together with fermentable carbohydrates in cured meat formulations is permitted for pH reduction (Bacus, 1979; Tanaka *et al.*, 1978). Typical LAPB associated with meats are *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. Excellent protection against *C. botulinum* toxin formation has been achieved using these LAPB and sucrose in nitrite-free treated bacon. A lowering of the addition level of nitrite to 40 ppm, together with these starter cultures has been approved by regulation for use in some products (Thompson, 1985).

In addition to the benefits of pH reduction, LAPB act to suppress the growth of pathogens through competitive effects and the production of antimicrobial substances (Daly *et al.*, 1973; Gilliland and Speck, 1977). Some of the bacteria produce antibacterial proteins called bacteriocins which prevent other bacteria such as *C. botulinum* from flourishing. The most studied bacteriocin is nisin and it is produced by *Lactococcus* spp. Nisin has proven anticlostridial activity in culture media (Scott and Taylor, 1981), but due to its poor solubility above pH 6.0 and incomplete diffusion throughout bacon, it has a limited anticlostridial effect (Taylor and Somers, 1985). At a level of 500 ppm, its extension of the shelf life of nitrite-free cooked meats is marginal, but the addition of

100-250 ppm nisin in combination with 120 ppm nitrite is more effective than addition of 156 ppm nitrite alone (Taylor *et al.*, 1985). Research into the use of bacteriocins or bacteriocinogenic LAPB for meat preservation is still in its infancy, and many difficulties must be overcome before LAPB can be used commercially to extend the storage life and to enhance the safety of meats (Stiles and Hastings, 1991).

Phenolic antioxidants have been known for many years to have antimicrobial activity against bacteria, molds, and viruses. The effectiveness of antioxidants and chelators as antimicrobial agents has been investigated in model and cooked meat systems by several researchers (Pierson *et al.*, 1981; Winarno *et al.*, 1971). In particular, it has been reported that the addition of BHA at 50 ppm exerts an inhibitory effect on the growth of *C. botulinum* types A and B in comminuted meats (Pierson *et al.*, 1980). BHT, TBHQ and PG were less effective agents (Robach and Pierson, 1978). The anticlostridial activity of EDTA (Winarno *et al.*, 1971) and various polyphosphates (Sofos, 1986) was investigated, but they did not provide effective bacteriostasis. Pierson and Reddy (1982) examined the effectiveness of 15 phenolic compounds for their activity against growth of and toxin production by *C. botulinum* types A and B in comminuted pork. Some of the phenolic compounds examined included esters of *p*-hydroxybenzoic acid and gallic acid, BHA, BHT, TBHQ, 8-hydroxyquinoline and phenol derivatives. 8-Hydroxyquinoline at a concentration of 200 ppm, alone or in combination with sodium nitrite at 40 ppm, inhibited the growth and toxin production of *C. botulinum* for 60 days at 27°C.

Kanner and Juven (1980) investigated the anticlostridial activity of SNC, a reaction

product in nitrite-cured meat, in meat model systems. The activity of SNC was found to be considerably less than that of nitrite at a 156 ppm concentration, and it therefore may not lend itself as an antimicrobial substitute for nitrite. Such compounds may also potentially participate in transnitrosation reactions with bacterial cells and spores or even meat pigments.

Finally, the use of radiation sterilization as an established technique of microbial inactivation and as a method of food preservation has been examined. Irradiation has been used for the sterilization of spices and herbs as well as for inhibiting the sprouting of potatoes and onions (Wasik, 1987). Many investigators have studied the effects of gamma irradiation on the sensory and microbiological properties of meat, poultry and seafood products (Colbey *et al.*, 1961; Chipault and Mizuno, 1966; Anellis *et al.*, 1972; Wierbicki and Heiligman, 1974; Wierbicki *et al.*, 1974; Shults *et al.*, 1977; Hussain *et al.*, 1978; Curzio and Quaranta, 1982; Urbain, 1982; Piccini *et al.*, 1986; Paul *et al.*, 1990). Low-dose irradiation at low temperatures eliminated or reduced the undesirable effects of radiation processing and resulted in an enhancement of the quality of products. Radiation sterilization has also been found effective against the outgrowth of *C. botulinum* spores in meats cured with reduced nitrite levels. Szczawiński *et al.* (1989) and Wierbicki and Heiligman (1980) reported that bacon and ham products as well as meat model systems containing nitrite concentrations of 25-40 ppm were microbiologically similar to their conventionally cured-analogs, after radiation processing and upon subsequent refrigerated storage. Wierbicki and Heiligman (1974) reported that the colour

of irradiated meats with reduced levels of added nitrite, and without the addition of sodium nitrate, as a nitrite reservoir, faded more readily; lower preference scores were noticed in sensory studies. McCormick (1982) found that irradiated preserved meats were superior to thermally processed meats in terms of their shelf-life without greatly altering their aroma, taste or texture. These results suggest that radiation processing may potentially be used either to substitute for the antimicrobial action of nitrite or to reduce the addition level of nitrite required for its bacteriostasis.

Of the above antimicrobial agents/processes, lactates and radiation sterilization perhaps offer the best safe alternatives to nitrite. Use of lactic acid at levels of up to 2-3% may be regarded as safe. Low- to medium-dose radiation sterilization (0-20 kGy), especially at reduced temperatures, may provide an attractive option. Under these conditions, no adverse effects on colour and flavour of irradiated samples were noticed (Shahidi *et al.*, 1991*b*).

CHAPTER 3. MATERIAL AND METHODS

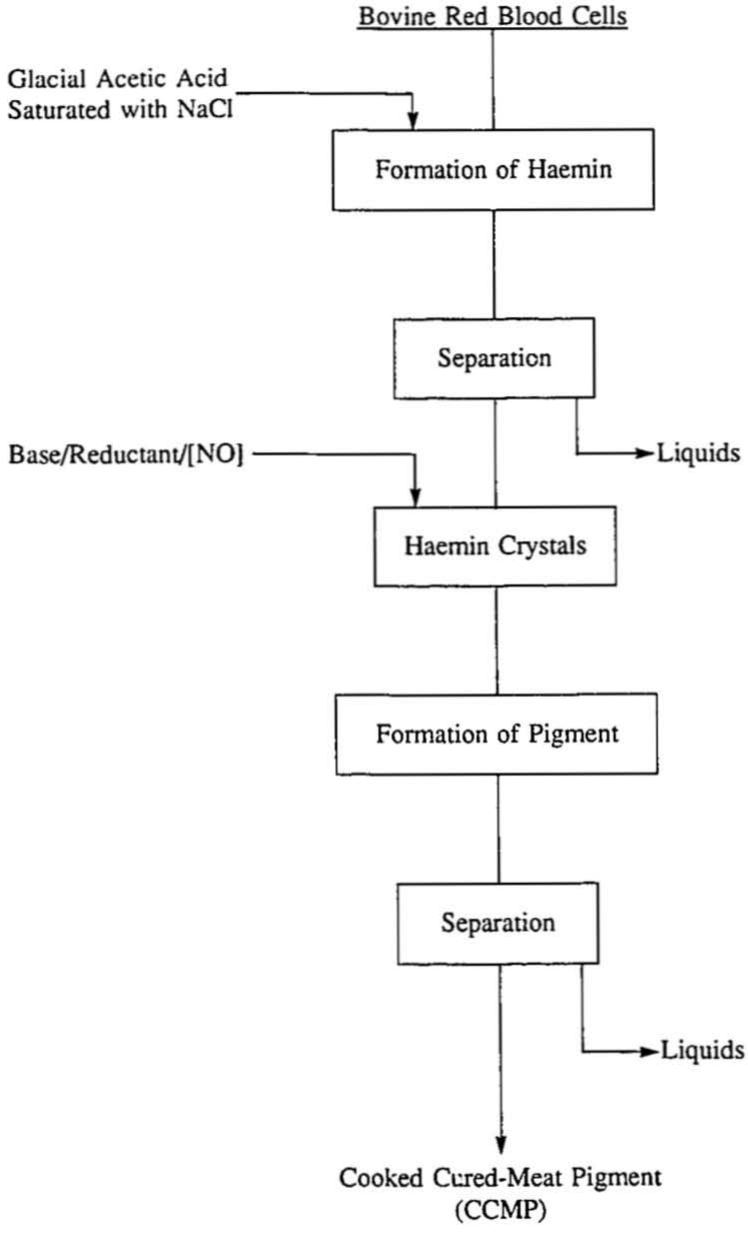
3.1 Production of the CCMP

3.1.1 Preparation of CCMP from Haemin

Haemin was purchased from the Sigma Chemical Co. (St. Louis, MO). Although any source of haemin is suitable, the bovine variety was used in all experiments, since it is readily available as a by-product in production of plasma, a food ingredient. The CCMP was prepared from haemin and nitric oxide (Canadian Liquid Air (CLA), St. John's, NF) as described by Shahidi *et al.* (1985*b*) with slight modifications (Figure 3.1). A typical procedure for the preparation of CCMP from haemin is described below.

Haemin (600 mg) was dissolved in 100 mL of a 0.04 M Na_2CO_3 solution (Fisher Scientific Co., Montréal, PQ) in a volumetric flask. The haemin reagent was stored in the dark for 30 min before use and shaken periodically to ensure complete dissolution. Sodium tripolyphosphate (STPP, Albright and Wilson Americas, Toronto, ON), 150 mg, and sodium ascorbate (Sigma), 400 mg, were added to 50-mL Corning centrifuge tubes. Nine millilitres of a 0.2 M sodium acetate buffer (Fisher), pH 6.5, were added to each tube. The contents were mixed using a Fisher Vortex Genie 2 to ensure complete dissolution of the solids. One millilitre aliquots of the haemin solution were added to each tube. Tubes were then transferred to an AtmosBag (Aldrich Chemical Co., Inc., Milwaukee, WI) which was flushed twice with nitrogen (K-grade, CLA) to remove oxygen from the headspace gases. Under a blanket of nitrogen, nitric oxide was bubbled into each tube for approximately 45 s in order to produce CCMP. During NO addition,

Figure 3.1 Preparation of the cooked cured-meat pigment (CCMP) from haemin and nitric oxide.


Bovine Red Blood Cells
Glacial Acetic Acid
Saturated with NaCl
Formation of Haemin
Separation
Liquids
Base/Reductant/[NO]
Haemin Crystals
Formation of Pigment
Separation
Liquids
Cooked Cured-Meat Pigment
(CCMP)

CCMP precipitates out of solution due to a drop in pH. Tubes were then capped, removed from the AtmosBag, and stored in the dark until used. Generally, CCMP was stored for less than three days.

Immediately before their use, pigment samples were centrifuged using an IEC clinical centrifuge (Damon/IEC Division, Needham Heights, MA) at 3000 rpm (905xg) for 5 min. The CCMP was recovered as a precipitate from the mixture after centrifugation. Tubes were opened, excess nitric oxide was released and the supernatant was discarded. Two millilitres of a 2% (w/v) sodium ascorbate or a 2% (w/v) ascorbic acid (AA) solution (Fisher) were added to each tube to wash CCMP and to ensure elimination of any traces of nitrite or nitrous acid from the mixture. Tubes were then capped, vortexed for 30 s, centrifuged at 3000 rpm (905xg) for 3 min and supernatant again discarded. The CCMP was applied to muscle food systems as described in section 3.3.

3.1.2 Preparation of CCMP from BRBC

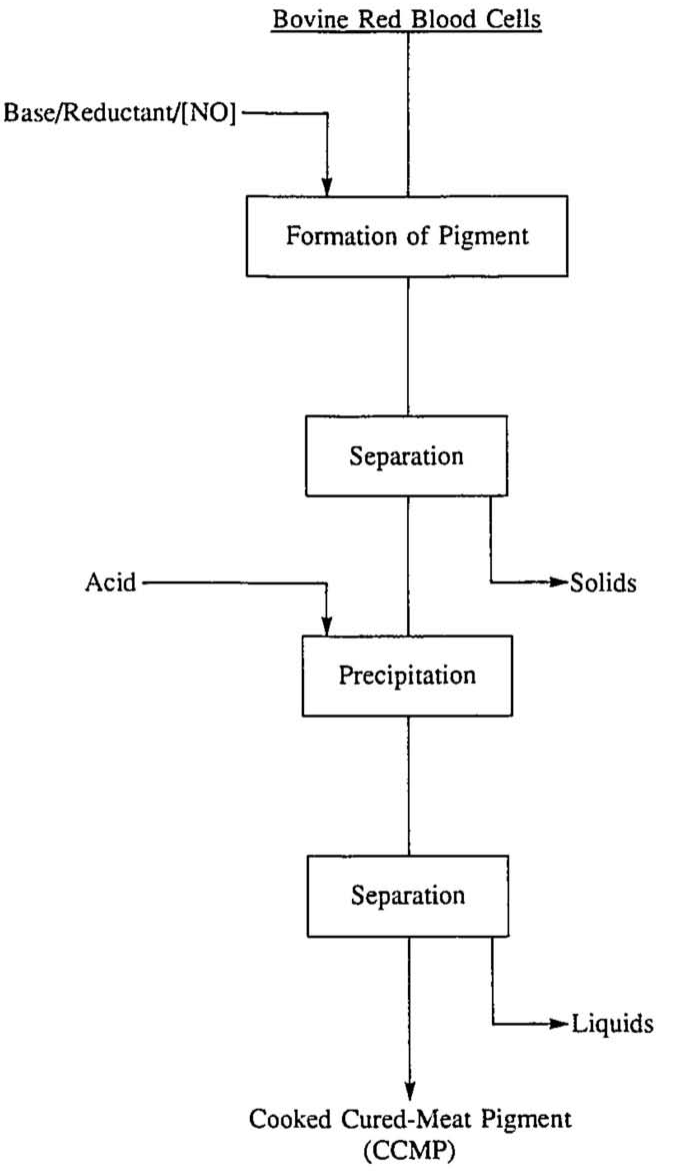
BRBC were collected from a slaughterhouse after blood plasma separation. They were transferred to Nasco Whirl-Pak polyethylene bags (Systems Plus, New Hamburg, ON) and stored at -15°C. Packages of BRBC were thawed overnight at 4°C and then stirred before use.

BRBC (10 g) were added to 90 mL of an 8:1 (v/v) mixture of distilled water/sodium hydroxide solution (Fisher) containing reductant(s) into which a nitrosating agent was introduced. Reducing agents, namely AA, erythorbic acid (EA, Fisher) and

ascorbyl palmitate (AP, Hoffmann-La Roche Ltd., Toronto, ON), were added to the reaction mixture at a haem to reductant molar ratio of 1:5, 1:10, or 1:20. Sodium nitrite (Fisher), the nitrosating agent employed, was added at a haem to sodium nitrite molar ratio of 1:10. The reaction mixture was heated at 85±2°C (75 and 80°C were also tested) for 15 min in a thermostated water bath (Lab-Line Instruments, Inc., Melrose Park, IL) with intermittent stirring, cooled in an ice bath to room temperature, and centrifuged for 2 min at 3000 rpm (905xg). The supernatant was acidified to pH 4.0 using an Accumet pH meter (Model 801, Fisher) with a 0.1 M citric acid solution. Acetic (0.1 M), hydrochloric (0.1 M), phosphoric (0.2 M) or sulphuric (0.05 M) acids were also tested as possible acidifying agents. All acids were obtained from Fisher Scientific Co. During acidification, CCMP as well as solubilized proteins were precipitated. After centrifugation for 2 min at 3000 rpm (905xg), the supernatant containing any residual nitrite from the curing process was discarded. A flow diagram of the direct preparation of CCMP from BRBC is outlined in Figure 3.2.

Yield and purity of the preformed pigment obtained from heat-treated BRBC/nitrite solutions were determined after acidification of the cooled reaction mixture to pH 4.0. This was followed by exhaustive extraction and recovery of the pigment from the resulting precipitate using 4:1 acetone/water solutions according to the procedure described by Hornsey (1956). The definitions of yield and purity are provided below.

Figure 3.2 Direct preparation of the cooked cured-meat pigment (CCMP) from bovine red blood cells.


Bovine Red Blood Cells
Base/Reductant/[NO]
Formation of Pigment
Separation
Solids
Acid
Precipitation
Separation
Liquids
Cooked Cured-Meat Pigment
(CCMP)

$$\text{Yield (mole \%)} = \frac{\text{Moles of CCMP formed}}{\text{Moles of haemin equivalents in BRBC used}}$$

The moles of CCMP formed are determined by extracting the pigment from the precipitate in a 4:1 (v/v) acetone/water solution and then measuring the absorbance of the extract at a wavelength of 540 nm. Based on the absorbance of the pigment at this wavelength and a molar extinction coefficient of nitrosylhaemochromogen in 4:1 (v/v) acetone/water of 11.3 $mM^{-1} \cdot cm^{-1}$ (Hornsey, 1956), the concentration of CCMP was determined according to Beer's Law. The moles of CCMP formed was calculated by multiplying the concentration of the pigment extract and the volume of solvent used. The moles of haemin equivalents in the BRBC was determined from an iron analysis of the cells using an atomic absorption spectrophotometer. Details of the method are described in section 3.6.1.

$$\text{Purity (\%)} = \frac{\text{Concentration of CCMP in the 4:1 (v/v) acetone/water}}{\text{Concentration of acid haematin}}$$

The purity of the pigment formed is a percentage of the concentration of CCMP extracted from the precipitate, as described above, to the concentration of this extract after conversion of the pigment to acid haematin by the addition of 1 drop of concentrated HCl. The concentration of acid haematin so obtained is determined from the absorbance of the extract at a wavelength of 640 nm and the molar extinction coefficient of acid haematin in 4:1 (v/v) acetone/water of 4.80 $mM^{-1} \cdot cm^{-1}$ (Hornsey, 1956).

3.2 Preparation of Comminuted Meat Systems

3.2.1 Pork

Boneless pork loins were obtained from the Newfoundland Farm Products Corp. (St. John's, NF) and their subcutaneous fat was trimmed. Loins were comminuted twice using a Hobart 4146 meat grinder (Hobart MFG Co. Ltd., Don Mills, ON) with a 7.9 mm and then with a 4.8 mm plate, or using an Oster meat grinder (Model KBZ3, Braun AG, Frankfurt, Germany) with an 8.0 mm and then with a 3.0 mm plate. Comminuted meat was used directly or transferred to polyethylene pouches (Eastern Paper Co., St. John's, NF), sealed by using a Multivac vacuum packager (Model A300/32, Wolfertschwenden, Germany) and then stored at -20°C until used.

3.2.2 Beef

Ground lean beef was purchased from a local supermarket on the day required and used as such.

3.2.3 Chicken

Chicken breasts were supplied by the Newfoundland Farm Products Corp. Skin, subcutaneous fat, bones and any blood spots were removed from samples. Meat was comminuted using an Oster meat grinder, vacuum packaged and stored at -20°C, as described earlier.

Mechanically separated chicken (MSCM), which was also supplied by the Newfoundland Farm Products Corp., was prepared by deboning the flesh of chicken backs and necks using a Poss deboner (Model PDE 500, POSS Limited, Toronto, ON).

Recovered meat was vacuum packaged and stored at -20°C, as previously above.

3.2.4 Lamb

Legs of lamb was purchased frozen from a local supermarket. The meat was thawed, trimmed of most of its subcutaneous fat and ground using the Oster meat grinder. Recovered meat was vacuum packaged and stored at -20°C, as previously described.

3.2.5 Meats used in Frankfurter and Salami Preparation

Comminuted meats from beef, pork and chicken including some mechanically-deboned meats, internal organs and fat were supplied by Maple Leaf Foods, Inc. (Toronto, ON) for frankfurter and salami preparations.

3.2.6 Cod (*Gadus morhua*)

Cod was obtained frozen at -20°C from Fisheries Product International (St. John's, NF). Before use, fillets were thawed and comminuted using the Oster meat grinder, as previously described.

Cod surimi, which is the washed flesh of minced cod to which sorbitol at 4% (w/w) and STPP at 0.3% (w/w) has been added, was a product of Terra Nova Fisheries (Clarenville, NF). The surimi was obtained as a frozen block and was stored at -60°C until used.

3.2.7 Harp Seal (*Phoca groenlandica*)

Beater (3 weeks to 1 year in age), bedlamer (1-4 years in age) and harp (4 years and older) seals, hunted in the Newfoundland coastal areas, were bled, skinned, eviscerated and trimmed of blubber fat. Seal carcasses were placed inside plastic bags

and stored in insulated iced containers for up to 3 days during transport to the laboratory and subsequent holding. Carcasses were washed with cold water for approximately 30 s to remove residual blood. Carcasses were packaged in polyethylene bags (W. Ralston (Canada) Ltd., Montréal, PQ) and frozen at -40°C until used. Seal muscle tissue was recovered, after size reduction of frozen carcasses into *ca.* 25 x 10 cm pieces using a band saw (Newfoundland Farm Products Corp.), by mechanical separation. Mechanically separated seal meat (MSSM) was prepared using the Poss deboner at the Newfoundland Farm Products Corp. Recovered seal meat was passed through the separator a second time to provide a homogeneous sample. MSSM was transferred to polyethylene pouches, vacuum packed and stored at -20°C, as previously described.

Seal surimi was prepared by washing MSSM with water. The seal meat was combined with 4°C distilled water at a meat to water ratio of 1:3 (w/v). The slurry was stirred manually for 10 min and then filtered through layers of cheese cloth with 1 mm diameter holes. Recovered washed meat (*i.e.* seal surimi) was transferred to polyethylene pouches, vacuum packaged and stored at -20°C, as described above.

3.3 Application of Pigments to Comminuted Meat Systems

3.3.1 Application of CCMP or PCCMP to Prepared Meats

Ground meats were mixed with 20% by weight of distilled water and 550 ppm sodium ascorbate in all experiments. Sodium nitrite and preformed CCMP were added directly to meat samples at levels ranging from 0 to 156 ppm and 0 to 72 ppm,

respectively. Powdered cooked cured-meat pigment (PCCMP), as described in section 3.5.3, was added to meat systems at concentrations of 35, 40 and 50 ppm. Meat slurries were thoroughly homogenized. Meat systems were then cooked at 85±2°C in a thermostated water bath for 40 min, while stirring occasionally with a glass rod. After cooling to room temperature, cooked meat samples were homogenized in a Waring blender (Fisher) for 30 s and then vacuum packaged as previously described.

3.3.2 Application of CCMP to Frankfurter and Salami Products

Soya protein isolates, flours, spices, and other carbohydrate-based binders were used with the meats supplied by Maple Leaf Foods, Inc. in frankfurter and salami preparations. A nitrite-cured control and a nitrite-free test sample were prepared for each of the above products. The control sample contained meat, water, binders and spices as well as sodium erythorbate and sodium nitrite at concentrations of 750 and 200 ppm, respectively. Nitrite-free test samples contained the above meat/binder/spice formulations as well as sodium erythorbate, CCMP, STPP, SHP (BDH Inc., Toronto, ON), and BHA (Sigma) at concentrations of 750, 18, 3000, 3000 and 30 ppm, respectively. The quantity of meat and adjuncts used in the formulations are of a proprietary nature. Meat formulations were stuffed in cellulose casings, smoked according to Maple Leaf's processing schedule, and subsequently evaluated by chemical and sensory tests.

3.4 Application of the CCMP to Solid Cuts of Pork

3.4.1 Preparation of Meat Model Systems

Longissimus dorsi muscles from chilled carcasses of hogs were obtained 24 hours post-mortem from the Newfoundland Farm Products Corp., and were used for all experiments. Subcutaneous fat as well as *Psoas major* muscle tissue was trimmed from muscle slabs. Loins were cut into 500 g cylindrical pieces approximately 15 cm long x 8 cm diameter, and were then placed on aluminium supports in the bottom of 1 L Pyrex beakers.

3.4.2 Preparation of Nitrite-Free Curing Pickle

Twenty grams of STPP were dissolved in 1 L of 4°C double-distilled water. The temperature of the water was allowed to equilibrate for 24 h before use. A 500-mL aliquot was transferred to a 1-L Erlenmeyer flask. CCMP, prepared from the modified haemin-nitric oxide synthesis, as described previously, was transferred to and dissolved in the STPP working solution at various concentrations (*i.e.* Solution A). Effect of pigment concentration on the degree of CCMP penetration into pork muscle was investigated at 12, 24, 36, 48 and 72 ppm levels of addition. The final concentration of CCMP in each system was based on the mass of meat and pickle used.

One hundred grams of sodium chloride (BDH) were dissolved in 1 L of 4°C double-distilled water. The water was allowed to equilibrate for 24 h before use. Four grams of sodium ascorbate were then dissolved in this solution. A 500-mL aliquot was transferred to a 1-L Erlenmeyer flask (*i.e.* Solution B). Solution B was added slowly to

solution A and mixed well using a magnetic stirring pad (Fisher). The resultant pickle was divided into two equal portions.

This procedure was repeated when preparing pickle for use in the 10 and 18°C experiments. A control pickle containing 200 ppm sodium nitrite was also prepared and applied to pork muscles.

3.4.3 Application of Pickle to Meat Systems and Determination of the Extent of CCMP Penetration Achieved

Each portion of the pickle was transferred to a 1 L beaker containing 500 g of prepared *longissimus dorsi* pork muscle. Parafilm (Fisher) was placed over top of each vessel which was then wrapped with aluminium foil to limit CCMP from further penetration of oxygen and light, respectively. Systems were marinated at 4°C in a low temperature incubator (Model 307, Fisher) for selected periods of time for a total of 7 days.

After various storage periods, marinated loins were removed from their pickle, strained and then transferred to polyethylene vacuum pouches. Meats were vacuum-packaged and then cooked in an 85°C thermostated water bath for 60 min. After cooling to room temperature, meats were removed from the bags, weighed and the drip loss recorded. A visual inspection of the interior surface was carried out after meats were sliced horizontally and vertically to examine the extent of CCMP diffusion through the muscle. The degree of penetration was recorded in all directions as outlined in Figure 3.3. Hunter **L, a, b** values of the cooked pickled products were recorded in selected

cases.

The shape occupied by the *longissimus dorsi* muscles used is depicted in Figure 3.3. Since the cross-section of the meat is elliptical, the volume of the muscle is given by the following equation.

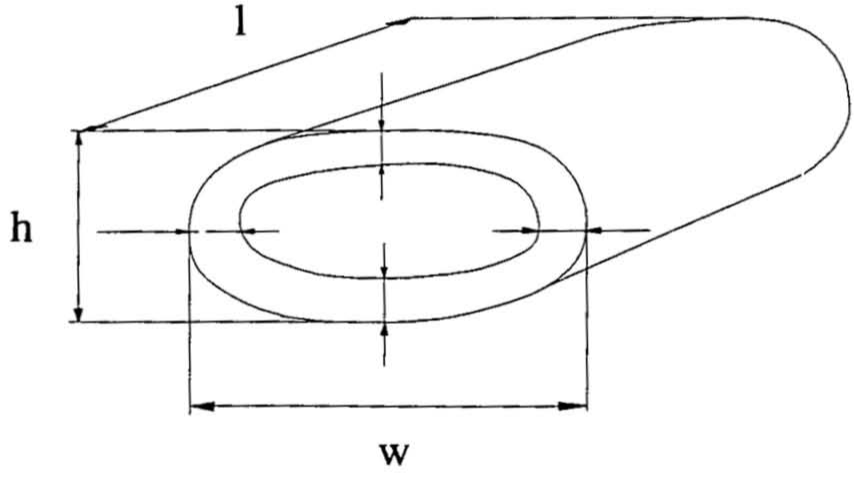
$$V_{meat} = \frac{w\ h\ \pi\ l}{4}$$

where, V_{meat} is the meat's volume (cm^3), w is the width (cm), h is the height (cm) and l is the length (cm). The degree of CCMP penetration can be approximated by the volume of muscle tissue which is pink in colour, as detected after thermal processing, at different storage periods. The boundary between pink and brown sections of treated meats was very sharp and relatively uniform along the cross-section of meat pieces, thus facilitating the measurements. The % pigment penetration was then calculated from the following equation.

$$\%\ \text{CCMP Penetration} = \frac{(V_{meat} - V_{brown})}{V_{meat}} \times 100$$

where, V_{brown} represents the volume occupied by the brown uncured portion of the muscle as determined by measurements made during visual examinations.

Figure 3.3 Shape of the *longissimus dorsi* muscle system used for the pigment penetration study and type of measurements recorded.


l
h
w

3.5 Stabilization of the CCMP

3.5.1 Washing of the CCMP Prior to its Encapsulation

The CCMP, prepared from haemin and nitric oxide, was recovered from tubes after centrifugation as described in section 3.1.1. The precipitate of one tube was added to comminuted pork at a 12 ppm concentration using a small volume of water from a wash bottle. Three other tubes containing precipitated CCMP were mixed with 2 mL of a 2% (w/v) AA solution. The mixtures were vortexed for 20 s. The CCMP did not dissolve in this solution. The tubes were then centrifuged at 3000 rpm (905xg) for 5 min, and the supernatants were discarded. Another tube containing the pigment was retained for subsequent application to meat. The washing procedure was repeated and pigments were retained after a second and a third wash. All pigment precipitates were applied to ground pork at a 12 ppm level as described above. The colour characteristics of treated systems were evaluated by Hunter **L, a, b** values as described in section 3.6.3. Finally, a pigment sample which was washed twice with the AA solution was encapsulated forming PCCMP and then added to meat at 12 ppm level. All samples were cooked at 85°C for 40 min and when cooled, their colour characteristics were examined.

Freshly prepared CCMP as well as a pigment sample which was washed twice with the AA solution were encapsulated forming a PCCMP, using N-LOK (National Starch and Chemical Corp., Bridgewater, NJ) as the wall material, and then applied to pork at a 50 ppm level. The preparation of PCCMP is described in section 3.5.3. The colour characteristics of meat mixtures were evaluated by their Hunter **L, a, b** values.

3.5.2 Storage of CCMP Under a Nitric Oxide Atmosphere

Pigment precipitates (100 mg) prepared from haemin and nitric oxide were washed with 3 x 20 mL of a 2% (w/v) AA solution and then transferred to amber-coloured ampules. The ampules were centrifuged at 3000 rpm (905xg) for 5 min, and the supernatants were discarded. Precipitates were then covered with 30 mL of the AA solution to which a slow stream of NO had been passed through for 15 min immediately prior. The ampules were frozen in liquid nitrogen and then sealed with a flame. Sealed CCMP tubes were opened after 3, 6 and 9 months of storage. The quality of the pigment was checked by monitoring its absorbance at 540 and 563 nm using a Shimadzu UV-260 spectrophotometer (Shimadzu, Kyoto, Japan). The absorption intensities at these wavelengths as well as any relative changes in the absorption spectra were noted.

CCMP stored during this period was also applied to comminuted pork at a 12 ppm level of addition in an effort to monitor its colouring potency. The CCMP precipitates were collected by centrifugation after decanting of the supernatant, as previously described. The colour characteristics of treated meats, after cooking, were evaluated by their Hunter **L, a, b** values.

3.5.3 Preparation of PCCMP from CCMP

A Büchi Mini Spray Dryer (Model 190, Büchi Laboratory-Techniques Limited, Flawil, Switzerland) was used for preparation of the PCCMP. Nitrogen was used as the spray flow gas to minimize contact between the preformed pigment and oxygen. Optimized spray drying conditions were: inlet ≥ 150°C; outlet 98°C; feed flow 5.5

$mL \cdot min^{-1}$ and nitrogen pressure 375 kPa(gauge).

An emulsion of the CCMP (50 mg) and encapsulating agents (3.28 g for a 1.5% (w/w) payload) was formulated prior to spray drying. The payload is a mass fraction of the pigment to be spray dried to the quantity of encapsulating agents and pigment used. The optimum payload determined was based on examination of Hunter **L**, **a**, **b** values of a typical set of PCCMP-treated samples compared to their CCMP-treated and nitrite-cured counterparts. A 1.5% (w/w) payload was employed for most experiments. The encapsulating agents (or wall materials) tested were N-LOK, β-cyclodextrin (Toyomenka America Inc., New York, NY), modified β-cyclodextrin (Lot RR3-HE Series, American Maize Products, Co., Hammond, IN), gum acacia (Aldrich) and Maltrin series M-040, M-100, M-150, M-200, M-250, M-500 and M-700 (Grain Processing Corp., Muscatine, IA). Wall materials were used individually or in combination and in some experiments, STPP, sodium acid pyrophosphate (SAPP, Albright and Wilson) and AP were added to the mixtures.

To prepare the emulsion, the wall materials were first dissolved or dispersed in water. Addition of a few drops of sodium hydroxide (Fisher) helped to increase the solubility of β-cyclodextrin as well as other wall materials. The pigment was then introduced into this mixture together with AA at a CCMP/AA ratio of 1:2 (w/w). The mixture was diluted with water generally to 3.5% (w/w) and in some cases to 10.0% (w/w) solids. Higher percentage solids under the conditions employed did not allow dissolution of CCMP in the mixtures. This solution was thoroughly stirred to ensure

uniform dispersion of the pigment. The vessel containing the emulsion was covered with parafilm and aluminum foil to minimize exposure of pigment to oxygen and light. The slurry was then spray dried under the operating conditions stated above, unless otherwise specified.

3.6 Quality Assessment of the CCMP and the PCCMP

3.6.1 Iron Analysis of BRBC

All glassware used for the iron analysis was washed in warm water with a metal-free non-ionic detergent (Acationox), rinsed with distilled water and placed in Nalgene bucks filled with a 3% (v/v) HNO_3 solution to soak for 24 h. After the acid wash, all glassware was rinsed with deionized water and then soaked in water for an additional 24 h. The cleaned glassware was dried in a forced-air Isotemp oven (300 Series, Model 338F, Fisher) and covered with parafilm when it had cooled.

Approximately 1.2 g of BRBC were accurately weighed into a 125-mL Erlenmeyer flask to which 20 mL each of concentrated HCl and HNO_3 were added. The mixture was gently heated on a hot plate at a low setting until frothing stopped. The acid solution was then heated at a medium setting until all solids were digested and NO_2/N_2O_4 gases expelled. Twenty mL of concentrated H_2SO_4 were added and the digestion proceeded until the solution was clear and appeared pale green in colour. After cooling, the acid digest was quantitatively transferred to a 50-mL volumetric flask and filled to mark with deionized water. A 1-mL aliquot was transferred to a 10-mL volumetric flask and further diluted. Sample absorbances due to the presence of iron were measured with a Perkin-

Elmer 2380 atomic absorption spectrophotometer (Perkin-Elmer Corp., Montréal, PQ). A calibration curve of absorbance against iron concentration was constructed using an iron standard (1000 ppm concentrate, Fisher) at concentrations of 2, 3, 4 and 5 ppm since Beer's law is only linear over this range. A path length of 10 cm was used. The haem content in BRBC was determined assuming a total Fe:haem of 1.

3.6.2 Absorption Spectroscopy

3.6.2.1 Determination of Haemoprotein Content in Fresh Meats

The concentration of haemoprotein pigments in comminuted meats was determined by the method of Rickansrud and Henrickson (1967) with slight modifications. Haem pigments were extracted from 20 g portions of comminuted meat into 50 mL of a 0.001 M acetate buffer, pH 4.5, at 4°C. For MSSM, only 5 g sample portions were used. The mixture was homogenized using a Polytron homogenizer (Brinkmann Instruments (Canada) Limited, Mississauga, ON) for 2 min and, then centrifuged for 15 min at 3000 rpm (905xg). Further extraction of the haemoprotein pigments from the sediment was carried out using 50 mL of fresh buffer. Samples were again homogenized and centrifuged as described above. Supernatants were combined, filtered by gravity through filter paper (Whatman No. 3, Fisher) into a 100 mL volumetric flask which was then filled to mark. To 20 mL of the haem pigment solution, 1-mL aliquots of a 13.2 mM $K_3Fe(CN)_6$ and a 17.6 mM KCN solution (Fisher) were added forming a cyanomet-myoglobin/cyanomethaemoglobin derivative. The haem pigment concentration, expressed as Mb equivalents, was determined spectrophotometrically using an HP 8452A diode

array spectrophotometer (Hewlett-Packard (Canada) Ltd., Montréal, PQ) at a wavelength of 540 nm. The molar extinction coefficient of cyanomethaemoglobin, 11.3 mM per L, was used (Drabkin, 1950) and the molecular weight of Mb was assumed to be 17 000 mg per mmol.

3.6.2.2 Absorption Characteristics of CCMP

The CCMP synthesized from haemin and nitric oxide, or PCCMP from various preparations, was dissolved or extracted in a 4:1 (v/v) acetone/water solution. A few mg of ascorbic acid were added to extracts in an effort to prevent oxidative deterioration of the pigment. To avoid problems of turbidity arising from interference of insoluble wall materials, acetone/water extracts of PCCMP were filtered through Whatman No. 3 filter paper before spectral analysis . Absorption spectra of extracts were recorded in the visible range using either a Beckman DU-70 (Beckman Instruments (Canada) Ltd., Toronto, ON), Shimadzu UV-260 spectrophotometer or a HP 8452A diode array spectrophotometer.

Meat pigments from nitrite-cured, CCMP-treated, and PCCMP-treated comminuted pork, after cooking, were extracted into 4:1 (v/v) acetone/water according to the method of Hornsey (1956) and absorption spectra were recorded in the visible range.

3.6.3 Colorimetry

A Gardner colorimeter (Model XL-20 Tristimulus Colorimeter, Gardner Laboratory Inc., Bethesda, MD) was used to determine tristimulus colour parameters, namely Hunter **L** (lightness/darkness, 100 for white and 0 for black), **a** (red +; green, -) and **b** (yellow,

+; blue, -) values of treated cooked meat samples. A white ceramic tile with specifications **L** = 92.0, **a** = -1.1, and **b** = 0.7 was used to standardize the colorimeter. Reflectance measurements were made at 3 to 8 different locations on the meat surface. Data reduction of Hunter **a** and **b** values yielded hue [arctan (**b/a**)] and chroma [$(\mathbf{a}^2 + \mathbf{b}^2)^{1/2}$] parameters as an index to help to characterize the colour of the cooked treated meats.

3.6.4 Colour Stability of Treated Cooked Meat Samples

Comminuted meat samples treated with either CCMP, PCCMP or sodium nitrite were prepared as described earlier. Meat systems were transferred to 12 x 10 cm polyethylene bags, their contents evenly distributed and then vacuum packaged. The packaged samples were placed next to one another in rows of 2 on a table in a 4°C Foster walk-in refrigerator. A set of two 30-Watt fluorescent Daylite lamps with a distance of 5 cm between each bulb was placed 25 cm directly above the surface of packaged samples. Care was taken to ensure that the packages did not overlap one another. Samples were subjected to intense fluorescent lighting (375 lux) and were withdrawn after different storage times during an 18 h period for colour evaluation. Hunter **L**, **a**, **b**, chroma and hue angle values were determined.

3.6.5 Sensory Analyses of Frankfurter and Salami Products

Flavour of both wiener and salami products was evaluated sensorially under red light to eliminate any influence from colour differences. Evaluations were done after one day and 30 days of refrigerated storage at 4°C using a triangle test. A total of 12

panellists was used, including some from the meat industry.

3.6.6 Volatile N-Nitrosamines

3.6.6.1 Apparatus

A Varian gas chromatograph (GC, Model 2700) coupled to a thermal energy analyzer (TEA) detector (Thermo Electron Corp., Waltham, MA, Model 502) was used for the analysis of volatile N-nitrosamines. The GC conditions were as follows: column, 6 ft x 1/8 in (o.d.) Ni tubing packed with 20% Carbowax 20M and 2% NaOH on 80-100 mesh acid-washed Chromosorb P; carrier gas, Ar, 30 $mL \cdot min^{-1}$; GC oven temperature, 170°C; injection port temperature, 220°C; TEA furnace temperature, 450 °C; TEA vacuum chamber pressure 1 mm Hg; TEA cold trap, stainless steel trap, immersed to 1/3 its depth in liquid nitrogen; recorder, 1 mV span.

A Varian Mat mass spectrometer (MS, Model 311A) equipped with an electron-impact ionization source and coupled to a Varian Aerograph gas chromatograph (Model 1400) was used for MS measurements. The GC column was similar to that used for GC-TEA analysis. The MS was operated in the selective ion monitoring mode for the molecular ion of N-nitrosodimethylamine (NDMA). Operating conditions were: source temperature, 250°C; emission current, 2 mA; electron voltage, 71 eV. The GC was operated under isothermal (115°C) conditions.

3.6.6.2 Analysis of Volatile N-Nitrosamines

A 20-25 g portion of homogenized sample was analyzed by a low-temperature vacuum distillation method. A suspension of the sample in 200 mL of a 5% (w/v)

solution of $Ba(OH)_2$ together with 100 ng of N-nitrosodi-*n*-propylamine (NDPA) as internal standard, was distilled under vacuum at 45-50°C. For samples that foamed excessively during vacuum distillation, the $Ba(OH)_2$ solution was replaced with 190 mL of 1% (w/v) sulphamic acid and 10 mL of 1 N H_2SO_4. The aqueous distillate was made alkaline and then extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed successively with an acidic buffer (to remove amines) and then with dilute alkali. The organic layer was dried by passing it through a layer of anhydrous sodium sulphate, and then concentrated to 1.0 mL using macro and micro Kuderna-Danish concentrators as described by Sen *et al.* (1985). The concentration of volatile N-nitrosamines in the final extract was carried out by analyzing 6-8 µL of the extract by GC-TEA. This procedure allowed for detection as well as quantitation of NDMA.

A GC-MS methodology was used to confirm the identity of the detected NDMA. Prior to GC-MS confirmation, the extract obtained from the above step was mixed with 10 mL of anhydrous *n*-pentane (nitrosamine-free) and the mixture was passed through a short column (1 cm x 4 cm) of basic alumina containing 3% water (Sen, 1978). The column was washed with 25 mL of anhydrous *n*-pentane and the washings were discarded. The column was then eluted with 50 mL of CH_2Cl_2 and the eluate carefully concentrated to 0.5 mL using a Kuderna-Danish concentrator (macro and micro Snyder column). A 2-5 µL aliquot of the extract was used for GC-MS confirmation.

To ensure absence of contamination due to the presence of N-nitrosamines in reagents, a reagent blank was run as described above except no food sample was included.

Each bottle of CH_2Cl_2 was also tested for N-nitrosamine contamination before use.

3.7 Effect of Irradiation on Pork Systems Containing the CCMP

Pork meat slurries were prepared by the addition of 20% (w/w) distilled water and 550 ppm sodium ascorbate. Sodium nitrite, preformed CCMP, STPP and SAPP were added directly to meats at various levels as specified. The mixtures (triplicates of each treatment), were homogenized and then cooked in Mason jars at 85±2°C in a thermostated water bath for 40 min. After cooling to room temperature (~23°C), cooked meat samples from each jar were homogenized. They were then divided into 3 x 100 g batches and vacuum packaged in polyethylene pouches. One pouch of each triplicate treatment was kept as a control and the other two sets were irradiated. Samples were stored over dry ice and shipped to Atomic Energy of Canada Limited (AECL) at Pinawa, MB for radiation processing.

Prior to irradiation, each sample was thawed overnight at 4°C and placed in a 2-L beaker containing crushed ice. Samples were irradiated in a Gammacell 220 (AECL) at a dose rate of 0.180 $kGy \cdot min^{-1}$ for 28.0 or 55.6 min to obtain 5 and 10 kGy irradiation doses, respectively. The dose rate represents a measure of the total irradiation applied to samples, and not that which is absorbed. Therefore, to measure the absorbed dose, radiometric dye films (Far West Technology Inc., Goletta, CA) sealed in polyethylene bags were attached to samples during radiation processing. Absorbance of the exposed films was then read at 600 nm and the absorbed radiation dose was calculated from a

standard curve. The average dose absorbed by samples for the 5 and 10 kGy experiments was 4.21 (84%) and 8.84 kGy (88 %), respectively. The colour and flavour characteristics of all samples were then analyzed.

The colour of meat samples, both before and after irradiation, was determined using the colorimeter. The flavour and oxidative stability of treated meats were monitored by determining their TBA values, as will be described. All irradiation, TBA and Hunter colour determinations were replicated 3-5 times and means ± standard deviations were determined.

3.8 The TBA Test

The distillation method of Tarladgis *et al.* (1960), as described by Shahidi *et al.* (1987*a*) or as modified by Zipser and Watts (1962) was used in this study.

3.8.1 Effect of Nitrite and Sulphanilamide on Malonaldehyde Quantitation During the TBA Test: Aqueous Model Systems

A stock solution of the malonaldehyde precursor 1,1,3,3-tetramethoxypropane was prepared in water at a concentration equivalent to 0.220 mg malonaldehyde/mL. Aliquots of this solution between 0 and 1 mL were added to distilled water in a 500-mL round-bottom flask. The total volume was 97.5 mL, and to this solution 2.5 mL of 4 N HCl was added and the mixture was distilled for approximately 20 min until 50 mL of distillate was collected. A 5-mL aliquot of the distillate was added to 5 mL of a 0.02 M aqueous solution of TBA. This mixture was heated in a boiling water bath for 35 min to ensure maximum colour development. A blank consisting of 5 mL of distilled water

and 5 mL of the TBA reagent was prepared and treated similarly. Once cooled to room temperature, the absorbance of the coloured complex so obtained was measured against the blank at 532 nm using a Shimadzu UV-260 spectrophotometer.

In another set of experiments, 5 mg of sodium nitrite, equivalent to 50 ppm in the system, was added to each of the above solutions containing HCl and the procedure was repeated as previously described. In a third set of experiments, 2 mL of a 5 mg/mL (w/v) solution of sulphanilamide in 0.1 N HCl was added to the malonaldehyde precursor and enough water to give a total volume of 98.5 mL. This sulphanilamide level represents a 100 ppm concentration in the system. To this mixture 1.5 mL of 4 N HCl was added and the procedure was continued as described above. A fourth set of experiments was performed, similar to those in the third set but included the addition of 5 mg of sodium nitrite to each solution prior to distillation.

3.8.2 Model System for Fluorescence Study

An aqueous model system consisting of the malonaldehyde precursor (0.1 mM) and sulphanilamide (1 mM) was prepared in 0.1 M HCl. The mixture was heated for 30 min in a boiling water bath and then cooled to room temperature. The excitation and fluorescence spectra were recorded using a Perkin-Elmer LS-5 fluorescence spectrophotometer at a sensitivity setting of 35 and with both excitation and emission slit widths set at 5 nm.

3.8.3 Effect of Nitrite and Sulphanilamide on Malonaldehyde Quantitation During the TBA Test: Meat Model Systems

Ground pork (120 g) was mixed with 20% by weight of distilled water and then cooked at 85±2°C in a thermostated water bath for 40 min while stirring occasionally with a glass rod. To 10.0 g portions of the cooked meat, varying amounts of the malonaldehyde precursor (up to 0.5 mL of a stock solution equivalent to 0.2124 mg malonaldehyde/mL) were added and mixed thoroughly. These mixtures were distilled and a 5-mL aliquot of the distillate was reacted with the TBA reagent. The absorbance readings at 532 nm of the chromogenic complex formed were corrected based on the absorbance due to the endogenous malonaldehyde in meat. A typical procedure for the TBA test is described below.

Ten grams of cooked pork were weighed and transferred to a 500-mL round-bottom flask to which 97.5 mL distilled water, 2.5 mL 4 N HCl, a few drops of Antifoam A (Sigma), and several glass beads were added. The mixture was distilled and 50 mL of distillate was collected over a 20-min period. Five millilitres of the distillate were transferred to a plastic tube and then mixed with 5 mL of freshly prepared 0.02 M TBA reagent. The TBA reagent was prepared by adding the appropriate quantity of TBA (Sigma) to water followed by warming in a water bath to ensure its complete dissolution. Tubes containing the reaction mixtures were capped and immersed in a boiling water bath for 35 min for colour development. A blank consisting of 5 mL of distilled water and 5 mL of TBA reagent was prepared and treated similarly. After heating, samples were

cooled to room temperature, and the absorbance of the pink-coloured complex was measured against the blank at 532 nm using a Shimadzu UV-260 spectrophotometer.

In another set of experiments, ground pork was mixed with 20% distilled water and 150 ppm of sodium nitrite and then cooked as described previously. Aliquots of the malonaldehyde precursor stock solution were added to meat samples at levels given above. The distillation procedure was followed with or without addition of sulphanilamide to the meat systems. The absorbance readings at 532 nm of the TBA-malonaldehyde complex formed were corrected, based on the absorbance due to the endogenous malonaldehyde in meat. A typical procedure for the TBA test of cured meats which involves sulphanilamide addition is described below.

Ten grams of nitrite-cured pork were weighed and transferred to a 500 mL round-bottom flask to which 96.5 mL distilled water, 1.5 mL of 4 N HCl, 2 mL of a 0.5% (w/v) solution of sulphanilamide in 20% HCl (v/v) and aliquots of the malonaldehyde stock solution were added. The resultant mixture was thoroughly mixed and then distilled. An aliquot of the distillate was reacted with the TBA reagent as described above.

Finally, two sets of pork samples were cooked containing 0, 25, 50, 100, 150 and 200 ppm levels of sodium nitrite. The distillation procedure was performed as above with and without sulphanilamide addition. The absorbance readings at 532 nm were recorded.

3.8.4 Model Systems for Absorbance Study

Stock solutions of 1,1,3,3-tetramethoxypropane (1mM), sulphanilamide (10 mM) and TBA (10 mM) were prepared in 0.1 M HCl. One mL aliquots of the malonaldehyde

precursor solution were transferred to glass tubes, and 1 mL of TBA and sulphanilamide solutions were added either individually or in combination. Eight mL of 0.1 M HCl was then added to each tube to reach a volume of 10.0 mL. Systems were capped, heated in a boiling water bath for 30 min, and then cooled to room temperature. A 1-mL aliquot from each system was transferred to a clean tube containing 9.0 mL of 0.1 M HCl. Tubes were vortexed, and absorption spectra of the chromogenic complexes formed were recorded.

In a second set of experiments, a 1-mL aliquot of the sulphanilamide stock solution was added to the heated TBA-malonaldehyde model system and a 1-mL aliquot of the TBA stock solution was added to the heated sulphanilamide-malonaldehyde model system. All absorption spectra for these systems were recorded. Finally, a 1-mL aliquot of the TBA stock solution was transferred to a clean tube containing 1 mL of the sulphanilamide solution. Eight mL of 0.1 M HCl was added, the system was heated and its absorption spectrum recorded as described above.

3.9 Preparation of Adducts of Malonaldehyde with Sulphanilamide, TBA and Their Combination

3.9.1 Synthesis and Purification of the TBA-Malonaldehyde Adduct (TMT)

The TMT adduct was synthesized in a model system of 1,1,3,3-tetramethoxypropane and TBA in 1.45 M HCl solution as described by Sinnhuber *et al.* (1958) with slight modifications. 1,1,3,3-Tetramethoxypropane (6.25 mmol) and TBA (12.5 mmol) were transferred to a 500-mL boiling flask and 250 mL of a 1.45 M HCl

solution were added. A condenser was attached, and the mixture was refluxed for 90 min. After cooling to room temperature, the resultant coloured products were suction filtered on a fine sintered-glass funnel. They were washed with 100 mL of 0.6 M HCl, briefly with hot water, and subsequently with 20 mL of 95 % (v/v) ethanol, 100 mL of 1:1 (v/v) ethanol and diethyl ether (Fisher), and finally with 100 mL of diethyl ether. The dark purple crystals were dried on a watch glass in a vacuum oven at 60°C for 24 h.

To purify the product, 1.0 g of the finely ground crystals was boiled in 200 mL of 0.6 M HCl for 40 min, cooled to 60°C, and then suction filtered on a fine sintered-glass funnel as described by Sinnhuber *et al.* (1958). Crystals were washed with 100 mL of 0.6 M HCl, 25 mL of cold water, 25 mL of ethanol, and finally with 100 mL of diethyl ether. The dark purple crystals were again dried on a watch glass in a vacuum oven at 60°C for 24 h. The procedure of Sinnhuber *et al.* (1958) was used because a suitable solvent for classical recrystallization was not found.

3.9.2 Synthesis and Purification of the Sulphanilamide-Malonaldehyde Adduct (SMS)

The SMS adduct was synthesized in a similar manner as described for TMT except that sulphanilamide (12.5 mmol) was used in place of TBA. A 0.03 M HCl solution was used instead of a 1.45 M HCl solution for synthesis and purification of SMS, as it afforded higher yields.

3.9.3 Synthesis and Purification of the Sulphanilamide-Malonaldehyde-TBA Adduct (SMT)

The SMT adduct was synthesized and purified in a similar manner as described

for TMT except that the reaction flask contained 1,1,3,3-tetramethoxypropane, TBA and sulphanilamide in an equimolar (6.25 mmol) ratio.

3.9.4 Spectroscopic Analyses of Complexes

3.8.4.1 UV-VIS Spectroscopy

Ultraviolet-visible (UV-VIS) absorption characteristics of the adducts were monitored using a Hewlett-Packard 8452A photodiode array spectrophotometer. Solutions of TMT and SMT were prepared by transferring 10-30 mg of dried crystals to a 1 L volumetric flask, dissolving them in 10 mL of dimethyl sulphoxide (DMSO) and then filling the flask to mark with 0.1 M HCl. Crystals of SMS (10-30 mg) were dissolved directly in 1 L of 0.1 M HCl. Aliquots of these stock solution were transferred to a 100-mL volumetric flask and filled with 0.1 M HCl. The UV-VIS absorption characteristics of these solutions were compared to those of complexes formed from model systems, as described in section 3.8.4.

3.9.4.2 Infrared (IR) Spectroscopy

Infrared (IR) spectral data of each product in a potassium bromide disk were obtained using a Mattson Polaris Fourier transform IR spectrophotometer.

3.9.4.3 Nuclear Magnetic Resonance (NMR) Spectrometry

Nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz with a General Electric GN-300 spectrometer. ^{1}H and $^{13}C\{^{1}H\}$ NMR data were collected at room temperature in DMSO-d_6 or in DMSO-d_6/D_2O mixtures. Chemical shifts are reported relative to tetramethylsilane (TMS) used as an internal standard. In addition,

attached proton tests (APT) and two-dimensional heteronuclear correlation (HETCOR) NMR experiments were performed to further elucidate the chemical structure of the adducts.

3.9.4.4 Mass Spectrometry (MS)

All mass spectra were measured using an electron ionization (EI) mode at 70 eV with a VG 7070 HS Micromass double-focusing mass spectrometer.

3.10 Determination of Dominant Aldehydes of Cooked Pork Systems

A Perkin-Elmer 8500 gas chromatograph and HS-6 headspace sampler were used for analysis of cooked pork samples. A high polarity Supelco SP-2330 fused-silica capillary column (30 m x 0.25 mm ID, 0.20μm film, Supelco Canada Ltd., Oakville, ON) was used. Helium was the carrier gas employed at an inlet column pressure of 17.5 psi(gauge). A split ratio of 7:1 was used. The oven temperature was maintained at 50°C for 5 min and then programmed to increase to 115°C at 10°C·min^{-1}, to remain at 115°C there for 1 min, and then to increase to 200°C at 30°C·min^{-1}. The injector and flame ionization detector (FID) temperatures were 250°C.

For headspace (HS) analyses, 2.0 g portions of homogenized pork samples were transferred to 5-mL glass HS vials. The vials were capped with a teflon-lined silicon septum, crimped and then frozen at -60°C until used. To avoid heat shock after removal from storage, frozen vials were tempered at room temperature for 30 min and then preheated in the HS-6 magazine assembly at 90°C for an equilibrium time of 45 min.

Pressurization time of the HS vials was 5 s, and the volume of the vapour phase drawn was approximately 1.5 mL. Chromatogram peak areas were expressed as integrator count units. Individual volatile compounds were tentatively identified by comparing relative retention times of GC peaks with those of commercially available standards. Quantitative determination of dominant aldehydes was carried out using 2-heptanone, as an internal standard.

3.11 Statistical Analyses

Analysis of variance and Tukey's studentized range test (Snedecor and Cochran, 1980) were used to determine differences in mean values based on data collected from replications of various experiments. Significance was determined at a 95% level of probability.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Iron Analysis of BRBC

The calibration curve for the iron standards as measured by the atomic absorption spectrophotometer is presented in Figure 4.1. Based on absorbance readings from six samples and after dilution factors were taken into consideration, the %Fe in BRBC samples was determined to be 1014±8 ppm (See Appendix for sample calculations). This corresponds to a haem content of 1.18% in BRBC.

4.2 Direct Preparation of the CCMP from BRBC

The effects of concentration and type of reducing agent on the yield and purity of the preformed CCMP are presented in Table 4.1 (See section 3.1.2 for definition of yield and purity and the Appendix for sample calculations). The CCMP was not produced in the absence of reductants. Reducing agents, namely ascorbic acid (AA), erythorbic acid (EA) and/or ascorbyl palmitate (AP), maintained the iron atom of the iron-porphyrin complex (*i.e.* haemoglobin) in its ferrous state and hastened conversion of nitrite to nitric oxide.

Incorporation of AP (reductant) into the reaction mixture at a molar ratio of 5:1, or greater, of reductant to haem gave a low yield of CCMP. Ascorbic acid and EA were significantly ($P<0.05$) more effective reducing agents when used at a reductant to haem molar ratio of at least 10:1. The maximum yield of the pigment was 95% (Table 4.1). Addition of AP to systems already containing either AA or EA did not significantly ($P>0.05$) affect the yield of the pigment. However, in preliminary studies, addition of AP

Figure 4.1 Calibration curve used for iron analysis of bovine red blood cell samples.

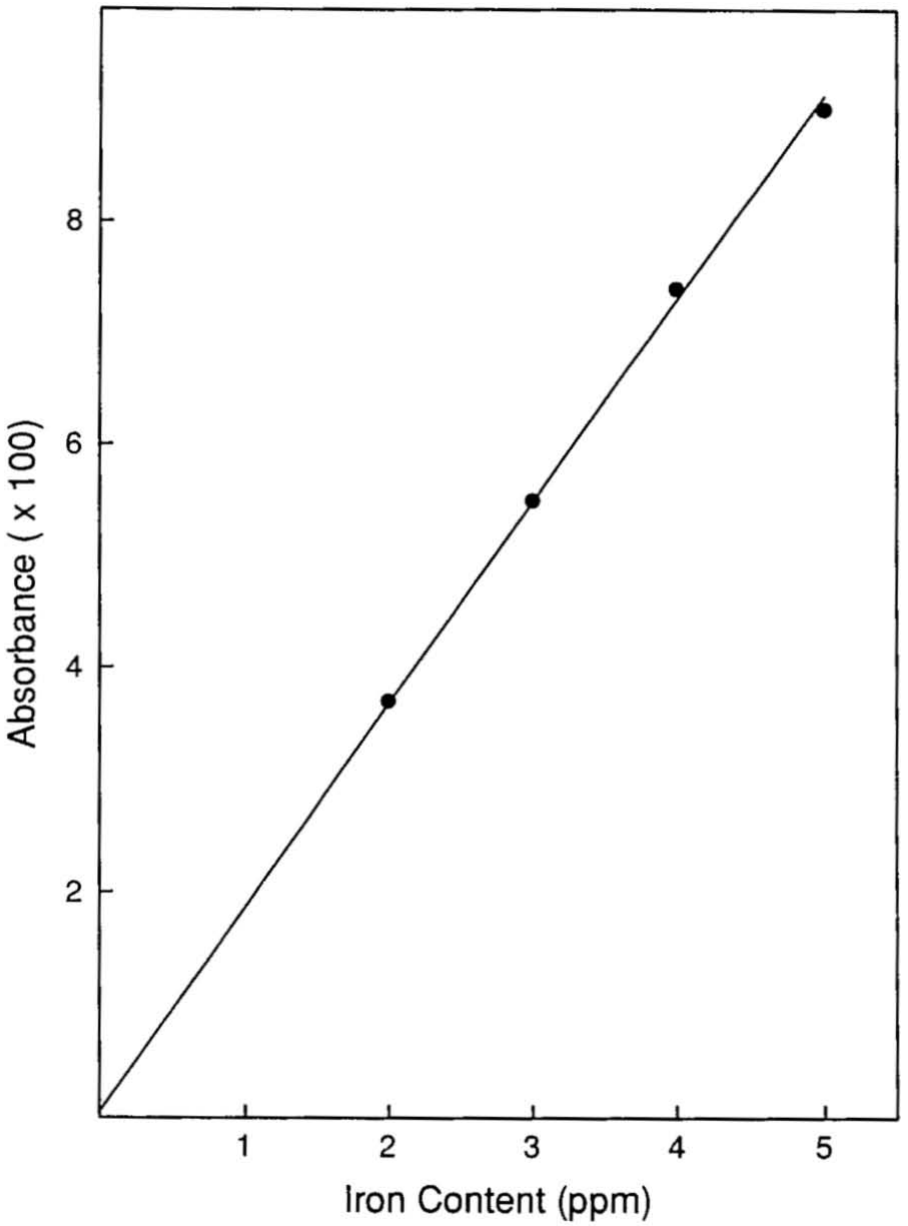

Absorbance (x 100)
2
4
6
8
1
2
3
4
5
Iron Content (ppm)

Table 4.1
Effect of various reducing agents on yield and purity of preformed cooked cured-meat pigment (CCMP).

Expt. No.	Treatment	[Reductant] / [Haem]	Yield[1] (%)	Purity[1] (%)
1	Ascorbyl Palmitate	5	51.9 ± 1.0^{c}	96.4 ± 1.6^{bc}
2	(1) + Ascorbic Acid	10	58.6 ± 8.7^{b}	97.8 ± 0.5^{abc}
3	(1) + Erythorbic Acid	10	44.8 ± 6.9^{c}	95.3 ± 1.0^{c}
4	Ascorbic Acid	10	94.0 ± 1.7^{a}	99.0 ± 0.6^{ab}
5	Erythorbic Acid	10	94.5 ± 2.0^{a}	99.0 ± 0.6^{ab}
6	Ascorbyl Palmitate	10	59.7 ± 2.4^{b}	97.1 ± 1.0^{abc}
7	(4) + (6)	20	94.9 ± 1.0^{a}	99.2 ± 1.0^{a}

[1]Results are means of three determinations ± standard deviation. Means sharing any of same letters in a column are not significantly different ($P>0.05$).

to the reaction mixture along with other reductants during the preparation of CCMP directly from BRBC was found to have a beneficial effect on the colour of treated pork samples after cooking (Shahidi, 1987). This may have resulted from stabilization of CCMP by AP during preparation and storage, possibly by a coating mechanism. At temperatures below 85°C, the yield of pigment was considerably less than those reported in Table 4.1. No significant difference ($P>0.05$) in pigment purity was evident by analysis of variance when reducing agents were used individually at a reductant to haem molar ratio of 10:1 or in combination at 20:1. A purity $\geq$ 98% was determined for the pigment in most cases.

In some preliminary experiments, use of sodium tripolyphosphate (STPP) in the reaction mixture for CCMP preparation was tested. The maximum yield was only 10.2%. Therefore the use of sodium hydroxide (NaOH) was examined. Its effect at different concentrations on yield and purity of pigment is presented in Table 4.2. Addition of NaOH resulted in a significant increase ($P<0.05$) in pigment yield but did not enhance its purity. The best yield, nearly 95%, was obtained using 0.2 M NaOH. The pigment purity was independent of base concentration over the range tested as indicated by analysis of variance.

Inorganic acids such as hydrochloric (0.1 M), phosphoric (0.2 M) and sulphuric (0.05 M) acids or organic acids such as acetic acid (0.1 M) or citric acid (0.1 M) were tested as acidifying agents. Pigment yields varied depending on the acid tested (Table 4.3). Citric and sulphuric acid usage in the precipitation step afforded pigment yields in

Table 4.2
Effect of sodium hydroxide concentration on yield and purity of preformed cooked cured-meat pigment (CCMP).[1]

NaOH [M]	Yield[2] (%)	Purity[2] (%)
0.0	66.1 ± 1.9[a]	98.8 ± 1.0[a]
0.1	83.5 ± 1.8[b]	99.4 ± 0.6[a]
0.2	94.9 ± 1.0[c]	99.2 ± 1.0[a]
0.3	88.6 ± 2.0[d]	97.0 ± 2.0[a]

[1]Reductants (ascorbic acid and ascorbyl palmitate) were present in the reaction at 1:1 (mol/mol) and a reductant to haem molar ratio of 20.
[2]Results are means of three determinations ± standard deviation. Means sharing any of same letters in a column are not significantly different (P>0.05).

Table 4.3
Effect of acidifying agents on yield and purity of cooked cured-meat pigment (CCMP).[1]

Acid	Concentration (M)	Yield[2] (%)	Purity[2] (%)
Citric	0.1	94.9 ± 1.0[a]	99.2 ± 1.0[a]
Acetic	0.1	65.1 ± 1.5[b]	96.5 ± 1.2[a]
Hydrochloric	0.1	87.5 ± 1.1[c]	96.5 ± 1.0[a]
Phosphoric	0.2	87.1 ± 1.0[c]	98.5 ± 1.0[a]
Sulphuric	0.05	91.4 ± 2.0[a]	97.2 ± 1.2[a]

[1]A reductant to haem molar ratio of 20 in 0.2 M NaOH solution was used. Other specifications were identical to those footnoted to Table 4.2.
[2]Results are means of three determinations ± standard deviation. Means sharing any of same letters in a column are not significantly different (P>0.05).

excess of 90%, but use of citric acid, a commonly used food acidulant/chelator, was favoured since the preformed CCMP would ultimately be applied to meat and meat products. The purities of precipitated CCMP were found to be independent of the acidulant employed (Table 4.3).

In order to determine the yield and purity of preformed CCMP, absorbance values of the pigment and its derivative, acid haematin, in a 4:1 (v/v) acetone:water mixture were measured at 540 and 640 nm, respectively, according to the method of Hornsey (1956). Their typical spectra are presented in Figure 4.2. The visible absorption spectrum of CCMP in acetone/water exhibited an absorption pattern characteristic of iron-porphyrin compounds with a red colour and had maxima at 563 (α), 540 (β) and 480 nm (Soret), $A_\beta/A_\alpha = 0.93$. Absorption characteristics of CCMP prepared from BRBC or from haemin (*i.e.*, before application to meat) were compared to those of pigments extracted from a nitrite-cured ham sample. After all pigments were dissolved/extracted into the acetone/water mixture, similar maxima were apparent in all cases. The visible absorption spectra of pigments extracted from cooked CCMP-treated pork systems were also qualitatively similar and had absorption maxima and shoulders at the same wavelengths as those of the nitrite-cured ham sample (Figure 4.3).

The CCMP, prepared directly from BRBC and after extraction/dissolution into 4:1 (v/v) acetone:water, deteriorated in the presence of light and air during a 6 h period as presented in Figure 4.4. Addition of small quantities of AA to the extracted pigment slowed the oxidation progression slightly. Similar findings for the CCMP prepared from

Figure 4.2 Absorption spectra of the preformed cooked cured-meat pigment in 4:1 (v/v) acetone:water, ——; acid haematin, — - —·.

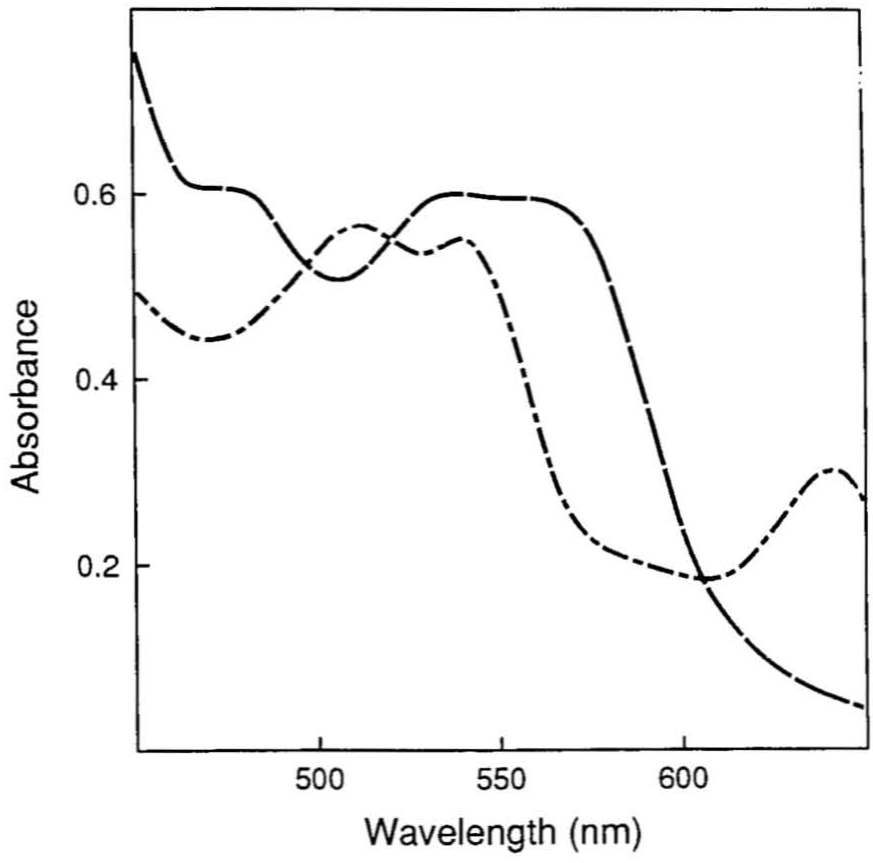

Absorbance
0.6
0.4
0.2
500
550
600
Wavelength (nm)

Figure 4.3 Absorption spectra of the preformed cooked cured-meat pigment (CCMP) from bovine red blood cells/sodium nitrite in 4:1 (v/v) acetone:water, - - - - - ; CCMP prepared from haemin/nitric oxide, —— - ; pigments extracted from nitrite-cured ham, ····· ; and pigments extracted from CCMP-treated pork, —— .

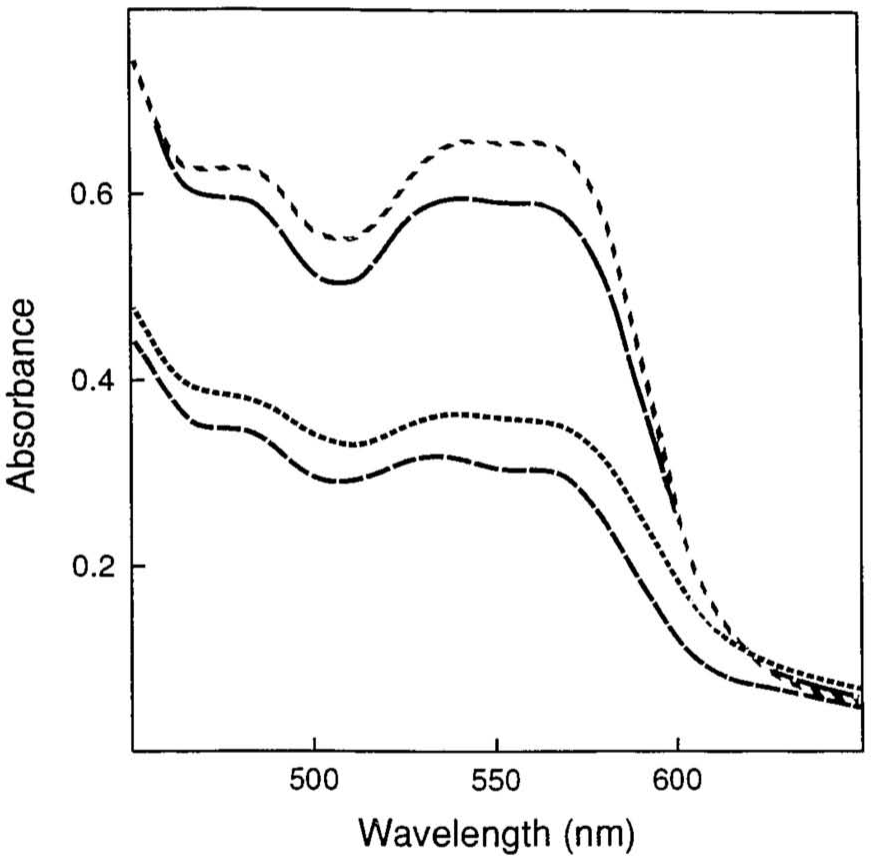
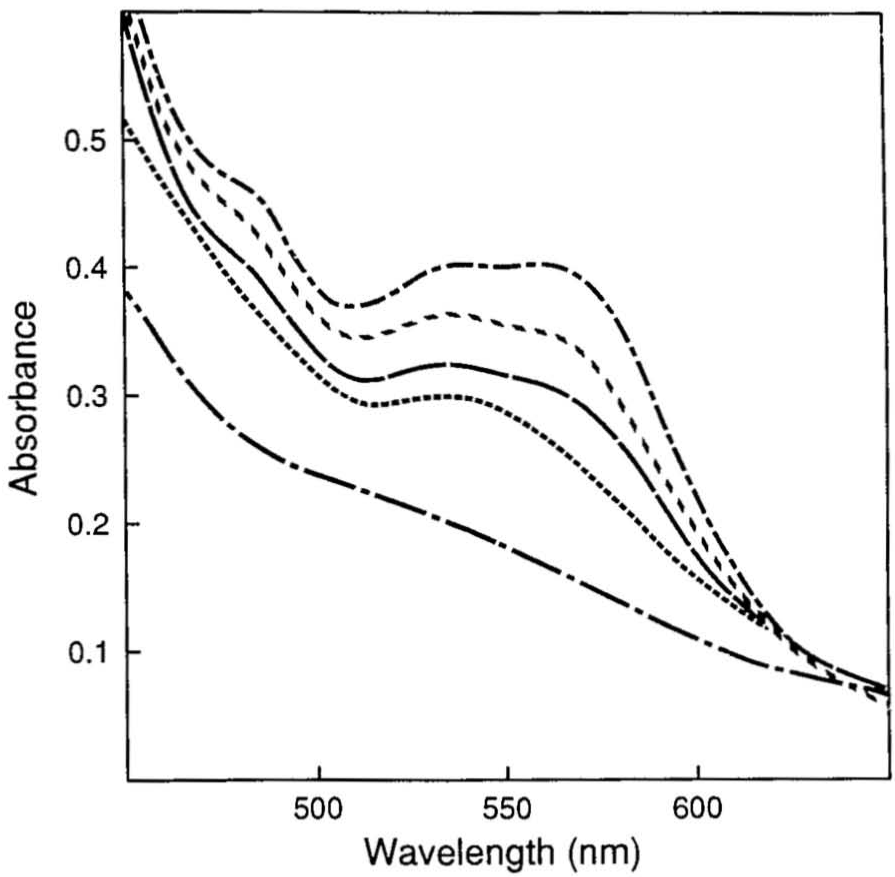

Absorbance
0.6
0.4
0.2
500
550
600
Wavelength (nm)

Figure 4.4 Progressive deterioration of the preformed cooked cured-meat pigment in 4:1 acetone:water: fresh, —·—·— ; partially decomposed, -·-·-·-, —·——·— , and ·····; and fully decomposed, ·——· .


Absorbance
0.5
0.4
0.3
0.2
0.1
500
550
600
Wavelength (nm)

haemin-NO synthesis, referred to as dinitrosyl ferrohemochrome, are reported by Shahidi *et al.* (1985*b*).

4.3 Application of CCMP to Comminuted Meats

4.3.1 Colour Characteristics of Nitrite-, CCMP- and PP-IX-Treated Pork

The colour characteristics of CCMP-treated ground pork, after cooking, were examined and compared to those of uncured and nitrite-cured samples by their Hunter **L**, **a**, **b** colour values (Figure 4.5). Addition of nitrite to fresh comminuted pork oxidized the haem iron to the ferric state and produced the brown-coloured nitrosylmetmyoglobin. Upon cooking, the bright pink colour, typical of nitrosylmyochromogen or the CCMP, was produced. No significant ($P>0.05$) differences in the Hunter **L** and **b** values of cured pork using sodium nitrite addition levels ranging between 25-156 ppm were evident, but a significant ($P<0.05$) increase in the Hunter **a** values was observed as greater nitrite levels were employed.

Addition of the preformed CCMP to comminuted pork at 3-30 ppm levels produced, upon cooking, a pink colour which was visually similar to that of nitrite-treated pork systems as judged by the experimenter. Although various levels of CCMP were employed, Hunter **a** and hue angle values of CCMP-treated pork samples at a 12-18 ppm level were not statistically ($P<0.05$) different from those of the cured control containing 156 ppm of sodium nitrite (Table 4.4). As was the case for nitrite-cured meats, Hunter **b** values of pigment-treated pork samples were not significantly ($P>0.05$) different from

Figure 4.5 Hunter **L**, **a**, **b** values of cooked ground pork treated with the cooked cured-meat pigment (CCMP), ● ; and sodium nitrite, ▲ .

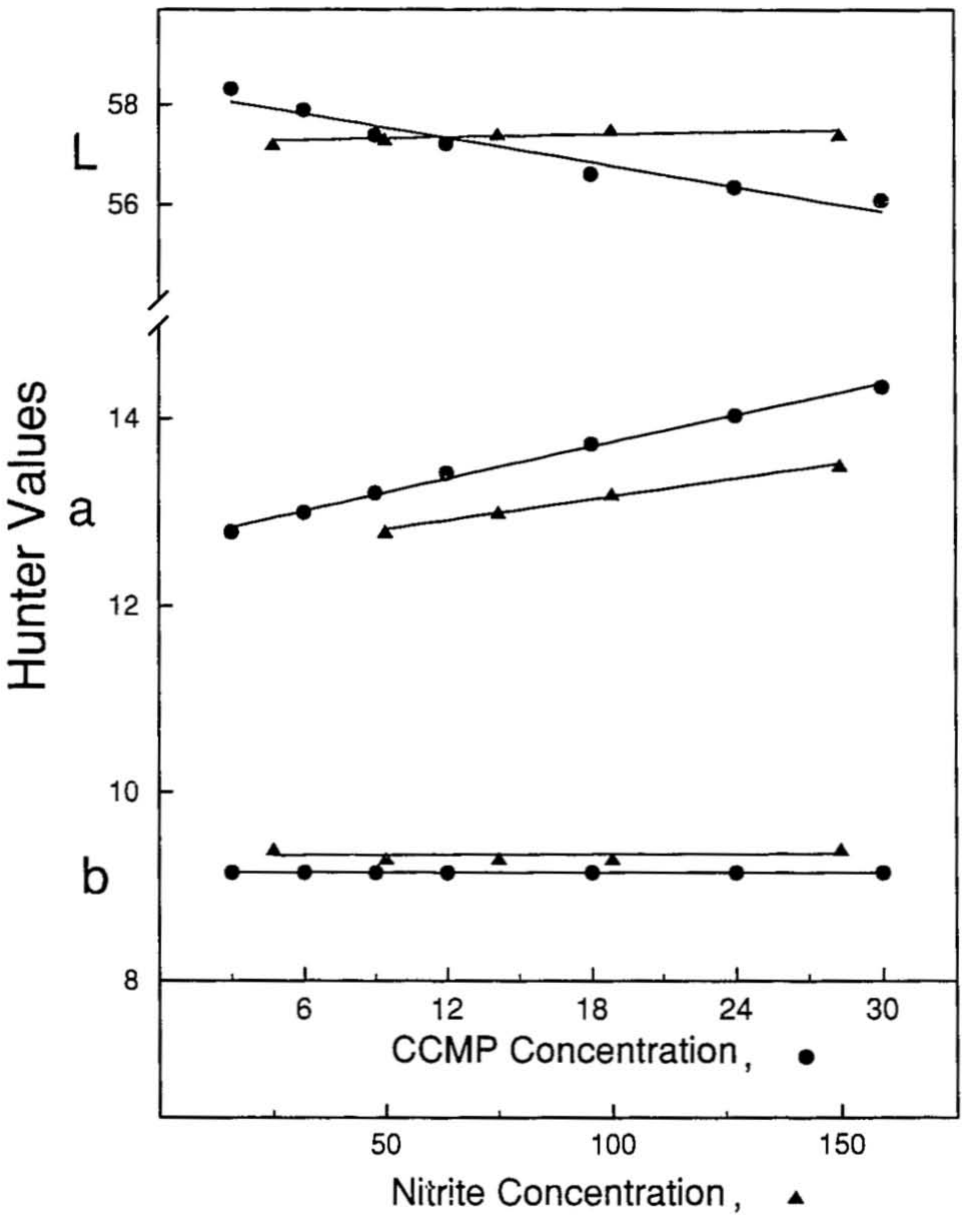

Hunter Values
L
a
b
58
56
14
12
10
8
6
12
18
24
30
CCMP Concentration, ●
50
100
150
Nitrite Concentration, ▲

Table 4.4
Dependence of Hunter colour values of meat on concentration of cooked cured-meat pigment (CCMP).[1]

CCMP (ppm)	Hunter Values[2]			Hue Angle ($tan^{-1}b/a$)
	L	a	b	
$NaNO_2$, 156 ppm	57.8 ± 0.2^{abc}	13.4 ± 0.2^{bcd}	9.2 ± 0.1^{b}	34.5 ± 0.5^{cd}
0	58.2 ± 0.5^{ab}	4.8 ± 0.1^{g}	11.9 ± 0.1^{a}	68.0 ± 0.4^{a}
3	58.4 ± 0.5^{a}	12.6 ± 0.2^{f}	9.1 ± 0.1^{b}	35.8 ± 0.5^{b}
6	57.9 ± 0.2^{abc}	12.8 ± 0.1^{ef}	9.1 ± 0.1^{b}	35.4 ± 0.4^{bc}
9	57.3 ± 0.3^{bcd}	13.0 ± 0.2^{def}	9.1 ± 0.1^{b}	35.2 ± 0.5^{bc}
12	57.1 ± 0.2^{cd}	13.2 ± 0.1^{cde}	9.1 ± 0.1^{b}	34.6 ± 0.3^{cd}
18	56.4 ± 0.2^{de}	13.5 ± 0.1^{bc}	9.2 ± 0.1^{b}	34.3 ± 0.3^{cd}
24	56.1 ± 0.4^{e}	13.8 ± 0.2^{ab}	9.1 ± 0.1^{b}	33.6 ± 0.4^{de}
30	55.8 ± 0.3^{e}	14.1 ± 0.2^{a}	9.1 ± 0.1^{b}	32.8 ± 0.4^{e}

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate.
[2]Results are means of three determinations ± standard deviation. Means sharing the same letters in a column are not significantly different ($P>0.05$) from one another as determined by Tukey's test.

one another. As the CCMP addition level increased, a corresponding decrease in Hunter **L** values was noted unlike their nitrite-cured counterparts, thus, denoting slightly darker products. Furthermore, as the concentration of CCMP increased, a significant ($P<0.05$) increase in Hunter **a** values and a decrease in hue angle values were observed, thereby, indicating a more intense pinkish colour in the products. This was presumably a consequence of increasing concentrations of nitrosylprotoporphyrin material in the products.

In order to illustrate the importance of iron in the porphyrin matrix for proper colour development of nitrite-free cured meats, protoporphyrin-IX (PP-IX) was added to comminuted pork. Smith and Burge (1987) had suggested possible use of PP-IX as a natural colorant for nitrite-free curing of meats. Addition of PP-IX to freshly comminuted pork at 60, 100, 150 and 250 ppm levels, imparted a purple-red colour to products prior to thermal processing. Upon cooking, PP-IX-treated meats turned dark brown as opposed to the typical pink colour of nitrite-cured meat. Although a significant ($P<0.05$) increase in Hunter **a** values of PP-IX-treated meats was observed, these samples were visually similar to that of cooked uncured meat rather than nitrite-cured or CCMP-treated meats. Meats treated with increasing levels of PP-IX also became significantly ($P<0.05$) darker in appearance as indicated by decreasing **L** values (Table 4.5) and were visually unappealing as judged by the experimenter. Use of PP-IX in comminuted meat systems does not mimic the pink colour imparted to meats by nitrite or the preformed CCMP upon thermal processing.

Table 4.5
Hunter **L**, **a**, **b** values of cooked cured-meat pigment- and protoporphyrin-IX-treated cooked ground pork.[1]

Treatment (ppm)	Hunter Values[2]			Hue Angle ($tan^{-1}b/a$)
	L	**a**	**b**	
No additives, 0	59.1 ± 0.2^{a}	5.6 ± 0.2^{e}	11.8 ± 0.1^{a}	64.6 ± 0.7^{a}
$NaNO_2$, 156	58.2 ± 0.4^{ab}	13.3 ± 0.3^{a}	8.6 ± 0.2^{c}	32.9 ± 0.7^{e}
CCMP, 12	57.4 ± 0.6^{b}	13.2 ± 0.2^{a}	8.7 ± 0.2^{c}	33.4 ± 0.6^{e}
CCMP, 18	57.1 ± 0.6^{b}	13.5 ± 0.3^{a}	8.5 ± 0.2^{c}	32.2 ± 0.7^{e}
PP-IX, 60	52.1 ± 0.4^{c}	6.8 ± 0.2^{d}	9.4 ± 0.2^{b}	54.1 ± 0.8^{b}
PP-IX, 100	49.1 ± 0.2^{d}	7.1 ± 0.2^{cd}	9.3 ± 0.1^{b}	52.6 ± 0.7^{b}
PP-IX, 150	46.2 ± 0.6^{e}	7.6 ± 0.2^{bc}	7.6 ± 0.1^{d}	45.0 ± 0.7^{c}
PP-IX, 250	43.2 ± 0.2^{f}	7.8 ± 0.2^{b}	6.2 ± 0.1^{e}	38.5 ± 0.7^{d}

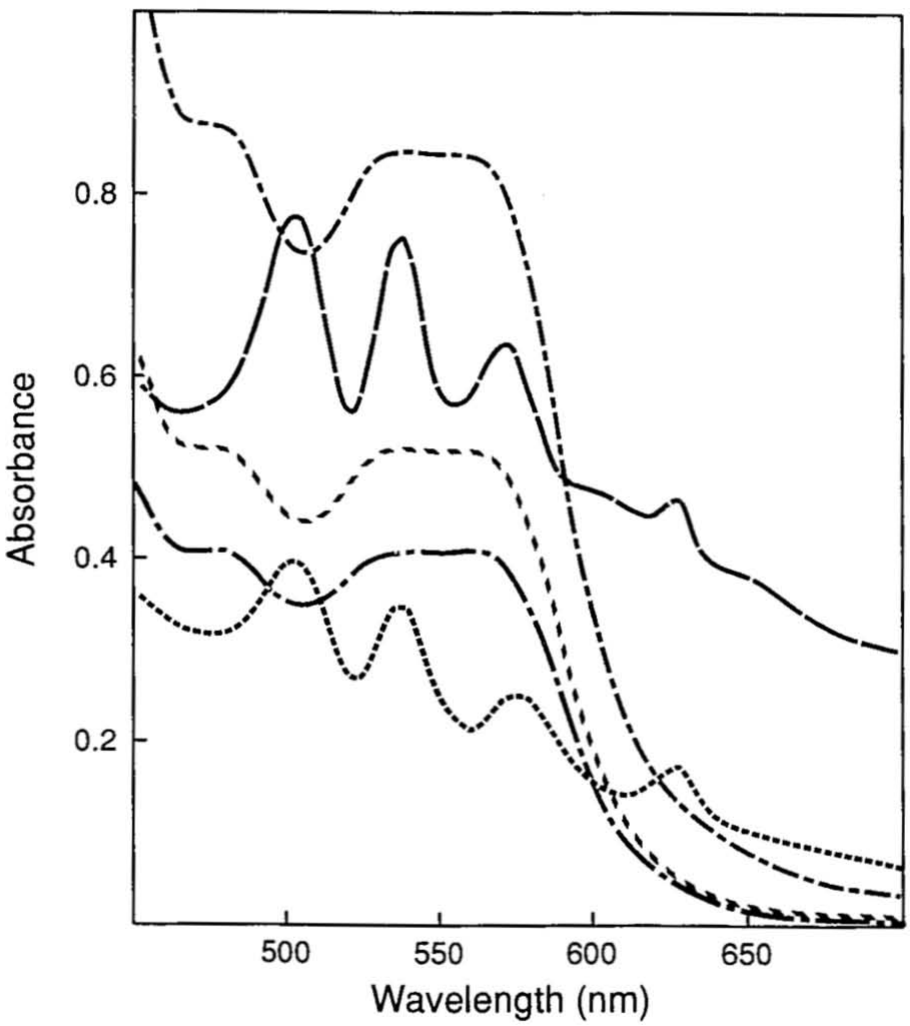
[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP -- cooked cured-meat pigment; PP-IX -- protoporphyrin-IX.
[2]Results are means of three determinations ± standard deviation. Means sharing the same letters in a column are not significantly different ($P>0.05$) from one another as determined by Tukey's test.

Further verification for this observation is obtained from visible spectroscopy of the pigments employed. The absorption spectrum of CCMP in a 4:1 (v/v) acetone:water solution was markedly different from that of PP-IX, but the spectrum of PP-IX was similar to the spectrum of pigments extracted from PP-IX-treated meats, after cooking (Figure 4.6). As expected, the absence of iron in the conjugated PP-IX precluded the development of the characteristic cured-meat colour in the final products (Giddings, 1977).

4.3.2 Sensory Evaluation of CCMP-Treated Frankfurter and Salami Products

The flavour of both frankfurter and salami products prepared in a pilot-scale study at Maple Leaf Foods was similar to their nitrite-cured counterparts. The panel members were unable to differentiate between the two sets of products. However, with respect to colour, panel members were able to differentiate between the two sets of samples under bright daylight. Generally nitrite-free samples had a slightly darker colour after 30 days which might be due to the presence of larger amounts of haem pigments in these products. O'Boyle *et al.* (1990) have also reported that pigment-treated wieners containing all-pork, pork with 10% beef, and all-chicken were somewhat darker and more red than their nitrite-cured counterparts. Adjusting the level of CCMP added to meats can easily control the degree of redness imparted by the pigment. Flavour scores, reported by a 15 member panel using triangle tests, showed that significant ($P<0.05$) differences were evident after one and two weeks of storage, but no significant flavour preference

Figure 4.6 Absorption spectra of preformed cooked cured-meat pigment (CCMP) in 4:1 (v/v) acetone:water, — - — - ; protoporphyrin-IX (PP-IX) in 4:1 (v/v) acetone:water, ——— - ; pigments extracted from CCMP-treated cooked pork, - - - - ; pigments extracted from nitrite-cured ham, - ——— - ; and pigments extracted from PP-IX-treated cooked pork, ---------- .


Absorbance
0.8
0.6
0.4
0.2
500
550
600
650
Wavelength (nm)

was ever perceived between test and control wieners. The flavour-difference comments were inconsistent and no specific flavour note could be pinpointed.

4.3.3 Influence of Native Haemoprotein Content on Colour Characteristics of Nitrite-Cured and CCMP-Treated Meats of Various Species

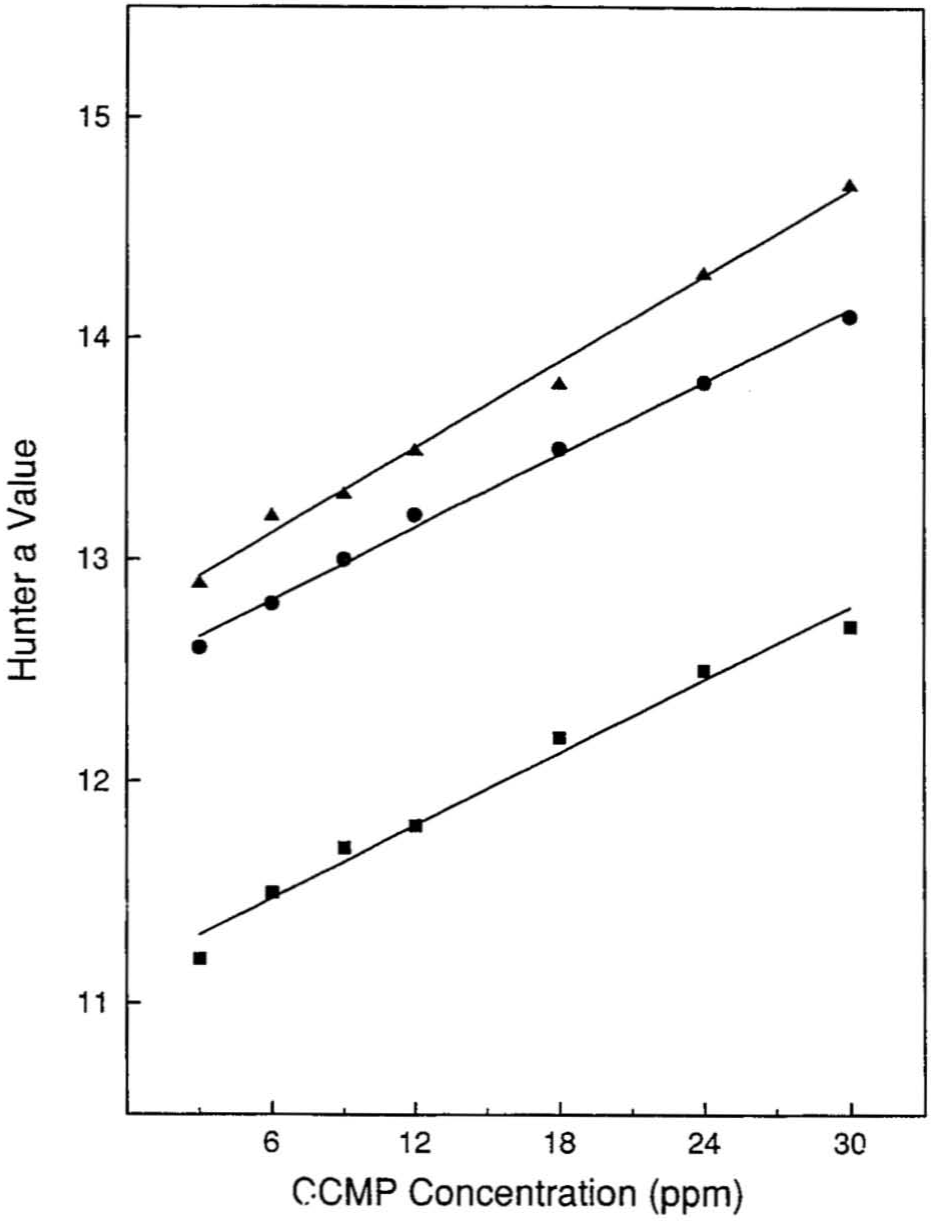
The colour intensity of CCMP-treated meats depended greatly on the initial myoglobin content of samples. The content of native haemoproteins in very pale, typical and dark-coloured pork muscles was determined to be 0.76, 1.22 and 1.76 mg myoglobin equivalents per gram wet tissue (*i.e.*, mg Mb eq/g tissue), respectively. Lawrie (1979) reported that the average content of Mb in pork muscles is only 0.6 mg Mb/g tissue, but in this study, a value of 0.76 mg Mb eq/g tissue was determined in the palest muscle tissues. Of the three types of pork examined, the one containing 1.76 mg Mb eq/g tissue exhibited after nitrite curing Hunter **a** values approximately 1 and 3.5 units higher than pork originally containing 1.22 and 0.76 mg Mb eq/g tissue, respectively (Table 4.6). Furthermore, hue angle values increased by 1.5 and 10 degrees, respectively, as the Mb concentrations in the samples decreased. Addition of different levels of CCMP to meats resulted in a linear increase in Hunter **a** values (Figure 4.7), but the final colour of CCMP-treated pork systems depended on the native Mb content (Pegg and Shahidi, 1990). For example, addition of the preformed CCMP at a 12 ppm level to the pork systems resulted in Hunter **a** values which increased by approximately 1.5 and 2.5 units as the Mb concentration increased from 0.76 to 1.76 mg Mb eq/g tissue. Hue angle values of CCMP-treated pork samples decreased as their nitrite-cured counterparts had, but only by

Table 4.6
Dependence of Hunter colour values of cooked ground pork systems on their myoglobin content.[1]

Myoglobin mg/g	Nitrite-Cured (156 ppm)			Pigment-Treated (12 ppm)		
	L	a	Hue Angle	L	a	Hue Angle
0.76 ± 0.02	64.2 ± 0.3	10.8 ± 0.2	43.1 ± 0.7	63.3 ± 0.4	11.8 ± 0.2	39.4 ± 0.7
1.22 ± 0.06	57.8 ± 0.5	13.4 ± 0.2	34.5 ± 0.5	57.1 ± 0.2	13.2 ± 0.2	34.6 ± 0.5
1.76 ± 0.06	56.7 ± 0.7	14.2 ± 0.4	33.0 ± 0.8	55.2 ± 0.3	14.2 ± 0.3	33.5 ± 0.7

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate to which either sodium nitrite or the cooked cured-meat pigment was added. Myoglobin (Mb) content was determined according to Rickansrud and Henrickson (1967) and its content is reported as mg Mb equivalents/g tissue.

Figure 4.7 Effect of concentration of cooked cured-meat pigment (CCMP) on the Hunter **a** values of cooked pork systems containing myoglobin (Mb) contents of 0.76, ■ ; 1.22, ● ; and 1.76, ▲ mg Mb equivalents/g tissue.


15
14
13
12
11
Hunter a Value
6
12
18
24
30
CCMP Concentration (ppm)

approximately 5 and 6 degrees, respectively. In order to attain a particular Hunter **a** value or hue angle measurement, the level of CCMP addition has to therefore be adjusted based on the Mb concentration of the muscle tissue used. Because the edible flesh of animal tissue may contain very low haemoprotein levels (such as those found in some fish species) to very high contents detected in seal or whale meat, the quantity of CCMP added will have to be adjusted in order to attain an attractive cured colour in the final product. Generally, it is expected that higher levels of CCMP will be required to impart optimum colour to meats containing high Mb contents.

The effect of sodium nitrite, as the reference curing agent, and CCMP on colour characteristics were compared with pork, beef, lamb, seal meat and seal surimi, mechanically deboned chicken meat and comminuted chicken breast meat, as well as cod surimi (Table 4.7). In each case, Hunter **L**, **a**, **b**, colour parameters, as well as chromaticity, hue angle and overall colour difference (ΔE) values obtained from Hunter data reduction, were determined. The ΔE value should be viewed with some scepticism since a wide variation in one of the Hunter parameters has a profound effect on the ΔE values between samples and the control. However, a large difference between the control and a test sample does not necessarily indicate that the colour characteristics of the test sample are less appealing. Since use of ΔE values in colorimetric measurements of foods is commonplace, they have been used in this study, but caution must be exercised as they are never judged solely on their own merit.

A close scrutiny of data presented in Table 4.7 revealed the following trends: (a)

Table 4.7
Effect of sodium nitrite and preformed cooked cured-meat pigment (CCMP) on Hunter **L**, **a**, **b** values, chromacity, hue angle and total colour difference (ΔE) of treated pork, lamb, beef, seal, poultry and cod surimi samples.[1]

Additive	Hunter Values					
	L	**a**	**b**	Chroma	Hue Angle	ΔE
			PORK			
None	58.3 ± 0.3^{a}	4.9 ± 0.1^{a}	12.1 ± 0.2^{a}	13.1	68.0	7.7
$NaNO_2$ (156)	58.6 ± 0.1^{a}	12.1 ± 0.1^{b}	9.3 ± 0.1^{b}	15.3	37.6	Ref.
CCMP (8)	57.9 ± 0.3^{b}	12.2 ± 0.2^{b}	9.0 ± 0.2^{b}	15.2	36.4	0.8
CCMP (12)	56.7 ± 0.2^{bc}	12.9 ± 0.2^{c}	8.9 ± 0.1^{b}	15.7	34.6	2.1
CCMP (24)	56.3 ± 0.1^{c}	13.5 ± 0.2^{d}	8.9 ± 0.2^{b}	16.2	33.4	2.7
			LAMB			
None	53.8 ± 0.3^{a}	5.3 ± 0.2^{a}	11.8 ± 0.1^{a}	12.9	65.8	10.5
$NaNO_2$ (156)	53.6 ± 0.2^{a}	15.3 ± 0.4^{bc}	8.6 ± 0.1^{b}	17.6	29.3	Ref.
CCMP (12)	52.7 ± 0.3^{b}	14.5 ± 0.2^{b}	8.5 ± 0.2^{b}	16.8	30.4	1.2
CCMP (24)	52.2 ± 0.4^{bc}	14.9 ± 0.3^{bc}	8.3 ± 0.1^{b}	17.1	29.1	1.5
CCMP (30)	52.0 ± 0.3^{bc}	15.3 ± 0.4^{bc}	8.3 ± 0.1^{b}	17.4	28.5	1.6
CCMP (36)	51.6 ± 0.3^{c}	15.5 ± 0.2^{c}	8.3 ± 0.2^{b}	17.6	28.2	2.0
			BEEF			
None	49.2 ± 0.5^{a}	5.7 ± 0.2^{a}	11.2 ± 0.3^{a}	12.6	63.0	12.7
$NaNO_2$ (156)	48.0 ± 0.5^{b}	18.1 ± 0.2^{b}	8.7 ± 0.1^{b}	20.1	25.7	Ref.
CCMP (12)	47.7 ± 0.3^{bc}	13.5 ± 0.2^{c}	8.8 ± 0.2^{b}	16.1	33.1	4.6
CCMP (24)	46.8 ± 0.4^{cd}	15.8 ± 0.1^{d}	8.6 ± 0.1^{b}	18.0	28.6	2.6
CCMP (36)	45.9 ± 0.3^{d}	18.0 ± 0.3^{b}	8.6 ± 0.2^{b}	20.0	25.5	2.1

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Table 4.7
Effect of sodium nitrite and preformed cooked cured-meat pigment (CCMP) on Hunter L, a, b values, chromacity, hue angle and total colour difference (ΔE) of treated pork, lamb, beef, seal, poultry and cod surimi samples.[1]

Additive	Hunter Values					
	L	a	b	Chroma	Hue Angle	ΔE
			SEAL MEAT			
None	24.2 ± 0.2^{ab}	7.8 ± 0.1^{a}	8.0 ± 0.1^{a}	11.2	45.7	14.0
$NaNO_2$ (156)	24.1 ± 0.1^{b}	15.4 ± 0.2^{b}	7.5 ± 0.2^{b}	17.1	26.0	6.4
$NaNO_2$ (500)	24.1 ± 0.2^{b}	21.8 ± 0.5^{c}	7.8 ± 0.2^{ab}	23.2	14.7	Ref.
CCMP (12)	24.0 ± 0.1^{b}	12.5 ± 0.1^{d}	7.5 ± 0.2^{b}	14.6	31.0	9.3
CCMP (24)	23.8 ± 0.1^{b}	14.2 ± 0.1^{e}	7.4 ± 0.1^{b}	16.0	30.6	7.6
CCMP (36)	24.9 ± 0.5^{a}	19.8 ± 0.2^{f}	8.0 ± 0.1^{a}	21.4	22.0	2.2
CCMP (48)	23.8 ± 0.3^{b}	22.3 ± 0.3^{c}	8.0 ± 0.2^{a}	23.7	19.7	0.6
			SEAL SURIMI			
None	28.9 ± 0.2^{a}	6.3 ± 0.1^{a}	8.1 ± 0.1^{a}	10.3	52.1	8.1
$NaNO_2$ (156)	28.8 ± 0.2^{a}	14.4 ± 0.2^{b}	7.3 ± 0.1^{c}	16.2	26.9	Ref.
CCMP (12)	29.3 ± 0.3^{a}	14.6 ± 0.3^{b}	7.7 ± 0.1^{b}	16.5	27.8	0.7
CCMP (24)	29.0 ± 0.2^{a}	14.8 ± 0.2^{b}	7.5 ± 0.1^{bc}	16.6	26.9	0.5
CCMP (36)	30.0 ± 0.2^{b}	15.6 ± 0.3^{c}	8.0 ± 0.1^{a}	17.5	27.1	1.8
			CHICKEN BREAST MEAT			
None	75.8 ± 0.2^{a}	1.9 ± 0.1^{a}	12.7 ± 0.1^{a}	12.8	81.5	3.9
$NaNO_2$ (156)	74.6 ± 0.3^{b}	5.0 ± 0.1^{b}	10.6 ± 0.1^{b}	11.7	64.8	Ref.
CCMP (6)	73.9 ± 0.4^{c}	5.3 ± 0.2^{b}	9.7 ± 0.2^{c}	11.1	61.3	1.0
CCMP (12)	72.0 ± 0.5^{d}	6.4 ± 0.1^{c}	9.6 ± 0.3^{c}	11.5	56.3	3.1
CCMP (24)	69.1 ± 0.1^{e}	7.4 ± 0.1^{d}	9.6 ± 0.2^{c}	12.1	52.4	6.1

.....continued on next page

Table 4.7
Effect of sodium nitrite and preformed cooked cured-meat pigment (CCMP) on Hunter L, a, b values, chromacity, hue angle and total colour difference (ΔE) of treated pork, lamb, beef, seal, poultry and cod surimi samples.[1]

Additive	Hunter Values					
	L	a	b	Chroma	Hue Angle	ΔE
			MECHANICALLY DEBONED CHICKEN MEAT			
Control	51.2 ± 0.1[a]	4.6 ± 0.1[a]	13.8 ± 0.1[a]	14.6	71.6	7.7
$NaNO_2$ (156)	52.9 ± 0.1[b]	11.4 ± 0.2[bc]	10.7 ± 0.2[b]	15.6	43.2	Ref.
CCMP (6)	52.6 ± 0.1[b]	11.1 ± 0.2[b]	10.8 ± 0.1[b]	15.5	44.2	0.4
CCMP (12)	51.5 ± 0.3[a]	11.3 ± 0.1[bc]	10.7 ± 0.2[b]	15.6	43.4	1.4
CCMP (24)	51.1 ± 0.2[a]	11.8 ± 0.3[c]	10.6 ± 0.2[b]	15.9	41.9	1.8
			COD SURIMI			
None	70.9 ± 0.4[a]	-2.0 ± 0.1[a]	7.5 ± 0.2[a]	7.8	89.0	0.3
$NaNO_2$ (156)	71.2 ± 0.2[a]	-2.1 ± 0.1[a]	7.5 ± 0.2[a]	7.8	88.9	Ref.
CCMP (12)	63.6 ± 0.2[b]	2.7 ± 0.2[b]	9.2 ± 0.3[b]	9.6	73.6	9.2
CCMP (24)	57.1 ± 0.1[c]	5.7 ± 0.2[c]	9.2 ± 0.1[b]	10.8	58.2	16.2
	[58.3 ± 0.3][d]	[4.9 ± 0.1][d]	[12.1 ± 0.2][c]	[13.1]	[68.0]	[15.4]
CCMP (36)	55.4 ± 0.4[e]	8.0 ± 0.2[e]	8.8 ± 0.2[b]	11.9	47.7	18.8
CCMP (60)	48.3 ± 0.3[f]	8.8 ± 0.1[f]	9.2 ± 0.2[b]	12.7	46.3	25.4

[1]All systems contained 20% (w/w) distilled water and 550 ppm sodium ascorbate. Values in parantheses indicate ppm concentration of adjuncts used. Hue angle is defined as $\tan^{-1}(b/a)$. Results are mean values of six determinations ± standard deviation. Values in each column for each species with same symbols are not different (P>0.05). Hunter values in brackets are for uncured pork.

Hunter **a** values of cured samples of red meat species, seal and poultry increased significantly ($P<0.05$) as a result of curing with nitrite; a similar increase in chromaticity and a decrease in hue angle values were noted. (b) Addition of CCMP to all muscle foods (Table 4.7) resulted in an increase in Hunter **a** and a decrease in Hunter **L** values as compared to their uncured counterparts. A corresponding increase in chromaticity and a decrease in hue angle values were also noticed. (c) Observed changes in Hunter **L** and **a** values, chromaticity and hue angle values depended on the concentration of added CCMP. Generally an increase in the level of CCMP added gave a parallel increase in Hunter **a** and hue angle values and a decrease in Hunter **L** and chromaticity values. (d) Calculated ΔE values depended on the amount of added CCMP and type of muscle tested. (e) No pink cured pink colour could be duplicated in cod surimi and ΔE values increased with an increase in the concentration of added CCMP.

The above observations may be explained by consideration of the following points. An increase in Hunter **a** values as a consequence of nitrite curing occurs due to a reddening effect exerted by nitrite and a nitrosylhaemochromogen formed in the products, albeit to an extent depending on the Mb content in muscles. No pink colour was observed in nitrite-cured cod surimi after thermal processing. This was due to a lack of Mb in the muscle tissue, and will be explained in detail later in this section. Increase in Hunter **a** values could be achieved to some extent by the use of varying amounts of CCMP for muscles containing some residual haemoproteins. The amount of CCMP required for colour duplication of nitrite-cured samples depended primarily on the content

of Mb originally present in muscle tissues. Generally a larger amount of CCMP was required for darker meats such as that of seal (Table 4.8). The Hunter **L** values of CCMP-treated muscle tissues were lower as compared with those of their nitrite-cured counterparts and this decrease became more pronounced as the level of CCMP addition to muscles increased. This is not unexpected, since addition of CCMP enhances the total haem content of macerated tissues, thereby producing a darker colour in products. Effects of CCMP and nitrite concentrations on chromaticity and hue angle values of muscle tissues followed the expected trends and depended on their apparent colours (hue angle) and their intensities (chroma).

The ΔE values depended on species and type of muscle tissue examined. The ΔE values generally corresponded with differences in observed Hunter **a** values of muscles treated with CCMP and that of their nitrite-cured reference sample. A similar conclusion could also be drawn using differences in hue angle values calculated from data reduction. These differences in hue angle originated primarily from variations in Hunter **a** values since their corresponding **b** values were generally unaffected.

Lack of appearance of a cured colour in cod surimi as a result of nitrite curing was due to the absence of any detectable amount of haemoproteins in muscle tissues (Shahidi *et al.* 1990). Hunter **L**, **a**, **b** values and calculated chromaticity, hue angle and ΔE values of the cod surimi sample treated with 24 ppm of CCMP were very similar to those of uncured pork (Table 4.7). Absence of haemoproteins in cod surimi muscle tissues which might be needed for some sort of, yet to be defined, interactions with added

Table 4.8
Total haemoprotein pigment content of muscle foods and the amount of preformed cooked cured-meat pigment (CCMP) required to achieve a cured colour in products.[1]

Species	Total Pigments (mg Mb eq/g)	CCMP (ppm)
Pork	1.2	8.0
Lamb	2.1	12.0
Beef	4.5	36.0
Seal	59.0	48.0
Seal Surimi	19.3	24.0
Chicken Breast Meat	0.4	6.0
Mechanically Deboned Chicken Meat	1.0	12.0
Cod Surimi	0.0	0.0

[1]All systems contained 20% (w/w) distilled water and 550 ppm sodium ascorbate. Total pigment content determined according to the method of Rickansrud and Henrickson (1967) and is reported as mg myoglobin equivalents/g tissue.

CCMP could be responsible for this observation.

4.3.4 Colour Stability of Nitrite-Cured and CCMP-Treated Pork

The colour stability of pork systems containing different concentrations of the preformed CCMP (3, 12, and 24 ppm) was examined and compared with that of a 156 ppm nitrite-cured sample (Figure 4.8). Hunter **a** values of treated meats decreased rapidly during the first 6 h of intense fluorescent lighting. Visually, the colour changed from the typical pink colour of cooked cured-meat to a brown colour characteristic of cooked uncured pork. The final Hunter **a** values depended on the level of CCMP which had been added to the systems (Pegg and Shahidi, 1989). In all cases, a similar decreasing trend was apparent regardless of whether samples were treated with CCMP or nitrite, but meats containing 12 ppm CCMP most closely resembled the fading characteristics of the nitrite-cured control. A three-dimensional representation of the effect of CCMP as well as nitrite concentration on Hunter hue angles during storage under fluorescent lighting is presented in Figure 4.9. Both systems faded rapidly during the first 6 h and the final Hunter hue angle values of treated samples increased by 15 to 20 degrees. The rate of colour fading of these systems appeared to be similar. These results, as presented by Shahidi and Pegg (1990*a*) and Shahidi *et al.* (1990), tend to suggest that the colour stability of nitrite-cured or pigment-treated meats is not affected by the presence of residual nitrite.

Figure 4.8 Colour stability of pork meat treated and cooked with the preformed cooked cured-meat pigment at 3 ppm, ▬ ▬ ▬ ; 12 ppm, ▬ ▬ ▬ ; 30 ppm, ▬ ▬ ▬ ; and with sodium nitrite at 156 ppm, ▬ ▬ ▬ .

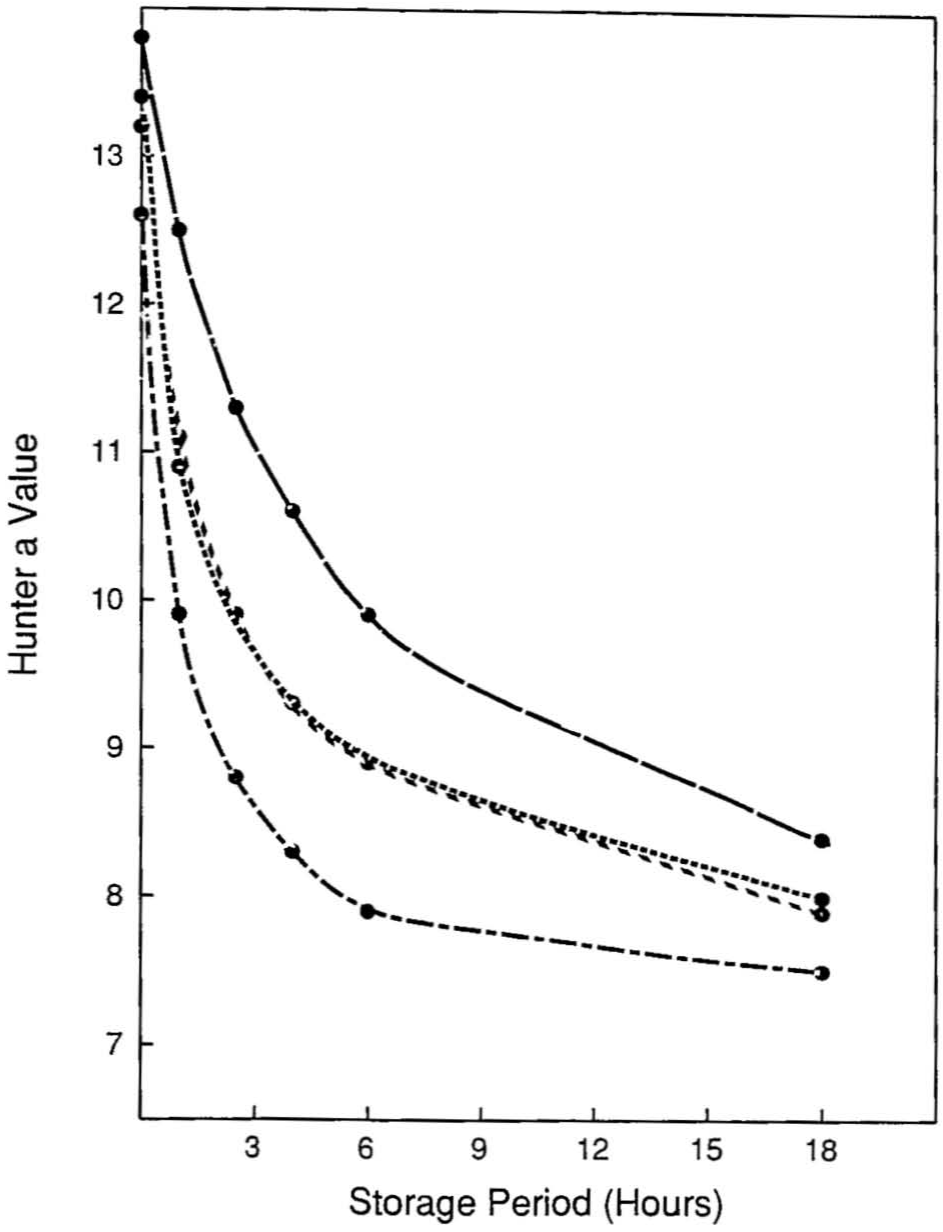
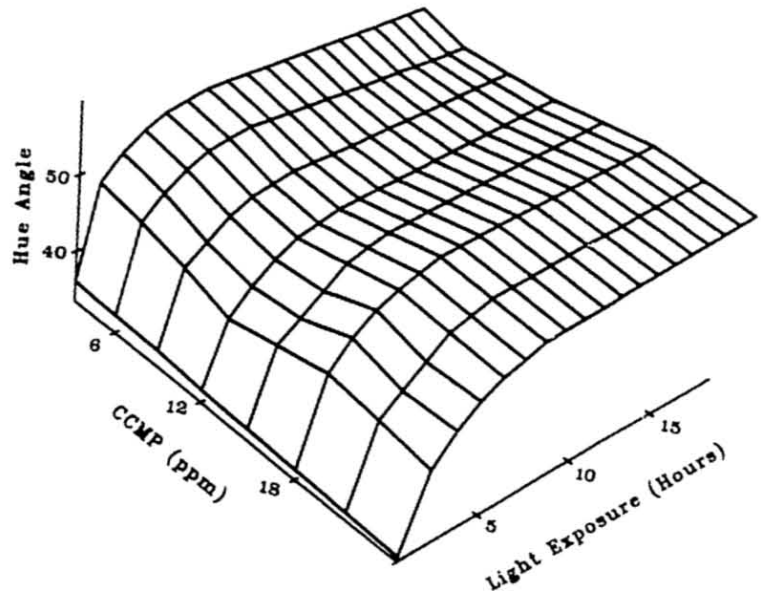
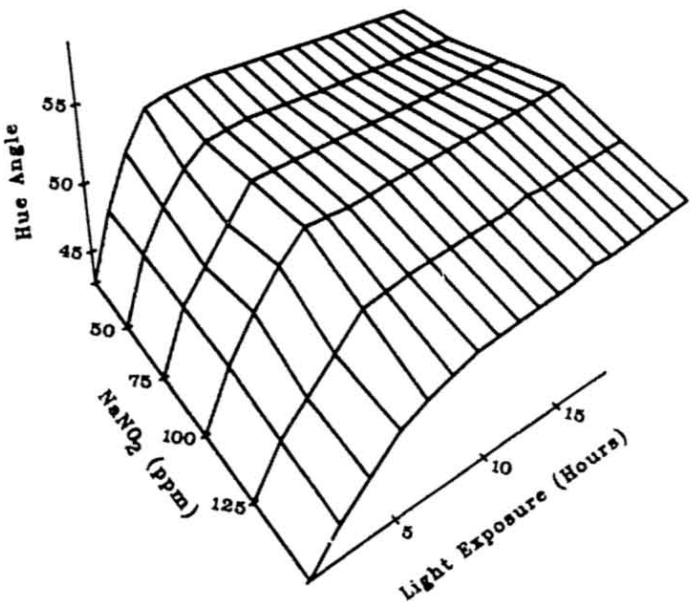

Hunter a Value
13
12
11
10
9
8
7
3
6
9
12
15
18
Storage Period (Hours)

Figure 4.9 Dependence of Hunter hue angle values of meats treated with varying concentrations of cooked cured-meat pigment (CCMP) or sodium nitrite during exposure to fluorescent lighting over an 18 h period.


Hue Angle
50
40
6
12
18
CCMP (ppm)
5
10
15
Light Exposure (Hours)


Hue Angle
55
50
45
50
75
100
125
$NaNO_2$ (ppm)
5
10
15
Light Exposure (Hours)

4.4 Application of CCMP to Solid Cuts of Pork

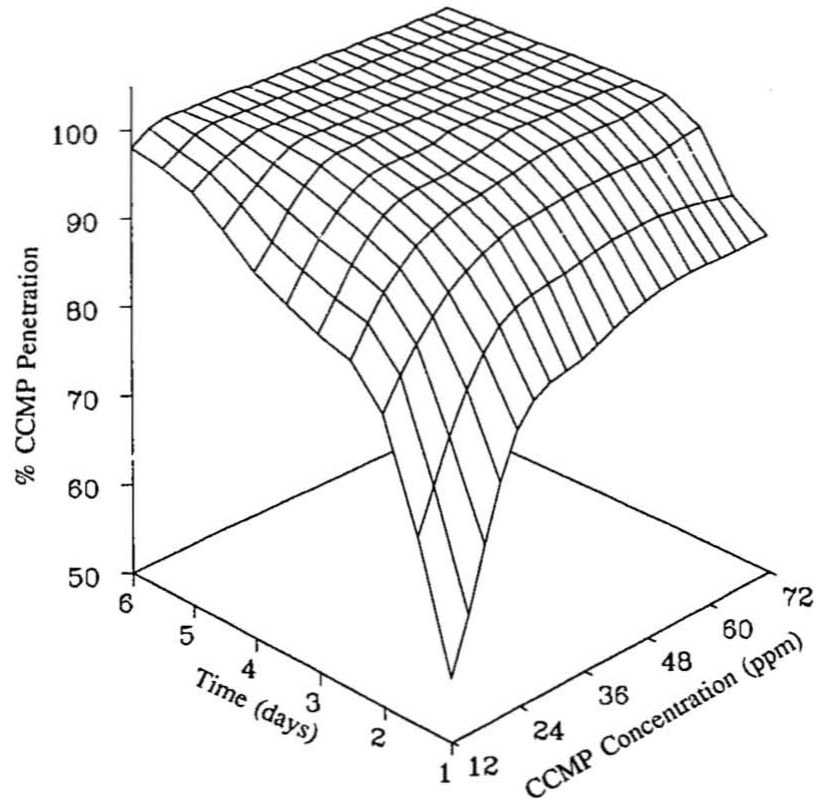
4.4.1 Preliminary Study

The preliminary study revealed that CCMP had successfully penetrated into the small pork cubes, by molecular diffusion, without need for pumping of the pickle into the pieces or tumbling of the cubes. A uniform pink colour with no evidence of red blotches was evident throughout the pieces. The CCMP-treated meat also had a more intense pink colour than its nitrite-cured counterpart which was also reflected in their Hunter **a** values. When CCMP-treated meat in the cellulose casing was sliced, a uniform colour was observed throughout as was the case for the nitrite-cured samples. These preliminary experiments suggest the potential for use of composite nitrite-free curing systems of small meat pieces for use in restructured-type products. These results disagree with those of O'Boyle *et al.* (1992) who reported that the preformed CCMP can not diffuse into the fibres of meat.

4.4.2 Effect of CCMP Concentration on the Extent of Diffusion

As larger concentrations of pigment were added to the pickle, less time was required for complete pigment penetration. A three dimensional diagram for the penetration of CCMP into pork *longissimus dorsi* muscles is presented in Figure 4.10. Of all pigment levels tested, only the systems containing 12 ppm did not show complete penetration into the meat after 6 days of marination. Only 98% penetration of CCMP into the muscle tissue was achieved. A penetration of ≥ 80% was evident after a 24 h period at 4°C in all systems containing ≥ 24 ppm of CCMP. For the highest pigment level

Figure 4.10 Effect of cooked cured-meat pigment (CCMP) concentration in pickle on the extent of CCMP penetration into excised *longissimus dorsi* muscles of pork held at 4°C for varying storage periods.


% CCMP Penetration
100
90
80
70
60
50
6
5
4
3
2
1
Time (days)
12
24
36
48
60
72
CCMP Concentration (ppm)

tested (72 ppm) complete penetration of CCMP into the product occurred after a 3 day period. These results show that below 24 ppm the concentration of CCMP used in the pickle has a marked effect on the length of time required for pigment to penetrate the muscle tissue.

The pink colour in the finished product was uniform with no red blotches suggesting that diffused CCMP does not precipitate prior to or during the heat processing. Hunter **a** values of CCMP-treated meats were within ± 1 unit of their nitrite-cured control. An attempt was made to quantitate the extent of pigment penetration across different cross-sectional depth of the interior of the meat. The method of Hornsey (1956) was used to extract CCMP from cooked muscle tissues for their subsequent quantitation, but no trend was evident across each section. This is not surprising because the pink colour of these meats was uniform. Chemical interactions between native myoglobin of the muscle tissue with CCMP, with respect to colour development during thermal processing, are unclear. Therefore, modelling of the diffusion behaviour is not feasible. During the early periods of pickling, heat processed CCMP-treated samples when sliced, revealed that fading of the pink colour can be viewed near the uncured brown region in which no penetration had occurred, but the region in which diffusion had occurred was visually uniform in colour. Although it was not feasible to study the penetration of CCMP into the meat, diffusion of sodium chloride into the interior of the meat was examined. Data revealed that a chloride ion gradient existed throughout the meat during the period of study (results not shown). Sodium chloride diffused faster into the meat than CCMP

which was expected due to the difference in molecular weight of these two additives. Again, contrary to the report of O'Boyle *et al.* (1992), who suggested that CCMP's molecular weight restricts its penetration into the fibres of meat, penetration occurred albeit at a slower rate.

4.4.3 Effect of Temperature on the Extent of Diffusion

The effect of temperature on the rate of CCMP penetration into excised *longissimus dorsi* muscles of pork was examined at 4, 10 and 18°C and is presented in Table 4.9. CCMP concentrations of 12 and 24 ppm were selected. For the system containing 12 ppm of CCMP, complete penetration was not achieved at higher temperatures. A possible explanation for this observation may be that CCMP is more sensitive to decomposition in the pickle at higher temperatures. Since an antibacterial agent such as sodium hypophosphite was not used in this composite nitrite-free curing system, the meats stored at 10 and 18°C spoiled after 5 and 4 days, respectively. A fermentative odour was noted suggesting that lactic-acid producing bacteria were involved. A substantial drop in the pH of the systems from 6.5 to 4.7 occurred progessively during pickling over 5 days due to the acid-producing bacteria. As the pH decreased, CCMP precipitated out of solution making it less available to penetrate into the meat.

4.5 Absence of N-Nitrosamines

Nitrite-free ingredients containing the preformed CCMP duplicated the colour,

Table 4.9
Effect of temperature on the rate of penetration of cooked cured-meat pigment (CCMP) into excised *longissimus dorsi* muscles of pork[1].

Days of Storage	% Pigment Penetration[2]					
	12 ppm CCMP			24 ppm CCMP		
	4°C	10°C	18°C	4°C	10°C	18°C
1	57	60	74	79	79	82
2	82	80	84	91	91	93
3	87	86	90	96	94	97
4	90	90	93	99	99	99
5	96	98	sp	100	100	sp
6	98	sp	--	--	--	--

[1]All cover pickles used contained 2.5% (w/v) sodium chloride, 0.5% (w/v) sodium tripolyphosphate, 0.1% (w/v) sodium ascorbate and the cooked cured-meat pigment (CCMP) at either 12 or 24 ppm level of addition. Meat to pickle ratio was 1:1 (w/v).
[2]Results are mean values of 2 determinations. All data points were within 5% of each other. sp - spoiled.

flavour and bacteriostatic effects of nitrite in meat model systems (Wood *et al.*, 1986; Shahidi *et al.*, 1987*a*; 1988; Shahidi and Pegg, 1990*a*; 1991*c*; 1992). Although use of these nitrite-free systems as an alternative to nitrite was proposed, the absence of carcinogenic N-nitrosamines in formulated products was not confirmed by the authors cited. The N-nitrosamines in some of the nitrite-cured samples were measured and compared with those in CCMP-treated material. Results indicated that only N-nitrosodimethylamine (NDMA) was present in some of the cooked nitrite-cured systems examined. Table 4.10 summarizes the content of volatile NDMA in cooked, nitrite-cured and pigment-treated pork, cod, and cod surimi. No measurable amount of NDMA was detected in the control, nitrite-cured or CCMP-treated pork systems, but in nitrite-cured pork-containing products trace quantities of NDMA have been reported (Sen *et al.*, 1979). No NDMA was detected in control fish systems. The occurrence of NDMA in nitrite-treated cod was expected since the formation of N-nitrosamines in cured fish has been shown to occur primarily in salt-water species (Sikorski and Kostuch, 1982). Only *ca.* 1 ppb of NDMA was detected which may reflect the very fresh nature and careful processing of the fish used in this study. The precursor of NDMA, dimethylamine (DMA), is formed in the muscles of fish as a result of activity of endogenous enzymes on trimethylamine N-oxide (TMAO). Perhaps only partial degradation of TMAO to DMA had occurred in the fish muscle tissue by the time of its use. Although NDMA was detected in nitrite-cured cod, CCMP-treated samples were devoid of it. This may imply that no disproportionation of CCMP had occurred or that it did not produce a sufficient

Table 4.10
Effects of nitrite and preformed cooked cured-meat pigment (CCMP) on the formation of N-nitrosodimethylamine (NDMA) in pork, cod or cod surimi systems.[1]

Muscle System	Treatment Mixture (ppm)	NDMA[2] (ppb)
Pork	No additive	ND
	$NaNO_2$, 156	ND
	CCMP, 12	ND
	CCMP, 24	ND
Cod	No additive	ND
	$NaNO_2$, 156	0.9
	CCMP, 12	ND
Cod Surimi	No additive	ND
	$NaNO_2$, 156	ND
	CCMP, 12	ND

[1]All meat systems were treated with 20% (w/w) distilled water and 550 ppm sodium ascorbate.
[2]ND - not detected. Detection threshold limit of TEA analyzer is 0.2 ppb.

quantity of nitric oxide to take part in possible nitrosylation reactions. The absence of N-nitrosamines in nitrite-cured cod surimi tends to suggest that washing of cod muscles offers an effective means to remove DMA, and its precursors from samples in order to avoid their nitrosylation.

The efficiency of the distillation and clean-up steps in determination of volatile N-nitrosamines, outlined in the experimental section, was routinely checked by spiking each sample with 10 ppb of the internal standard, N-nitrosodi-*n*-propylamine (NDPA), to ensure that possible loss of detectable N-nitrosamines did not occur. The recoveries of NDPA were found to vary in the range of 75-85%. Similar recovery studies were occasionally carried out using NDMA. Recoveries of 70-90% were highly satisfactory and compared favourably with those reported by other investigators (Stephany *et al.*, 1976). The detection limit of N-nitroso compounds for the TEA analyzer is 0.2 ppb. Although the GC-TEA technique is regarded as a reliable and specific method for determination of N-nitroso compounds in foods, other chemicals may interfere and give a false positive result, especially at low levels (Sen *et al.*, 1979). Because very low concentrations of NDMA were detected in this study, all results were confirmed by MS as unequivocal proof of the presence of NDMA.

The effect of added sodium nitrite and CCMP at 156 and 12 ppm levels, respectively, on NDMA formation in hybrid meat/fish systems is presented in Table 4.11. As expected, no NDMA was detected in the control samples, but addition of nitrite to hybrid pork systems containing 15 and 50% cod produced 0.3 and 1.0 ppb of NDMA,

Table 4.11
Effects of nitrite and cooked cured-meat pigment (CCMP) on the formation of N-nitrosodimethylamine (NDMA) in hybrid pork and cod or cod surimi systems.[1]

Muscle System	Treatment Mixture (ppm)	NDMA[2] (ppb)
Pork (85%) + Cod (15%)	No additive	ND
	$NaNO_2$, 156	0.3
	CCMP, 12	ND
Pork (85%) + Cod Surimi (15%)	No additive	ND
	$NaNO_2$, 156	0.2
	CCMP, 12	ND
Pork (50%) + Cod (50%)	No additive	ND
	$NaNO_2$, 156	1.0
	CCMP, 12	ND
Pork (50%) + Cod Surimi (50%)	No additive	ND
	$NaNO_2$, 156	0.2
	CCMP, 12	ND

[1]All meat systems were treated with 20% (w/w) distilled water and 550 ppm sodium ascorbate.
[2]ND - not detected. Detection threshold limit of TEA analyzer is 0.2 ppb.

respectively. The amounts of NDMA in hybrid systems were equal to or less than that found in nitrite-cured cod (Table 4.10). Again, no measurable amount of NDMA was detected in CCMP-treated hybrid products for the same reasons as stated for other pigment-treated systems. Unlike the nitrite-treated cod surimi, NDMA was noticed at 0.2 ppb in pork/cod surimi hybrid formulations both at 15 and 50% substitution; the origin of this remains unclear. The CCMP-treated counterparts were free of NDMA as noted for all other pigment-treated samples, including cod.

This study supports the view that nitrite-free curing systems containing preformed CCMP can be successfully employed in the preparation of processed meat products without the fear of N-nitrosamine formation. In addition, it has been demonstrated that nitrite-free curing of fish or fishery by-products in combination with red meats in the production of novel N-nitrosamine-free cured meats is now feasible. This would not only make use of underutilized fish protein, but it also has the potential to increase the nutritional and sensory quality of formulated products (Pensabene *et al.*, 1991).

4.6 Stabilization of the CCMP

4.6.1 Effect of Washing of the CCMP Prior to its Encapsulation

The effect of washing CCMP with a 2% (w/v) solution of AA on the colour obtained after treating ground pork with the washed pigments was determined. The colour characteristics of pigment-treated pork systems were measured by Hunter **L**, **a**, **b** values after thermal processing. Results in Table 4.12 show that a significant ($P<0.05$)

Table 4.12
Effect of washing on the removal of residual nitrite and the colour characteristics of cooked cured-meat pigment- (CCMP) and encapsulated CCMP-treated pork systems.[1]

Washings[2]	Hunter Values		
	L	a	b
0	56.7±0.2^{b}	11.7±0.2^{a}	9.7±0.1^{a}
1	56.0±0.2^{c}	11.3±0.1^{b}	9.7±0.1^{a}
2	55.8±0.1^{c}	11.1±0.1^{bc}	9.9±0.2^{a}
3	55.3±0.2^{d}	11.0±0.2^{bc}	9.8±0.1^{a}
Encapsulated†	57.6±0.2^{a}	12.0±0.1^{a}	9.8±0.2^{a}
Encapsulated‡	56.1±0.2^{c}	10.8±0.2^{c}	9.9±0.1^{a}

[1]All pork samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP was prepared from haemin and nitric oxide. Encapsulated CCMP was prepared from unwashed† and washed‡ pigment using N-LOK as the wall material.
[2]CCMP was washed with a 2% (w/v) ascorbic acid solution.

decrease was observed in the Hunter **a** values of meat treated with CCMP after one wash compared to those of an unwashed counterpart. No significant ($P>0.05$) difference was determined in the Hunter **a** values of treated meats after further washing of the pigment. A similar trend was observed for the Hunter **L** values of samples tested, and no significant ($P>0.05$) difference in the Hunter **b** values of pigment-treated meats was observed as a consequence of washing. Perhaps some residual nitrite or nitrous acid remained with the pigment precipitate after its preparation which was removed by washing of the CCMP. Encapsulation of unwashed pigment and its subsequent application to ground pork resulted in a Hunter **a** value of 12.0±0.1 after thermal processing, but a Hunter **a** values of 10.8±0.2 was observed for the encapsulated CCMP which had been washed twice with the AA solution (Table 4.12).

4.6.2 Storage of CCMP Under a Nitric Oxide Atmosphere

The stability of the CCMP stored for up to 9 months in amber-coloured ampules and under a positive pressure of nitric oxide was tested by examining its absorption at 540 and 563 nm (Hornsey, 1956). The absorbance values of these pigments did not change significantly ($P>0.05$) over the test period (Table 4.13). After 9 months of storage, the pigment was applied to ground pork at a 12 ppm level of addition. A control sample containing 12 ppm of freshly prepared CCMP was used for comparison. The colour characteristics of these pigment-treated samples were judged by their Hunter **L**, **a**, **b** values after thermal processing. The Hunter **L**, **a**, **b** values of the CCMP-treated control were 57.8±0.1, 11.8±0.1 and 9.2±0.1 and those of the 9-month old CCMP sample

Table 4.13
Stability of the preformed cooked cured-meat pigment (CCMP) stored under a nitric oxide atmosphere.[1]

Storage Period (Months)	$A_{540\ nm}$	$A_{563\ nm}$
0	0.345±0.005[a]	0.352±0.003[a]
3	0.342±0.004[a]	0.351±0.002[a]
6	0.343±0.005[a]	0.350±0.002[a]
9	0.339±0.006[a]	0.348±0.003[a]

[1]Hunter **L**, **a**, **b** values of meats treated with pigment after 9 months of storage were 57.4, 11.4 and 9.1, respectively. Nitrite-cured meat (156 ppm) had respective values of 58.0, 11.7 and 9.1.

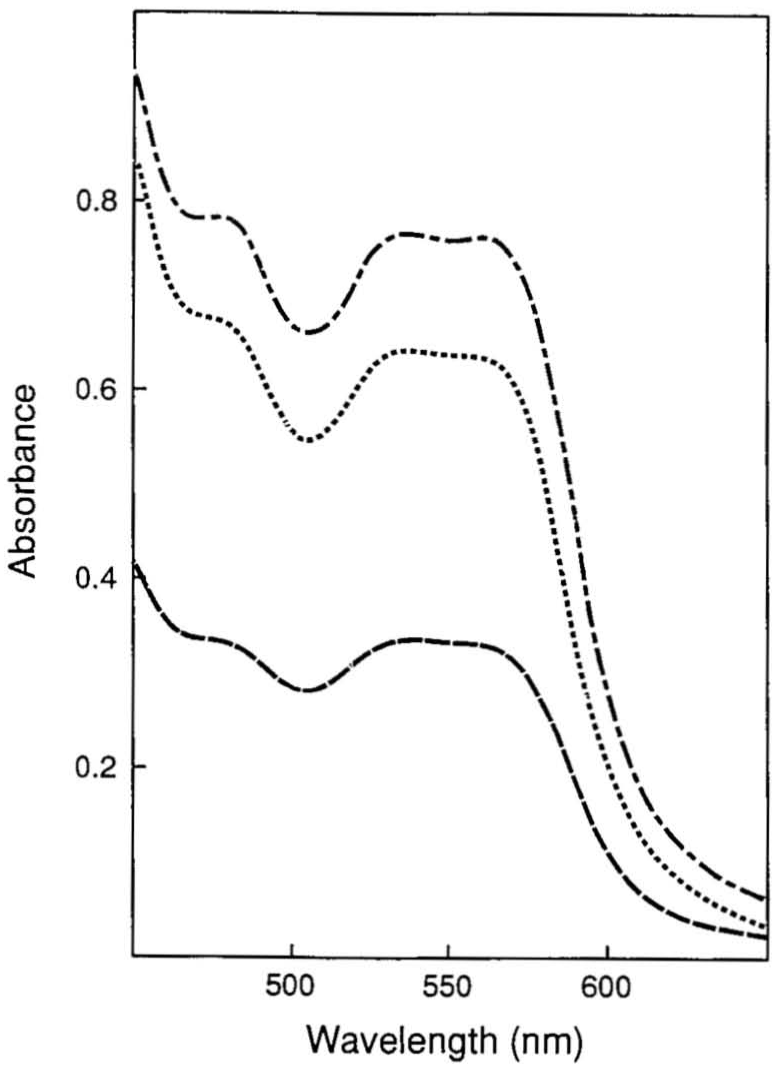
were 57.4±0.1, 11.4±0.1 and 9.1±0.2 (test sample). While Hunter **b** values were not significantly (P>0.05) different from one another, Hunter **L** and **a** values were marginally different from those of the meat cooked with a fresh sample of CCMP.

4.6.3 Preparation of the PCCMP from CCMP

Although a large number of experiments was performed and many different wall material combinations or encapsulating agents were tested, only some representative results are reported here. In all cases, the colour quality of meats treated with the PCCMP was compared to those treated with 12 ppm of freshly prepared CCMP as well as 156 ppm of sodium nitrite. It has previously been shown by Hunter colour values that the colour characteristics of pork treated with 12 ppm CCMP were indistinguishable from those of the nitrite-cured counterpart (Shahidi and Pegg, 1990*a*). In this study, colour parameters of treated meats which closely resembled those of nitrite-cured analogs (± 1 Hunter **a** value) were considered desirable. Although smaller differences observed by instrumental means were statistically significant (P<0.05), these were not easily detectable visually, as judged by the experimenter who was most familiar with these systems. Furthermore, the colour of such samples was generally found to be indistinguishable from those of nitrite-cured controls. Treated samples which did not satisfy this condition were always found to be undesirable and visually different from those cured with nitrite.

Figure 4.11 compares the typical absorption spectrum, in the visible region, of a PCCMP sample in acetone/water (Hornsey, 1956) with that of freshly prepared CCMP or that extracted from a nitrite-cured meat sample. All pigment solutions showed the

Figure 4.11 Absorption spectra of powdered cooked cured-meat pigment (PCCMP) prepared from N-LOK as the wall material, — - — - ; cooked cured-meat pigment (CCMP) from haemin/nitric oxide synthesis, ---------- ; and of pigments extracted from nitrite-cured ham, - — — — . All pigments were dissolved/extracted in acetone:water (4:1, v/v).


0.8
0.6
0.4
0.2
Absorbance
500
550
600
Wavelength (nm)

characteristic absorption spectrum of iron-porphyrin compounds with a red colour, and had maxima at 540 and 563 nm. The pigments extracted from cooked PCCMP-treated meats also exhibited a similar absorption pattern and maxima to those reported above (Figure 4.11). It might be reasonable to assume that microencapsulation and spray drying did not alter the chemical nature of the CCMP, but this was not verified. The most important variables in preparation of PCCMP were type of wall materials used, and the payload and the inlet temperature of the spray dryer. Other parameters such as feed flow rate and nitrogen pressure were less important.

Hunter **L**, **a**, **b** values of a typical set of PCCMP-treated meat samples were monitored in order to set the optimum payload of the pigment in encapsulating materials. The best encapsulated pigments used payloads of 1 to 1.5% (Table 4.14). As the payload was increased from 1 to 1.5%, a significant ($P<0.05$) increase in Hunter **a** value of meats treated with PCCMP was noted. At higher levels, less wall material was available to protect the preformed CCMP. In those, the effective colour imparted by the spray-dried pigment was significantly reduced ($P<0.05$). This was shown by a decrease in Hunter **a** values of treated meat samples (Table 4.14). A higher Hunter **a** value is preferred since it potentially allowed for use of a lesser amount of PCCMP in order to achieve a given final colour.

Of the several variables in the spray-drying conditions of CCMP, inlet temperature was shown to be of critical importance. Typical results indicated that an inlet of $\geq$ 150°C afforded the best quality PCCMP as judged by Hunter colour measurement of PCCMP-

Table 4.14
Effect of payload on Hunter **L**, **a**, **b** values of ground pork systems treated and cooked with powdered cooked cured-meat pigment (PCCMP)[1].

Experiment Number	Treatment (ppm)	Wall Materials	Payload (%)	Hunter Values[2]		
				L	**a**	**b**
1	No additive, 0	-	-	59.0 ± 0.2^{a}	4.7 ± 0.2^{d}	11.4 ± 0.1^{a}
2	$NaNO_2$, 156	-	-	58.4 ± 0.1^{b}	11.8 ± 0.2^{bc}	9.1 ± 0.1^{b}
3	CCMP, 12	-	-	57.9 ± 0.2^{b}	11.7 ± 0.2^{bc}	9.1 ± 0.1^{b}
4	PCCMP, 50	95% N-LOK[3]	0.67	52.0 ± 0.2^{d}	11.8 ± 0.2^{bc}	9.1 ± 0.1^{b}
5	(4)	(4)	1.0	52.1 ± 0.2^{d}	11.9 ± 0.2^{b}	9.1 ± 0.1^{b}
6	(4)	(4)	1.5	52.5 ± 0.1^{d}	12.9 ± 0.2^{a}	8.9 ± 0.1^{c}
7	(4)	(4)	2.0	53.8 ± 0.2^{c}	11.3 ± 0.2^{c}	8.7 ± 0.2^{c}
8	(4)	(4)	3.0	53.6 ± 0.2^{c}	11.3 ± 0.1^{c}	8.7 ± 0.2^{c}

[1]Percentage of solids in the mixture was 10% (w/v).
[2]All pork systems contained 20% (w/w) distilled water and 550 ppm sodium ascorbate. Results are mean values of three to six determinations ± standard deviation. Means sharing any of same letters in a column are not significantly ($P>0.05$) different from one another.
[3]Wall materials contained 2% sodium tripolyphosphate, 2% sodium acid pyrophosphate and 1% ascorbyl palmitate.

treated meats (Table 4.15). As the inlet temperature was increased from 130 to 150°C, a significant ($P<0.05$) increase in Hunter **a** value of PCCMP-treated pork systems was observed. Variations of the inlet temperature did not affect Hunter **L** or **b** values of treated meats. Because the spray performance depends on the inlet temperature chosen, at a given flow and aspiration rate, a temperature of 150-170°C may be used. For all remaining experiments, an inlet temperature of 150°C was selected.

The effect of concentration of PCCMP on colour intensity of treated meats was measured. Table 4.16 summarizes typical results for PCCMP-treated meats in which combinations of wall materials consisted of 95% N-LOK, 2% STPP, 2% SAPP and 1% AP. Results indicated that PCCMP-treated samples at 30-40 ppm levels resembled most the colour of nitrite-cured meats. Higher addition levels of PCCMP significantly ($P<0.05$) increased Hunter **a** values and decreased Hunter **L** values of treated samples, but the colour may not be visually unattractive. Nonetheless, the optimal addition level of spray-dried pigment to meat depends primarily on its original Mb content (Shahidi and Pegg, 1988; 1991*d*) as well as the conditions under which encapsulation was performed.

Based on the above experiments and under the best conditions specified above, the colour characteristics of PCCMP-treated meats were tested as a function of different wall materials employed for encapsulation. Typical results of the study are provided in Table 4.17. Generally carbohydrates are used in microencapsulation processes because of their low cost and good functionality. Simple starch hydrolysates, modified starches or various gums are used. Of the wall materials examined in this work, N-LOK, β-cyclodextrin and

Table 4.15
Effect of inlet temperature of spray dryer on Hunter **L**, **a**, **b** values of ground pork treated and cooked with powdered cooked cured-meat pigment (PCCMP).[1]

Inlet Temperature (°C)	Hunter Values[2]		
	L	**a**	**b**
130	52.7 ± 0.2[a]	11.8 ± 0.1[b]	9.1 ± 0.2[a]
150	52.5 ± 0.1[a]	12.9 ± 0.1[a]	8.9 ± 0.2[a]
150[3]	53.1 ± 0.2[a]	12.5 ± 0.2[a]	9.0 ± 0.2[a]
170	52.4 ± 0.1[a]	12.6 ± 0.2[a]	9.2 ± 0.2[a]

[1]Wall materials employed consisted of 95% N-LOK, 2% sodium tripolyphosphate, 2% sodium acid pyrophosphate and 1% ascorbyl palmitate. PCCMP was added at 50 ppm, unless otherwise specified.
[2]Results are mean values of three determinations ± standard deviation. Means sharing any of same letters in a column are not significantly (P>0.05) different from one another.
[3]PCCMP was added at a 35 ppm level.

Table 4.16
Effect of pigment concentration on the Hunter **L, a, b** values of cooked treated pork systems.[1]

Treatment (ppm)	Hunter Values[2]		
	L	**a**	**b**
No additive, 0	59.0 ± 0.2^{a}	4.7 ± 0.1^{d}	11.4 ± 0.1^{a}
$NaNO_2$, 156	58.4 ± 0.1^{b}	11.8 ± 0.2^{e}	9.1 ± 0.1^{b}
CCMP, 12	57.9 ± 0.2^{c}	11.7 ± 0.2^{e}	9.1 ± 0.1^{b}
PCCMP, 30	54.5 ± 0.2^{d}	12.2 ± 0.2^{bc}	9.1 ± 0.1^{b}
PCCMP, 50	52.5 ± 0.1^{e}	12.9 ± 0.1^{a}	8.8 ± 0.1^{c}

[1]All systems contained 20% (w/w) distilled water and 550 ppm sodium ascorbate. A payload of 1.5% (w/w) was used. Wall materials for PCCMP were N-LOK, 95%; sodium tripolyphosphate, 2%; sodium acid pyrophosphate, 2%; and ascorbyl palmitate, 1%. CCMP - cooked cured-meat pigment; PCCMP - powdered cooked cured-meat pigment.
[2]Results are mean values of six determinations ± standard deviations. Means sharing any of same letters in a column are not significantly ($P>0.05$) different from one another.

Table 4.17
Hunter **L**, **a**, **b** values of powdered cooked cured-meat pigment- (PCCMP) treated cooked pork systems as affected by wall materials.[1]

Experiment Number	Additives (ppm)	Wall Material(s)	Hunter Values[2]		
			L	**a**	**b**
1	No additive, 0	--	59.0 ± 0.2^{a}	4.7 ± 0.1^{k}	11.4 ± 0.1^{a}
2	$NaNO_2$, 156	--	58.4 ± 0.1^{ab}	11.8 ± 0.2^{ef}	9.1 ± 0.1^{d}
3	CCMP, 12	--	57.9 ± 0.2^{b}	11.7 ± 0.2^{efg}	9.1 ± 0.1^{d}
4	PCCMP, 50	N-LOK	54.0 ± 0.2^{ghi}	11.9 ± 0.2^{de}	9.2 ± 0.1^{cd}
5	(4)	β-cyclodextrin	54.7 ± 0.1^{cdef}	11.7 ± 0.1^{efg}	9.1 ± 0.1^{d}
6	(4)	Modified β-cyclodextrin	54.0 ± 0.2^{ghi}	9.0 ± 0.1^{d}	9.6 ± 0.1^{bc}
7	(4)	Maltrin M-250	53.3 ± 0.2^{jkl}	11.6 ± 0.1^{efg}	9.7 ± 0.2^{b}
8	(4)	Gum Acacia	53.4 ± 0.2^{ijkl}	11.1 ± 0.2^{h}	9.2 ± 0.2^{cd}
9	(4)	95% (4) + 5% (8)	53.2 ± 0.2^{kl}	12.4 ± 0.2^{bc}	9.1 ± 0.1^{d}
10	(4)	95% (4) + 5% (COMBO)	52.8 ± 0.2^{l}	12.9 ± 0.1^{a}	9.1 ± 0.1^{d}
12	(4)	85% (4) + 15% (5)	53.9 ± 0.2^{ghij}	11.3 ± 0.1^{gh}	9.2 ± 0.1^{cd}
13	(4)	80% (4) + 15% (5) + 5% (8)	54.0 ± 0.2^{ghi}	11.7 ± 0.1^{efg}	9.2 ± 0.1^{cd}

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Table 4.17
Hunter **L, a, b** values of powdered cooked cured-meat pigment- (PCCMP) treated cooked pork systems as affected by wall materials.[1]

Experiment Number	Additives (ppm)	Wall Material(s)	Hunter Values[2]		
			L	**a**	**b**
14	(4)	80% (4) + 15% (6) + 5% (8)	53.6 ± 0.1^{hijk}	11.4 ± 0.1^{fgh}	9.2 ± 0.1^{cd}
15	(4)	80% (4) + 15% (5) + 5% (COMBO)	53.2 ± 0.2^{kl}	12.6 ± 0.1^{ab}	9.0 ± 0.2^{d}
16	(4)	75% (4) + 15% (5) + 5% (8) + 5% (COMBO)	53.3 ± 0.2^{jkl}	12.0 ± 0.1^{cde}	8.9 ± 0.1^{d}
17	(4)	70% (4) + 30% (5)	53.2 ± 0.2^{kl}	11.9 ± 0.2^{de}	9.1 ± 0.1^{d}
18	(4)	98% (5) + 1% (STPP) + 1% (SAPP)	55.1 ± 0.2^{c}	10.5 ± 0.1^{i}	9.3 ± 0.2^{bcd}
19	(4)	95% (5) + 5% (COMBO)	53.4 ± 0.2^{ijkl}	11.4 ± 0.2^{fgh}	9.1 ± 0.1^{d}
20	(4)	95% (5) + 5% (8)	53.3 ± 0.1^{jkl}	12.3 ± 0.1^{bcd}	9.3 ± 0.1^{bcd}
21	(4)	90% (5) + 5% (8) + 5% (COMBO)	54.9 ± 0.2^{cd}	11.1 ± 0.2^{h}	9.6 ± 0.1^{bc}
22	(4)	95% (6) + 5% (COMBO)	54.0 ± 0.1^{ghi}	9.1 ± 0.1^{j}	9.2 ± 0.2^{cd}
23	(4)	95% (7) + 5% (COMBO)	54.4 ± 0.2^{defg}	12.3 ± 0.1^{bcd}	9.2 ± 0.2^{cd}

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Table 4.17
Hunter **L**, **a**, **b** values of powdered cooked cured-meat pigment- (PCCMP) treated cooked pork systems as affected by wall materials.[1]

Experiment Number	Additives (ppm)	Wall Material(s)	Hunter Values[2]		
			L	**a**	**b**
24	(4)	80% (7) + 15% (5) + 5% (COMBO)	54.1 ± 0.1^{fgh}	11.9 ± 0.1^{de}	9.2 ± 0.1^{cd}
25	(4)	80% (7) + 15% (6) + 5% (COMBO)	54.2 ± 0.5^{efgh}	11.0 ± 0.1^{h}	9.3 ± 0.1^{bcd}

[1]All samples were prepared with 20% (w/w) water and 550 ppm sodium ascorbate. COMBO refers to a combination of STPP/SAPP/AP (2:2:1, w/w/w). An average yield of 59-68% (maximum 76%) of encapsulated product was obtained in all cases. Samples were kept at a refrigerated temperature of 2-4°C. CCMP - cooked cured-meat pigment; PCCMP - powdered cooked cured-meat pigment; STPP - sodium tripolyphoshate; SAPP - sodium acid pyrophosphate; AP - ascorbyl palmitate.

[2]Results are mean values of three replicates ± standard deviation. Means sharing any of same letters in a column are not significantly ($P>0.05$) different from one another.

Maltrin M-250, when used individually, gave powders which upon addition to pork meat and subsequent heat processing afforded products which most closely resembled the colour characteristics of nitrite-cured ham. These treated meats were also indistinguishable from that of pork treated with 12 ppm of freshly prepared CCMP. In addition to Maltrin M-250, other grades of Maltrin namely M-040, M-100, M-150, M-200, M-500 and M-700 were tested. Only marginal differences were apparent with different Maltrins (results not shown). Modified β-cyclodextrin (etherified) with obscured hydroxyl groups (Parmerter *et al.*, 1969) and gum acacia, however, were less effective in protection of CCMP (Table 4.17).

Protection of CCMP may arise from either partial inclusion of the pigment in the central cavity of β-cyclodextrin or simply by it becoming surrounded by the carbohydrate-based material. In general, to form inclusion compounds, the material to be encapsulated is added to a warm aqueous solution of β-cyclodextrin. Equilibrium is reached with intense stirring. During slow cooling, the inclusion complex precipitates and afterwards is recovered by filtration. On the other hand, water may be removed from the system by freeze- or spray-drying (Szejtli, 1982). In this study, CCMP was added to a basic solution of β-cyclodextrin at room temperature. Because precipitation of the mixture under these conditions is unlikely (Szejtli, 1982), dehydration was accomplished by spray drying. The sensitivity of CCMP to oxidation necessitated a short preparation time to retard its possible decomposition. Despite the excellent encapsulating ability of β-cyclodextrin for different applications (Yamada *et al.*, 1980; Pitha, 1981; Szejtli, 1981;

Shaw *et al.*, 1984; Martin *et al.*, 1990), it is not yet permitted as a food ingredient in many countries.

Several wall materials were also employed in combination. Those tested in combination were N-LOK, β-cyclodextrin, modified β-cyclodextrin, or Maltrin M-250. Generally, addition of gum acacia to combinations containing the above wall materials improved their performance, as was noted in the Hunter **a** values of PCCMP-treated meats (Table 4.17). In all cases examined, addition of a 5% mixture of STPP/SAPP/AP (2:2:1, w/w/w) to the wall materials improved performance of the encapsulated pigment. Larger Hunter **a** values were evident when this mixture was present (Table 4.17).

In another set of experiments, the effect of storage of PCCMP on its performance in meat systems was monitored. Hunter **a** values of pigment-treated meats indicated that the colouring quality of stored PCCMP was primarily dictated by its initial colour properties (Table 4.18). The colour characteristics of meat samples treated with PCCMP which had been encapsulated with modified β-cyclodextrin remained less desirable as their Hunter **a** values were more than one unit below those of nitrite-cured counterparts. Samples containing STPP/SAPP/AP combinations or gum acacia had more desirable colouring properties, thus indicating that protected pigments retained their structural integrity.

Finally, the effect of intense fluorescent lighting on colour stability of pork systems treated with PCCMP was examined. Typical results using different encapsulating materials, as judged by initial Hunter **a** value of freshly encapsulated pigments, are shown

Table 4.18
Effect of storage on Hunter **L, a, b** values of cooked pork treated with powdered cooked cured-meat pigment (PCCMP).[1]

Experiment Number	Wall Material(s)	Storage Time (Months)	Hunter Values[2]		
			L	**a**	**b**
1	N-LOK	0	54.0 ± 0.2^{mn}	11.9 ± 0.2^{bcd}	9.2 ± 0.1^{ef}
		9	57.2 ± 0.1^{ab}	10.9 ± 0.2^{g}	9.7 ± 0.2^{abcd}
2	N-LOK (95%) + COMBO (5%)	0	52.8 ± 0.2^{p}	12.9 ± 0.1^{a}	9.1 ± 0.1^{f}
		2.5	55.2 ± 0.1^{ghij}	12.1 ± 0.2^{bd}	9.4 ± 0.2^{cdef}
		9	55.7 ± 0.1^{efgh}	12.0 ± 0.2^{bcd}	9.4 ± 0.2^{cdef}
		18	55.8 ± 0.1^{efg}	11.7 ± 0.2^{cde}	9.2 ± 0.2^{ef}
3	N-LOK (95%) + Gum Acacia (5%)	0	53.2 ± 0.2^{p}	12.4 ± 0.2^{ab}	9.1 ± 0.2^{f}
		2.5	53.2 ± 0.1^{p}	12.3 ± 0.1^{b}	9.2 ± 0.2^{ef}
		9	53.4 ± 0.2^{nop}	12.3 ± 0.2^{b}	9.2 ± 0.2^{ef}
4	β-cyclodextrin	0	54.7 ± 0.1^{jkl}	11.7 ± 0.1^{cde}	9.1 ± 0.1^{f}
		11	55.3 ± 0.2^{ghij}	11.2 ± 0.2^{efg}	9.2 ± 0.1^{ef}

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Table 4.18
Effect of storage on Hunter **L, a, b** values of cooked pork treated with powdered cooked cured-meat pigment (PCCMP).[1]

Experiment Number	Wall Material(s)	Storage Time (Months)	Hunter Values[2]		
			L	**a**	**b**
5	β-cyclodextrin (95%) + COMBO (5%)	0	55.1 ± 0.2^{hij}	11.5 ± 0.1^{def}	9.3 ± 0.1^{def}
		4	56.5 ± 0.2^{cd}	11.0 ± 0.2^{fg}	9.8 ± 0.1^{abc}
		18	56.2 ± 0.2^{def}	11.0 ± 0.2^{fg}	9.7 ± 0.2^{abcd}
6	β-cyclodextrin (95%) + Gum Acacia (5%)	0	55.0 ± 0.1^{ijk}	11.6 ± 0.2^{cde}	9.4 ± 0.1^{cdef}
		4	56.3 ± 0.2^{de}	11.2 ± 0.3^{efg}	9.6 ± 0.1^{abcde}
7	Modified β-cyclodextrin	0	54.0 ± 0.5^{mn}	9.0 ± 0.1^{i}	9.6 ± 0.1^{abcde}
		4	54.9 ± 0.3^{jk}	8.6 ± 0.2^{i}	9.7 ± 0.2^{abcd}
8	Maltrin M-250	0	53.3 ± 0.2^{op}	11.6 ± 0.1^{cde}	9.7 ± 0.2^{abcd}
		4	55.6 ± 0.1^{fgji}	10.9 ± 0.2^{g}	9.9 ± 0.1^{abc}
9	Maltrin (95%) + COMBO (5%)	0	54.4 ± 0.2^{klm}	12.3 ± 0.1^{b}	9.2 ± 0.2^{ef}
		11	56.0 ± 0.1^{def}	11.2 ± 0.3^{efg}	9.7 ± 0.2^{abcd}

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Table 4.18
Effect of storage on Hunter **L, a, b** values of cooked pork treated with powdered cooked cured-meat pigment (PCCMP).[1]

Experiment Number	Wall Material(s)	Storage Time (Months)	Hunter Values[2]		
			L	**a**	**b**
10	N-LOK (85%) + β-cyclodextrin (15%)	0	53.9 ± 0.1^{mno}	11.0 ± 0.1^{fg}	9.5 ± 0.2^{bcdef}
		9	55.6 ± 0.1^{fghi}	10.1 ± 0.1^{h}	9.6 ± 0.1^{abcde}
11	Maltrin (85%) + β-cyclodextrin (15%)	0	54.1 ± 0.1^{lm}	11.9 ± 0.1^{bcd}	9.2 ± 0.1^{ef}
		11	56.0 ± 0.1^{def}	11.2 ± 0.2^{efg}	9.7 ± 0.1^{abcd}
12	(10) (95%) + COMBO (5%)	0	54.2 ± 0.5^{lm}	11.6 ± 0.1^{cde}	9.3 ± 0.1^{def}
		11	57.3 ± 0.1^{ab}	11.2 ± 0.3^{efg}	9.7 ± 0.1^{abcd}
13	Gum Acacia	0	57.0 ± 0.3^{bc}	8.9 ± 0.1^{i}	9.9 ± 0.2^{abc}
		4	57.7 ± 0.3^{a}	8.7 ± 0.1^{i}	10.0 ± 0.1^{a}

[1]All samples were prepared with 20% (w/w) distilled water, 550 ppm sodium ascorbate and 50 ppm PCCMP. COMBO refers to a combination of STPP/SAPP/AP (2:2:1, w/w/w). PCCMP - powdered cooked cured-meat pigment; STPP - sodium tripolyphosphate; SAPP - sodium acid pyrophosphate; AP - ascorbyl palmitate.

[2]Results are mean values of three determinations ± standard deviation. Means sharing any of same letters in a column are not significantly ($P>0.05$) different from one another.

in Figure 4.12. In all cases, a drastic reduction in Hunter **a** values was evident during the first 6 h of fluorescent lighting. The ultimate Hunter **a** value, after an 18 h exposure was near that observed for meats cured with 156 ppm sodium nitrite (±1 Hunter **a** value). Nonetheless, this value depended, to some extent, on the initial Hunter colour values of the treated meat samples and also on the total concentration of pigments in the muscle tissue homogenates (Figure 4.12).

4.7 Effect of Irradiation on the Colour and Flavour Characteristics of CCMP-Treated Pork Systems

The Hunter **L**, **a**, **b** colour values of pork systems, cooked with different additives and irradiated at 5 or 10 kGy, as a function of storage time and refrigerated at 4°C were compared with those of unirradiated counterparts (Tables 4.19-4.21). Results indicated that all meat samples, regardless of chemical or radiation treatment, were less pinkish in appearance and their Hunter **a** values decreased over a 3 week storage period. For untreated meat samples which were not irradiated, Hunter **a** values followed an opposite, increasing trend. This may have been a consequence of sodium nitrate impurities in the system. The nitrate would gradually be reduced to sodium nitrite and ultimately to a nitrosating species such as dinitrogen trioxide (N_2O_3), leading to formation of a very slight pink colour in the product. Irradiation may have also increased the reducing potential of sodium ascorbate. Thus, irradiation of freshly prepared uncured cooked meats will have higher Hunter **a** values than unirradiated samples. Another possibility for the pinkish colour stems from reduction of denatured metmyoglobin in irradiated meats. This

Figure 4.12 Hunter a values of nitrite-cured, - - - - - ; and powdered cooked cured-meat pigment (PCCMP) treated meats (156 and 50 ppm addition levels, respectively), stored under fluorescent lighting (375 lux) at 4°C. Wall materials of PCCMP are N-LOK, —— —— ; N-LOK (85%) + β-Cyclodextrin (15%), ·····; N-LOK (80%) + β-Cyclodextrin (15%) + gum acacia (5%), — - — - ; and modified β-Cyclodextrin, - —— - .

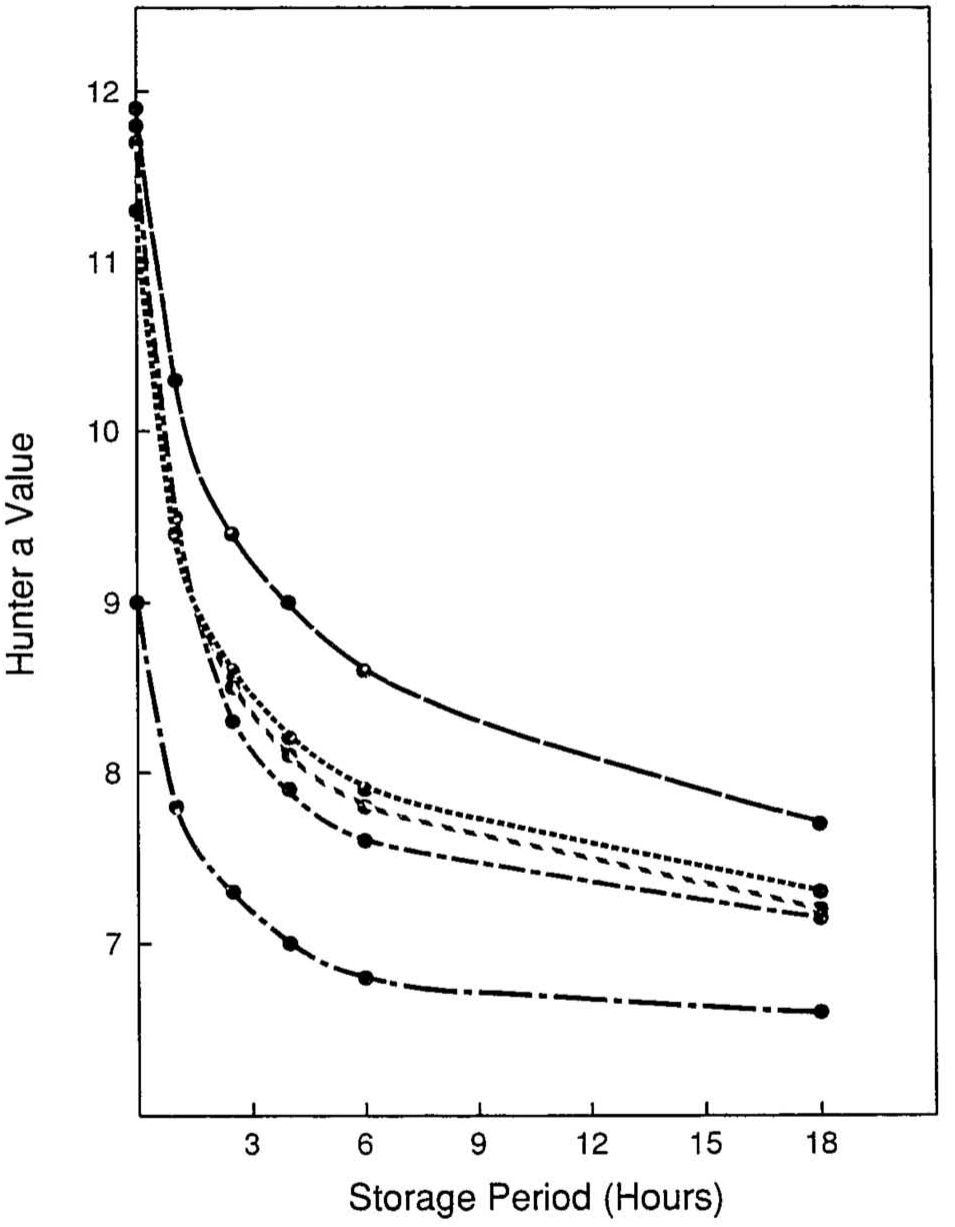

Hunter a Value
12
11
10
9
8
7
3
6
9
12
15
18
Storage Period (Hours)

Table 4.19
Hunter L, a, b colour values of treated and cooked pork systems during three weeks of storage.[1]

Additive(s) (ppm)	Storage (Days)	Hunter Values, 0 kGy		
		L	a	b
None (0)	0	64.0 ± 0.2^{bcs}	3.8 ± 0.1^{lv}	11.5 ± 0.1^{ax}
	7	65.2 ± 0.3^{abs}	4.7 ± 0.3^{iu}	11.0 ± 0.4^{abx}
	14	65.7 ± 0.4^{as}	5.2 ± 0.3^{hiu}	10.7 ± 0.2^{by}
	21	65.8 ± 0.5^{as}	5.6 ± 0.2^{hu}	11.0 ± 0.6^{aby}
$NaNO_2$ (156)	0	62.2 ± 0.5^{defs}	11.7 ± 0.2^{bu}	8.9 ± 0.1^{cy}
	7	63.2 ± 0.3^{cds}	10.4 ± 0.1^{efgu}	8.4 ± 0.2^{cdey}
	14	63.9 ± 0.5^{bcst}	10.2 ± 0.1^{fgu}	8.4 ± 0.1^{cdey}
	21	65.2 ± 0.8^{abs}	10.1 ± 0.1^{gu}	8.3 ± 0.1^{cdefz}
CCMP (12)	0	60.0 ± 0.2^{ijks}	11.8 ± 0.2^{bu}	8.5 ± 0.1^{cdx}
	7	61.6 ± 0.8^{efghs}	10.8 ± 0.2^{defu}	7.8 ± 0.1^{efz}
	14	61.9 ± 0.5^{dcfgs}	10.4 ± 0.2^{efgu}	7.7 ± 0.2^{fz}
	21	63.0 ± 0.4^{cdes}	10.8 ± 0.2^{defuv}	7.7 ± 0.1^{fy}
CCMP (12) + STPP (3000)	0	59.2 ± 0.5^{ks}	12.6 ± 0.3^{au}	8.2 ± 0.2^{defx}
	7	59.9 ± 0.7^{ijks}	11.6 ± 0.1^{bcu}	7.9 ± 0.2^{defx}
	14	60.4 ± 0.2^{hijks}	11.4 ± 0.3^{bcdu}	8.1 ± 0.2^{defx}
	21	61.0 ± 0.3^{fghis}	11.2 ± 0.3^{bcdu}	8.1 ± 0.2^{defx}
CCMP (12) + STPP (1500) + SAPP (1500)	0	59.3 ± 0.2^{jks}	12.6 ± 0.1^{au}	8.1 ± 0.1^{defy}
	7	59.8 ± 0.7^{ijks}	11.4 ± 0.2^{bcdu}	7.9 ± 0.2^{defx}
	14	60.7 ± 0.4^{ghijst}	11.0 ± 0.1^{cdeu}	7.8 ± 0.1^{efy}
	21	60.4 ± 0.4^{hijkt}	11.3 ± 0.1^{bcdu}	8.0 ± 0.2^{defy}

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP - cooked cured-meat pigment; STPP - sodium tripolyphosphate; and SAPP - sodium acid pyrophosphate. Results are means of 5 determinations ± standard deviation. Means in same column (a-k) or in the same row (s-z) with the same letters are not significantly ($P>0.05$) different.

Table 4.20
Effect of irradiation (5 kGy) on Hunter L, a, b colour values of treated and cooked pork systems during three weeks of storage.[1]

Additive(s) (ppm)	Storage (Days)	Hunter Values, 5 kGy		
		L	a	b
None (0)	0	63.8 ± 0.1^{cdes}	4.8 ± 0.1^{hu}	11.4 ± 0.1^{cx}
	7	64.7 ± 0.1^{bcs}	4.4 ± 0.1^{hiu}	11.7 ± 0.2^{bcx}
	14	66.0 ± 0.7^{abs}	4.2 ± 0.1^{ijv}	11.9 ± 0.2^{abx}
	21	66.3 ± 0.6^{as}	3.8 ± 0.1^{jv}	12.2 ± 0.2^{ax}
$NaNO_2$ (156)	0	62.7 ± 0.1^{efgs}	11.3 ± 0.2^{bcu}	9.5 ± 0.1^{dx}
	7	63.3 ± 0.5^{defs}	9.7 ± 0.1^{gv}	8.8 ± 0.1^{efx}
	14	64.5 ± 0.1^{cds}	9.5 ± 0.2^{gv}	8.7 ± 0.1^{efgx}
	21	65.0 ± 0.6^{abcs}	9.7 ± 0.1^{gv}	8.8 ± 0.1^{efy}
CCMP (12)	0	60.3 ± 0.6^{ijks}	11.7 ± 0.2^{abu}	8.8 ± 0.2^{efx}
	7	60.2 ± 0.7^{ijks}	11.0 ± 0.3^{cdu}	9.1 ± 0.1^{dex}
	14	61.5 ± 0.1^{ghis}	10.9 ± 0.2^{cdeu}	9.1 ± 0.1^{dexy}
	21	61.7 ± 0.3^{ghi}	10.9 ± 0.1^{cdeu}	9.1 ± 0.1^{dex}
CCMP (12) + STPP (3000)	0	59.0 ± 0.2^{ks}	12.1 ± 0.3^{au}	8.2 ± 0.2^{hx}
	7	60.0 ± 0.3^{jks}	10.8 ± 0.2^{cdefu}	8.1 ± 0.2^{hx}
	14	60.7 ± 0.5^{hijs}	10.5 ± 0.2^{defu}	8.2 ± 0.2^{hx}
	21	60.9 ± 0.5^{hijs}	10.5 ± 0.1^{defu}	8.2 ± 0.1^{hx}
CCMP (12) + STPP (1500) + SAPP (1500)	0	59.7 ± 0.4^{jks}	12.0 ± 0.3^{au}	8.5 ± 0.1^{fghx}
	7	60.9 ± 0.5^{hijs}	10.4 ± 0.2^{efv}	8.2 ± 0.1^{hx}
	14	60.8 ± 0.1^{hijs}	10.3 ± 0.2^{fv}	8.2 ± 0.1^{hx}
	21	62.0 ± 0.2^{fghs}	10.3 ± 0.1^{fv}	8.3 ± 0.1^{ghxy}

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP - cooked cured-meat pigment; STPP - sodium tripolyphosphate; and SAPP - sodium acid pyrophosphate. Results are means of 5 determinations ± standard deviation. Means in same column (a-k) or in the same row (s-z) with the same letters are not significantly ($P>0.05$) different.

Table 4.21
Effect of irradiation (10 kGy) on Hunter L, a, b colour values of treated and cooked pork systems during three weeks of storage.[1]

Additive(s) (ppm)	Storage (Days)	Hunter Values, 10 kGy		
		L	a	b
None (0)	0	63.6 ± 0.3^{cds}	4.7 ± 0.2^{fu}	11.4 ± 0.1^{bx}
	7	63.1 ± 0.5^{cdt}	3.6 ± 0.1^{gv}	11.2 ± 0.4^{bx}
	14	65.0 ± 0.4^{abs}	3.1 ± 0.2^{gw}	11.5 ± 0.2^{bx}
	21	66.1 ± 0.6^{as}	2.2 ± 0.5^{hw}	12.4 ± 0.1^{ax}
$NaNO_2$ (156)	0	62.3 ± 0.3^{des}	11.3 ± 0.2^{abu}	9.3 ± 0.1^{cx}
	7	63.4 ± 0.1^{cds}	9.8 ± 0.2^{ev}	8.7 ± 0.1^{defgxy}
	14	63.7 ± 0.2^{bct}	9.8 ± 0.2^{euv}	8.7 ± 0.1^{defgx}
	21	63.8 ± 0.6^{bcs}	9.9 ± 0.1^{deuv}	9.1 ± 0.1^{cdx}
CCMP (12)	0	60.3 ± 0.6^{fghs}	11.7 ± 0.2^{au}	8.8 ± 0.2^{cdefx}
	7	61.0 ± 0.4^{efgs}	10.2 ± 0.5^{cdeu}	8.7 ± 0.1^{defgy}
	14	61.1 ± 0.3^{efgs}	10.6 ± 0.3^{bcdeu}	8.8 ± 0.1^{cdefy}
	21	61.6 ± 0.3^{eft}	10.5 ± 0.1^{bcdev}	8.9 ± 0.1^{cdex}
CCMP (12) + STPP (3000)	0	58.9 ± 0.2^{is}	12.1 ± 0.1^{au}	8.4 ± 0.1^{efghx}
	7	60.3 ± 0.7^{fghs}	10.5 ± 0.3^{bcdeu}	8.1 ± 0.3^{hx}
	14	61.1 ± 0.4^{efgs}	10.5 ± 0.2^{bcdeu}	8.1 ± 0.2^{hx}
	21	61.1 ± 0.2^{efgs}	10.7 ± 0.4^{bcdu}	8.3 ± 0.2^{fghx}
CCMP (12) + STPP (1500) + SAPP (1500)	0	59.6 ± 0.4^{his}	12.1 ± 0.3^{au}	8.2 ± 0.1^{ghy}
	7	60.1 ± 0.3^{ghis}	10.7 ± 0.3^{bcdu}	8.2 ± 0.3^{ghx}
	14	59.9 ± 0.4^{ghit}	10.8 ± 0.4^{bcuv}	8.3 ± 0.1^{fghx}
	21	60.2 ± 0.4^{gijt}	10.7 ± 0.4^{bcduv}	8.6 ± 0.1^{defghx}

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP - cooked cured-meat pigment; STPP - sodium tripolyphosphate; and SAPP - sodium acid pyrophosphate. Results are means of 5 determinations ± standard deviation. Means in same column (a-k) or in the same row (s-z) with the same letters are not significantly ($P>0.05$) different.

hypothesis is consistent with reports by Giddings and Markakis (1972) who found that oxidized, brown, surface colour of vacuum packed meats became purple in appearance upon irradiation. In the presence of oxygen, a bright cherry red colour developed indicative of oxymyoglobin.

Radiation processing had little effect on the colour fading characteristics of nitrite-cured meat samples compared with controls (Tables 4.19-4.21), but irradiation of freshly prepared nitrite-cured meats had slightly lower Hunter **a** values. A similar observation was found for CCMP-treated meats. The presence of residual nitrite may not be a determining factor in colour stability of treated meats. Similar results were reported when pigment-treated and nitrite-cured meats were subjected to fluorescent lighting (Pegg and Shahidi, 1989*a*; Shahidi *et al.*, 1990), but this was contrary to the reports by Shults *et al.* (1977). They reported that the presence of sodium nitrate was critical for the colour and flavour stability of irradiated meats cured with 25 ppm sodium nitrite, but a radiation dosage of up to 74.4 kGy was used.

The flavour and oxidative stability of irradiated meat samples containing different additives, determined by TBA values, are reported in Table 4.22. The antioxidative effect of nitrite was somewhat enhanced in the irradiated samples as lower TBA values were found. Perhaps irradiation enhanced the conversion of sodium nitrite to nitric oxide in the presence of sodium ascorbate.

The TBA values of stored CCMP-treated meats were generally lower than those of controls with no additives (Table 4.22). Irradiation had a beneficial effect on the

Table 4.22
Effect of irradiation on the TBA values of treated and cooked pork systems during three weeks of storage.[1]

Additive(s) (ppm)	Irradiation Dose kGy	Storage Period, Days			
		0	7	14	21
None (0)	0	4.55 ± 0.05^{ay}	6.23 ± 0.08^{ax}	6.02 ± 0.10^{ax}	8.41 ± 0.12^{aw}
	5	0.89 ± 0.07^{cz}	2.71 ± 0.10^{cy}	3.95 ± 0.12^{cx}	6.59 ± 0.06^{bw}
	10	1.39 ± 0.08^{bz}	3.17 ± 0.09^{by}	4.85 ± 0.02^{bx}	6.62 ± 0.05^{bw}
$NaNO_2$ (156)	0	0.21 ± 0.03^{dy}	0.83 ± 0.04^{ew}	0.84 ± 0.04^{fgx}	0.74 ± 0.02^{ex}
	5	0.18 ± 0.03^{dy}	0.56 ± 0.05^{fgw}	0.47 ± 0.02^{hwx}	0.43 ± 0.05^{fx}
	10	0.17 ± 0.02^{dy}	0.47 ± 0.03^{gx}	0.60 ± 0.05^{hw}	0.45 ± 0.04^{fx}
CCMP (12)	0	0.11 ± 0.03^{dy}	1.62 ± 0.04^{dx}	1.59 ± 0.01^{ex}	4.23 ± 0.02^{cw}
	5	0.13 ± 0.03^{dy}	1.61 ± 0.04^{dx}	1.67 ± 0.03^{dex}	2.21 ± 0.03^{dw}
	10	0.23 ± 0.02^{dz}	1.65 ± 0.05^{dy}	1.79 ± 0.04^{dx}	2.30 ± 0.08^{dw}
CCMP (12) + STPP (3000)	0	0.13 ± 0.04^{dy}	0.68 ± 0.03^{efw}	0.65 ± 0.02^{ghw}	0.32 ± 0.03^{fgx}
	5	0.15 ± 0.04^{dy}	0.52 ± 0.01^{dx}	0.62 ± 0.05^{hw}	0.23 ± 0.03^{gx}
	10	0.15 ± 0.03^{dx}	0.54 ± 0.03^{fgw}	0.53 ± 0.04^{hw}	0.22 ± 0.03^{gx}
CCMP (12) + STPP (1500) + SAPP (1500)	0	0.11 ± 0.04^{dx}	0.78 ± 0.03^{ew}	0.89 ± 0.08^{fw}	0.23 ± 0.09^{gx}
	5	0.18 ± 0.03^{dx}	0.60 ± 0.04^{fgw}	0.52 ± 0.03^{hw}	0.22 ± 0.10^{gx}
	10	0.19 ± 0.02^{dx}	0.58 ± 0.03^{fgw}	0.66 ± 0.07^{ghw}	0.26 ± 0.08^{fgx}

[1]All samples were prepared with 20% (w/w) distilled water and 550 ppm sodium ascorbate. CCMP - cooked cured-meat pigment; STPP - sodium tripolyphosphate; SAPP - sodium acid pyrophosphate. Results are means of 3 TBA determinations ± standard deviation. Means in the same column (a-h) or in the same row (w-z) with same letters are not significantly ($P>0.05$) different.

oxidative stability of CCMP-treated systems; lower TBA values were measured during the 3 week storage period. Data from TBA and oxygen uptake studies showed that less oxidation took place in irradiated meats upon storage (Chang, *et al.*, 1961; Whitehair *et al.*, 1964; Greene and Watts, 1966). Piccini *et al.* (1986) reported a similar finding in irradiated seafood. The lipids in these systems may have been protected against radiation-induced oxidative changes by meat proteins and protein-carbohydrate reaction products. Proteins and protein-carbohydrate adducts have been reported to exert an antioxidant effect that increases with irradiation dosage (Diehl, 1982). Addition of STPP and SAPP to meat systems had beneficial effect; lower TBA values were measured. No undesirable odours were noticed in any of the samples tested.

4.8 Effect of Nitrite and Sulphanilamide on Malonaldehyde Quantitation During the TBA Test

4.8.1 Aqueous Model Systems

The absorption intensities of the TBA-malonaldehyde complex at 532 nm for each of four aqueous model systems consisting of malonaldehyde alone, with sodium nitrite, with sulphanilamide or both are given in Table 4.23. As larger aliquots of the 1,1,3,3-tetramethoxypropane stock solution (*i.e.* the precursor of malonaldehyde) were present in systems devoid of nitrite and sulphanilamide, a significant ($P<0.05$) increase in absorbance at 532 nm of the complex of the distillate with the TBA reagent was noticed. Distillation of 0.03 mM malonaldehyde (*i.e.* addition of the 1,1,3,3-tetramethoxypropane to the system equivalent to 0.22 mg malonaldehyde) with 5 mg of sodium nitrite

Table 4.23
Absorption intensity of the TBA-malonaldehyde complex in aqueous model systems.[1]

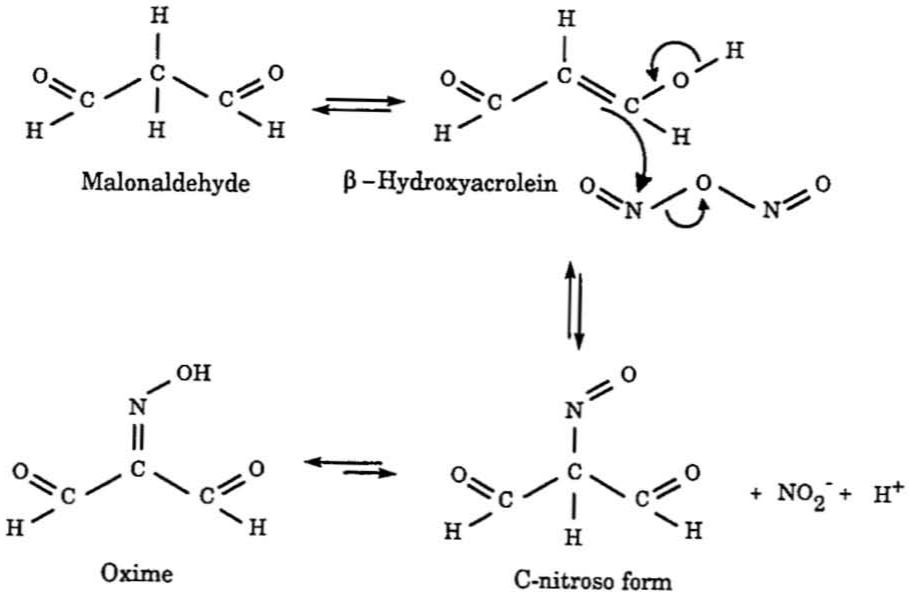
Malonaldehyde (mg)	Absorbance at 532 nm/Additive[2]			
	No additive	$NaNO_2$	$NaNO_2$ + Sulphanilamide	Sulphanilamide
0.044	0.62 ± 0.02^{ax}	0.00 ± 0.00^{ay}	0.57 ± 0.01^{az}	0.58 ± 0.02^{az}
0.088	1.23 ± 0.08^{bx}	0.00 ± 0.00^{ay}	1.07 ± 0.01^{bz}	1.00 ± 0.05^{bz}
0.132	1.87 ± 0.03^{cx}	0.01 ± 0.00^{ay}	1.77 ± 0.03^{cz}	1.50 ± 0.09^{cz}
0.176	2.38 ± 0.04^{dx}	0.01 ± 0.00^{ay}	2.09 ± 0.05^{dz}	2.00 ± 0.15^{dz}
0.220	2.98 ± 0.12^{ex}	0.01 ± 0.00^{ay}	2.73 ± 0.09^{ez}	2.65 ± 0.13^{ez}

[1]Results are mean values of three replicates ± standard deviation. Means in the same column (a-e) or in the same row (x-z) with same letters are not significantly ($P>0.05$) different.
[2]$NaNO_2$ and sulphanilamide were added at 5 mg and 10 mg levels (equivalent to 50 and 100 pm in the system), respectively.

eliminated the TBA reaction of the distillate. Lack of chromogen formation suggested that all malonaldehyde had reacted with and thus was unavailable to react with the TBA reagent. According to March (1992), the most probable mechanism for the nitrosation of dicarbonyl compounds such as malonaldehyde involves attack of the enol form of the dialdehyde (*i.e.*, β-hydroxyacrolein) by a nitrosating species. The initial reaction product is a C-nitroso compound which tautomerizes to a more stable oxime. The nitrosating species derived from nitrite is dinitrogen trioxide (N_2O_3). It exists in equilibrium with nitrous acid in water and has long been recognized as a nitrosating agent in aqueous solutions of nitrous acid at low acidity (Williams, 1983). The free nitrosonium ion (*i.e.*, $^{+}N{=}O$) is also known to exist in these solutions (Ridd, 1978) and may act as the nitrosating species. The mechanism for the nitrosation of malonaldehyde is summarized in Figure 4.13. A nitrite level of 50 ppm was used in this study, because it represents the minimum nitrite concentration required for adequate colour and flavour development in cured pork (MacDonald *et al.*, 1980*c*; National Academy of Sciences, 1982). It also represents the typical residual nitrite level in processed meat products.

Sulphanilamide interfered with the reaction between malonaldehyde and sodium nitrite when it was added at a 100 ppm level to the above systems prior to distillation. Distilled malonaldehyde, released from its diacetal precursor, reacted with the TBA reagent to produce the typical pink chromogen, but absorbance values of the complex formed for the malonaldehyde concentrations tested were significantly ($P<0.05$) different from those systems devoid of nitrite and sulphanilamide (*i.e.* the control system with no

Figure 4.13 Proposed mechanism for the nitrosation of malonaldehyde.


Malonaldehyde
β-Hydroxyacrolein
Oxime
C-nitroso form
+ NO_2^- + H^+

additives). Absorbance readings were 87.0 to 94.6% of those when only malonaldehyde was used (Table 4.23). Sulphanilamide scavenges nitrite from the system by its reaction with nitrous acid or one of its derivatives, such as N_2O_3. A diazonium complex is formed, thereby allowing malonaldehyde to react with the TBA reagent without the interference of nitrite (Figure 4.14).

Sulphanilamide addition to malonaldehyde systems in the absence of nitrite produced a bright yellow-coloured solution. Absorbance readings of the distilled malonaldehyde after reaction with the TBA reagent of these systems were significantly ($P<0.05$) different from those systems devoid of sulphanilamide. Absorbance readings at 532 nm were 82.0 to 93.5% of those when only malonaldehyde was used. These results together with those described above indicate that sulphanilamide reacted with malonaldehyde in some manner. Shahidi *et al.* (1985*a*) postulated that an enamine/imine or N,N′-disubstituted 1-amino-3-iminopropene was formed (Figure 4.15). Sawicki *et al.* (1963) reported that aromatic primary amines react with malonaldehyde to yield N,N′-disubstituted 1-amino-3-iminopropenes, although they did not test sulphanilamide.

The 6π electrons of the -N=C-C=C-N- moiety of a 1-amino-3-iminopropene derivative of malonaldehyde and sulphanilamide may be delocalized by the aromatic rings of sulphanilamide, thereby giving rise to fluorescent activity as analyzed by a spectrofluorometric method. Excitation and fluorescence maxima for the derivative were observed at 395 and 460 nm, respectively (Figure 4.16). These excitation/emission data correspond well with those of 1-amino-3-iminopropene fluorophores reported by Chio and

Figure 4.14 Proposed mechanism for the nitrosation of sulphanilamide.

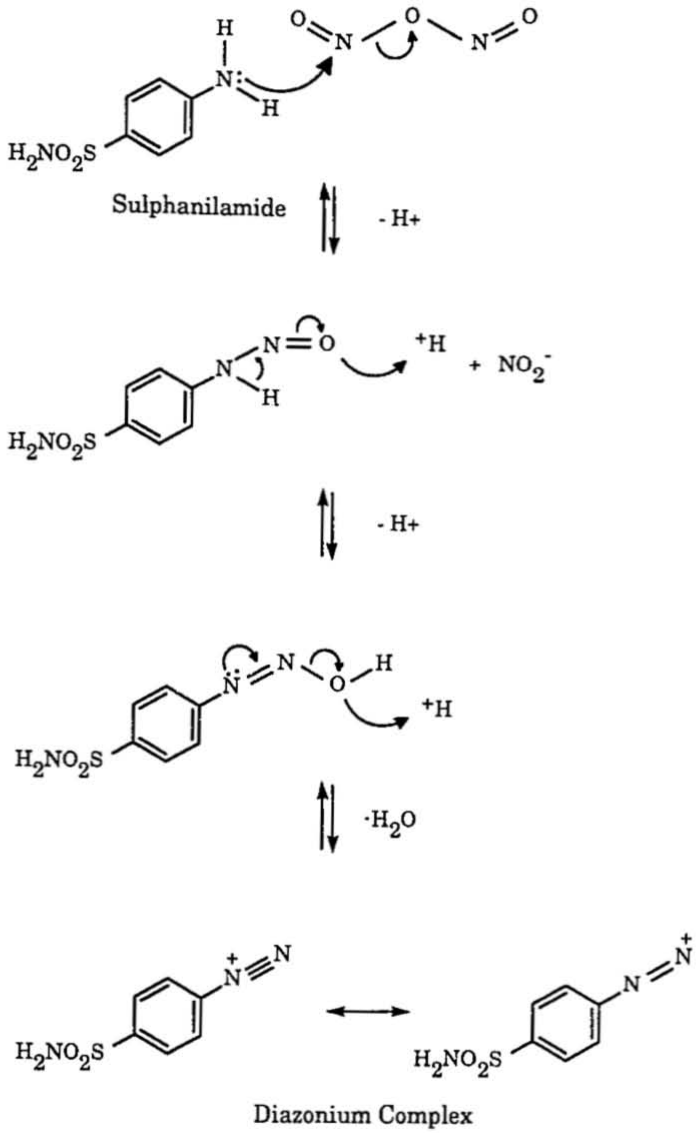

Sulphanilamide
- H+
+H
+ NO2-
- H+
+H
·H2O
Diazonium Complex

Figure 4.15 Proposed mechanism for the formation of a 1-amino-3-iminopropene derivative of sulphanilamide and malonaldehyde.

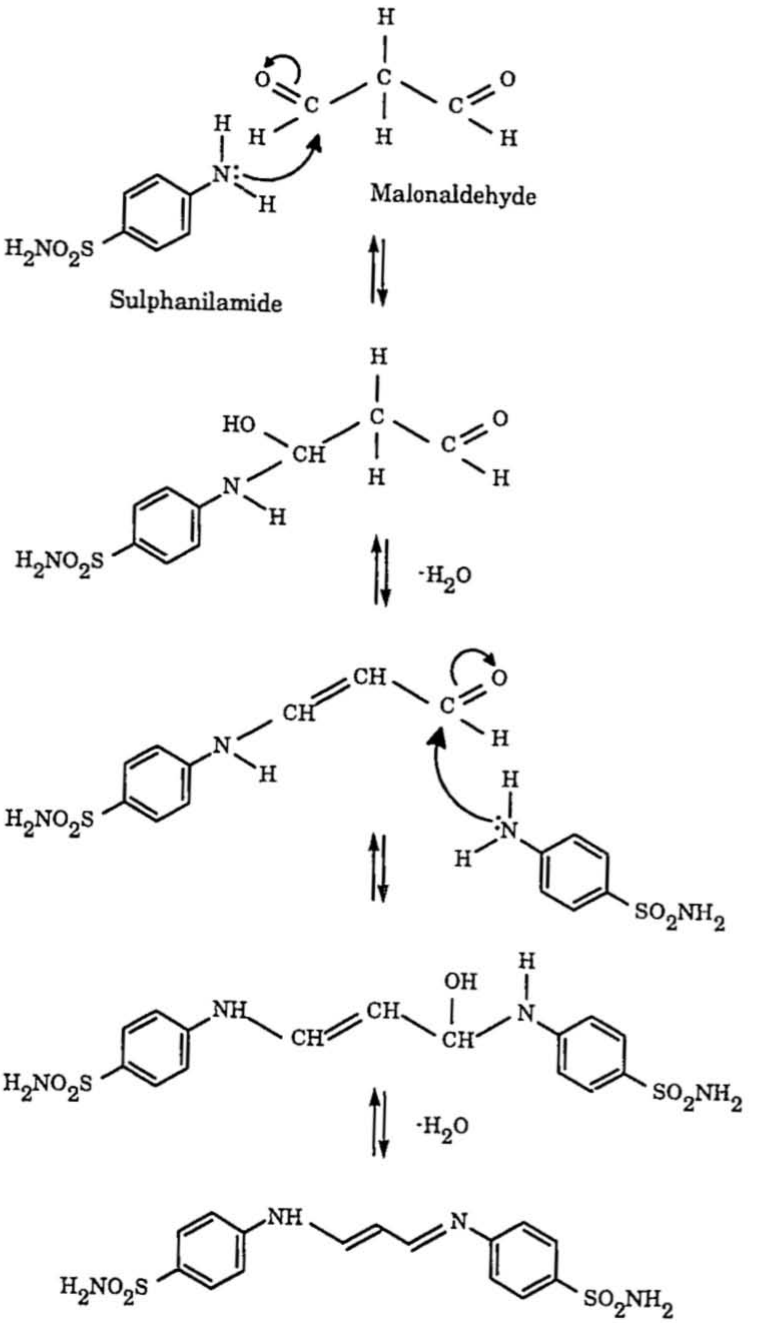
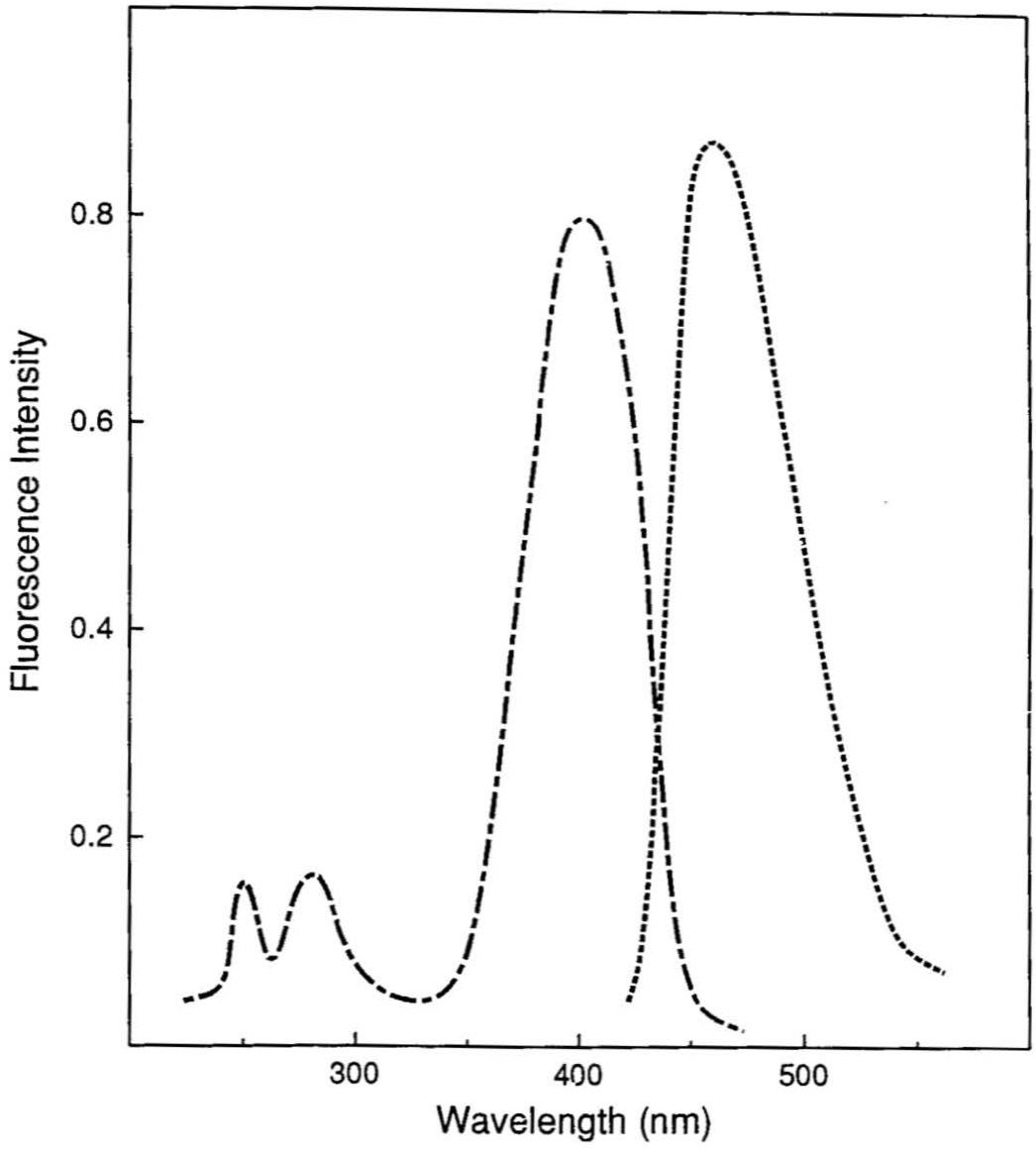

Malonaldehyde
Sulphanilamide
H_2NO_2S
SO_2NH_2
$-H_2O$
$-H_2O$

Figure 4.16 Excitation, ----, and emission, ····· , spectra of the 1-amino-3-iminopropene adduct of sulphanilamide and malonaldehyde.


Fluorescence Intensity
0.8
0.6
0.4
0.2
300
400
500
Wavelength (nm)

Tappel (1969*a*).

4.8.2 Meat Model Systems

The effect on the absorption intensity of the TBA-malonaldehyde complex formed after spiking cooked pork systems with the malonaldehyde precursor before distillation was examined. Figure 4.17 illustrates the relationship between the quantity of malonaldehyde added to meats and the corresponding absorption data at 532 nm for the TBA-malonaldehyde complex formed, after correction for the absorbance due to endogenous malonaldehyde in meat. For a fixed concentration of added malonaldehyde precursor the order of absorption readings for uncured and cured pork with 150 ppm of sodium nitrite was:

Uncured Meat > Cured Meat + Sulphanilamide > Cured Meat

This trend indicates that a significantly ($P<0.05$) better recovery of malonaldehyde was attained when sulphanilamide was added to the nitrite-cured pork systems prior to distillation.

Variation of the absorption data at 532 nm for the TBA-malonaldehyde complex for two sets of pork samples cooked with or without 25, 50, 100, 150 and 200 ppm sodium nitrite is shown in Figure 4.18. For both sets, the corresponding absorption readings of meats cured with 100, 150 and 200 ppm of sodium nitrite were significantly ($P<0.05$) larger when sulphanilamide was added to the mixture prior to distillation, thereby lending support to the findings of Zipser and Watts (1962) who originally proposed the benefits of using sulphanilamide. A reversal of this trend was noticed when

Figure 4.17 Absorbance of the TBA-malonaldehyde complex at 532 nm versus malonaldehyde concentration added to uncured meat, ● ; cured meat with 150 ppm $NaNO_2$, ▲ ; and cured meat with 150 ppm $NaNO_2$ and sulphanilamide, ■ .

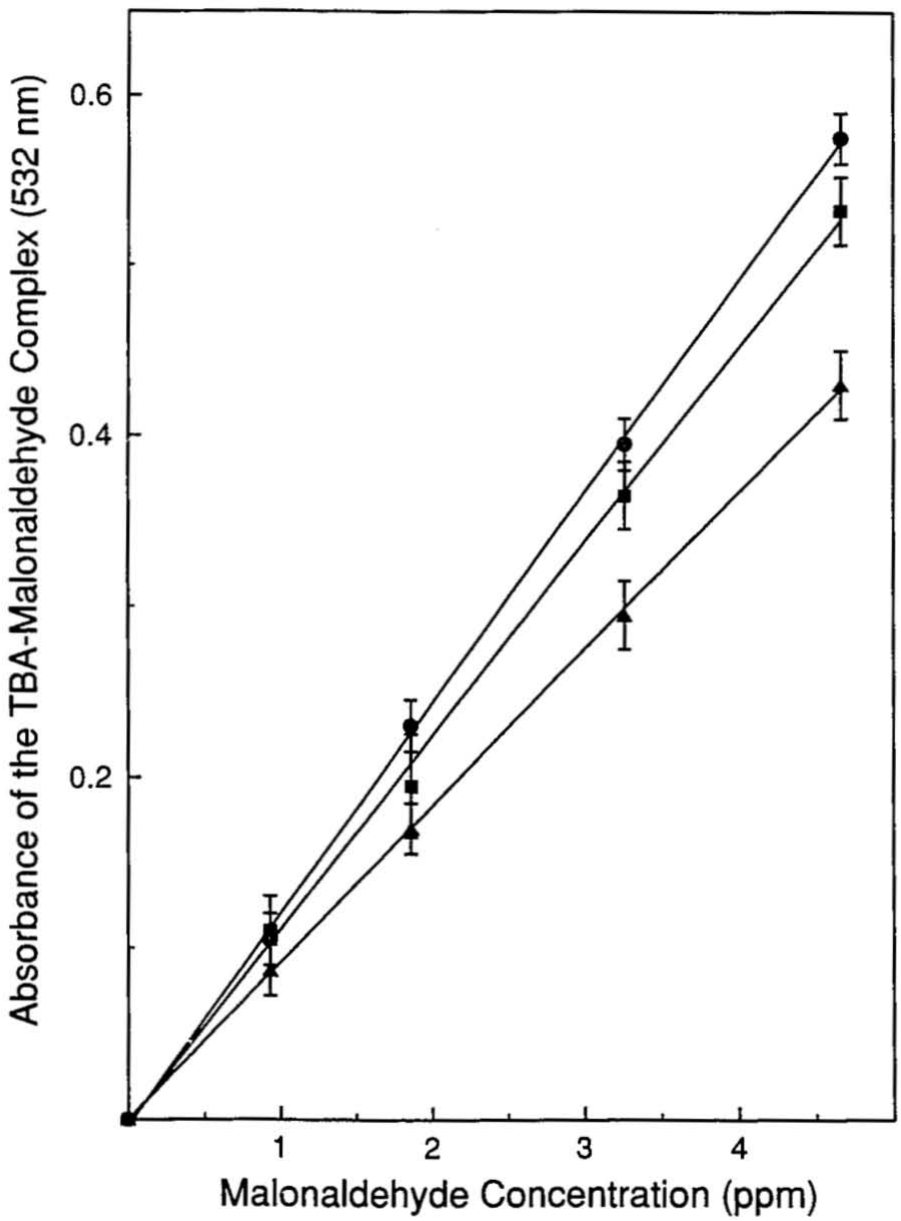
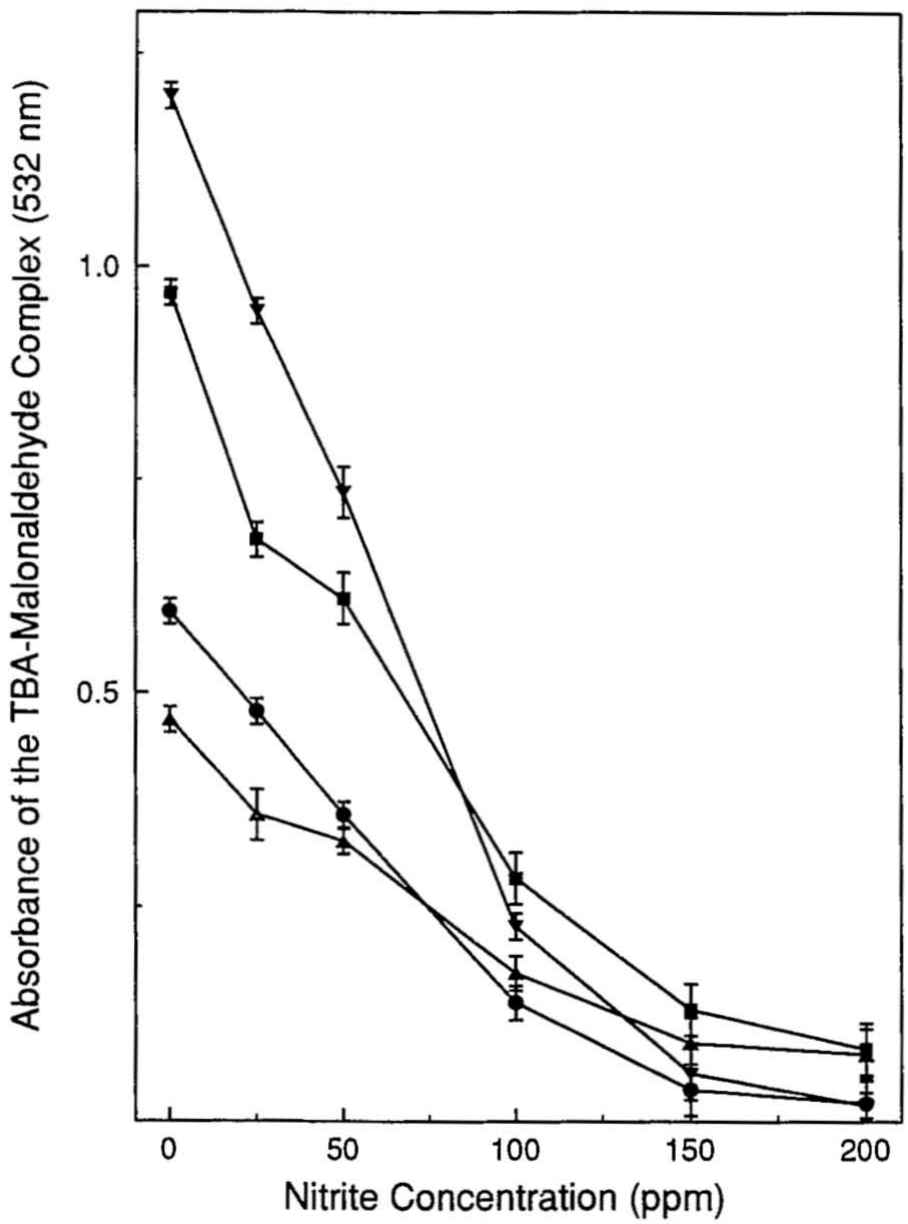

Absorbance of the TBA-Malonaldehyde Complex (532 nm)
0.6
0.4
0.2
1
2
3
4
Malonaldehyde Concentration (ppm)

Figure 4.18 Absorbance of the TBA-malonaldehyde complex at 532 nm versus the concentration of $NaNO_2$ for two sets of meats with different original TBA values without sulphanilamide addition, ▼ and ● , and with sulphanilamide addition, ■ and ▲ .

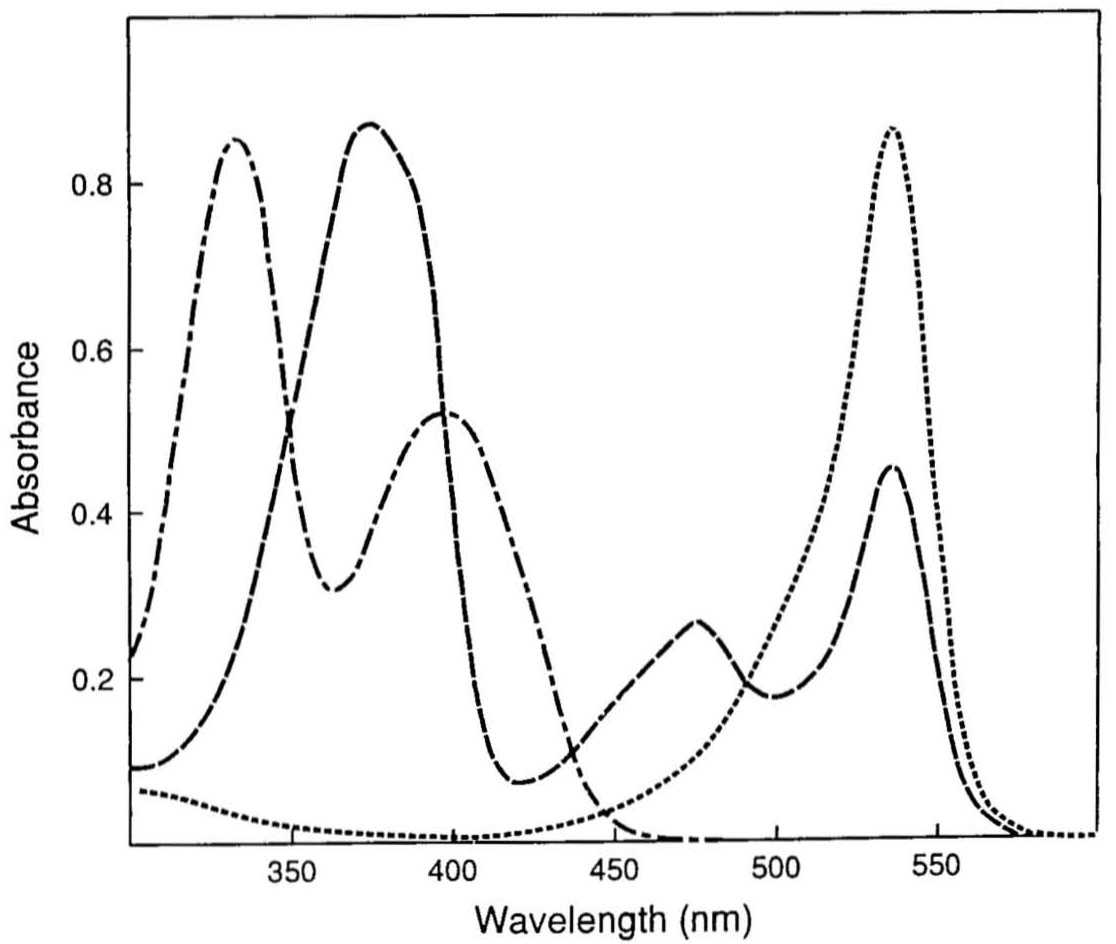

Absorbance of the TBA-Malonaldehyde Complex (532 nm)
1.0
0.5
0
50
100
150
200
Nitrite Concentration (ppm)

sulphanilamide was added to either uncured meat or meats cured with 25 or 50 ppm of sodium nitrite. These differences were significant ($P<0.05$). Interaction of sulphanilamide with malonaldehyde prevents an accurate determination of the extent of rancidity development in cured meats when less than 100 ppm of sodium nitrite is used in the preparation of cured pork.

4.8.3 Interactions of Malonaldehyde with Sulphanilamide, TBA, or Their Combinations in Aqueous Model Systems: Absorption Study

Absorption spectra of complexes formed from the reaction of malonaldehyde with sulphanilamide, TBA, or their mixtures in aqueous model systems are presented in Figure 4.19. The concentrations of the TBA and sulphanilamide solutions used in this study were ten time that of the malonaldehyde precursor. Excess levels were chosen in an attempt to ensure that all malonaldehyde present had completely reacted with these compounds. The absorption spectrum of the TBA-malonaldehyde complex exhibited its characteristic maximum at 532 nm, and showed a small but broad absorption band between 370 and 380 nm with a maximum at 372 nm. The absorption spectrum of the sulphanilamide-malonaldehyde-TBA system had maxima at 532, 472 and 372 nm. The spectrum of the proposed N,N′-disubstituted 1-amino-3-iminopropene derived from sulphanilamide and malonaldehyde exhibited absorption maxima at 396 and 332 nm, but these maxima disappeared from the spectrum when an aliquot of the TBA reagent was added. The 396 and 332-nm bands were lost and the appearance of maxima at 372, 472 and 532 nm was noted. However, when an aliquot of the sulphanilamide solution was

Figure 4.19 Absorption characteristics of model systems of TBA-malonaldehyde, ·····, sulphanilamide-malonaldehyde, - - — - -, and sulphanilamide-malonaldehyde-TBA ————.


Absorbance
0.8
0.6
0.4
0.2
350
400
450
500
550
Wavelength (nm)

added to the TBA-malonaldehyde model system, loss of absorption maxima at 532 and 372 nm did not occur, but the appearance of a new band at 472 nm was noted. Qualitatively, the absorption spectra of the sulphanilamide-malonaldehyde model system to which an aliquot of the TBA reagent was introduced, the TBA-malonaldehyde model system to which an aliquot of the sulphanilamide solution was added and the sulphanilamide-malonaldehyde-TBA model system itself were remarkably similar.

The absorption spectrum of a TBA-sulphanilamide model system was examined and found to have no detectable absorbance in the UV range above 320 nm. Lack of absorption in the visible spectrum tends to suggest that the 472 nm absorbance in the sulphanilamide-malonaldehyde-TBA model system may be due to a mixed chromogenic complex formed among these three compounds. Therefore, reaction products from the reaction of TBA and malonaldehyde (*i.e.*, TMT), sulphanilamide and malonaldehyde (*i.e.*, SMS) and sulphanilamide, malonaldehyde and TBA (*i.e.*, SMT) in model systems were formed, isolated and characterized (Pegg *et al.*, 1992) and as summarized below.

4.8.4 Interactions of Malonaldehyde with Sulphanilamide and TBA: Structure of Adducts

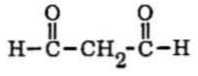
4.8.4.1 Characterization of the TMT Adduct

The TMT isolated from the reaction of TBA and malonaldehyde, in a model system, had a deep purple colour and appeared as needle-like crystals under a microscope. The compound did not melt but decomposed when a temperature of 350°C was attained. Similar findings were reported by Sinnhuber *et al.* (1958). The TMT crystals were not

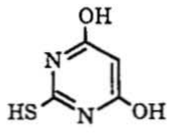
readily soluble in dilute acid solutions, and were dissolved in a small volume of dimethyl sulphoxide (DMSO) and then diluted with 0.1 M HCl. The UV-VIS spectrum of this solution exhibited a pink colour with an absorption maximum at a wavelength of 532 nm (ε = 125,000 $M^{-1} \cdot cm^{-1}$). Unlike the TBA-malonaldehyde system used in the absorbance study, a broad absorption band between 370 and 380 nm with a maximum at 372 nm was absent from the spectrum of TMT. This band is most likely due to a one-to-one adduct of TBA and malonaldehyde as opposed to the usual two-to-one complex. The one-to-one adduct is an intermediate reaction product in the proposed mechanism of TMT formation as described by Nair and Turner (1984) and illustrated in Figure 2.5 (Pegg and Shahidi, 1991). Lack of a 372-nm absorption in the spectrum of purified TMT suggests that this two-to-one complex is thermodynamically stable in the acid medium, because hydrolytic breakdown products are not evident. The proposed chemical structure of TMT is presented in Figure 4.20.

The IR spectrum of TMT exhibited bands characteristic of group frequencies associated with the proposed molecule (Table 4.24 and Appendix). The three vibrational bands diagnostic of secondary amides were present (*i.e.*, C=O stretch, 1630 cm^{-1}; N-H bend, 1496 cm^{-1}; and C-N stretch, 1299 cm^{-1}) as was the C=S stretch (1127 cm^{-1}) of the thioamide moiety. In the IR spectrum of TBA, a strong absorption at 1163 cm^{-1} was interpreted as being the C=S stretch of the many resonance contributors (Goel *et al.*, 1985). A weak vibrational band at 2550 cm^{-1} was also observed in the IR spectrum of the parent molecule. This band is characteristic of S-H stretching, but it was absent from

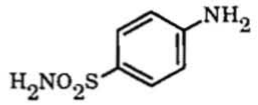
Figure 4.20 Formation of TMT, SMS and SMT from malonaldehyde, TBA, and sulphanilamide.



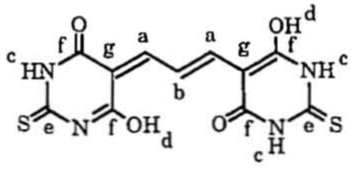
Malonaldehyde



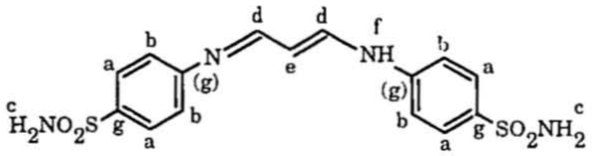
2-Thiobarbituric Acid



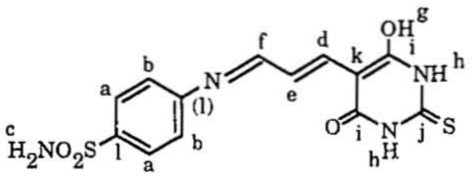
Sulphanilamide



TMT



SMS



SMT

Table 4.24
The FTIR (in KBr) data for complexes of malonaldehyde with TBA and sulphanilamide.

Compound	Wavenumber, cm^{-1}	Assignment
TMT	1630, 1671 (sh) vs	ν C=O (Amide I)
		ν C=C
	1496 (vs)	δ N-H (Amide II)
	1360 (vs)	δ_{ip} O-H
	1299 (s)	ν C-N
	1214 (s)	ν C-O
	1176 (m)	ν C-O
	1127 (s)	ν C=S
	1002 (m)	δ_{op} =C-H
SMS	3168 (s)	ν =C-H
	3060 (s)	ν =C-H
	1637 (vs)	δ NH_2
	1579, 1600 (sh) (vs)	ν C=C of aromatic ring
	1491 (m)	ν C=C of aromatic ring
	1336 (vs)	ν_{as} SO_2
	1197 (m)	ν C=C of aromatic ring
	1152 (vs)	ν_s SO_2
	909 (m)	ν S-N
	835 (m)	δ_{op} =C-H
	612 (s)	δ SO_2
SMT	3369 (m)	ν_{as} NH_2
	3209 (s)	ν =C-H
	3087 (s)	ν =C-H
	1638 (s)	ν C=O (Amide I)
		ν C=C
	1489, 1510 (sh) (vs)	δ N-H
	1385, 1412 (sh) (vs)	ν C-O
	1337 (s)	ν_{as} SO_2
	1302 (s)	ν C-N (Amide III)
	1196, 1185 (sh) (s)	ν C=C of aromatic ring
		ν C-O
	1153 (vs)	ν SO_2
	1130 (vs)	ν C=S

[1]vs - very strong; s = strong; m = medium; sh = shoulder.
[2]ν = stretching; ν = bending; ν_{as} - asymmetric stretching; ν_s = symmetric stretching; δ_{ip} = in plane bending; δ_{op} = out of plane bending.

the spectrum of TMT. Although sulphydryl groups were not detected in the complex in its solid state, they may exist as resonance contributors of the molecule in acidic solution. Raman spectroscopy may offer a means of clarifying the existence of this functional group in TMT, because it shows a strong signal for S-H stretching.

The 300-MHz ^{1}H NMR spectrum of TMT dissolved in DMSO-d_6 revealed four types of resonances. A doublet and a triplet at δ 7.71 and 8.55, with a relative integration equivalent to two protons and one proton, respectively, and a coupling constant of 13.8 Hz, were diagnostic of the *trans*-vinyl protons of the malonaldehyde moiety in TMT. A broad temperature- and concentration-dependent resonance at δ 5.1 which rapidly exchanged with D_2O was assigned to the amide protons of the substituted pyrimidine moiety. A sharp peak at δ 11.5 with a relative integration equivalent to four protons was assigned to the hydroxyl groups of TMT, but an integration of only two protons was expected. Addition of D_2O resulted in the disappearance of this signal, thereby indicating that these protons were exchangeable.

The $^{13}C\{^{1}H\}$ NMR spectrum revealed five resonances for the proposed 11-carbon complex. The attached proton test (APT) spectrum showed that three of the five nonequivalent carbons of the molecule were quaternary. Carbon resonances at δ 158.0 and 118.1 correlated with the vinyl ^{1}H resonances at δ 7.71 and 8.55, respectively. The broad, but weak, signal at δ 162.5 was assigned to the thioamide group as reported by Nair and Turner (1984). Signals of the quaternary carbons at δ 175.8 and 101.5 were assigned as the amide carbons and the remaining two equivalent ring carbons,

respectively. Assignments of $^{13}C\{^1H\}$ NMR signals of the substituted pyrimidine in TMT are supported by $^{13}C\{^1H\}$ NMR data obtained for the TBA parent molecule. The $^{13}C\{^1H\}$ NMR spectrum of TBA dissolved in DMSO-d_6 exhibited five signals for the three nonequivalent carbon atoms at δ 82.0, (162.2, 166.0), and (175.1, 180.8). Signals at δ 162.2 and 166.0 are most probably based on tautomeric forms of the thioamide group, while signals at δ 175.1 and 180.8 are due to tautomeric forms of the amide functional group. In contrast to the spectrum of TBA, only five $^{13}C\{^1H\}$ NMR signals were observed for the five nonequivalent carbon atoms of TMT, thus suggesting a limited number of tautomers. A summary of the ^1H and $^{13}C\{^1H\}$ NMR assignments for TMT is presented in Figure 4.20 and Table 4.25.

The normalized mass spectrum of TMT ($C_{11}H_8N_4O_4S_2$) obtained by electron impact revealed a base peak with *m/z* of 144, signifying that the major fragment ion was the TBA parent molecule. Although a base peak with *m/z* of 143 was expected to be the major fragment ion based on the proposed structure of the complex and typical fragmentation pathways, hydrogen migration may have occurred between fragments in the mass spectrometer resulting in the observed 144 peak. The molecular ion was detected at *m/z* of 324 with a modest intensity of 1.5%. During some scans, an ion with *m/z* of 338 was detected at an intensity of approximately 0.3%, but it could not be assigned. Major fragment ions included *m/z* of 180 (61%), 170 (11%), 156 (10%), 137 (9%), 122 (13%), 116 (47%), 109 (17%), 93 (31%), 86 (12%), 69 (49%), 65 (15%), 64 (22%), 59 (68 %), 43 (81%), and 42 (95%) (see Appendix).

Table 4.25
The 300 MHz ^{1}H and 75 MHz ^{13}C NMR (in DMSO-d_6) data for complexes of malonaldehyde with TBA and sulphanilamide.

TMT				
^{1}H Data			^{13}C Data	
δ (ppm), TMS		Assignment	δ (ppm), TMS	Assignment
5.1 (s)	br., ex., var.	c	101.5	g
7.71 (d, 2H)	J = 13.8 Hz	a	118.1	b
8.55 (t, 1H)	J = 13.8 Hz	b	158.0	a
11.5 (s, 4H)	ex.	d	162.5	e
			175.8	f
SMS				
^{1}H Data			^{13}C Data	
δ (ppm), TMS		Assignment	δ (ppm), TMS	Assignment
3.43 (s)	br., ex., var.	f	100.2	e
6.56 (t, 1H)	J = 11.3 Hz	e	117.7	b
7.44 (s, 4H)	ex.	c	127.7	a
7.57 (d, 4H)		b	141.1	g
7.92 (d, 4H)		a	159.6	d
8.92 (d, 2H)		d		

.....continued on next page

Table 4.25
The 300 MHz ^{1}H and 75 MHz ^{13}C NMR (in DMSO-d_6) data for complexes of malonaldehyde with TBA and sulphanilamide.

SMT					
^{1}H Data				^{13}C Data	
δ (ppm), TMS			Assignment	δ (ppm), TMS	Assignment
3.44	(s)	br., ex., var.	h	103.5	k
7.34	(s, 2H)	ex.	c	107.9	e
7.51	(d, 2H)		b	117.0	b
7.57	(dd, 1H)	J = 13.2 Hz	e	127.6	a
		with D_2O appears		139.8	l
		more like a t		141.8	l
7.82	(d, 2H)		a	157.5	d/f
8.15	(d, 1H)		d	162.4	j
8.77	(dd or t, 1H)	J = 13.2 Hz	f	177.3	i
11.8	(s, 2H)	ex.	g		

[1]s = singlet; d = doublet; dd = doublet of doublet; t = triplet; br. = broad peak; ex. = exchangeable; var. = variable peak; and J = coupling constant. For assignment letters refer to Figure 4.20.

4.8.4.2 Characterization of the SMS Adduct

Reaction of sulphanilamide and malonaldehyde, in a model system, yielded a bright yellow chromogen which possibly was due to the formation of condensation products of an enamine/imine or N,N′-disubstituted 1-amino-3-iminopropene structure (Shahidi *et al.*, 1991*a*). Excitation and fluorescence spectra of this molecule (*i.e.* SMS) were characteristic of 1-amino-3-iminopropene derivatives produced from the reaction of malonaldehyde with amino compounds as mentioned above (Chio and Tappel, 1969*a*; Arya *et al.*, 1974). The SMS isolated had a yellow colour, and appeared as small crystals under a microscope. A melting point of 203°C was determined. Its UV-VIS spectrum in 0.1 M HCl showed absorption bands at wavelengths of 396 nm ($\varepsilon \approx 10{,}500\ M^{-1}\cdot cm^{-1}$), 332 nm ($\varepsilon = 28{,}000\ M^{-1}\cdot cm^{-1}$) and 256 nm ($\varepsilon = 14{,}000\ M^{-1}\cdot cm^{-1}$) confirming that the adduct was of a highly conjugated nature. The absorption spectrum of SMS crystals dissolved in 0.1 M HCl was similar to that of the sulphanilamide and malonaldehyde model system used in the absorbance study of section 4.8.3. Dissolved SMS was not stable in the acidic solution, because a slow decrease in the intensity of the 396-nm band occurred over time. Formation of the adduct is most probably initiated by attack of the nucleophilic amino group of sulphanilamide at carbon-1 of malonaldehyde, followed by dehydration, forming an enamine/imine compound. This reversible reaction is followed by an identical reaction of the intermediate one-to-one complex with a second molecule of sulphanilamide forming a N,N′-disubstituted 1-amino-3-iminopropene complex. The proposed chemical structure of this adduct is presented in Figure 4.20.

The IR spectrum of SMS exhibited bands representative of group frequencies associated with the proposed molecule (Table 4.24 and Appendix). Vibrational bands of the aromatic C=C and C=N conjugated system were observed at 1600, 1579, 1491, and 1411 cm^{-1}. The N-H bending of the sulphonamide group was detected at 1637 cm^{-1}. Characteristic asymmetric and symmetric stretching bands of SO_2 groups were noted at 1336 and 1152 cm^{-1}, respectively. Group frequencies of SMS were almost identical to those found in the IR spectrum of the sulphanilamide parent molecule, but the vibrational bands of SMS were generally broader in nature. Strong asymmetric and symmetric stretching of primary amino bonds of sulphanilamide at 3478 and 3376 cm^{-1}, respectively, were absent in the spectrum of SMS. The asymmetric and symmetric signals of the sulphonamide group at 3360 cm^{-1} (shoulder) and at 3266 cm^{-1}, respectively, were obscured by two strong IR absorption signals at 3168 and 3060 cm^{-1}. These bands may be due to =C-H stretching of the extended conjugation of the aromatic system of SMS.

The 300-MHz ^1H spectrum of SMS dissolved in DMSO-d_6 displayed five resonances. Two AA′BB′ doublets centred at δ 7.57 and 7.92 were diagnostic of the aromatic protons of sulphanilamide. Integration of these doublets, relative to the δ 7.44 signal, indicated a total of eight protons, thereby suggesting that the complex contained two molecules of sulphanilamide. A singlet at δ 7.44, with a relative integration of four protons, was assigned to the $-NH_2$ protons of the sulphonamide groups of sulphanilamide. Further evidence for this assignment was obtained from the $^1H/D_2O$ exchange/DMSO-d_6

NMR spectrum of the complex. The intensity of the signal at δ 7.44 decreased substantially after D_2O addition to the NMR tube indicating that these protons were exchanging with the solvent. Assignments made above for the protons of SMS were supported by those determined for the sulphanilamide parent molecule, but the signals in SMS were shifted down-field by 0.5-1.0 ppm compared to those of sulphanilamide itself. The ^1H spectrum of sulphanilamide dissolved in DMSO-d_6 also showed a singlet at δ 5.82. This signal had a relative integration denoting two protons and disappeared upon D_2O addition. It was assigned to the primary amino group of sulphanilamide which was not observed in the SMS molecule. This suggests that cross-linking of the carbonyl moieties of malonaldehyde occurs with the primary amino groups of sulphanilamide. A doublet centred at δ 8.92 and a triplet at δ 6.56, with a relative integration equivalent to two and one protons, respectively, and a coupling constant of 11.3 Hz were assigned as being characteristic of the *trans*-vinyl protons of malonaldehyde.

The $^{13}C\{^1H\}$ NMR spectrum of SMS dissolved in DMSO-d_6 revealed five resonances for the 15-carbon adduct, thereby suggesting that the compound had considerable symmetry. Assignments for signals from the $^{13}C\{^1H\}$ NMR spectrum were aided by APT data which showed the presence of one or two quaternary carbons (δ 141.1 and 141.2) and four tertiary carbons (δ 100.2, 117.7, 127.7, and 159.6). The vinyl protons of malonaldehyde at δ 6.56 and 8.92 correlated with $^{13}C\{^1H\}$ resonances at δ 100.2 and 159.6, respectively. Carbon atoms bonded to the aromatic protons at δ 7.57 and 7.92 were assigned to $^{13}C\{^1H\}$ resonances at δ 117.7 and 127.7, respectively. The

$^{13}C\{^1H\}$ NMR spectrum of the sulphanilamide parent molecule assisted in elucidating these assignments. Two equivalent tertiary carbon atoms of sulphanilamide adjacent to the sulphonamide group gave a signal at δ 127.5 which was identical to that found in SMS. The remaining two tertiary carbon atoms adjacent to the primary amino group of sulphanilamide gave a signal at δ 112.5. Weak $^{13}C\{^1H\}$ NMR signals produced by the sulphonamide- and amino-ipso carbon atoms of sulphanilamide at δ 152.0 and 130.0, respectively, were absent from the spectrum of SMS. A $^{13}C\{^1H\}$ signal at or near δ 152.0 was expected in the spectrum of SMS even though the cross-linking of sulphanilamide with malonaldehyde at the primary amine changed the chemical environment of this carbon atom. The $^{13}C\{^1H\}$ signals were detected however at δ 141.1 and 141.2, but it is unclear whether this represents one or two quaternary carbon atoms. It is conceivable that the signal at δ 141.1 and 141.2 is representative of the carbon atoms of the sulphonamide group in SMS while the signal for the amino/imino carbon atoms was obscured by ^{14}N quadrupole broadening. A summary of the ^1H and $^{13}C\{^1H\}$ NMR assignments for SMS is presented in Figure 4.20 and Table 4.25.

The normalized mass spectrum of the SMS adduct ($C_{15}H_{16}O_4N_2S_2$) obtained by electron impact revealed that the base peak had *m/z* of 172, which is characteristic of the molecular mass of sulphanilamide. With typical fragmentation patterns, a major fragment at *m/z* of 171 was expected from the sulphanilamide groups of SMS, but as was the case for TMT hydrogen migration of fragments may have occurred in the mass spectrometer resulting in the observed 172 signal. Another major mass ion at *m/z* of 156 from the

fragmentation of sulphanilamide moieties was expected and found in the spectrum. The molecular ion was detected at *m/z* of 380 with a modest intensity of 1%. No higher *m/z* signals were noted. Major fragment ions included *m/z* of 156 (75%), 108 (33%), 93 (24%), 92 (53%), 66 (12%), 65 (44%), 64(40%), 48 (21%), and 43 (15%) (See Appendix).

4.8.4.3 Characterization of the SMT Adduct

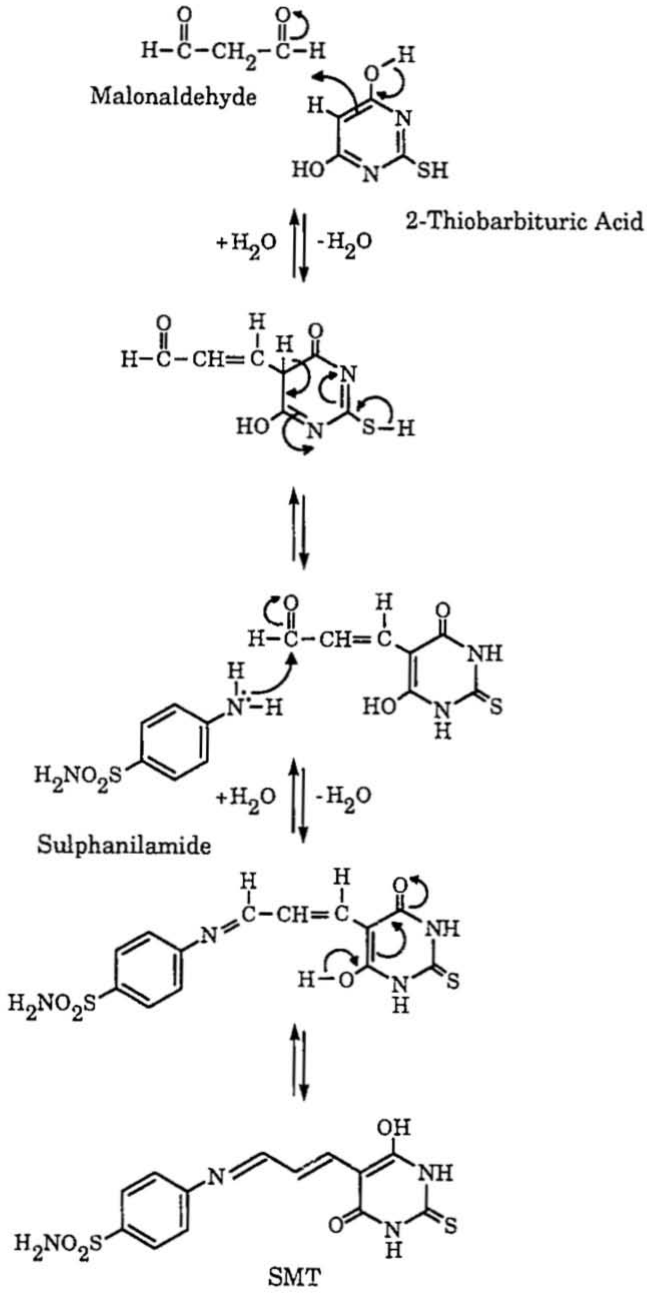
The reaction of TBA and sulphanilamide with malonaldehyde, in a model system, produced an orange- rather than the characteristic pink-coloured solution. The SMT isolated had an orange colour, and appeared as small crystals under a microscope. The compound did not melt but decomposed when a temperature of 350°C was attained. The SMT crystals were not readily soluble in dilute acid solutions, similar to TMT, and were dissolved in a small volume of DMSO and then diluted with 0.1 M HCl. The UV-VIS spectrum of this solution showed absorption maxima at wavelengths of 532, 472, 372 and 278 nm. The absorption spectrum of dissolved SMT crystals was similar to that of the sulphanilamide-malonaldehyde-TBA model system used in the absorbance study of section 4.8.3, except that a bathochromic shift of the 278-nm band to 266 nm occurred. A greater absorbance of dissolved SMT at the 372-nm band, relative to other maxima detected in the absorbance study, suggested that some of the SMT had hydrolyzed at the amine junction forming the one-to-one TBA-malonaldehyde intermediate and free sulphanilamide. Molar extinction coefficients for absorption maxima of SMT were not determined, because the intensity of the maxima decreased too rapidly with time for their

accurate determination. The 472- and 532-nm bands were detected in the spectrum of the SMT solution even after 4 weeks of storage. The proposed chemical structure and the mechanism of formation of SMT is presented in Figures 4.20 and 4.21, respectively.

The IR spectrum of SMT exhibited vibrational bands diagnostic of group frequencies associated with both TMT and SMS (Table 4.24 and Appendix). The three vibrational bands characteristic of the secondary amide group of the substituted pyrimidine moiety of TBA were observed at 1638, 1489 (1510, shoulder), and 1301 cm^{-1}. The C=S stretching of the thioamide group was noted at 1130 cm^{-1}. No vibrational band due to S-H stretching was detected at 2550 cm^{-1} . Characteristic asymmetric and symmetric stretching bands of the SO_2 moiety of sulphanilamide and SMS were observed at 1337 and 1130 cm^{-1}, respectively. Strong asymmetric and symmetric stretching bands of the primary amino group of sulphanilamide at 3478 and 3376 cm^{-1}, respectively, were absent from the spectrum, as was the case for SMS. Absence of these signals suggests that cross-linking between sulphanilamide and malonaldehyde occurs at the primary amino groups of sulphanilamide. The asymmetric stretching band of the sulphonated $-NH_2$ of sulphanilamide was observed at 3367 cm^{-1}, but its symmetrical counterpart was obscured by broad signals at 3209 and 3087 cm^{-1}. These are possibly due to a =C-H rocking of the aromatic ring.

The 300-MHz ^1H NMR spectrum of SMT dissolved in DMSO-d_6 showed eight resonances. Two AA′BB′ doublets centred at δ 7.82 and 7.51 with a relative integration equivalent to four protons were diagnostic of the two sets of equivalent aromatic protons

Figure 4.21 Proposed mechanism for the formation of SMT from sulphanilamide, malonaldehyde and TBA.


Malonaldehyde
2-Thiobarbituric Acid
+H2O
-H2O
Sulphanilamide
SMT

of the sulphanilamide moiety. A singlet at δ 7.34 with a relative integration equivalent to two protons, was assigned to the $-NH_2$ protons of the sulphonamide group of the sulphanilamide moiety. Further evidence for this assignment was obtained from the $^1H/D_2O$ exchange/DMSO-d_6 NMR spectrum of the complex. The intensity of the signal at δ 7.34 decreased substantially after D_2O addition to the NMR tube indicating that these protons were exchanging with the solvent. Assignments made above for the protons of SMT are supported by those determined for SMS and the parent sulphanilamide molecule. A broad peak at δ 3.44 was assigned to the amide protons of the substituted moiety of TBA, but its position and intensity varied slightly depending on the concentration of SMT in the tube and the temperature at which the NMR experiment was performed. A singlet at δ 11.8 with a relative integration equivalent to two protons was assigned to the hydroxyl group of the TBA moiety of SMT. The time-average C_{2v} symmetry of the complexes of TMT and SMS is absent from the proposed structure of SMT, as can be seen in Figure 4.20. Consequently, the *trans*-vinyl protons from malonaldehyde's contribution to SMT were more difficult to assign. Three chemical shifts for the vinyl protons, each with a relative integration of one proton and with a coupling constant of 13.2 Hz, were detected at δ 7.57, 8.15, and 8.77. The doublet centred at δ 8.15 was assigned to the vinyl proton adjacent to the substituted pyrimidine moiety. The ^1H signal in the spectrum at δ 7.57 was partially obscured by the resonance of aromatic protons of sulphanilamide at δ 7.51. The δ 7.57 signal appeared to be a doublet of a doublet which would be characteristic of the central proton in the malonaldehyde moiety, but in the

spectrum of the $^1H/D_2O$ exchange/DMSO-d_6 NMR experiment this resonance appeared more like a triplet than as a doublet of doublets. The δ 8.77 signal in the ^1H spectrum appeared as a triplet but may have actually been an overlapping doublet of doublets. In the $^1H/D_2O$ exchange/DMSO-d_6 NMR experiment, the δ 8.77 signal appeared as a doublet, indicating that exchange of hydrogen atoms with those of deuterium had occurred.

The $^{13}C\{^1H\}$ NMR spectrum revealed nine resonances for the proposed 13-carbon complex which is indicative of a decrease in symmetry of SMT compared with TMT and SMS. The APT spectrum showed that five of the nine nonequivalent carbons in the molecule were quaternary in nature, while the other four were tertiary. Signals of the quaternary carbons at δ 177.3, 162.4, and 103.5 were assigned to the positions determined in their TBA parent molecule. Aromatic protons at δ 7.51 and 7.82 correlated with $^{13}C\{^1H\}$ resonances at δ 117.0 and 127.6, respectively, and are similar to those assignments for SMS. Quaternary carbons detected at δ 139.8 and 141.8 may actually be tautomers of one signal representative of the ipso carbon of the sulphonated moiety. As was the case for SMS, the signal from the amino/imino-ipso carbon was obscured by ^{14}N quadrupole broadening. Only two $^{13}C\{^1H\}$ NMR signals were detected for the three carbon atoms of the malonaldehyde moiety in SMT. The resonance at δ 107.9 was assigned to the central carbon atom of the malonaldehyde group, and the signal at δ 157.5 for the tertiary carbon atoms was assigned to the other two as accidentally degenerate. Alternatively, the δ 157.5 signal may be assigned to carbon atom next to the substituted

pyrimidine moiety of TBA, and the signal from the other carbon atom adjacent to the nitrogen atom of the sulphanilamide group was obscured by ^{14}N quadrupole broadening. A summary of the ^{1}H and $^{13}C\{^{1}H\}$ NMR assignments is presented in Figure 4.20 and Table 4.25.

The normalized mass spectrum of the SMT adduct ($C_{13}H_{12}N_4O_4S_2$) obtained by electron impact showed the base peak at *m/z* of 65. This ion was also detected as a major fragment in the mass spectra of both sulphanilamide and SMS. The molecular ion was detected at *m/z* of 352 with a modest intensity of 1%. No higher *m/z* signals were noted. Major fragment ions included *m/z* of 180 (23%), 172 (80%), 156 (82%), 144 (54%), 128 (24%), 116 (28%), 108 (56%), 93 (21%), 92 (93%), 80 (18%), 69 (39%), 64 (22%), 63 (22%), 59 (54%), 44 (20%), 43 (54%), 42 (57%), and 41 (34%). Fragment ions of TMT and SMS as well as their parent molecules TBA and sulphanilamide, respectively, are noted in the mass spectrum of SMT (Appendix).

4.8.4.4 Implications of Interaction of Sulphanilamide with Malonaldehyde in Determination of Oxidative State of Nitrite-Cured Meats

To determine the oxidative state of nitrite-cured meats by the TBA test, sulphanilamide is added to samples prior to analysis in order to react with residual nitrite present. Sulphanilamide addition prevents the nitrosation of malonaldehyde, thereby allowing distilled malonaldehyde to react with the TBA reagent. Results of a study by Kolodziejska *et al.* (1990) on this topic agreed with the above statements. Addition of sulphanilamide to malonaldehyde model systems containing sodium nitrite allowed the

distilled malonaldehyde to react with the TBA reagent, but the TBA values determined were lower than those when nitrite and sulphanilamide were absent from the malonaldehyde system. Similar conclusions were reached when meat model systems containing nitrite and sulphanilamide were tested. The latter results suffered from errors ranging between 6 and 20%, but according to Kolodziejska *et al.* (1990) the reaction of malonaldehyde with sulphanilamide is reversible and therefore all malonaldehyde present will react with the TBA reagent forming the typical two-to-one TBA-malonaldehyde complex. Although it is true that the formation of Schiff bases is reversible, the above authors failed to note that the visible absorption spectrum of the sulphanilamide-malonaldehyde-TBA model system was markedly different from that of its counterpart devoid of sulphanilamide. The appearance of the new band at 472 nm in the sulphanilamide-malonaldehyde-TBA system, as it has now been fully documented, suggests the presence of a second complex due to multiple interactions between malonaldehyde with both sulphanilamide and TBA. The complex is a condensation product of one molecule of each of sulphanilamide and TBA cross-linked with the highly reactive three carbon moiety of malonaldehyde.

4.9 Hexanal Content in Uncured, Nitrite-Cured and CCMP-Treated Cooked Pork

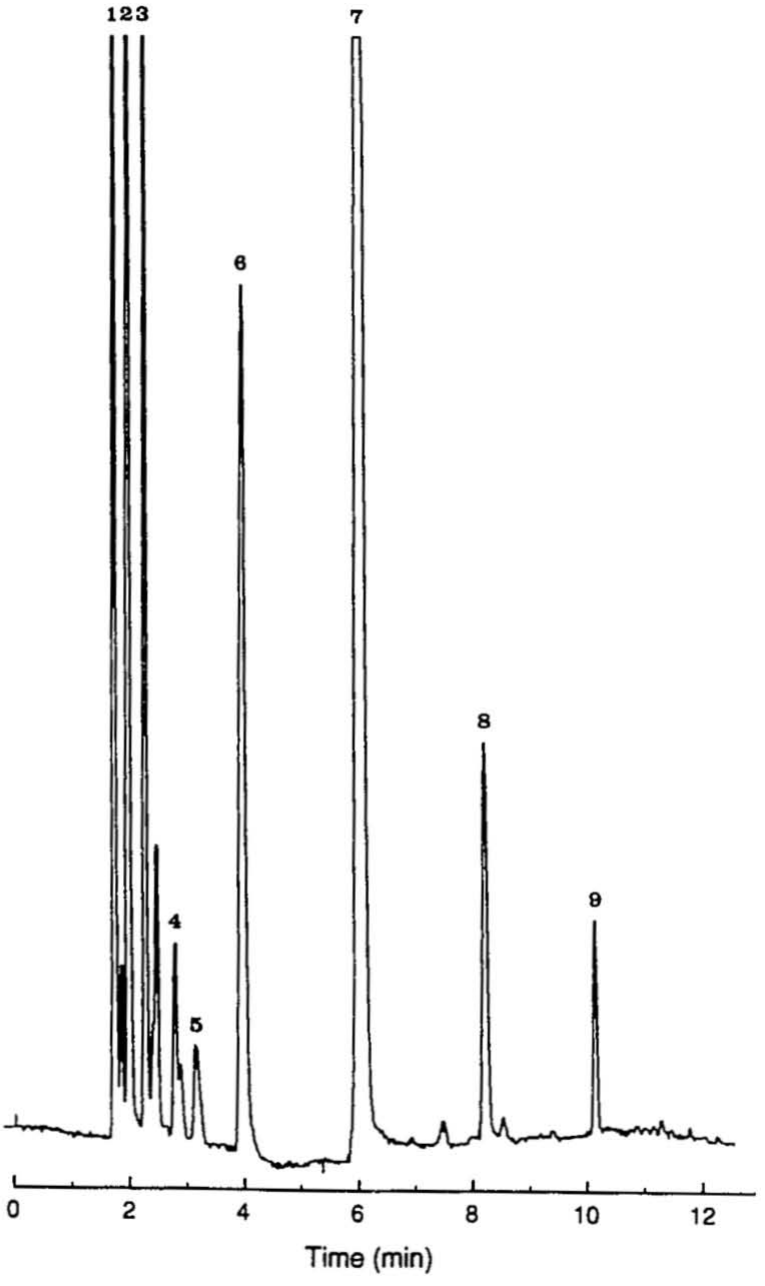
To characterize the pork used, a proximate analysis of the fresh meat was carried out. The pork contained 73.2±0.6% moisture, 20.2±0.4% crude protein, 5.6±0.4% total lipids and 1.0±0.3% ash. Since variations in moisture and fat levels of cooked samples

may affect the stability of water- and fat-soluble flavour precursor compounds, respectively, the amount of water and lipid in samples was monitored to see if their content varied during the storage period (Ang and Lyon 1990). No significant ($P>0.05$) changes in either level were observed during 3 weeks of refrigerated storage.

A typical chromatogram of the headspace (HS) volatiles of cooked pork after 5 days of storage is presented in Figure 4.22. The rapid gas chromatographic-flame ionization detection method and capillary column used in this investigation did not allow for the analysis of all possible compounds related to meat flavour deterioration (MFD), but most of the HS volatiles determined were low-molecular-weight aldehydes. All volatiles were eluted from the column within a 20-min period. The automated sampling features of the HS-6 analyzer and integration of the microprocessor controlled chromatographic and data management systems facilitated reproducibility between replicates. The dominant aldehydes detected were pentanal (peak #6) and hexanal (peak #7). Uncooked pork samples contained negligible amounts of these aldehydes, as determined in preliminary tests. Other aldehydes tentatively identified by retention time matching included acetaldehyde (peak #1), propanal (peak #2), isobutanal (peak #3), butanal (peak #4), isopentanal (peak #5), heptanal (peak #8) and octanal (peak #9).

The HS volatile profiles detected during the study period were qualitatively similar, but were quantitatively different. The numbers assigned to peaks in Figure 4.22 were only used to mark the major components and do not reflect the total number of peaks observed. The peak areas of several volatile compounds increased substantially

Figure 4.22 A headspace-gas chromatogram of the flavour volatiles of cooked ground pork after 5 days of storage at 4°C.


1 2 3
7
6
8
9
4
5
0
2
4
6
8
10
12
Time (min)

during the early stages of storage. Pentanal (peak #6) and hexanal (peak #7) levels increased by 350 and 650%, respectively, by day 6, reached a maximum and then declined. The increase in the content of pentanal, hexanal and total volatiles observed during the first 6 days of storage is presented in Figure 4.23. Many studies have illustrated the increase in hexanal content during the first several days of storage (0 to 5) of cooked muscle foods and its correlation with TBA values or sensory scores (Morrissey and Apte 1988; Ang and Lyon 1990; Spanier *et al.* 1992*b*), but after this period, the content of hexanal is not reported. Because aldehydes are quite reactive, they continually oxidize. Palamand and Dieckmann (1974) subjected hexanal to autoxidation and reported that the aldehyde underwent oxidation, polymerization and degradation resulting in the production of a large number of flavour-active compounds, most notably hexanoic acid. A decrease in the concentration of hexanal in the pork systems after 6 days of storage may be due to hexanal's interaction with various components in the meat matrix or oxidation to hexanoic acid.

Based on the use of 2-heptanone, the concentrations of pentanal and hexanal in the pork volatiles reached a maximum of 8.0 and 29 ppm, respectively, on day 6 (Figure 4.24). The increase in pentanal and hexanal concentrations was linear over this period (*i.e.*, days 0 to 6), after which, a decreasing trend was observed. A given hexanal level may correspond with two different points during storage of cooked meats. Caution should therefore be exercised when using hexanal as an indicator of lipid oxidation and MFD, but hexanal levels do correspond well with a single point during the early stages of

Figure 4.23 The content of pentanal, hexanal and total volatiles detected by headspace-gas chromatography in cooked ground pork during the first 6 days of storage at 4°C.

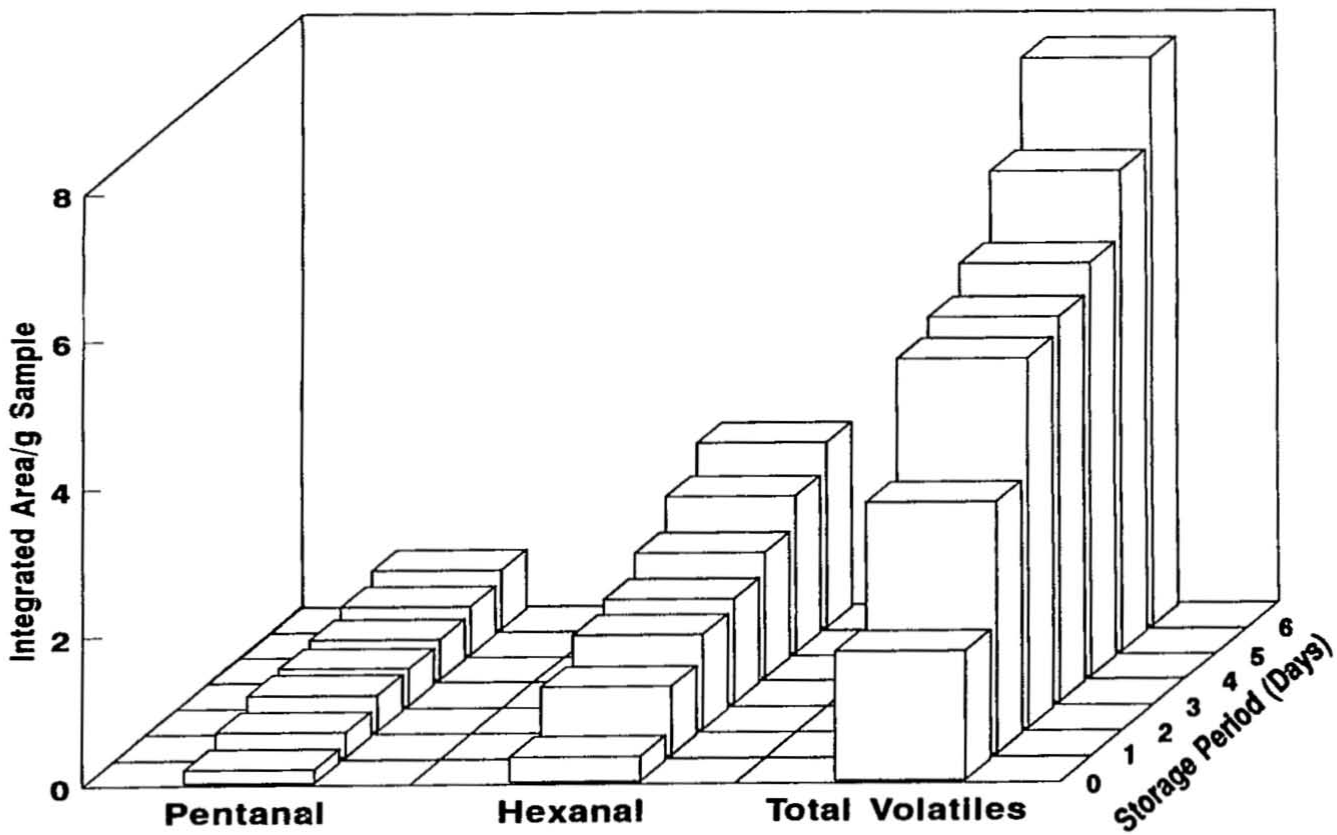
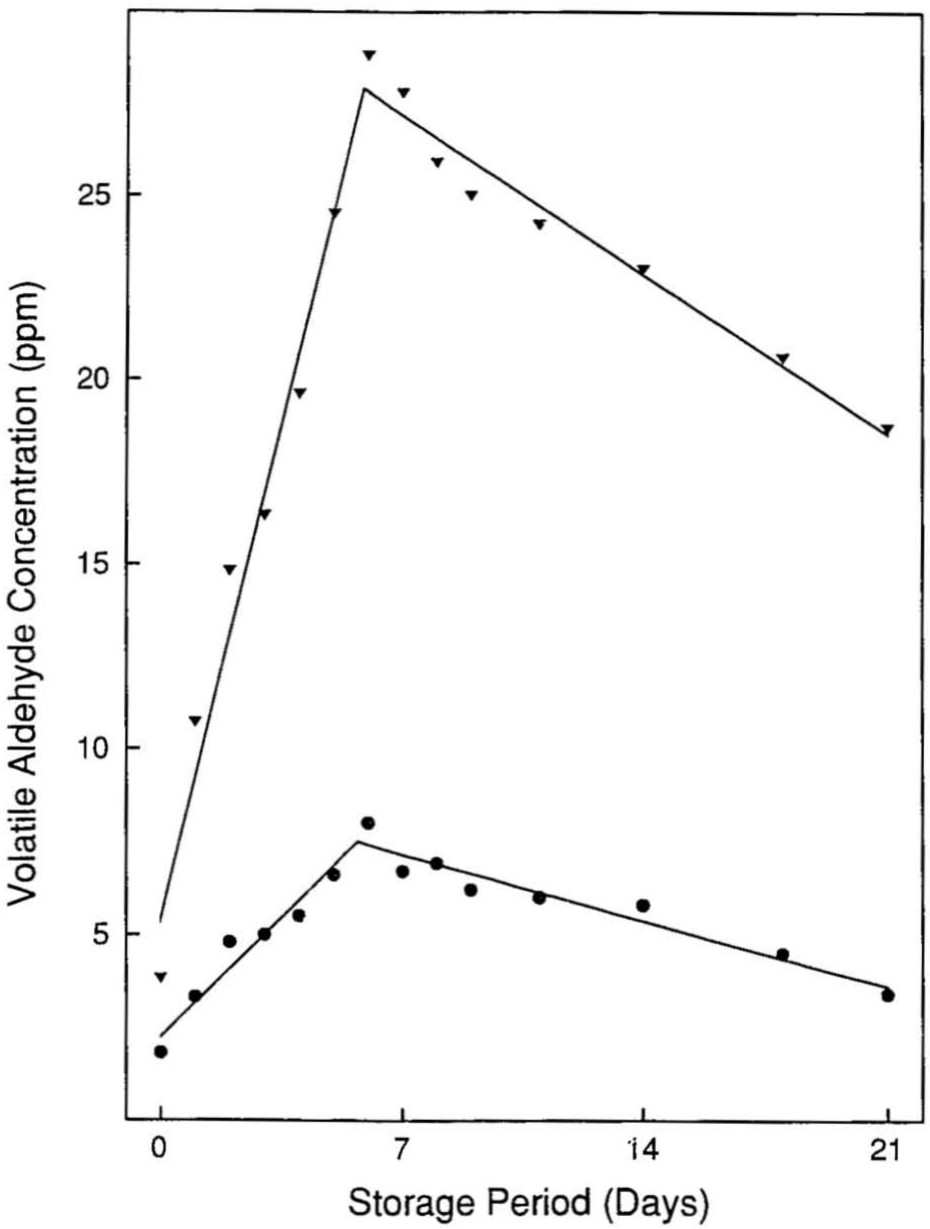

Integrated Area/g Sample
0
2
4
6
8
Pentanal
Hexanal
Total Volatiles
0
1
2
3
4
5
6
Storage Period (Days)

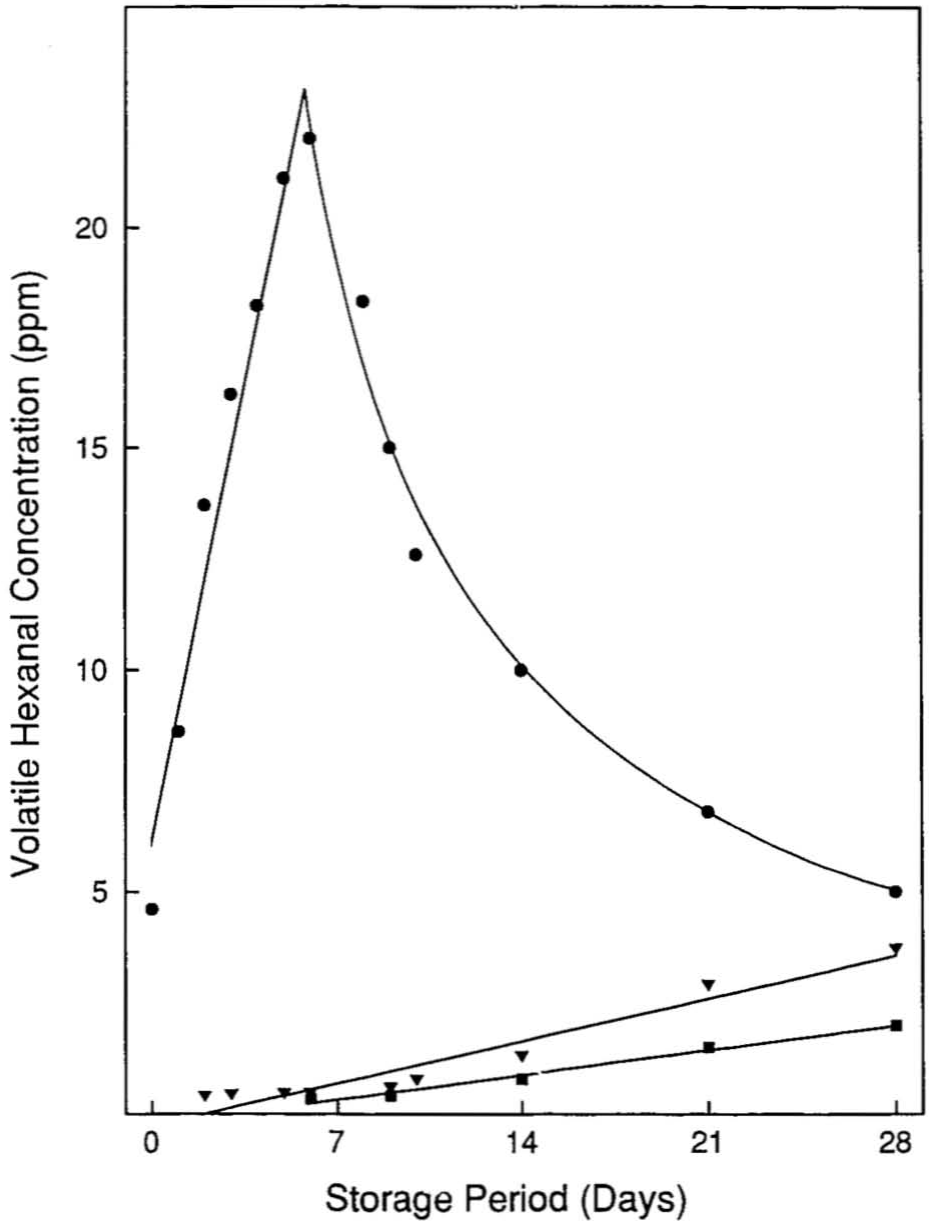
Figure 4.24 The concentration of pentanal, ● , and hexanal, ▼ , in cooked pork volatiles during storage at 4°C.


Volatile Aldehyde Concentration (ppm)
25
20
15
10
5
0
7
14
21
Storage Period (Days)

storage.

As mentioned previously, nitrite-free composite curing systems containing the CCMP have mimicked the characteristics of their nitrite-cured counterparts (Wood *et al.*, 1986; Shahidi *et al.*, 1987*a*,*b*, 1988; Shahidi, 1989*a*; Shahidi and Pegg, 1990*a*, 1991*b*, 1992). The preformed CCMP, added to meat model systems, has been shown to have a weak antioxidative effect (Shahidi *et al.*, 1988), but its suppression of hexanal generation has not been reported. The volatiles of cooked uncured, nitrite-cured and nitrite-free treated pork systems, stored for a 4 week period at 4°C, were examined using the static HS-GC methodology outline above (Ang and Young, 1989). As indicated earlier, hexanal concentrations in cooked uncured pork systems increased by 650% after 6 days of refrigerated storage and then declined. For nitrite-cured samples, hexanal levels were depressed indicating that nitrite successfully retarded hexanal generation and MFD (Figure 4.25). During the 4 week storage period, the hexanal level in nitrite-cured pork increased slowly and by day 28 was 2.0 ppm which represents only 9% of the level reached on day 6 by the uncured sample. For nitrite-free cured samples, hexanal concentrations followed a similar trend to their nitrite-cured analogs; by day 28, the hexanal content was 3.5 ppm which is only 16% of the level of the uncured sample after 6 days of refrigerated storage. Use of TBHQ instead of BHA as an antioxidant in the nitrite-free curing mixture at a 30 ppm level has been reported to provide better protection to pork systems against lipid oxidation and hexanal generation (Shahidi *et al.*, 1988; Shahidi, 1992), but its use in Canada is prohibited. After 28 days of storage at 4°C, hexanal levels in the CCMP-

Figure 4.25 The content of hexanal in uncured, ● , nitrite-free treated, ▼ , and nitrite-cured, ■ , cooked ground pork during four weeks of storage at 4°C.


Volatile Hexanal Concentration (ppm)
20
15
10
5
0
7
14
21
28
Storage Period (Days)

treated and nitrite-cured systems continued to increase. For the nitrite-free systems, a maximum hexanal level was eventually reached and, in some cases, a moderate decline was noted, again suggesting that caution should be exercised when evaluating the hexanal content of cooked treated meat systems.

Although hexanal was used as an index of lipid oxidation and MFD, it is not intended to imply that it is mainly responsible for the characteristic off-flavour of stored meat. The relationship between hexanal concentration and off-flavour notes, perceived by sensory means, is statistical and does not offer any physiological explanation of changes that occur in meat upon storage. Nonetheless, hexanal detection by the HS-GC method has potential for use as an indicator for quality control during the processing and storage of meat products. Hexanal concentrations may also be used for evaluating frozen and cured-meat products where oxidation proceeds slowly, or when the TBA methodology may lead to erroneous results.

SUMMARY AND CONCLUSIONS

The CCMP was prepared from BRBC and sodium nitrite in aqueous solutions and at a temperature of 85°C. The effect of reductants and sodium hydroxide addition to the reaction mixture on the pigment so obtained was investigated. Reductants employed were ascorbic acid, erythorbic acid or ascorbyl palmitate. The best yield of the pigment was 95% and its purity was greater than 98% in most cases. The absorption characteristics of this pigment were similar to those of extracted pigments from a nitrite-cured sample of ham or of pigment prepared from haemin and nitric oxide.

The colour characteristics of comminuted muscle samples of different species treated with varying levels of CCMP were compared to those of their nitrite-cured counterparts. Muscle samples tested include beef, chicken, lamb, pork and seal as well as surimi from cod and seal. The effects of protoporphyrin-IX (PP-IX) addition to ground pork, as a colorant for nitrite-free curing of meats were also investigated. The colour characteristics of PP-IX-treated pork were found to resemble those of uncured meat rather than the typical pink colour of cured pork as determined by tristimulus colour parameters. Hunter **L**, **a**, **b** values of CCMP-treated meats depended on the original myoglobin content of muscles as well as the addition level of CCMP. The presence of some myoglobin in muscle tissue was determined to be necessary in order to impart a cured colour to meats. Cod surimi treated with CCMP had a dull uncured rather than a typical cured colour noted for other meat tissues. The colour stability of CCMP-treated pork systems was similar to their nitrite-cured analogs, thereby suggesting that the presence of residual

nitrite in cured meats may not play an important role in colour stability under extreme conditions. Pilot-scale preparation of CCMP-treated frankfurter and salami products was successful. Flavour characteristics were indistinguishable from those of their nitrite-cured counterparts even after 30 days of refrigerated storage, but the colour of CCMP-treated samples was slightly redder in appearance than the nitrite-cured control when examined under bright daylight.

Addition of CCMP to solid cuts of pork *via* pickling was successful in conferring the characteristic cured-meat colour throughout the muscles after thermal processing. The concentration of pigment used in the pickles had a pronounced effect on the extent of CCMP penetration as well as its rate of penetration into the muscle tissue. Increasing the temperature of systems did not enhance the rate of penetration of CCMP into meats and had a detrimental effect on the process in some cases.

The absence of N-nitrosamines in cooked nitrite-free systems containing CCMP was confirmed using a GC-TEA methodology. No N-nitrosamines were detected in CCMP-treated cod, cod surimi or mixtures containing pork with 15 or 50% cod or cod surimi, but counterpart samples cured with sodium nitrite (156 ppm) and sodium ascorbate (550 ppm) contained N-nitrosodimethylamine at 1.0 ppb levels or less. These results demonstrate that nitrite-free curing of meat and meat/fish systems with compositions containing the preformed CCMP is successful in yielding products devoid of volatile N-nitrosamines.

The CCMP may be stabilized effectively by either storage under a nitric oxide

atmosphere or by its encapsulation in food-grade starch-based wall materials. Although stabilized pigments prepared by both methods conferred similar colour characteristics to meats, the encapsulated CCMP may be more practical for use by processors. Amongst the wall materials tested, β-cyclodextrin, N-LOK, and Maltrin M-250 served as the best encapsulating agents. The colour characteristics of pigment-treated pork systems were analyzed by comparing their Hunter **L**, **a**, **b** values to nitrite-cured counterparts. The presence of gum acacia or a mixture of sodium tripolyphosphate, sodium acid pyrophosphate and ascorbyl palmitate at a 5% level in the wall materials improved the colour of meats treated with the PCCMP. Some encapsulated pigments remained stable during 18 months of refrigerated storage. Spectral characteristics of PCCMP were similar to those of CCMP. When PCCMP-treated pork systems were placed under intense fluorescent lighting, the colour stability of these meats was similar to that of nitrite-cured and CCMP-treated products.

The effects of 5 and 10 kGy irradiation on the colour and oxidative stability of meats treated with nitrite or a nitrite-free curing system were investigated. The nitrite-free curing system contained CCMP, sodium ascorbate and sodium tripolyphosphate with or without sodium acid pyrophosphate. Radiation processing had no detrimental effect on the colour or flavour of treated samples. Polyphosphate addition to meats had a beneficial effect on the oxidative stability of irradiated samples, but had a slight detrimental effect on their colour stability.

The absorbance of the TBA-malonaldehyde complex at 532 nm when the

malonaldehyde was derived from aqueous model systems and meat model systems was measured. Addition of sulphanilamide played a beneficial role in evaluating the oxidative state of cured meats prepared with the addition of ≥ 100 ppm of sodium nitrite. Sulphanilamide prevented the underestimation of the TBA values, but the measured TBA values still suffered from errors ranging from 6 to 20%. In the absence of nitrite, sulphanilamide reacts with malonaldehyde forming a 1-amino-3-iminopropene complex. Fluorescence spectroscopy showed that this complex had an excitation maximum at 395 nm and an emission maximum at 460 nm. The TBA test for evaluating the oxidative state of cured meats should only be performed when the content of nitrite in the cure or final product is known.

Reaction of distilled malonaldehyde with the TBA reagent formed the typical pink complex (TMT) with absorption maxima at 372 and 532 nm. Addition of sulphanilamide to a solution of the malonaldehyde precursor, 1,1,3,3-tetramethoxypropane, produced a bright yellow complex (SMS) with absorption maxima at 332 and 396 nm. Addition of TBA to a model system of malonaldehyde and sulphanilamide resulted in the disappearance of the characteristic absorption bands of SMS and the appearance of maxima at 372, 472 and 532 nm. The appearance of the new band at 472 nm in the sulphanilamide-malonaldehyde-TBA system, suggests the presence of a second complex due to multiple interactions between malonaldehyde with both sulphanilamide and TBA. The structure of the above complexes, recovered as crystalline products, were elucidated using ultraviolet-visible (UV-VIS), Fourier transform infrared (IR), nuclear magnetic

resonance (NMR), and mass spectroscopic (MS) techniques.

Pentanal and hexanal were the dominant volatile aldehydes generated from cooked pork during 3 weeks of refrigerated storage as determined by a headspace-gas chromatographic (HS-GC) methodology. Hexanal concentrations may serve as an index of meat flavour deterioration (MFD) during the early stages of storage; its concentration increased more rapidly than any other aldehyde. During the first 6 days, contribution of pentanal and hexanal to the total volatile aldehydes increased linearly by 350 and 650%, respectively, after which, their concentrations declined quite markedly. Reactions of pentanal and hexanal with meat components or their further oxidation may be responsible for this observation. Caution should be exercised when using hexanal as an indicator of lipid oxidation and MFD because a given hexanal level may correspond with two points during the storage period of cooked meats. The hexanal and pentanal concentrations of CCMP-treated and nitrite-cured pork systems were depressed even after 4 weeks of refrigerated storage. The rate of generation of carbonyl compounds in these systems was similar but at a slower rate relative to their uncured counterpart indicating that lipid oxidation was suppressed.

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APPENDIX

Determination of the Iron Content in BRBC

The absorption of iron by atomic absorption spectroscopy is linear with concentration according to Beer's Law up to 5 ppm. For one of the samples, 1.243 g of BRBC were digested with the concentrated acids as described in section 3.6.1. The absorbance of an aliquot of the resultant 50-mL solution was 0.048 (*i.e.* an average of 3 measurements). The absorbance of a blank sample containing no BRBC was 0.002. Based on the standard curve of Figure 4.1 constructed from a 1000 ppm certified iron concentrate, the concentration of iron in the aliquot examined was 2.52 ppm. Assuming that the density of the iron solution tested was 1.00 $g \cdot mL^{-1}$, and because the iron content is so small, this concentration represents 2.52 mg of Fe per L of solution. Considering dilution factors,

$$\%Fe_{BRBC} = \frac{2.52 \text{ mg of Fe}}{1000 \text{ mL}} \times 50 \text{ mL} \times \frac{10 \text{ mL}}{1 \text{ mL}} \times \frac{1}{1243 \text{ mg}_{BRBC}} \times 100$$

$$= 0.1014$$

$$\% \text{ Haemin Equivalents in BRBC} = \%Fe_{BRBC} \times \frac{MW_{Haemin}}{AW_{Fe}}$$

$$= 0.1014 \times \frac{651.96}{55.847}$$

$$= 1.184$$

Yield and Purity of the CCMP Prepared from BRBC and Sodium Nitrite: Sample Calculation

$$\text{Yield (mol \%)} = \frac{\text{Moles of CCMP formed}}{\text{Moles of haemin equivalents in BRBC used}}$$

A total of 320 mL of 4:1 (v/v) acetone:water was used for exhaustive extraction of CCMP from the pigment precipitate after the acidification step. A 10-mL aliquot of the pigment extract was diluted with an additional 30 mL of 4:1 (v/v) acetone:water before spectral analysis. For one of the experiments (Table 4.1), the absorbance of the pigment extract at a wavelength of 540 nm was 1.514. Using Beer's Law, a path length of 1 cm and the molar extinction coefficient of nitrosylhaemochromogen in 4:1 (v/v) acetone:water of 11.3 $mM^{-1} \cdot cm^{-1}$ (Hornsey, 1956), the concentration of CCMP in the solution was calculated as follows:

$$A = \varepsilon_{540}\, c\, l$$

where,

A	=	absorbance
ε_{540}	=	molar extinction coefficient, $mM^{-1} \cdot cm^{-1}$
l	=	path length, cm
c	=	concentration of CCMP, mM

Hence,

$$c = \frac{A}{\varepsilon_{540}\, l} = \frac{1.514}{(11.3)(1)} = 0.134 \text{ mM}$$

Correcting for dilution,

$$\text{the mmol of CCMP prepared} = \frac{0.314 \text{ mmol}}{\text{L}} \times \frac{40 \text{ mL}}{10 \text{ mL}} \times 0.32 \text{ L}$$

$$= 0.172$$

Based on the iron analysis previously described, the % haemin equivalents in BRBC was 1.184. In 10 g of BRBC there are 0.1184 g haemin equivalents which represents 0.1815 mmol.

$$\text{Yield (mol \%)} = \frac{0.172 \text{ mmol}}{0.1815 \text{ mmol}} \times 100$$

$$= 94.5$$

$$\text{Purity (\%)} = \frac{\text{Concentration of CCMP in the 4:1 (v/v) acetone:water}}{\text{Concentration of acid haematin}}$$

After the addition of 1 drop of concentrated HCl to the cuvette, the absorbance of the acid haematin extract, so obtained, at a wavelength of 640 nm was 0.650. Using Beer's Law, a path length of 1 cm and the molar extinction coefficient of acid haematin in 4:1 (v/v) acetone:water of 4.8 $mM^{-1} \cdot cm^{-1}$ (Hornsey, 1956), the purity of CCMP in the solution was calculated as follows:

$$c = \frac{A}{\varepsilon_{640}\, l} = \frac{0.650}{(4.8)(1)} = 0.135 \text{ mM}$$

Since the concentration of CCMP was determined to be 0.134 mM in this diluted solution,

$$\text{Purity (\%)} = \frac{0.134 \text{ mmol}}{0.135 \text{ mmol}} \times 100$$

$$= 99.0$$

Figure A.1 The IR spectrum of the TMT complex prepared from TBA and malonaldehyde.

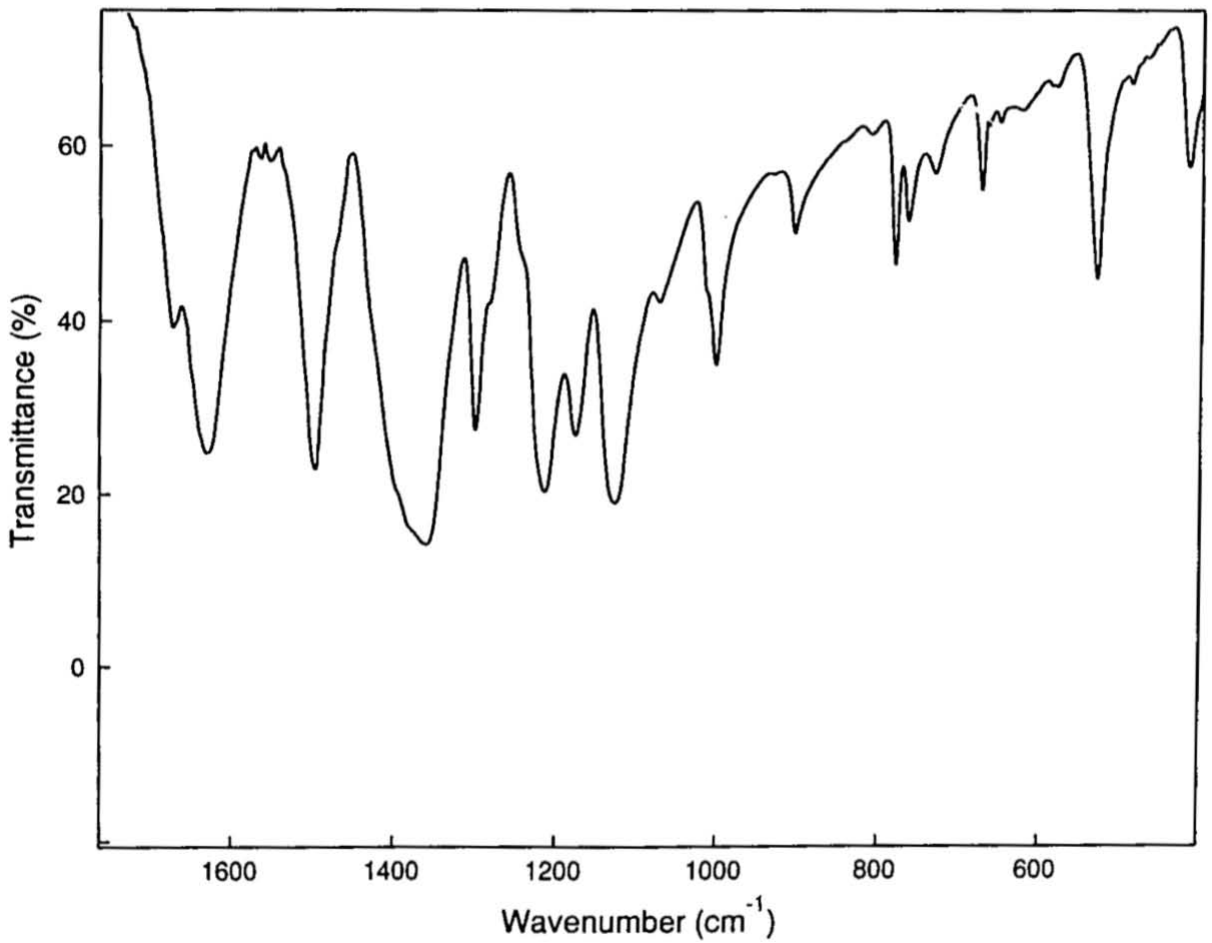

Transmittance (%)
60
40
20
0
1600
1400
1200
1000
800
600
Wavenumber (cm-1)

Figure A.2 The IR spectrum of the SMS complex prepared from sulphanilamide and malonaldehyde.

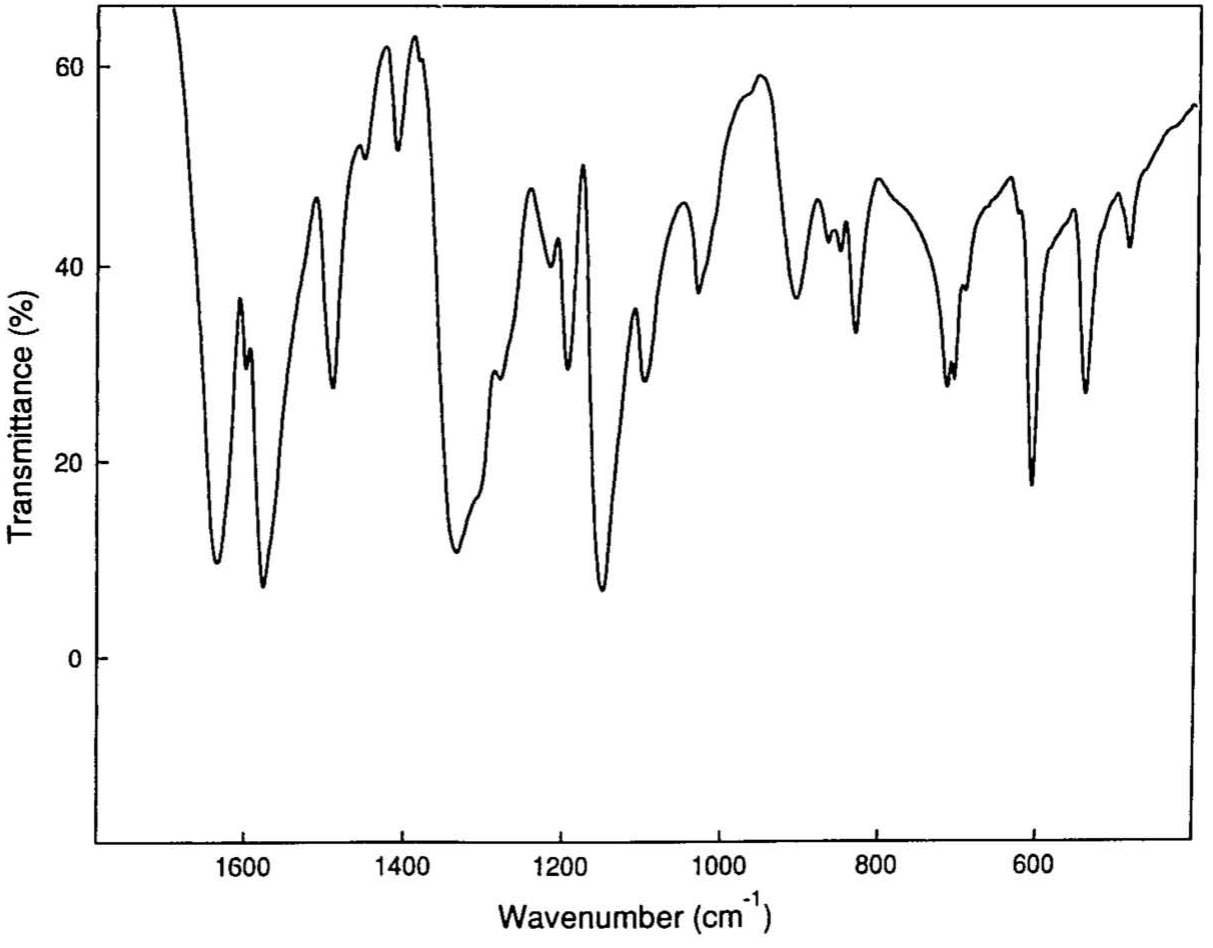

Transmittance (%)
60
40
20
0
1600
1400
1200
1000
800
600
Wavenumber (cm-1)

Figure A.3 The IR spectrum of the SMT complex prepared from malonaldehyde with sulphanilamide and TBA.

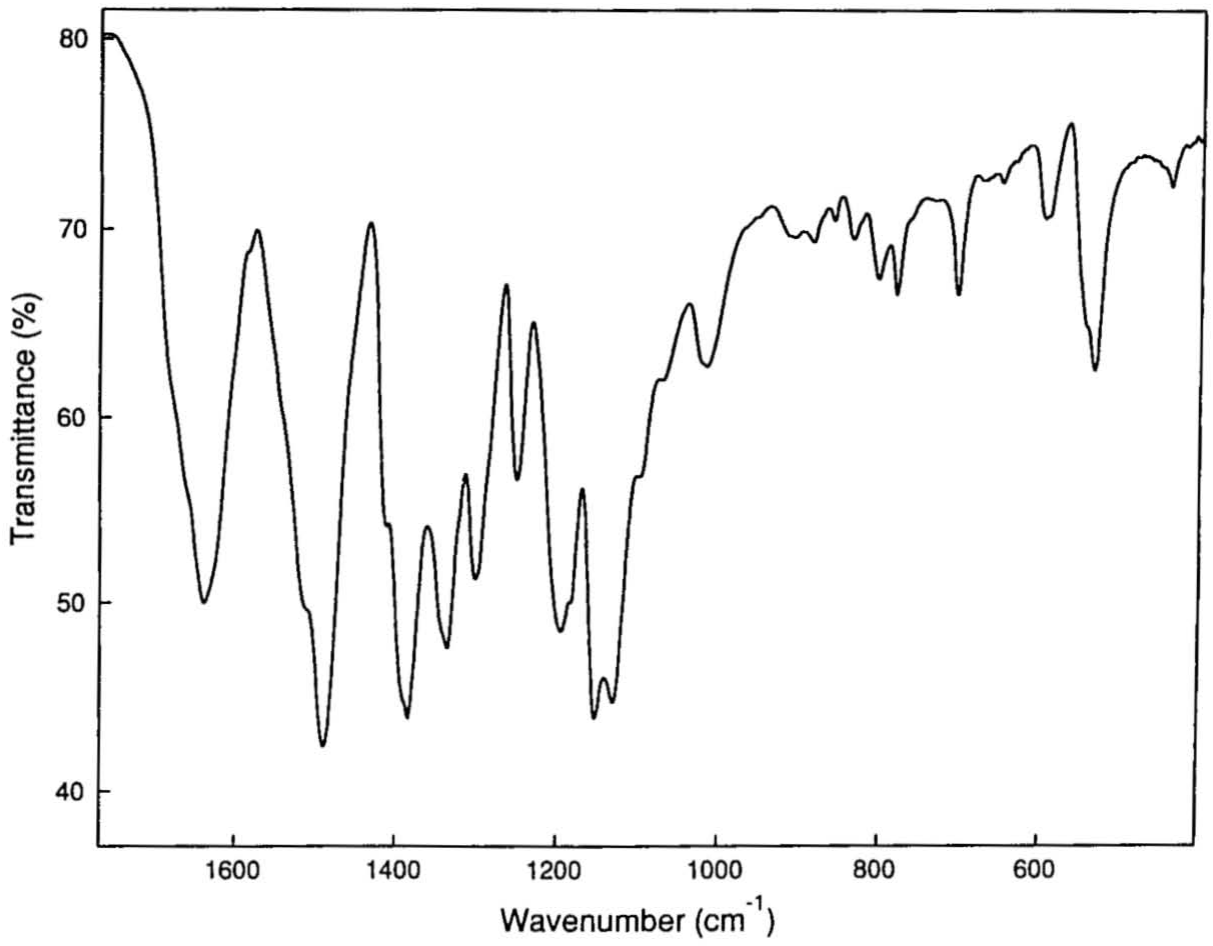

80
70
60
50
40
Transmittance (%)
1600
1400
1200
1000
800
600
Wavenumber (cm-1)

Figure A.4 The ^{1}H spectrum of the TMT complex in DMSO-d_6 prepared from TBA and malonaldehyde.

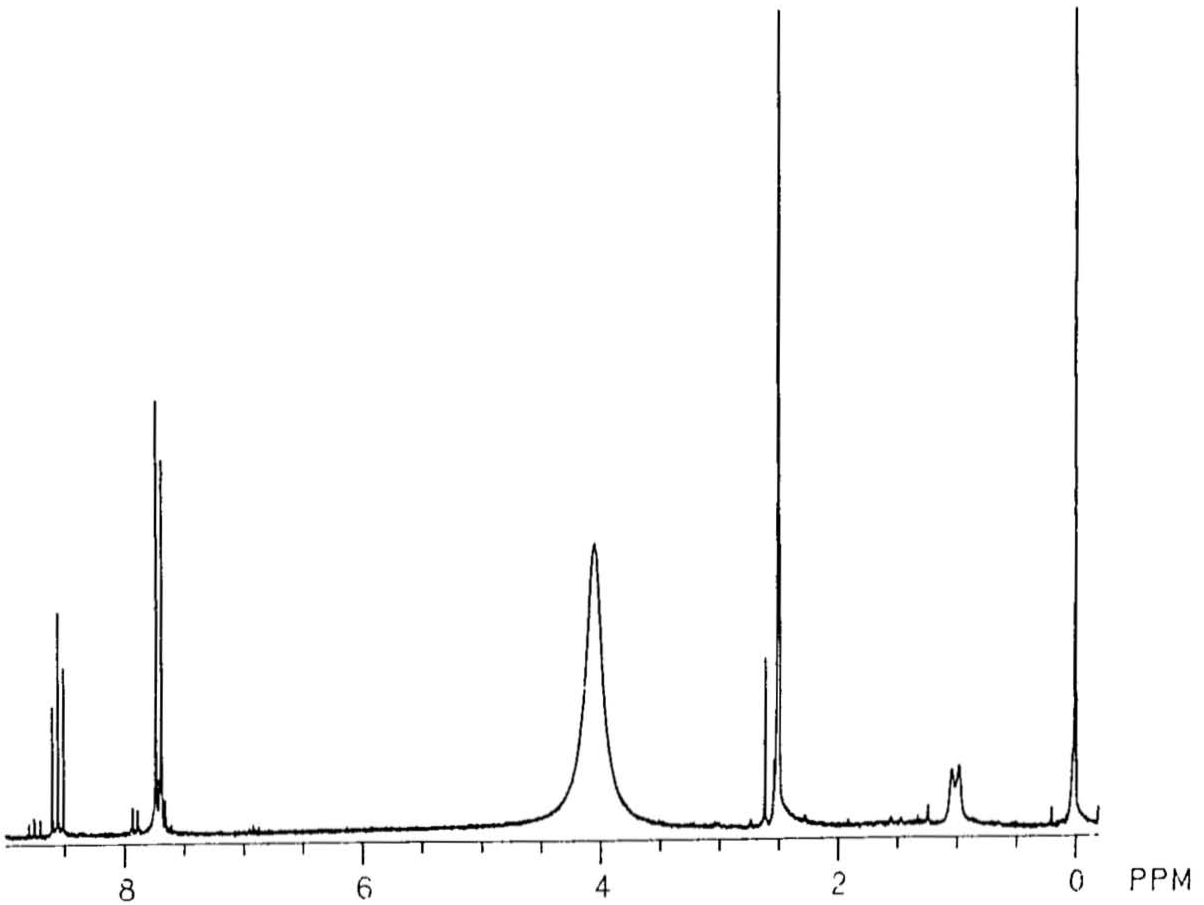

8
6
4
2
0
PPM

Figure A.5 The $^{13}C\{^1H\}$ spectrum of the TMT complex in DMSO-d_6 prepared from TBA and malonaldehyde.

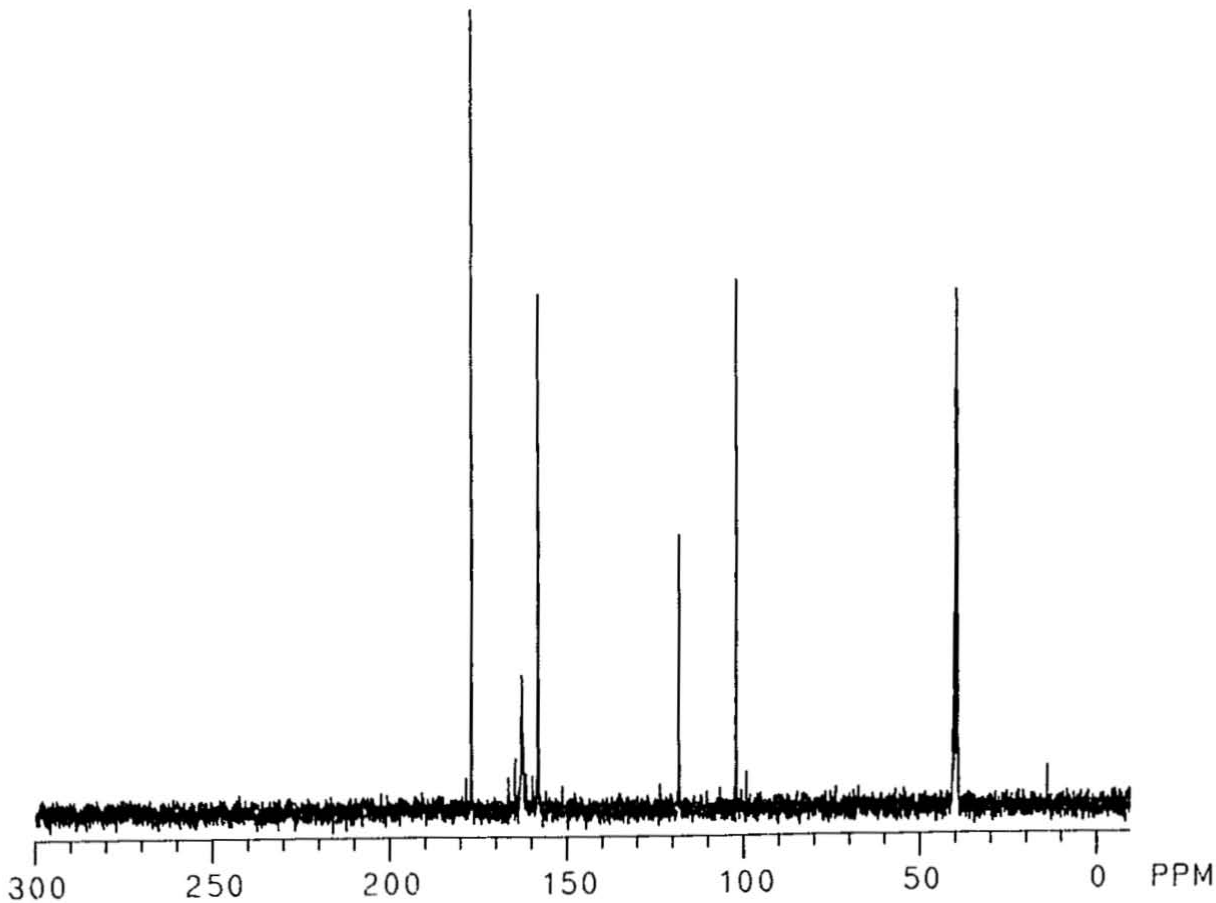

300
250
200
150
100
50
0
PPM

Figure A.6 The ^{1}H spectrum of the SMS complex in DMSO-d_6 prepared from sulphanilamide and malonaldehyde.

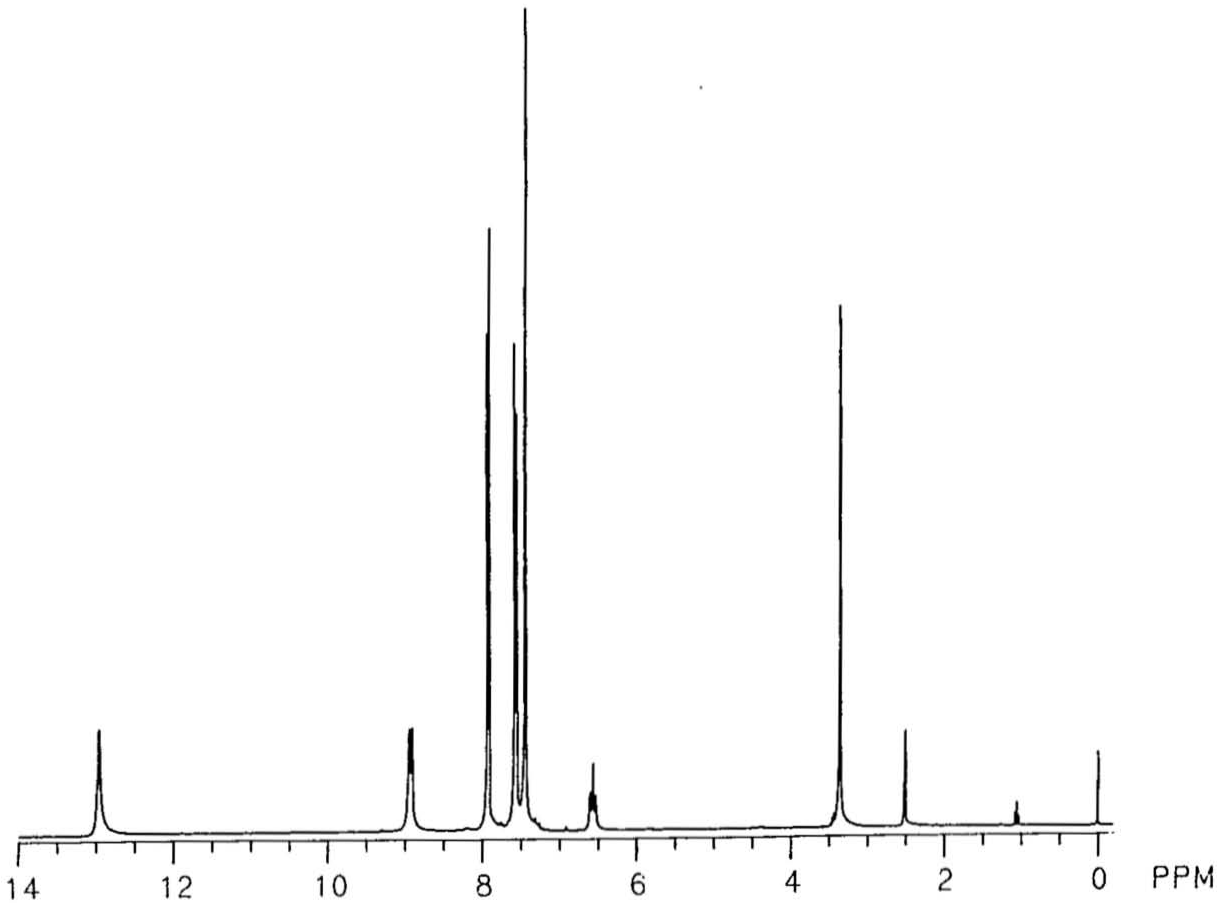

14
12
10
8
6
4
2
0
PPM

Figure A.7 The $^{13}C\{^{1}H\}$ spectrum of the SMS complex in DMSO-d_6 prepared from sulphanilamide and malonaldehyde.

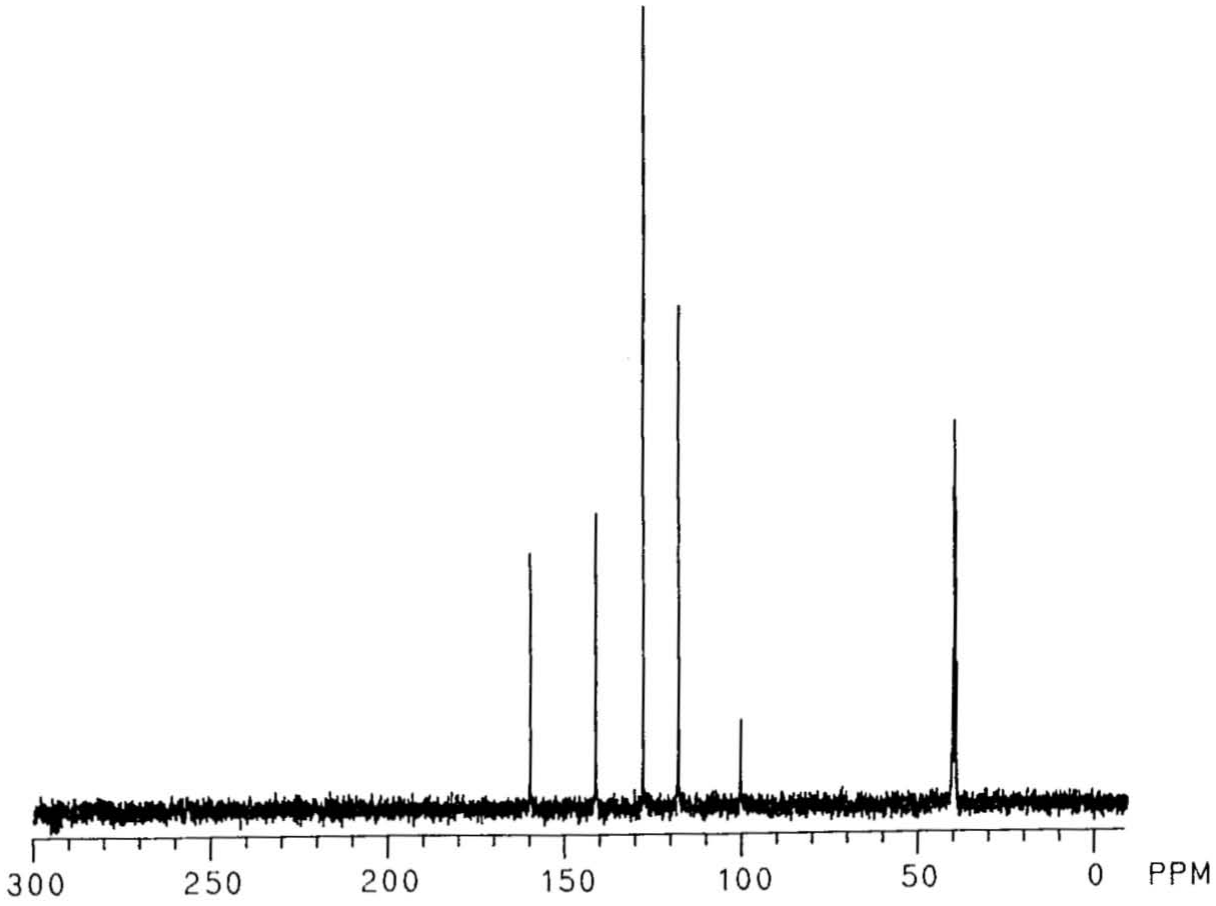

300
250
200
150
100
50
0
PPM

Figure A.8 The ^{1}H spectrum of the SMT complex in DMSO-d_6 prepared from malonaldehyde with sulphanilamide and TBA.

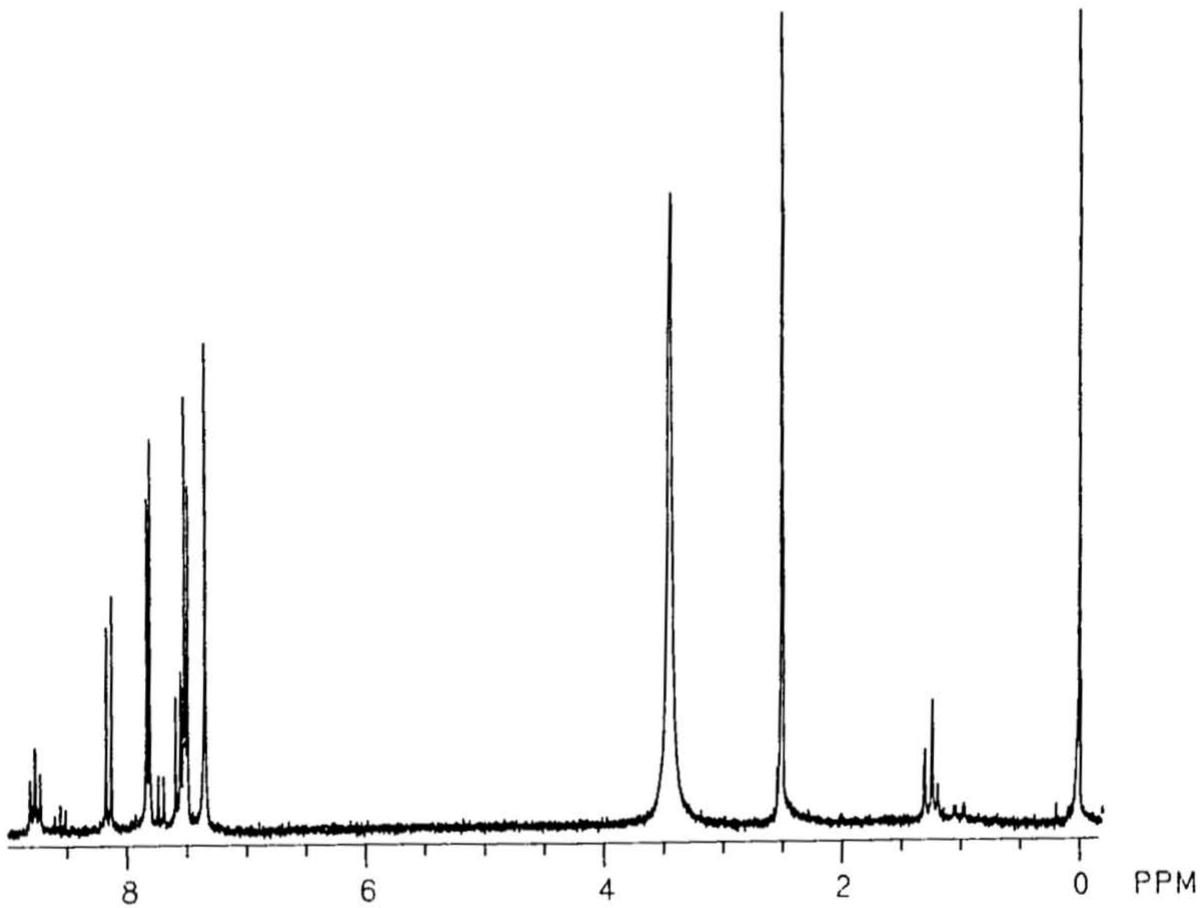

8
6
4
2
0
PPM

Figure A.9 The $^{13}C\{^{1}H\}$ spectrum of the SMT complex in DMSO-d_6 prepared from malonaldehyde with sulphanilamide and TBA.

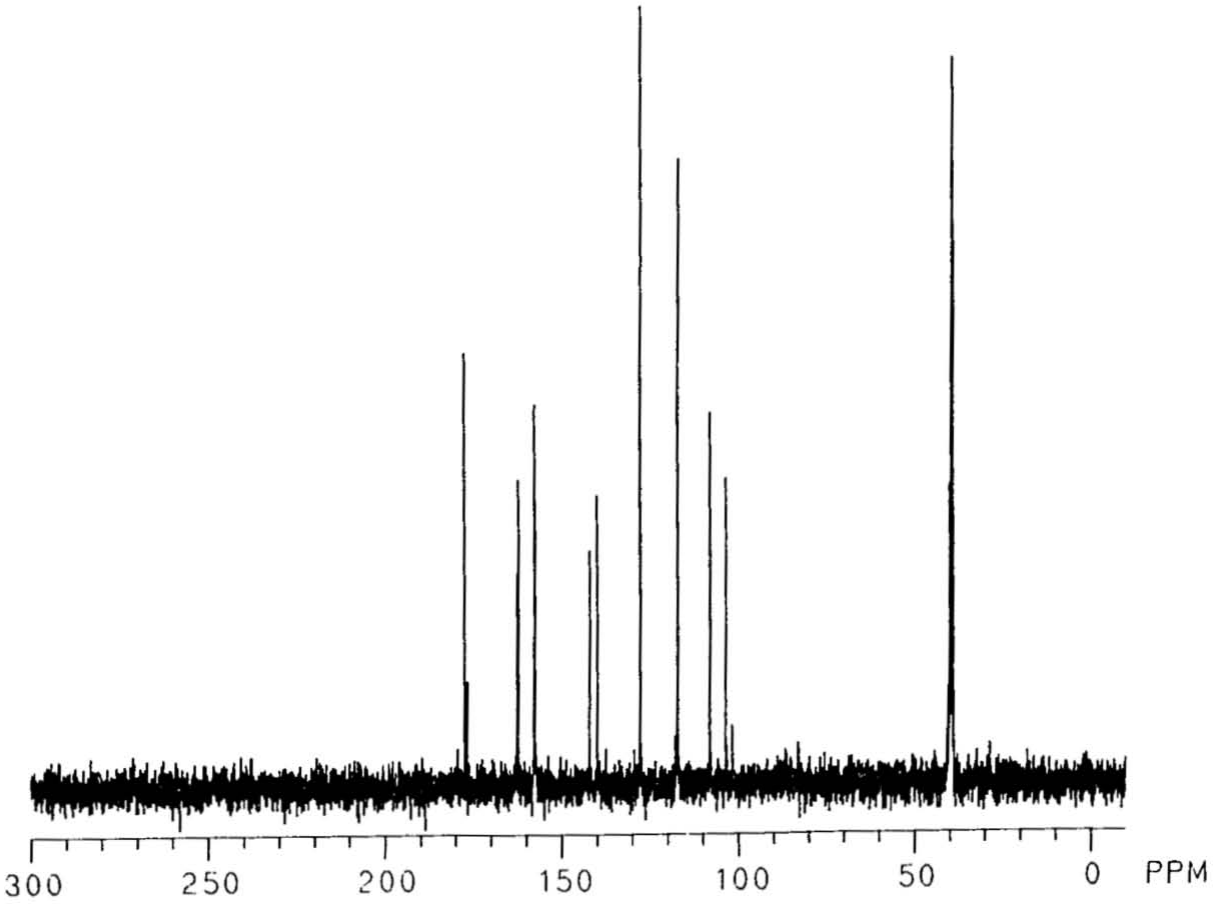

300
250
200
150
100
50
0
PPM

Figure A.10 A summary of the $^{13}C\{^{1}H\}$ and ^{1}H signals and their assignments for the TMT, SMS and SMT complexes prepared from malonaldehyde (M) with TBA (T), sulphanilamide (S) or their combination with respect to resonances observed in the parent molecules.

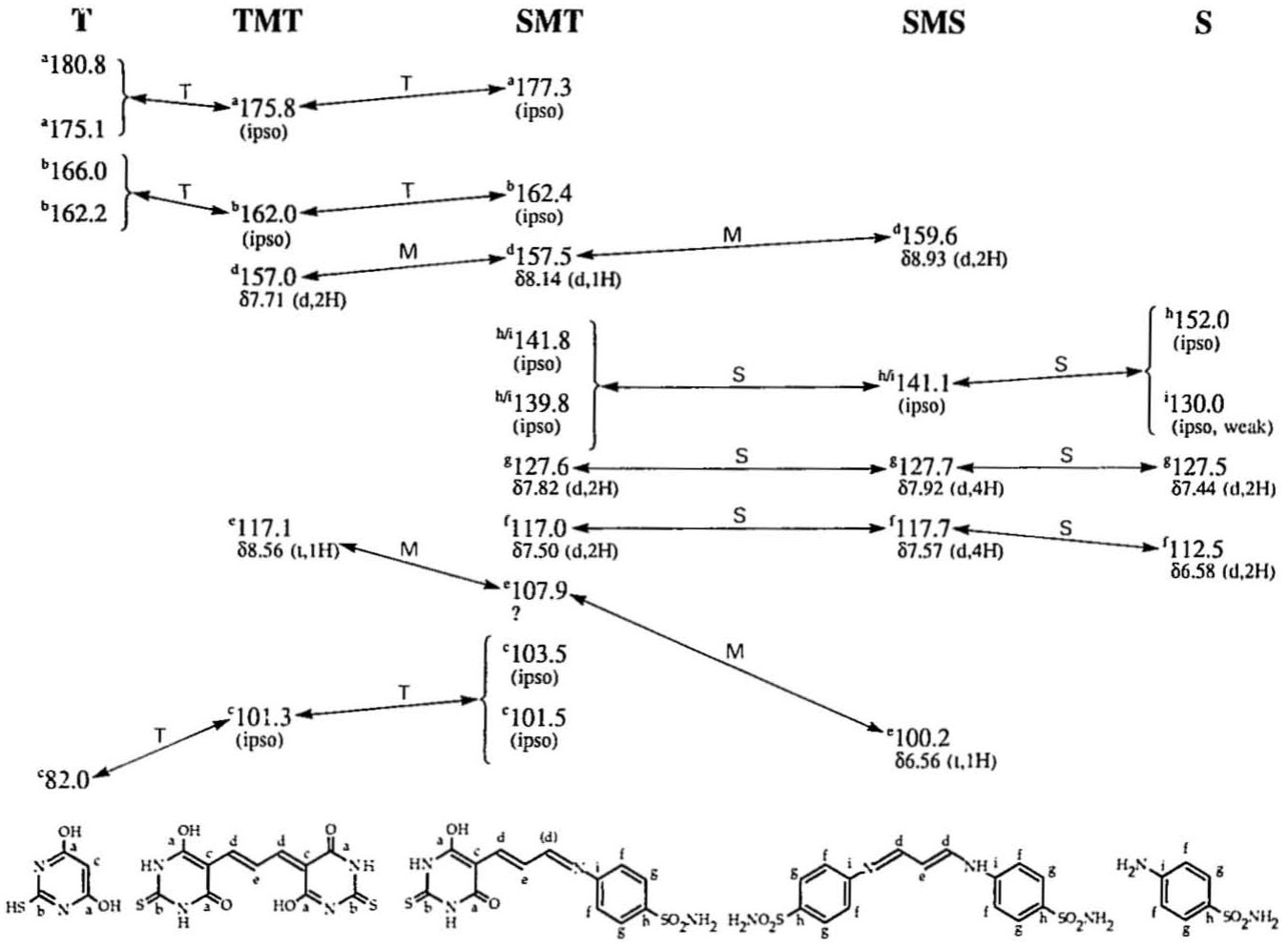
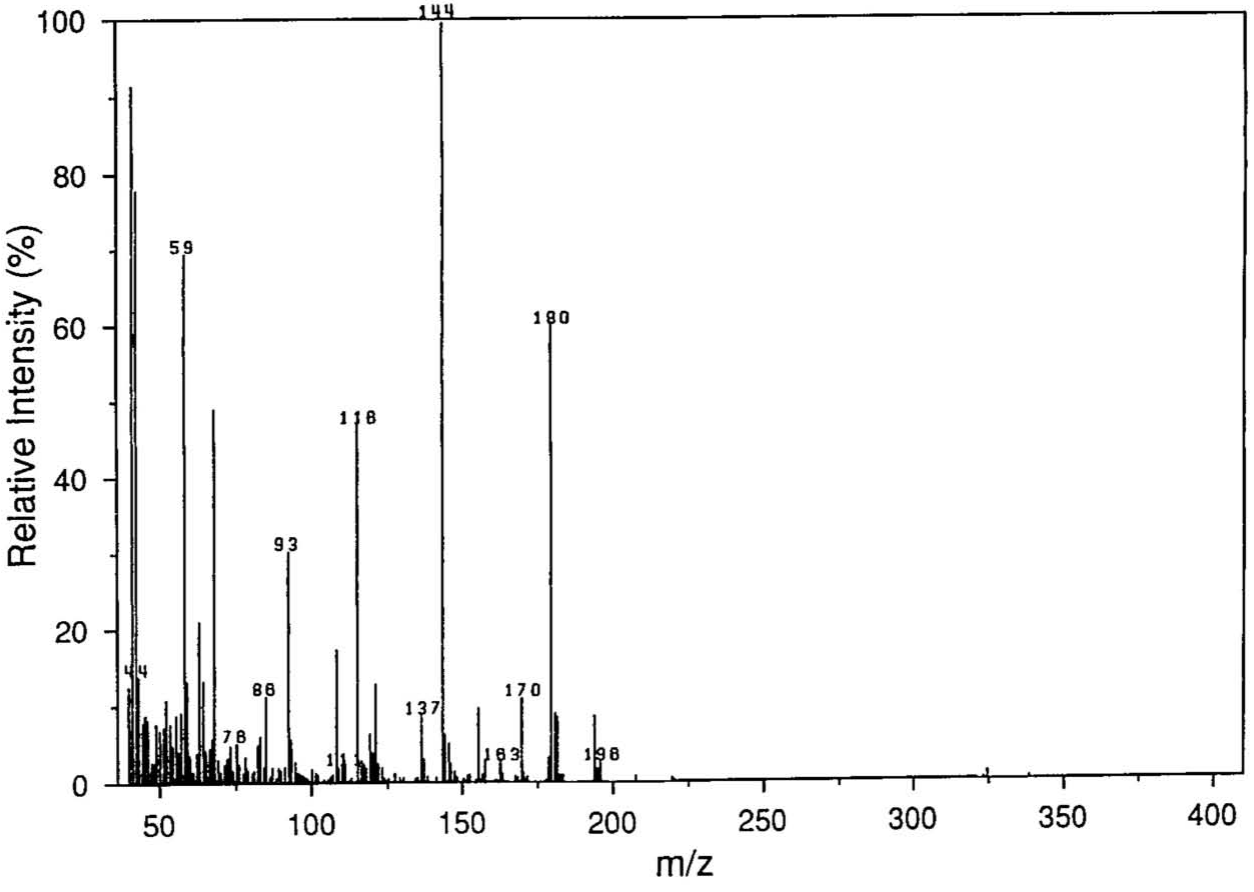

T
TMT
SMT
SMS
S
[a]180.8
[a]175.1
T
[a]175.8
(ipso)
T
[a]177.3
(ipso)
[b]166.0
[b]162.2
T
[b]162.0
(ipso)
T
[b]162.4
(ipso)
M
[d]157.0
δ7.71 (d,2H)
M
[d]157.5
δ8.14 (d,1H)
M
[d]159.6
δ8.93 (d,2H)
[h/i]141.8
(ipso)
[h/i]139.8
(ipso)
S
[h/i]141.1
(ipso)
S
[h]152.0
(ipso)
[i]130.0
(ipso, weak)
[g]127.6
δ7.82 (d,2H)
S
[g]127.7
δ7.92 (d,4H)
S
[g]127.5
δ7.44 (d,2H)
[e]117.1
δ8.56 (t,1H)
M
[f]117.0
δ7.50 (d,2H)
S
[f]117.7
δ7.57 (d,4H)
S
[f]112.5
δ6.58 (d,2H)
[e]107.9
?
M
[c]103.5
(ipso)
[c]101.5
(ipso)
T
[c]101.3
(ipso)
T
[c]82.0
M
[e]100.2
δ6.56 (t,1H)

Figure A.11 The mass spectrum of the TMT complex prepared from TBA and malonaldehyde.


Relative Intensity (%)
100
80
60
40
20
0
144
59
180
118
93
86
170
137
78
163
198
50
100
150
200
250
300
350
400
m/z

Mass Spectral Data for TMT

MASS	%AR. MOD.	%AR. BASE	% TIC	ABS AREA	MASS	%AR. MOD.	%AR. BASE	% TIC	ABS AREA
40.92	12.96	12.96	1.308	325	94.06	5.58	5.58	0.564	140
41.99	95.33	95.33	9.626	2391	95.07	2.47	2.47	0.250	62
43.07	80.54	80.54	8.132	2020	96.06	1.28	1.28	0.129	32
44.08	14.23	14.23	1.437	357	97.07	0.96	0.96	0.097	24
45.09	7.78	7.78	0.785	195	98.05	0.76	0.76	0.076	19
46.07	9.05	9.05	0.914	227	98.97	0.60	0.60	0.060	15
47.05	8.25	8.25	0.833	207	99.92	0.68	0.68	0.068	17
48.01	2.59	2.59	0.262	65	100.94	1.95	1.95	0.197	49
48.96	2.59	2.59	0.262	65	101.98	1.20	1.20	0.121	30
49.88	7.30	7.30	0.737	183	103.03	1.00	1.00	0.101	25
50.95	7.18	7.18	0.725	180	104.05	0.12	0.12	0.012	3
52.02	7.02	7.02	0.709	176	105.07	0.60	0.60	0.060	15
53.05	11.56	11.56	1.167	290	106.06	0.56	0.56	0.056	14
54.09	7.58	7.58	0.765	190	107.05	0.84	0.84	0.085	21
55.12	4.94	4.94	0.499	124	107.99	1.12	1.12	0.113	28
56.11	8.29	8.29	0.837	208	108.97	17.03	17.03	1.719	427
57.10	4.15	4.15	0.419	104	109.93	1.99	1.99	0.201	50
58.00	8.89	8.89	0.898	223	110.96	2.47	2.47	0.250	62
58.94	68.26	68.26	6.892	1712	112.02	2.43	2.43	0.246	61
59.87	12.84	12.84	1.296	322	113.03	0.40	0.40	0.040	10
60.92	3.39	3.39	0.342	85	114.04	0.40	0.40	0.040	10
61.99	1.40	1.40	0.141	35	115.05	0.16	0.16	0.016	4
63.05	3.87	3.87	0.390	97	116.02	46.49	46.49	4.694	1166
64.07	21.61	21.61	2.182	542	117.01	2.71	2.71	0.274	68
65.10	14.67	14.67	1.481	368	117.99	2.31	2.31	0.233	58

.....continued on next page

Mass Spectral Data for TMT

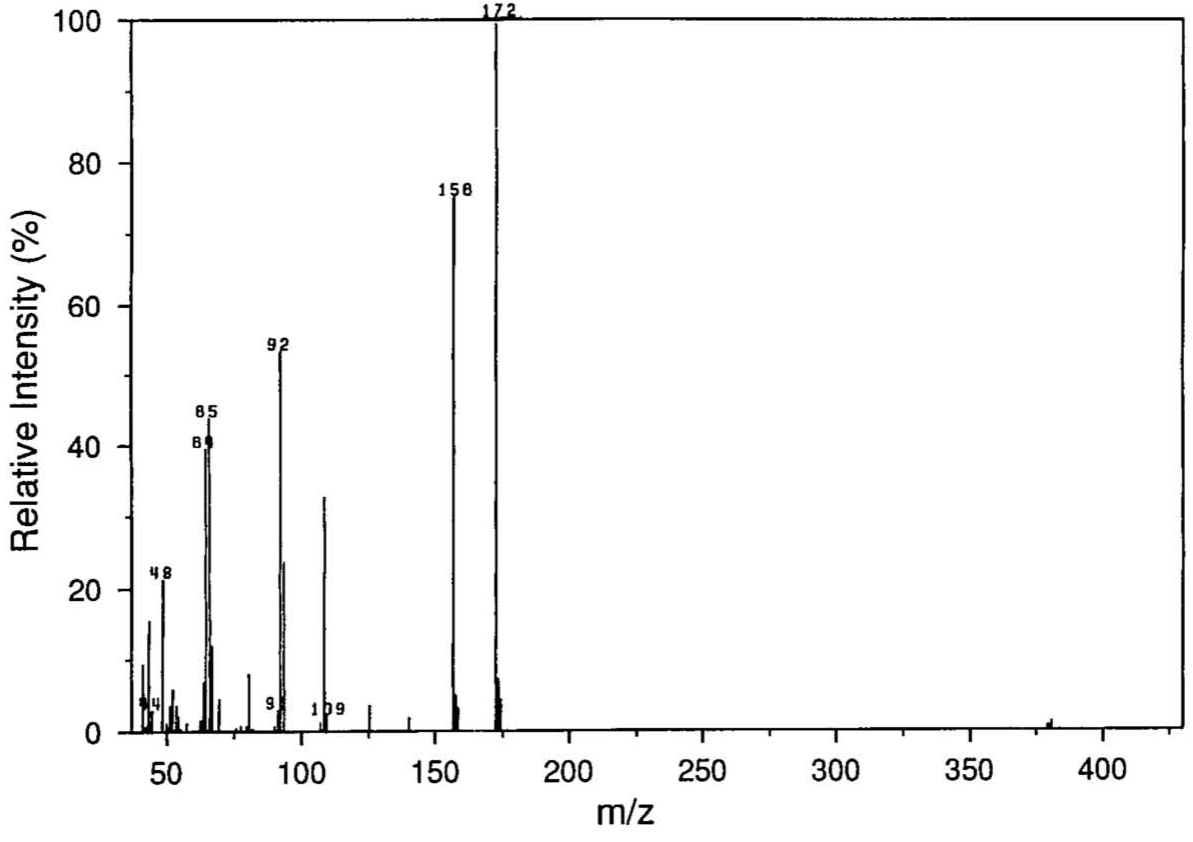
66.09	4.43	4.43	0.447	111	118.97	1.83	1.83	0.185	46
67.06	4.86	4.86	0.491	122	119.94	6.58	6.58	0.664	165
68.03	5.86	5.86	0.592	147	120.97	3.87	3.87	0.390	97
68.96	48.88	48.88	4.936	1226	122.00	13.36	13.36	1.349	335
69.91	3.07	3.07	0.310	77	123.03	2.31	2.31	0.233	58
70.99	2.03	2.03	0.205	51	124.04	2.03	2.03	0.205	51
71.99	2.55	2.55	0.258	64	125.03	0.56	0.56	0.056	14
73.03	3.27	3.27	0.330	82	126.04	0.36	0.36	0.036	9
74.04	4.98	4.98	0.503	125	127.03	0.12	0.12	0.012	3
75.05	1.59	1.59	0.161	40	128.00	1.12	1.12	0.113	28
76.05	5.02	5.02	0.507	126	128.97	0.12	0.12	0.012	3
77.04	2.39	2.39	0.242	60	129.95	0.72	0.72	0.072	18
78.02	1.16	1.16	0.117	29	130.95	0.52	0.52	0.052	13
78.97	3.63	3.63	0.366	91	133.03	0.28	0.28	0.028	7
79.91	1.71	1.71	0.173	43	134.03	0.08	0.08	0.008	2
80.97	1.04	1.04	0.105	26	135.03	0.60	0.60	0.060	15
81.99	1.71	1.71	0.173	43	136.02	0.32	0.32	0.032	8
83.04	5.30	5.30	0.535	133	136.99	9.25	9.25	0.934	232
84.07	6.22	6.22	0.628	156	137.96	3.11	3.11	0.314	78
85.08	2.19	2.19	0.221	55	138.95	0.92	0.92	0.093	23
86.01	11.72	11.72	1.184	294	139.91	0.12	0.12	0.012	3
87.02	0.76	0.76	0.076	19	141.99	0.36	0.36	0.036	9
87.99	2.15	2.15	0.217	54	144.01	100.00	100.00	10.097	2508
88.98	0.60	0.60	0.060	15	145.00	6.18	6.18	0.624	155
89.93	1.95	1.95	0.197	49	146.00	5.06	5.06	0.511	127
90.99	1.83	1.83	0.185	46	147.02	2.67	2.67	0.270	67
92.02	2.31	2.31	0.233	58	148.01	1.32	1.32	0.133	33
93.04	31.58	31.58	3.188	792	148.98	0.80	0.80	0.081	20

.....continued on next page

Mass Spectral Data for TMT

149.97	0.28	0.28	0.028	7
150.95	0.48	0.48	0.048	12
151.96	0.80	0.80	0.081	20
152.98	0.88	0.88	0.089	22
156.00	9.57	9.57	0.966	240
157.00	1.00	1.00	0.101	25
157.99	2.83	2.83	0.286	71
158.97	0.28	0.28	0.028	7
159.92	0.12	0.12	0.012	3
160.98	0.12	0.12	0.012	3
161.99	0.40	0.40	0.040	10
162.99	2.71	2.71	0.274	68
164.00	0.92	0.92	0.093	23
165.01	0.20	0.20	0.020	5
167.96	0.76	0.76	0.076	19
168.94	0.80	0.80	0.081	20
169.93	10.49	10.49	1.059	263
170.94	1.12	1.12	0.113	28
171.97	0.56	0.56	0.056	14
177.97	0.40	0.40	0.040	10
178.93	3.75	3.75	0.378	94
179.91	60.96	60.96	6.155	1529
180.93	9.57	9.57	0.966	240
181.96	8.77	8.77	0.886	220
182.96	1.12	1.12	0.113	28
184.00	0.76	0.76	0.076	19
192.99	0.16	0.16	0.016	4
193.99	9.77	9.77	0.986	245
194.98	2.15	2.15	0.217	54
195.98	2.75	2.75	0.278	69
196.97	0.12	0.12	0.012	3
197.97	0.12	0.12	0.012	3
205.99	0.16	0.16	0.016	4
207.98	0.64	0.64	0.064	16
209.93	0.08	0.08	0.008	2
219.89	0.44	0.44	0.044	11
220.93	0.32	0.32	0.032	8
221.91	0.08	0.08	0.008	2
322.87	0.48	0.48	0.048	12
323.91	1.48	1.48	0.149	37
337.96	0.32	0.32	0.032	8

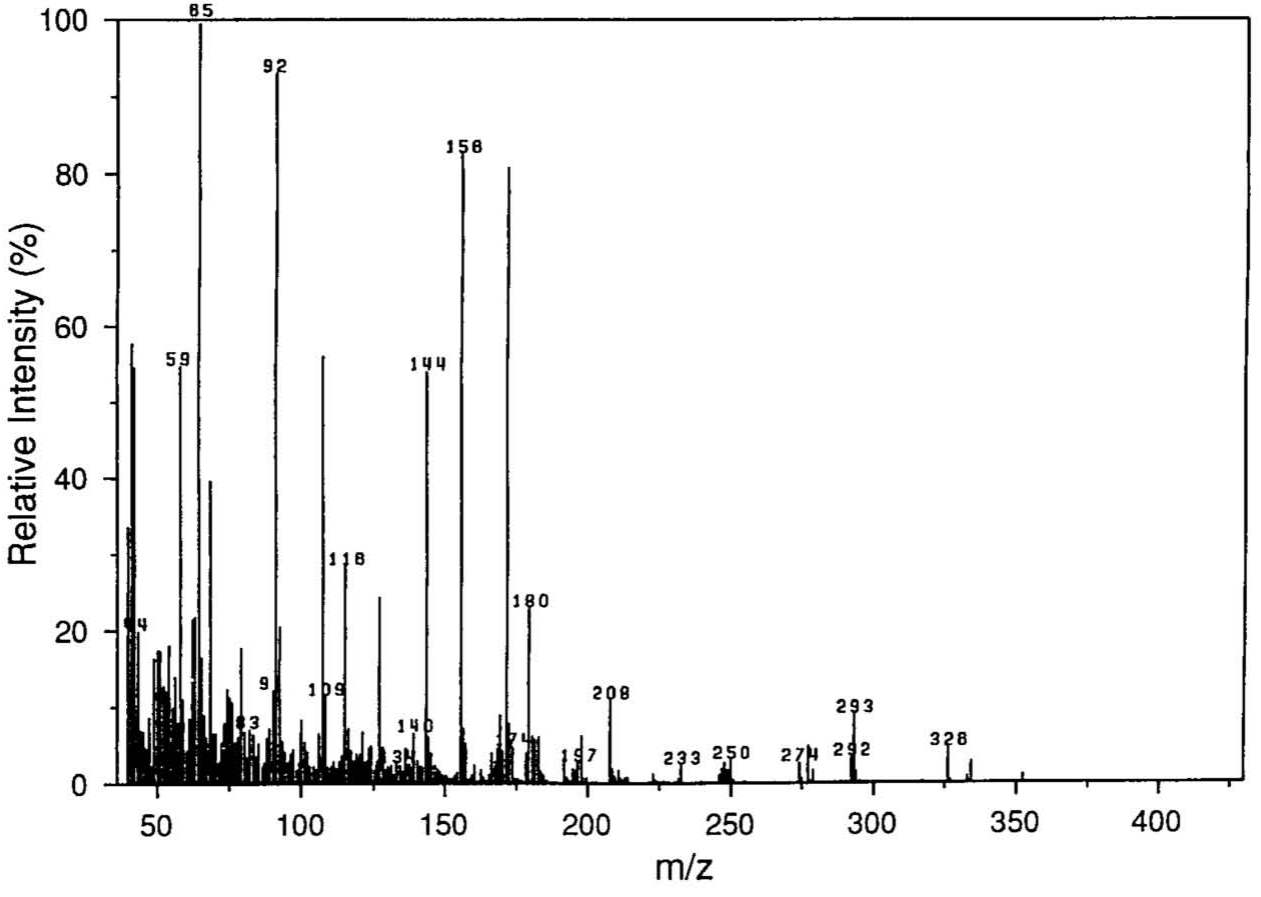
Figure A.12 The mass spectrum of the SMS complex prepared from sulphanilamide and malonaldehyde.


Relative Intensity (%)
100
80
60
40
20
0
50
100
150
200
250
300
350
400
m/z
172
158
92
85
48
109

Mass Spectral Data for SMS

MASS	%AR. MOD.	%AR. BASE	% TIC	ABS AREA
40.94	9.35	9.35	1.857	26
42.02	0.72	0.72	0.143	2
43.08	15.47	15.47	3.071	43
44.08	2.88	2.88	0.571	8
47.99	21.22	21.22	4.214	59
49.89	1.08	1.08	0.214	3
50.96	3.60	3.60	0.714	10
52.03	5.76	5.76	1.143	16
53.09	3.60	3.60	0.714	10
54.12	2.16	2.16	0.429	6
55.14	0.36	0.36	0.071	1
57.13	1.08	1.08	0.214	3
62.02	1.44	1.44	0.286	4
63.07	6.83	6.83	1.357	19
64.04	39.57	39.57	7.857	110
65.12	43.88	43.88	8.714	122
66.09	11.87	11.87	2.357	33
68.93	4.32	4.32	0.857	12
75.06	0.36	0.36	0.071	1
77.05	0.72	0.72	0.143	2
78.96	0.72	0.72	0.143	2
79.92	7.91	7.91	1.571	22
80.98	0.36	0.36	0.071	1
89.94	0.72	0.72	0.143	2
90.99	2.88	2.88	0.571	8
92.03	53.24	53.24	10.571	148
93.06	23.74	23.74	4.714	66
107.07	1.08	1.08	0.214	3
108.03	32.73	32.73	6.500	91
109.01	2.16	2.16	0.429	6
125.04	3.60	3.60	0.714	10
139.94	1.80	1.80	0.357	5
156.02	74.82	74.82	14.857	208
157.03	5.04	5.04	1.000	14
157.99	3.24	3.24	0.643	9
172.00	100.00	100.00	19.857	278
173.01	7.19	7.19	1.429	20
174.03	4.32	4.32	0.857	12
378.96	0.72	0.72	0.143	2
379.97	1.08	1.08	0.214	3

Figure A.13 The mass spectrum of the SMT complex prepared from malonaldehyde with sulphanilamide and TBA.


Relative Intensity (%)
m/z
100
80
60
40
20
0
50
100
150
200
250
300
350
400
85
92
158
59
144
118
180
208
293
328
109
140
233
250
292

Mass Spectral Data for SMT

MASS	%AR. MOD.	%AR. BASE	% TIC	ABS AREA	MASS	%AR. MOD.	%AR. BASE	% TIC	ABS AREA
40.94	33.61	33.61	2.137	363	94.06	5.46	5.46	0.347	59
42.01	57.31	57.31	3.645	619	95.08	4.07	4.07	0.259	44
43.09	54.26	54.26	3.451	586	96.08	2.59	2.59	0.165	28
44.09	19.91	19.91	1.266	215	97.08	3.70	3.70	0.236	40
45.11	6.85	6.85	0.436	74	98.06	4.35	4.35	0.277	47
46.07	6.67	6.67	0.424	72	99.00	1.67	1.67	0.106	18
47.06	4.54	4.54	0.289	49	99.94	2.59	2.59	0.165	28
48.00	8.52	8.52	0.542	92	100.99	8.15	8.15	0.518	88
48.97	2.04	2.04	0.130	22	102.03	5.37	5.37	0.342	58
49.89	16.30	16.30	1.036	176	103.06	3.98	3.98	0.253	43
50.97	17.41	17.41	1.107	188	104.08	2.04	2.04	0.130	22
52.03	17.22	17.22	1.095	186	105.09	2.04	2.04	0.130	22
53.08	12.69	12.69	0.807	137	106.08	1.57	1.57	0.100	17
54.12	11.94	11.94	0.760	129	107.08	6.39	6.39	0.406	69
55.14	18.06	18.06	1.148	195	108.04	55.65	55.65	3.539	601
56.14	9.81	9.81	0.624	106	109.00	11.30	11.30	0.718	122
57.13	13.89	13.89	0.883	150	109.97	1.94	1.94	0.124	21
58.03	7.78	7.78	0.495	84	111.01	2.13	2.13	0.135	23
58.96	54.35	54.35	3.456	587	112.04	2.69	2.69	0.171	29
59.89	10.93	10.93	0.695	118	113.06	1.85	1.85	0.118	20
60.96	4.26	4.26	0.271	46	114.07	2.69	2.69	0.171	29
62.04	8.43	8.43	0.536	91	115.10	3.61	3.61	0.230	39
63.08	21.48	21.48	1.366	232	116.05	28.43	28.43	1.808	307
64.07	21.76	21.76	1.384	235	117.08	7.04	7.04	0.448	76

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Mass Spectral Data for SMT

65.13	100.00	100.00	6.359	1080	118.05	4.17	4.17	0.265	45
66.08	16.39	16.39	1.042	177	119.01	2.69	2.69	0.171	29
67.08	8.80	8.80	0.559	95	119.98	3.70	3.70	0.236	40
68.03	6.02	6.02	0.383	65	121.02	3.70	3.70	0.236	40
68.96	39.44	39.44	2.508	426	122.05	6.57	6.57	0.418	71
69.92	6.30	6.30	0.400	68	123.07	2.69	2.69	0.171	29
71.00	6.48	6.48	0.412	70	124.08	4.44	4.44	0.283	48
72.02	2.59	2.59	0.165	28	125.09	4.91	4.91	0.312	53
73.06	5.19	5.19	0.330	56	126.07	1.57	1.57	0.100	17
74.06	7.69	7.69	0.489	83	127.06	2.78	2.78	0.177	30
75.08	12.31	12.31	0.783	133	128.05	24.35	24.35	1.549	263
76.08	11.20	11.20	0.712	121	129.04	4.72	4.72	0.300	51
77.07	10.56	10.56	0.671	114	130.01	1.57	1.57	0.100	17
79.01	5.93	5.93	0.377	64	132.05	2.31	2.31	0.147	25
79.95	17.69	17.69	1.125	191	133.06	0.93	0.93	0.059	10
81.02	6.67	6.67	0.424	72	134.07	2.50	2.50	0.159	27
82.03	3.15	3.15	0.200	34	135.07	2.31	2.31	0.147	25
83.09	6.94	6.94	0.442	75	136.07	1.48	1.48	0.094	16
84.10	6.30	6.30	0.400	68	137.03	4.44	4.44	0.283	48
85.10	3.43	3.43	0.218	37	138.03	2.41	2.41	0.153	26
86.02	5.09	5.09	0.324	55	138.99	1.94	1.94	0.124	21
87.03	2.04	2.04	0.130	22	139.99	6.48	6.48	0.412	70
88.00	2.59	2.59	0.165	28	141.04	2.96	2.96	0.188	32
88.98	5.74	5.74	0.365	62	142.06	2.13	2.13	0.135	23
89.93	7.04	7.04	0.448	76	143.08	2.04	2.04	0.130	22
90.99	12.04	12.04	0.765	130	144.05	53.70	53.70	3.415	580
92.03	92.59	92.59	5.888	1000	145.07	5.93	5.93	0.377	64
93.05	20.56	20.56	1.307	222	146.07	3.80	3.80	0.241	41

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Mass Spectral Data for SMT

147.09	2.31	2.31	0.147	25	212.05	0.46	0.46	0.029	5
148.05	1.76	1.76	0.112	19	213.09	0.65	0.65	0.041	7
149.03	1.48	1.48	0.094	16	214.03	0.65	0.65	0.041	7
149.99	0.93	0.93	0.059	10	222.01	0.19	0.19	0.012	2
151.02	1.02	1.02	0.065	11	223.06	1.11	1.11	0.071	12
152.06	0.46	0.46	0.029	5	224.08	0.28	0.28	0.018	3
153.05	0.56	0.56	0.035	6	225.03	0.09	0.09	0.006	1
154.08	0.74	0.74	0.047	8	226.07	0.19	0.19	0.012	2
155.07	1.30	1.30	0.082	14	227.08	0.09	0.09	0.006	1
156.07	82.04	82.04	5.217	886	228.12	0.09	0.09	0.006	1
157.05	7.13	7.13	0.453	77	231.02	0.09	0.09	0.006	1
158.03	5.09	5.09	0.324	55	232.06	0.46	0.46	0.029	5
159.04	0.56	0.56	0.035	6	233.10	2.22	2.22	0.141	24
160.00	1.02	1.02	0.065	11	239.98	0.09	0.09	0.006	1
161.05	2.31	2.31	0.147	25	246.10	0.93	0.93	0.059	10
162.05	0.19	0.19	0.012	2	247.09	1.85	1.85	0.118	20
163.06	1.67	1.67	0.106	18	248.08	2.59	2.59	0.165	28
164.08	0.65	0.65	0.041	7	249.05	1.67	1.67	0.106	18
165.07	0.19	0.19	0.012	2	250.04	2.87	2.87	0.183	31
166.10	1.02	1.02	0.065	11	251.08	0.28	0.28	0.018	3
167.09	3.80	3.80	0.241	41	255.06	0.09	0.09	0.006	1
168.07	2.69	2.69	0.171	29	274.12	2.59	2.59	0.165	28
169.04	4.44	4.44	0.283	48	275.07	0.46	0.46	0.029	5
170.00	8.89	8.89	0.565	96	277.11	4.81	4.81	0.306	52
171.02	4.17	4.17	0.265	45	278.06	0.19	0.19	0.012	2
172.06	80.28	80.28	5.105	867	279.04	1.67	1.67	0.106	18
173.08	7.78	7.78	0.495	84	292.08	3.15	3.15	0.200	34

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Mass Spectral Data for SMT

174.08	4.63	4.63	0.294	50	293.07	8.89	8.89	0.565	96
175.08	0.46	0.46	0.029	5	294.10	1.39	1.39	0.088	15
176.09	0.46	0.46	0.029	5	295.05	0.09	0.09	0.006	1
177.08	0.28	0.28	0.018	3	317.03	0.09	0.09	0.006	1
179.02	3.80	3.80	0.241	41	326.08	4.44	4.44	0.283	48
179.97	22.96	22.96	1.460	248	327.06	0.28	0.28	0.018	3
181.02	6.20	6.20	0.395	67	333.05	0.83	0.83	0.053	9
182.03	5.65	5.65	0.359	61	334.09	2.69	2.69	0.171	29
183.07	6.02	6.02	0.383	65	352.08	0.93	0.93	0.059	10
184.09	1.39	1.39	0.088	15					
185.09	1.02	1.02	0.065	11					
186.08	0.28	0.28	0.018	3					
192.05	3.15	3.15	0.200	34					
193.04	0.65	0.65	0.041	7					
194.12	0.37	0.37	0.024	4					
195.12	1.85	1.85	0.118	20					
196.06	1.57	1.57	0.100	17					
197.07	2.59	2.59	0.165	28					
198.06	6.20	6.20	0.395	67					
199.04	0.46	0.46	0.029	5					
200.01	0.65	0.65	0.041	7					
203.03	0.19	0.19	0.012	2					
208.04	10.83	10.83	0.689	117					
209.04	1.85	1.85	0.118	20					
210.01	0.74	0.74	0.047	8					
211.05	1.67	1.67	0.106	18					

Figure A.14 The electron distribution of ferrous and ferric iron. The 3d orbitals are 5-fold degenerate in both cases. Although unoccupied in the free ionic forms, the 4s and the 3-4p orbitals are involved in the electron distribution of their coordination complexes. Adapted from Giddings (1977).

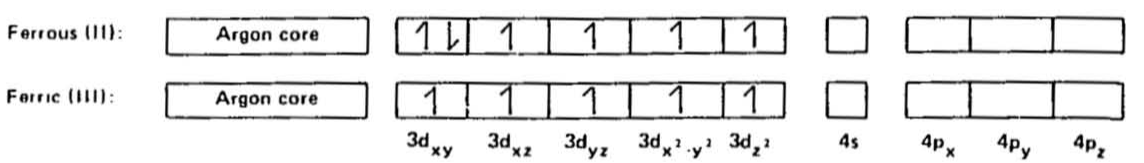

Ferrous (II):
Argon core
↑↓
↑
↑
↑
↑
Ferric (III):
Argon core
↑
↑
↑
↑
↑
3d_xy
3d_xz
3d_yz
3d_x²-y²
3d_z²
4s
4p_x
4p_y
4p_z

Figure A.15 Molecular orbital energy level diagram for both σ and π bonds in an octahedral complex. The superscripts • and * denote nonbonding and antibonding molecular orbitals, respectively. The electrons originally belonging to the iron as well as those belonging to the ligands are placed in the lowest orbitals until all the electrons have been accommodated. Each ligand usually supplies 2 σ and 4 π electrons making a total of 36 electrons which completely fills the nonbonding and bonding orbitals. The electrons from the iron are accommodated in $t_{2g}(\pi^{\bullet})$ and $e_g(\sigma^{\bullet})$ antibonding $e_g(\sigma^{\bullet})$ orbital which is raised in energy relative to the weakly antibonding $t_{2g}(\pi^{\bullet})$ orbital. Adapted from Giddings (1977).

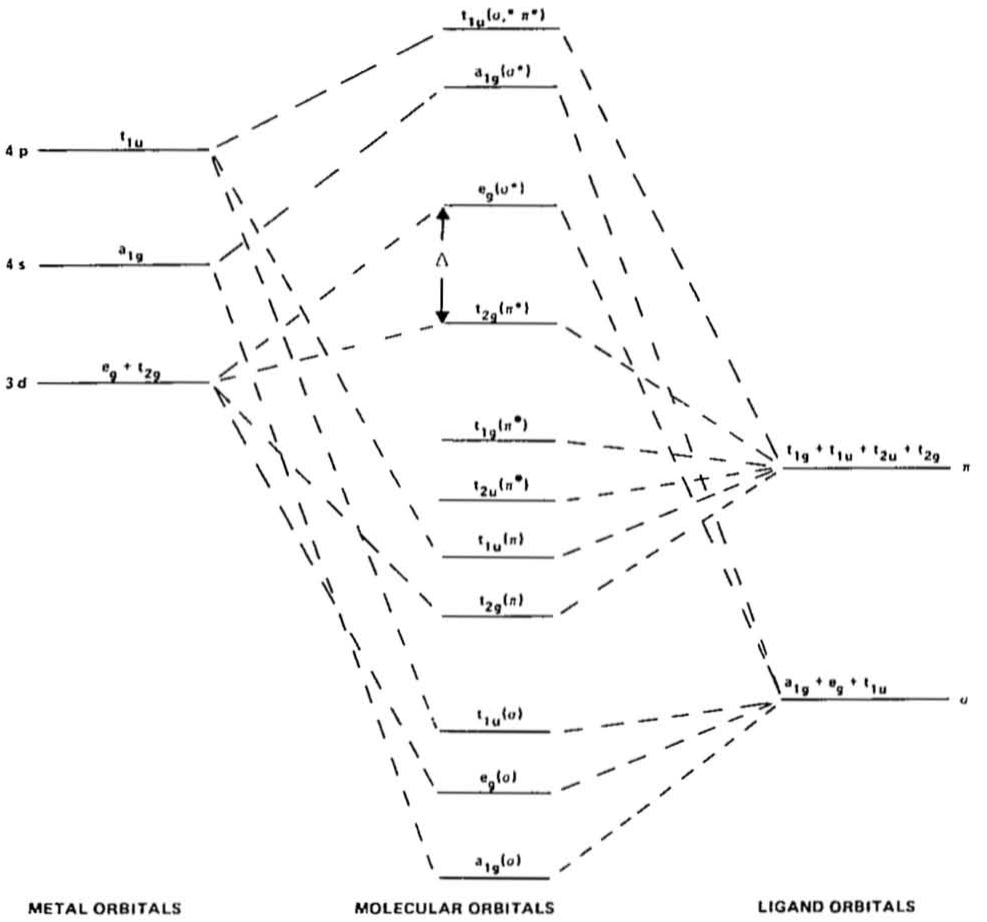

4 p
4 s
3 d
t_{1u}
a_{1g}
$e_g + t_{2g}$
$t_{1u}(\sigma^* \pi^*)$
$a_{1g}(\sigma^*)$
$e_g(\sigma^*)$
Δ
$t_{2g}(\pi^*)$
$t_{1g}(\pi^*)$
$t_{2u}(\pi^*)$
$t_{1u}(\pi)$
$t_{2g}(\pi)$
$t_{1u}(\sigma)$
$e_g(\sigma)$
$a_{1g}(\sigma)$
$t_{1g} + t_{1u} + t_{2u} + t_{2g}$
π
$a_{1g} + e_g + t_{1u}$
σ
METAL ORBITALS
MOLECULAR ORBITALS
LIGAND ORBITALS

Figure A.16 Splitting of the 3d orbitals in ligand fields having octahedral, tetragonal or rhombic symmetry. To a first approximation the six-coordinated Mb/Hb complexes are octahedral, but they generally exhibit tetragonal or rhombic distortion with additional loss of degeneracy. The iron orbitals spatially directed toward the six ligand of the e_g set are at a higher energy level than those of the t_{2g} set. The energy differential (Δ) depends largely on the sixth coordination site ligand, and is greater for strong field ligands such as NO than for weak field ligands. The deoxyferrous complexes, having no sixth ligand, exhibit a square-pyramidal symmetry. Adapted from Giddings (1977).

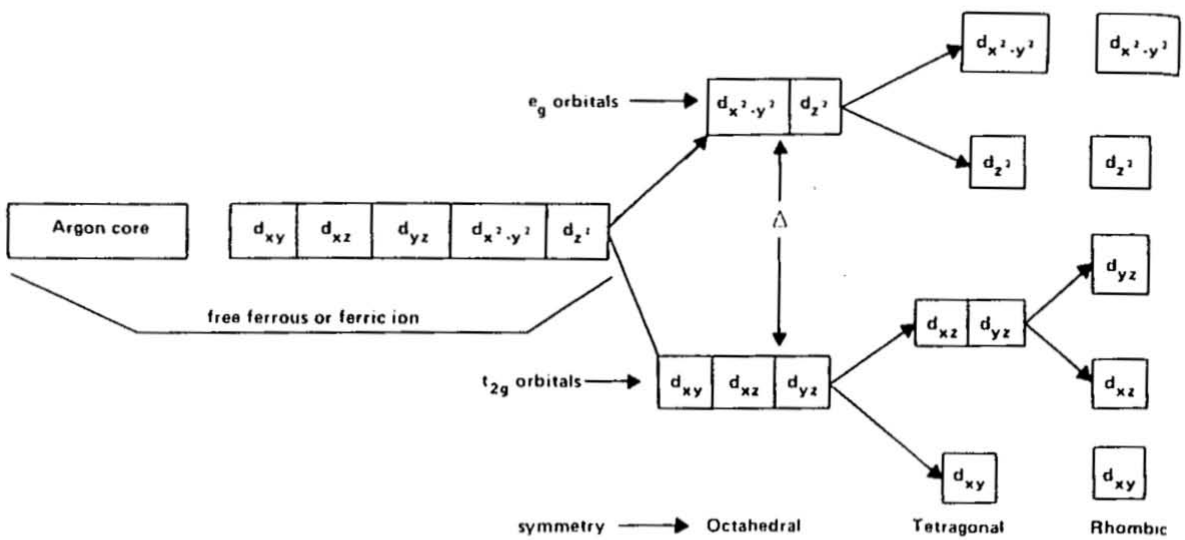
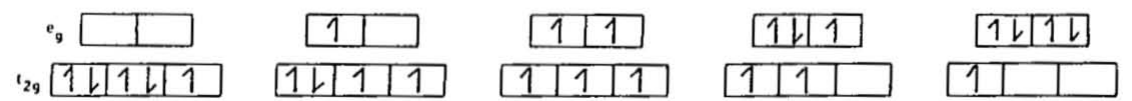

Argon core
d_{xy} d_{xz} d_{yz} $d_{x^2-y^2}$ d_{z^2}
free ferrous or ferric ion
e_g orbitals
t_{2g} orbitals
Δ
$d_{x^2-y^2}$ d_{z^2}
d_{xz} d_{yz}
d_{xy}
$d_{x^2-y^2}$ d_{z^2} d_{yz} d_{xz} d_{xy}
symmetry
Octahedral
Tetragonal
Rhombic

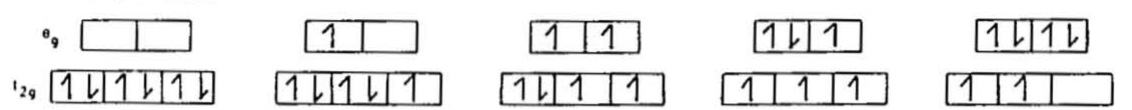
Figure A.17 The distribution of ferric and ferrous iron 3d electrons in d orbitals of six-coordinate octahedral complexes. The spin alignments (*i.e.* ↑↓) are those that have maximum total magnetic spin (S) for each case. The high spin ferriMb complexes such as ferriMb itself, having a water molecule as the sixth ligand, have S = 5/2. The low spin ferriMb complexes have S = 1/2. Low spin ferroMb complexes such as NOMb have S = 0 while deoxyferrous Mb, with a five coordinate square pyramidal symmetry, is the only high spin ferroMb complex having S = 2. Furthermore, certain derivatives such as ferrMbOH can exist in a thermal equilibrium mixture of high and low spin forms, giving a net spin of 3/2 intermediate between the 2 forms. Adapted from Giddings (1977).

FERRIC (Fe^{3+}), $(3d)^5$



t_{2g}^5	$t_{2g}^4 e_g$	$t_{2g}^3 e_g^2$	$t_{2g}^2 e_g^3$	$t_{2g} e_g^4$
$S = \frac{1}{2}$	$S = \frac{3}{2}$	$S = \frac{5}{2}$	$S = \frac{3}{2}$	$S = \frac{1}{2}$

FERROUS (Fe^{2+}), $(3d)^6$



t_{2g}^6	$t_{2g}^5 e_g$	$t_{2g}^4 e_g^2$	$t_{2g}^3 e_g^3$	$t_{2g}^2 e_g^4$
$S = 0$	$S = 1$	$S = 2$	$S = 2$	$S = 1$