CHAPTER 2

LITERATURE REVIEWS

2.1 Roselle (*Hibiscus sabdariffa* Linn.)

2.1.1 Introduction

Roselle (*Hibiscus sabdariffa* Linn.) is a shrub belonging to the family-Malvaceae. It is thought of native to Asia (India to Malaysia) or Tropical Africa. In English, roselle is called as Rozelle, Sorrel, Red sorrel, Jamaica sorrel, Indian sorrel, Guinea sorrel, Sour-sour, Queensland jelly plant, Jelly okra, Lemon bush and Florida cranberry. In Thai, it is called Krajeab or Krajeab-dang and it is known by these names in the pharmaceutical and food-flavoring.

The species *H. sabdariffa* comprises a large number of cultivated types which, on the basis of their growth habit or end use, are classified broadly under two varieties, *H. sabdariffa* var. *sabdariffa* and *H. sabdariffa* var. *altissima Wester*. Roselle is cultivated in various parts of Nakhon Pathom, Pathum Thani, Nonburi, Suphanburi, Samutsakorn, Pijit, Kanjanaburi, Ratchaburi, Rayong and Nakornnayok during July to September. The propagation is done by seeds or by rooting shoot cuttings. The edible fleshy calyces are collected after 15 to 20 days of flowering. Rest of the crop is left in the field until seeds are ready for threshing. The calyces can be dried and stored in air-tight container.

2.1.2 Phytochemistry of roselle

The leaf is reported to contain protein, fat, carbohydrate, fibre, ash, calcium, phosphorus, iron, thiamine, β-carotene, riboflavin, niacin and ascorbic acid. The calyces are rich in acid and pectin (Wong et al., 2002). Analysis of calyces has shown the presence of crude protein and minerals such as iron, phosphorus, calcium, manganese, aluminium, magnesium, sodium and potassium. Mucilage, calcium citrate, ascorbic acid, gossypetin and hibiscin chlorideare also present in calyces.

The seeds contain protein (18.8 to 22.3 %), fat (19.1 to 22.8 %) and dietary fibre (39.5 to 42.6 %). The seeds were found to be a good source of minerals like phosphorus, magnesium, calcium, lysine and tryptophan. Seed oil is rich in unsaturated fatty acids (70 %), of which linoleic acid constituted 44 %. The physicochemical analysis of the fresh calyces and leaves are given in Table 2.1 and phytochemicals present in the various parts of the plant are presented in Table 2.2.

Table 2.1 Physicochemical constituents of the fresh calyces and leaves of roselle

Constituents	Calyces (fresh)	Leaves (fresh)
Moisture	9.2 g	86.2 %
Protein	1.145 g	1.7-3.2 %
Fat	2.61 g	1.1 %
Fibre	12.0 g	10 %
Ash	6.90 g	1 %
Calcium	12.63 mg	0.18 %
Phosphorus	273.2 mg	0.04 %
Iron	8.98 mg	0.0054 %
Carotene	0.029 mg	-
Thiamine	0.117 mg	-
Riboflavin	0.277 mg	-
Niacin	3.765 mg	-
Ascorbic Acid	6.7 mg	-

Source: Shivali and Kamboj (2009)

Table 2.2 Phytochemicals of roselle

Part of the plant	Chemical constituents
Flower	Carbohydrates, arabinans, mannose, sucrose, thiamin, xylose, mucilage, niacin, pectin, proteins, fat, arabinogalactans, rhamnogalacturans, riboflavin, β-carotene, phytosterols, citric acid, ascorbic acid, fruit acids, maleic acid, malic acid, hibiscic acid, oxalic acid, tartaric acid, (+)-allooxycitronic acid-lactone, allohydroxycitric-acid, glycolic acid, utalonic acid, protocatechuic acid, cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-xyloglucoside, delphinidin, delphinidin-3-glucoside, delphinidin-3-sambubioside, delphinidin-3-xyloglucoside, delphinin, gossypetin, gossypetin-3-glucoside, hibiscetin, hibiscin, hibiscitrin, sabdaretin, sabdaritrin, fibre, resin, minerals and ash.

Table 2.2 Phytochemicals of roselle (cont.)

Leaf	α-Terpinyl acetate, anisaldehyde, *β*-carotene, *β*-sitosterol, *β*-D-galactoside, *β*-sitosteryl benzoate, niacin, fat, isoamyl alcohol, iso-propyl alcohol, methanol, 3-methyl-1-butanol, benzyl alcohol, ethanol, malic acid, fibre and ash.
Seed	Starch, cholesterol, cellulose, carbohydrates, campesterol, *β*-sitosterol, ergosterol, propionic acid, pentosans, pelargonic acid, palmitoleic acid, palmitic acid, oleic acid, myristic acid, methanol, malvalic acid, linoleic acid, sterculic acid, caprylic acid, formic acid, stearic acid, cis-12,13-epoxy-cis-9-octadecenoic acid, isopropyl alcohol, isoamyl alcohol, ethanol, 3-methyl-1-butanol, fibre and minerals.
Fruit	α-Terpinyl acetate, pectin, anisaldehyde, ascorbic acid, calcium oxalate, caprylic acid, citric acid, acetic acid, ethanol, formic acid, pelargonic acid, propionic acid, isopropyl alcohol, methanol, benzyl alcohol, 3-methyl-1-butanol, benzaldehyde and minerals.
Root	Tartaric acid and saponin.

Source: Shivali and Kamboj (2009)

Roselle is implicated in many biological activities that may impact positively on human health (Ali, 2004). Their use for therapeutic purposes has long been supported by epidemiological evidence, but only in recent years some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been verified by controlled *in vitro*, *in vivo*, or clinical research studies (Ali, 2004).

1) Antioxidant activity. The antioxidant and free radical scavenging effects of extract obtained from dried roselle petals were investigated (Tsai et al., 2002; Tsai and Huang, 2004). Moreover, the fractions of the ethanol extract (chloroform soluble fraction and ethyl acetate soluble fraction) of dried flower showed scavenge hydrogen peroxide and inhibitory effects on superoxide anions radicals $\left(O_2^-\right)$ (Farombi and Fakoya, 2005).

2) Anticancer. Anthocyanins can cause cancer cell apoptosis, especially in HL-60 cells (Chang et al., 2005). Anti-oxidative activity of anthocyanins was evaluated by their effects on LDL oxidation in cell free system and anti-apoptotic abilities in RAW 264.7 cells (Chang et al., 2006a). The study showed that anthocyanins of this plant may be used to inhibit LDL oxidation and oxLDL-mediated macrophage apoptosis, serving as a chemopreventive agent. Inhibitory

effect of protocatechuic acid on tumour promotion in mouse skin demonstrated that protocatechuic acid possesses potential as a cancer chemopreventive agent against tumour promotion (Tseng et al., 1998).

3) Other activities. Delphinidin 3-sambubioside, a anthocyanins isolated from the dried calyces of *H. sabdariffa* can induce a dose-dependent apoptosis in human leukemia cells (HL-60) as characterized by cell morphology, DNA fragmentation, activation of caspase 3, 8 and 9, and inactivation of poly(ADP)ribose polymerase (Hou et al., 2005). Ethanol and aqueous extracts of its calyces possess antipyretic activity in experimental animals (Reanmongokol and Itharat, 2007).

Investigation of the anti-inflammatory activity showed that its extract had no effect on rat paw edema but had an inhibitory effect on yeast induced pyrexia and a significant effect on the hot plate reaction time. Polysaccharides from its flowers can stimulate proliferation and differentiation of Human Keratinocytes (Brunold et al., 2004). The study also showed that raw polysaccharides and all acidic fractions cause a strong induction of proliferation of human keratinocytes while the neutral polymers were ineffective.

Antibacterial activity of gossypetin isolated from *H. sabdariffa* was carried out and results revealed that the activity may be due to polyphenolic nature of the flavonoid gossypetin (Mounnissamy et al., 2002). Investigation on nootropic acitivity of its calyces in mice indicated that the extract of calyces might prove to be useful memory restorative agent in the treatment of dementia seen in elderly which may be due to its anti-acetylcholinesterase property (Joshi and Parle, 2006).

2.2 Anthocyanins

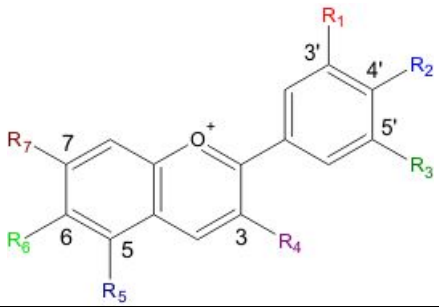
2.2.1 Introduction

Anthocyanins are a class of flavonoids which are prominent in many colored plants. The intense absorption of anthocyanins at visible wavelengths of light imparts color (orange, red, and blue) to plant tissues including flowers, vegetables, and fruits. As a result of their intense colors, they have a history of use as dyeing agents and food additives. There are 18 common base anthocyanidin (aglycone) species which differ in their patterns of hydroxylation and methylation (Figure 2.1). In addition, there are well over 600 glycosides and a rapidly expanding list of identified acylated derivatives (Delgado-Vargas and Paredes-López, 2003). In plants, anthocyanins occur in glycosylated forms, generally linked with glucose, galactose, arabinose, rhamnose, xylose, or fructose. The sugar moiety is most often found on the 3 or 5 position, but

can also occur at the 7, 3', or 5' positions. Cyanidin, delphinidin, and pelargonidin are the most common anthocyanins in nature with cyanidin glycosides reportedly present in nearly 90 % of all fruits (Prior, 2003). Contributing to the colorful appearance of fruits, vegetables, and flowers, anthocyanins help them to attract animals, leading to seed dispersal and pollination.

2.2.2 Distribution and content of anthocyanins in fruits and vegetables

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom. They are accumulated in fruit such as blackberry, red and black raspberries, blueberries, bilberries, cherries, currants, blood orange, elderberries, grapes, and vegetables such as roselle, red onion, radish, red cabbage, eggplant, purple sweet potato. Although they are accumulated mostly in flowers and fruits, but are also present in leaves, stems and storage organs (Delgado-Vargas and Paredes-López, 2003). Among different plants or even cultivars in the same plant, the total anthocyanins content varies considerably, affected by genes, light, temperature, and agronomic factors. In general that level of anthocyanins in fruits is much higher than in vegetables (Table 2.3).



Anthocyanidin	R1	R2	R3	R4	R5	R6	R7	main color
Cyanidin	-OH	-OH	-H	-OH	-OH	-H	-OH	magenta
Delphinidin	-OH	-OH	-OH	-OH	-OH	-H	-OH	purple, blue
Pelargonidin	-H	-OH	-H	-OH	-OH	-H	-OH	orange, salmon
Malvidin	$-OCH_3$	-OH	$-OCH_3$	-OH	-OH	-H	-OH	purple
Peonidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	-OH	magenta
Petunidin	-OH	-OH	$-OCH_3$	-OH	-OH	-H	-OH	purple
Rosinidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	$-OCH_3$	red

Figure 2.1 Basic structures of anthocyanins (flavylium cation)

Source: Delgado-Vargas and Paredes-López (2003)

Mean distribution of the six most common anthocyanidins in the edible parts of plants is as follow: Cyaniding; Cy (50 %), Pelargonidin; Pg (12 %), Peonidin; Pn (12 %), Delphinidin; Dp (12 %), Petunidin; Pt (7 %), and Malvidin; Mv (7 %) (Kong et al., 2003). The three non-methylated anthocyanidins; Cyanidin, Delphinidin and Pelargonidin are the most widespread in nature. Most species contain a limited number of anthocyanins pigments (apples, plums, pears), but in some cases-like red grapes, may contain a mixture of more than 20 pigments (Clifford, 2000). Overall, cyanidin aglycone occurs in about 90 % of fruits, and is the most frequently appearing aglycone (Prior, 2004) (Tables 2.4).

Table 2.3 Total anthocyanins content in selected common fruits and vegetables

Food source	Anthocyanins content in mg per 100 g	Literature sources
Açaí	320	Schauss et al., 2006
Blackcurrant	130-400	Clifford, 2000
Black raspberry	589	Clifford, 2000
Chokeberry	1,480	Bridle and Timberlake, 1997
Cherry	350-400	Eder, 2000
Eggplant	750	Clifford, 2000
Roselle	340	Chumsri et al., 2008
Orange, blood	200	Clifford, 2000
Onion, red	up to 250	Franke et al., 2004
Purple corn	1,640	Lieberman, 2007
Purple sweet potato	17-30	Brown, 2006
Red cabbage	25	Wu et al., 2006
Red grape	888	Eder, 2000; Clifford, 2000
Red wine	24-35	Clifford, 2000
Red radish	110-600	Giusti et al., 1998
Strawberries	127-360	Clifford, 2000

Source: Adapted from Horbowicz et al. (2008)

Anthocyanins in fruits and vegetables are naturally presented in glycosylated forms. However, many of the analytical procedures convert the glycosides into aglycones and thus results in Table 2.4 are reported as aglycones. Therefore results summarized in this table is after

conversion the glycoside values into aglycone forms based on molecular weight to make data consistent across the database.

In plants, the following three classes of anthocyanidin glycosides are common: 3-monoglycosides, 3-diglycosides, and 3,5-diglycosides. 3-Glycosides occur about two and half times more frequently than 3,5-diglycosides (Kong et al., 2003). Considering that glucoside form is the most abundant comparing to other glycosides, cyanidin 3-glucoside is the most widespread anthocyanins in nature (Prior, 2004; Kong et al., 2003).

Table 2.4 Content of main anthocyanidins in selected fruits and vegetables

Species	Milligram per 100 gram						Literature sources
	Cy	**Pg**	**Mv**	**Pt**	**Pn**	**Dp**	
Banana						7.4	Harnly et al., 2006
Black bean			6.5	9.6		12.0	Franke et al., 2004 Wu et al., 2006
Bilberry	112.6		54.4	51.1	51.1	161.9	Kähkönen et al., 2003
Blueberries	17.0		61.4	26.4	11.4	47.4	Franke et al., 2004
Egg plant						13.8	Wu et al., 2006
Radish		25.7					Harnly et al., 2006 Wu et al., 2006
Red bean	1.2	2.4					Wu et al., 2006
Red cabbage	72.9					0.1	Wu et al., 2006
Red onion	6.2				1.2	2.3	Franke et al., 2004 Wu et al., 2006 Arabbi et al., 2004
Red grape	1.5	1.1	34.7	2.9	2.1	3.7	Franke et al., 2004 Wu et al., 2006
Roselle	25 (%)					75 (%)	Wong et al., 2002
Strawberries	2.0	31.3				0.3	Franke et al., 2004 Wu et al., 2006

Source: Adapted from Horbowicz et al. (2008)

2.2.3 Extraction and determination of anthocyanins

The most widely used solvents for the extraction of anthocyanins are aqueous solutions of acetone, methanol, and ethanol. The extraction of anthocyanins is the first step in determination of total as well as individual anthocyanins in any type of plant tissue. Extraction procedures have generally involved the use of acidic solvents, which denature the membranes of cell tissue and simultaneously dissolve pigments. The acid tends to stabilize anthocyanins, but it may also change the native form of the pigment in the tissue by breaking associations with metals, co-pigments, or other factors. Extraction with solvents containing hydrochloric acid may result in pigment degradation during concentration, and is one of the reasons why acylations with aliphatic acids had been overlooked in the past (Rodriguez-Saona and Wrolstad, 2001).

The qualitative and quantitative determination of anthocyanins in plant extracts can be achieved by a variety of classical (spectrophotometric) or contemporary methods – HPLC coupled with a various types of mass spectrometers or NMR apparatus.

Spectrophotometric measurements. As was described earlier, anthocyanins absorption spectra are dependent of pH. At pH equal or below than 2, anthocyanins solutions show two maxima of absorption, first in the ultraviolet region (approximately 260 to 280 nm) and second in the visible region (approximately 415 and 490-540 nm). The wavelengths of these absorbance peaks can differ slightly by a few nanometers among various anthocyanins, depending on structure of each anthocyanins. The maximum of absorption at 520 to 540 nm in the visible region is the most common wavelength used in the spectrophotometric measurement of total anthocyanins (Giusti and Wrolstad, 2001).

Spectrophotometric procedures, however, are unsatisfactory for precise characterization of the particular anthocyanins present in plant materials. Variability in the sample extraction media (water, acidic water, acidic organic solvent, etc.), chemical properties of anthocyanins, and co-pigmentation of anthocyanins with other secondary metabolites present in the sample solution can cause an inaccurate correlation between absorbance and actual anthocyanins concentration (Bridle and Timberlake, 1997). Moreover, the spectrophotometric method does not provide any specificity as far as the molecular identity of the anthocyanins composition present in the material is concerned.

Analysis of anthocyanins and anthocyanidins by HPLC. HPLC separation combined with diode array detection (DAD) is the most common contemporary method for qualitative and quantitative analysis of anthocyanins (Durst and Wrolstad, 2001). Single wavelength detection is

performed at range 520 to 540 nm, while data acquisition between 200 and 800 nm is useful when DAD detection is available. Although UV-visible spectra of anthocyanins are quite similar, a tentative identification of compounds can be achieved by comparison with the spectra of reference compounds.

Reversed-phase (RP) columns: 4.6 mm (internal diameter) and 100 to 300 mm (length), are the mostly used in HPLC analysis of anthocyanins. Columns are usually maintained at ambient temperature, and elution systems are binary, using aqueous acidified solvents such as acetic acid, perchloric acid, or formic acid in an organic solvent such as methanol or acetonitrile (Zhang et al., 2004). In the reversed-phase system, the more polar phenolic acids elute first, followed by the 3, 5-diglycosylated anthocyanins, 3-monoglycosylated anthocyanins, aglycones, and last the acylated anthocyanins. Anthocyanidins elution order on a reversed-phase column is delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Durst and Wrolstad, 2001). The retention of glycosides in comparison to that of the aglycones is decreased by the presence of sugars, with diglucosides preceding monoglucosides. Subsequently, acylation increases the retention time of anthocyanins.

2.2.4 Stability of anthocyanins

Many factors, such like the pH, temperature, light, presence of other phenolic compounds, enzymes, metal ions, sugars, ascorbic acid, and oxygen have impact on the stability of anthocyanins. In aqueous solution, anthocyanin undergoes structural transformations that are pH-dependent (Figure 2.2) (Delgado-Vargas and Paredes-López, 2003). It has been found that four major anthocyanins forms exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone. At pH < 2, anthocyanins exist primarily in the form of the red flavylium cation. Process of a flavylium salt salvation in a slightly acidic or neutral aqueous solution resulted in formation of neutral or quinonoidal bases. Hydration of the flavylium cation gives the colorless carbinol pseudobase at pH values ranging from 3 to 6. This can further equilibrate to an open form, the colorless chalcone pseudobase.

Processing and storage under low temperature can improve the stability of anthocyanins. Temperature has been reported to induce a slow destruction of the pigments. When temperature is increased, anthocanins can be transformed into unstable formation of chalcone, and the chalcone is further degraded to brown products.

Light is usually deleterious to anthocyanins components. Acylated anthocyanins are less affected by light, with only slight difference on pigment stability when exposed to light as

compared to stored in the dark (Giusti et al., 1999). Oxygen and hydrogen peroxide can easily oxidize anthocyanins, and this mechanism is often accelerated by the presence of ascorbic acid. The negative impact of ascorbic acid on anthocyanins has been of great concern because of the universality of ascorbic acid in fruit and vegetable juices (Delgado-Vargas and Paredes-López, 2003). The interaction of ascorbic acid and oxygen may be mediated by H_2O_2 because the mechanism of ascorbic acid oxidation produces peroxide, and peroxide is known to destroy anthocyanins.

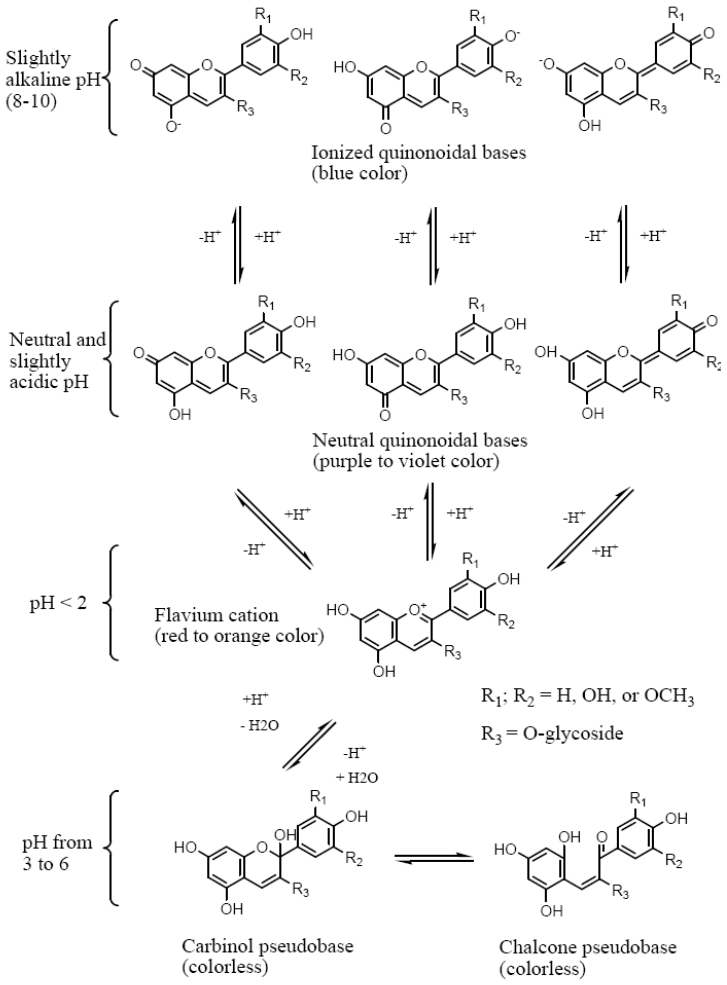


Figure 2.2 Inter-conversion pathways of various forms of anthocyanins in acidic aqueous medium

Source: Delgado-Vargas and Paredes-López (2003)

Increased sugar level may affect the rate of anthocyanins destruction. It was found that sucrose addition improved the color characteristics of frozen strawberries (Wrolstad et al., 1990). Tsai et al. (2004) reported, according to the degradation index (DI), half life and activation energy

of anthocyanin degradation, that sucrose is a good anthocyanin protector. In another study, the anthocyanins stability of grape, elderberry and blackcurrant extracts was lower in all sucrose (10 %) added systems as compared to the control at pH values of 3, 4 and 5, whereas the browning index did not change with addition of sugar (Malien-Aubert et al., 2001). Addition of 20 g/L of sucrose to a drink model system (pH 3) containing red cabbage and grape extracts did not influence the thermal and photo stability of the anthocyanins (Duhard et al., 1997).

The mechanism could be associated with the inhibition of enzymatic activities of phenolooxidase and peroxidase. Anthocyanins are very reactive toward metals, and form stable complexes with tin, copper, and irons. For instance, cyanidin 3-glucoside forms a stable colored complex in the presence of aluminum ions at pH 5.5.

2.2.5 Bioactivity of anthocyanins

In recent years, numerous studies have suggested that anthocyanins are protective against many chronic degenerative diseases (Prior, 2004; Kong et al., 2003). For the most part, the beneficial qualities of anthocyanins have been primarily attributed to their antioxidant capacities (Kong et al., 2003). Cancer is generally believed to be initiated in part by oxidative mechanisms acting upon genetic materials such as DNA and RNA. The oxidation of macromolecules such as lipids and proteins may result in altered cellular processes associated with cancer development and progression (Rao and Agarwal, 2000). Anthocyanins have been observed to prevent oxidation of these structures in vitro (Rice-Evans et al., 1996; Hou, 2003, Kong et al., 2003) and a growing body of evidence now suggests a possible association between anthocyanins and cancer prevention (Hou, 2003). Furthermore, an increased level of oxidation in the blood is assumed to be associated with cardiovascular disease (CVD). Elevated oxidation in the serum/plasma results in a variety of reactions including the oxidative modification of LDL particles, initiation of inflammation, activation of nitric oxide synthase (NOS), increased platelet aggregation, and increased foam cell production (Parthasasrathy et al., 2001). Recently, anthocyanins has been observed to reduce serum oxidation in the postprandial hyperlipidemic state (Kay and Holub, 2002; Kong et al., 2003) and to enhance the resistance of LDL to oxidative modification (Natella et al., 2002). The antioxidant characteristics of anthocyanins have been associated with a variety of properties including free radical scavenging, chelation of trace metals, and inhibition of lipid peroxidation and DNA oxidation. Additionally, anthocyanins consumption has been observed to directly increase the hydrophilic and lipophilic antioxidant capacity of the blood serum/plasma in many investigations (Seeram and Nair, 2002).

Antioxidant activity. Anthocyanins are highly reactive radical scavengers in various *in vitro* environments. Anthocyanins not only scavenge radicals, but through their ability to bind heavy metals such as iron, zinc, and copper, also prevent the formation of radicals (Rice-Evans et al., 1996; Rao and Agarwal, 2000). Anthocyanins may also exert antioxidant abilities through the protection or enhancement of endogenous antioxidants or through the induction of antioxidant enzymes such as glutathione-*S*-transferase (GST) and superoxide dismutase (SOD) (Ross and Kasum, 2002).

Structural characteristics effecting antioxidant activity. The structural characteristics responsible for the antioxidant effect of anthocyanins are generally associated with the number of free hydroxyls around the pyrone ring, greater number of hydroxyls showed greater antioxidant capacity. The antioxidant capacity of a polyphenolic is dictated not only by the number of free hydroxyls, but also by the basic structural orientation of the compound. The ring orientation will determine the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical and the ability of the compound to support an unpaired electron. The conjugation of the anthocyanins ring structure is also important. The C_2-C_3 double bond of the C-ring is consistently associated with a higher antioxidant capacity, reportedly having a stabilizing effect on the phenoxy radical (Middleton et al., 2000; Zheng and Wang, 2003). The positioning of hydroxyls in relation to one another is also a very important determinant of the antioxidant capacity of anthocyanins. Hydroxyl groups in close proximity, such as the ortho-hydroxyls of the B-ring, appear to greatly enhance the antioxidant capacity of the anthocyanins (Zheng and Wang, 2003) in experimental (*in vitro*) models; however, the availability of the highly reactive ortho-hydroxyls in a biological system (*in vivo*) has yet to be established. Conceptually, this site on the B-ring could form bonds with many compounds within biological fluids thus inhibiting the ability of this reactive site to participate in oxidation, metal chelation, or protein binding *in vivo*.

Glycosylation and antioxidant capacity. Anthocyanins are found in plants in glycosylated forms. Glycosylation is reported to influence the antioxidant capacity of anthocyanins/flavonoids (Seeram and Nair, 2002). It is generally stated in the literature that glycosylation decreases the antioxidant capacity of anthocyanins by reducing free hydroxyls and metal chelation sites; however, contradictory results have been reported (Kähkönen and Heinonen, 2003). It is important to note that the effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed such as aqueous-soluble or lipid-soluble phases.

Glycosylation diminishes the antioxidant capacity of the anthocyanins in an artificial membrane system by decreasing the number of free hydroxyls and metal chelation sites. More importantly, glycosylation will decrease the accessibility of the flavonoids to membranes as a result of the increased polarity (increased water-solubility) associated with the sugar moiety. The physiological relevance of this effect has not been sufficiently established *in vivo*. Aglycones are less water-soluble and therefore have an increased partitioning into the lipid-soluble phase of the artificial membrane system. One would assume that the increased antioxidant capacity of anthocyanidins (aglycones) in this environment would therefore be partly a result of the increased lipid solubility of the aglycones over the glycosides. Conversely, other assay systems such as the oxygen radical absorbance capacity (ORAC) assay (Wang et al., 1997), the ferric reducing assay, and certain lipid oxidation models (Kähkönen and Heinonen, 2003) have found some glycosides to have higher antioxidant capacities than their respective aglycones. Therefore, the *in vitro* effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed (aqueous-soluble or lipid-soluble phase). Additionally, since anthocyanins aglycones have not been identified in the blood or urine, the physiological relevance of the antioxidant capacity of aglycones in the circulation is questionable. This being said, as anthocyanins glycosides are generally believed to be cleaved by colonic microflora, the aglycones could have physiological relevance within the colon with the glycosides having more systemic relevance. It is clear that the respective *in vivo* antioxidant capabilities of the anthocyanins aglycones versus their glycoside derivatives require further investigation.

Effect of pH on antioxidant activity. Anthocyanins exist in equilibrium in a variety of protonated, deprotonated, and hydrated forms. These range from colored quinonoid forms, to the flavylium ion, and to colorless hemi-acetal forms (Kähkönen and Heinonen, 2003). The expression of the predominant form is generally pH dependent. There is little evidence regarding the effect of pH on the biological activity of these compounds. Although in spite of the loss of color of anthocyanins at physiological (pH 7), evidence presented by Narayan et al. (1999) suggests that anthocyanins glycosides retain their antioxidant activity.

Compartmentalization and antioxidant activity. Results of trials aimed at determining the link between antioxidant consumption, antioxidant status, and oxidative-associated disease have been inconsistent (Willett, 2000). Although anthocyanins have shown promise in many *in vitro* antioxidant models, it has yet to be established if these compounds can reach their target of suspected action and if high enough concentrations are attained to elicit a biologically significant

response. Youdim et al. (2000) have shown evidence of the incorporation of anthocyanins into cells and cell membranes. In a cell culture experiment, using human aortic endothelial cells, cyanidin glycosides from the elderberry were observed to be incorporated into both the plasma membrane and cytosol. The cells containing anthocyanins were determined to have significant protection against oxidation induced by reactive oxygen species. Subsequently, Bagchi et al. (2004) reported the cellular uptake of berry anthocyanins by endothelial cells. Although uptake was indicated in these studies, the mechanism by which anthocyanins enter intracellular compartments has yet to be determined.

2.3 Reactive oxygen and nitrogen species

2.3.1 Production of reactive oxygen and nitrogen species

A free radical is by definition an atom or a molecule which contains an unpaired electron in its outer orbit. Radicals are therefore highly reactive and can consequently cause harmful effects on cell structures. Free radicals and other oxygen-related reactive compounds, such as singlet oxygen $\left({}^1O_2\right)$ and hydrogen peroxide $\left(H_2O_2\right)$ are collectively termed reactive oxygen species (ROS). Although ${}^1O_2$ and H_2O_2 are not radicals, the oxidizing ability of ${}^1O_2$ is increased due to its altered spin, whereas H_2O_2 can form ROS in the presence of transition metals (Fenton reaction). Oxides of nitrogen, i.e. nitric oxide (NO) and nitrogen dioxide $\left(NO_2\right)$ molecules fall into the definition of free radicals, since they possess odd numbers of electrons (Wiseman and Halliwell, 1996).

Reactive oxygen species are generated during normal aerobic metabolism in the reaction where molecular oxygen $\left(O_2\right)$ is reduced to water (a four-electron transfer) by cytochrome oxidase, about 1 to 3 % is reduced to ROS, especially O_2^- (one electron) and H_2O_2 (two electrons) (Figure 2.3). The intermediates of this reaction are superoxide anion $\left(O_2^{\bullet -}\right)$, hydrogen peroxide and hydroxyl radical $\left(OH^\bullet\right)$. The most reactive and harmful form of these oxygen radicals is the hydroxyl radical (Halliwell and Gutteridge, 1999; Davis et al., 2001).

The production of $^\bullet OH$ radicals is difficult to demonstrate in vivo because of their extremely high reactivity as compared with that of O_2^- radicals (lifetime of 2 to 4 μs) and H_2O_2 (lifetime of 1 ms). The $^\bullet OH$ radicals can be formed from H_2O_2 by Fenton reactions that are catalyzed by traces of redox-active metals, like iron and copper, and, therefore, the concentrations of free “catalytic” metals in cells tend to approach zero (Halliwell and Gutteridge, 1999).

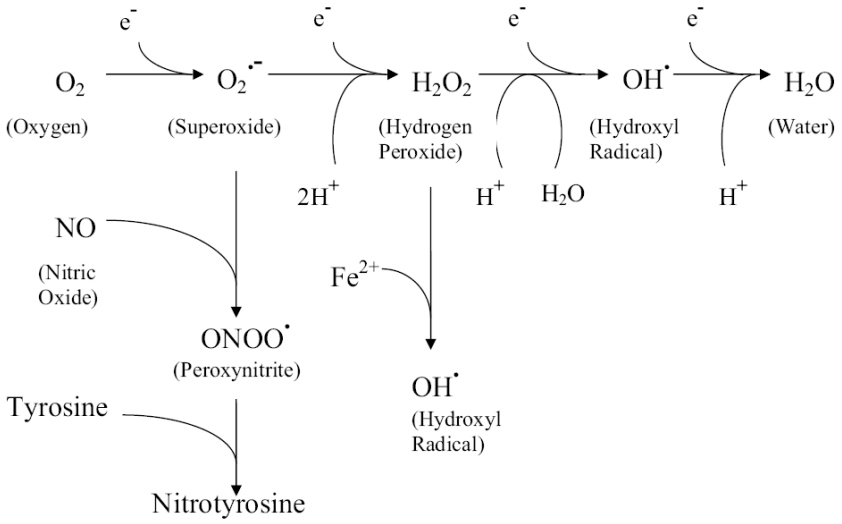


Figure 2.3 The formation of reactive oxygen species and nitric oxide

Source: Halliwell and Gutteridge (1999), Davis et al. (2001)

2.3.2 Sources and chemistry of reactive oxygen species

Reactive oxygen species (ROS) are those produced directly or indirectly from molecular oxygen (Halliwell and Gutteridge, 1999). Thus, superoxide $\left(O_2^{\bullet -}\right)$, produced by electrons "leaking" from the electron transport chain of oxidative metabolism or from the oxidative burst of activated phagocytes, is a major contributor of ROS *in vivo* and as much as 2 kg may be produced in the human body per year. This is not necessarily damaging because this species is not very reactive and "superoxide dismutase" (SOD) enzymes exist which convert superoxide to molecular oxygen and hydrogen peroxide. Hydrogen peroxide is more reactive but can also be degraded to harmless products by enzymes such as catalase. If the production of these species exceeds the ability to catalyze their degradation then damage may occur. Both superoxide and hydrogen peroxide are frequently regarded as poorly reactive. However, they may be involved in the production of more reactive species.

Hydrogen peroxide may form more damaging ROS such as hydroxyl radical either by reaction with superoxide (Haber-Weiss chemistry) or transition metals such as copper and iron. Hydroxyl radical is so reactive that it will attack whatever biological molecule exists nearby, initiating free radical chain reactions. Hydroxylation of aromatic structures has been used to probe the involvement of hydroxyl radical in protein damage *in vivo* (Pennathur et al., 2001). Hydrogen peroxide is also utilized by myeloperoxidase to produce hypochlorous acid which is responsible for chlorinating and, indirectly, nitrating tyrosine residues of proteins (van Dalen et al., 2000).

Hypochlorous acid also behaves directly as an oxidising agent producing p-hydroxyphenylacetaldehyde from tyrosine (Hazen et al., 1996).

The ability of superoxide to produce highly reactive species is exemplified by its reaction with nitric oxide to produce peroxynitrite. The consequences of this are discussed later since peroxynitrite is derived from nitric oxide and is therefore generally regarded as a reactive nitrogen species. Despite this, it will be shown that peroxynitrite is capable of exhibiting ROS-like reactivity through the production of reactive intermediates which behave like the hydroxyl radical, and through the formation of dityrosine.

Free radical chain reactions initiated by ROS may propagate through lipid compartments via the initial formation of a lipid radical $\left(L^{\bullet}\right)$. This will give rise to lipid peroxides $\left(LOO^{\bullet}\right)$ through the reaction of the lipid radical with oxygen. This species is a major contributor to free radical chain reactions in lipid structures as it effectively abstracts hydrogen atoms from nearby lipids, especially bis-allylic hydrogens of polyunsaturated fatty acids, to form a second lipid radical and a lipid hydroperoxide $\left(LOOH\right)$. The relatively unstable peroxide bond may initiate further free radical chain reactions via formation of alkoxyl radicals $\left(LO^{\bullet}\right)$ thus magnifying the effects of the initiating radical.

2.3.3 Sources and chemistry of reactive nitrogen species

The term reactive nitrogen species (RNS) describes an array of compounds which are typically higher oxidation products of nitric oxide and are derived from nitric oxide either directly (peroxynitrite) or indirectly (nitrylchloride) through the activation of a secondary nitric oxide product to a reactive tertiary product. Therefore, various reactions of nitric oxide with other species have been proposed to give rise to the RNS *in vivo*. Perhaps the most widely studied is peroxynitrite $\left(ONOO^{-}\right)$, a potent oxidizing agent which can react with a wide range of biological molecules.

Although various figures are quoted (Goldstein and Czapski, 1995), its rate of production from nitric oxide and superoxide is known to approximate a diffusion controlled reaction rate and thus peroxynitrite is thought to be rapidly formed wherever concurrent production of nitric oxide and superoxide occurs. Evidence indicates that peroxynitrite is sufficiently stable to diffuse large distances on a cellular scale, until it might reach a target molecule such as protein or DNA (Squadrito and Pryor, 1998).

The exact reaction mechanisms of peroxynitrite are not clear although the products derived from its breakdown exhibit behavior similar to both RNS and ROS such that the products

of the reaction of peroxynitrite with biological molecules may not reflect its nitric oxide origins. It is commonly expected that a RNS imparts a nitrogenous modification upon a target molecule but this may not always be the case. For example, peroxynitrite has been shown to produce both 3NT and dityrosine depending upon the concentration or flux of peroxynitrite used such that at low flux, dityrosine is formed and at higher flux 3NT is formed (Pfeiffer et al., 2000; Goldstein et al., 2000). This apparent biphasic reaction mechanism of peroxynitrite has important implications in the study of RNS-induced damage *in vitro*. Specifically, it indicates that experiments examining peroxynitrite should mimic its *in vivo* production. Many experiments have used bolus addition of synthetic peroxynitrite to an experimental solution to investigate nitration/anti-nitration effects. This is not satisfactory since, if peroxynitrite is produced through the reaction of nitric oxide with superoxide *in vivo*, then experiments using a simultaneous generator of $^{\bullet}NO$ and $O_2^{\bullet -}$ or independent generators of $^{\bullet}NO$ and superoxide should be used. This has implications for the experimentally derived product profile of peroxynitrite. The observations of Goldstein et al. (2000) and Jourd'heuil et al. (2001) indicate also that the exact flux of $^{\bullet}NO$ and $O_2^{\bullet -}$ can influence the degree of nitration versus oxidation produced by peroxynitrite suggesting that where chemical production of nitric oxide and superoxide is used, the ratio of the two reactants can influence the results obtained. Thus, the chemistry associated with peroxynitrite is extremely complex and renders it virtually impossible to propose a standard set of experimental conditions which might mimic a heterogeneous *in vivo* situation.

Other factors can influence the product profile of peroxynitrite through the formation of intermediates which follow alternative reaction mechanisms. Peroxynitrite is thought to produce reactive tertiary products through the interaction with other species present *in vivo* such as carbon dioxide. This is thought to produce an intermediate nitrosoperoxycarbonate species which favors nitration of targets due to a decomposition mechanism which produces nitrogen dioxide and the carbonate radical. Alternatively, protonation of peroxynitrite yields peroxynitrous acid which is unstable and spontaneously decomposes into poorly described intermediates which behave mostly like hydroxyl radical. Figure 2.4 shows the species thought to be produced from peroxynitrite. This could explain dityrosine and aromatic hydroxylation products which are also frequently observed following treatment with peroxynitrite. Peroxynitrite can also rapidly react with other biological targets such as glutathione and glucose to produce meta-stable intermediates which release nitric oxide. This could represent a means of scavenging peroxynitrite *in vivo* but would have to compete with carbon dioxide.

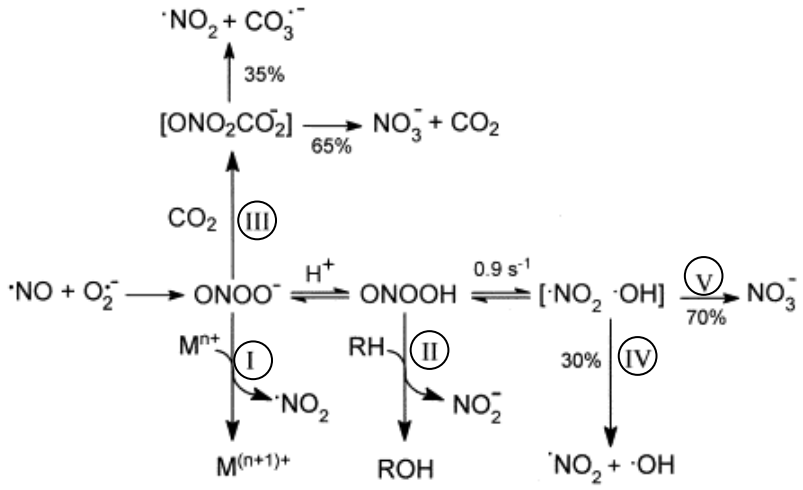


Figure 2.4 Peroxynitrite reaction pathways. Numbers I to V indicate possible fates of peroxynitrite:

i) direct reactions include the one-electron oxidation of transition metal centers (Fe, Mn, Cu)

ii) the two-electron oxidation with a target substrate (RH)

iii) the formation of nitroso-peroxocarboxylate

iv) that rapidly decomposes to secondary radicals in 35% yield. Peroxynitrous acid undergoes homolysis at 0.9 s^{-1} to yield free radicals in 30% yields

v) or rearrange to nitrate

Source: Radi et al. (2001); Uppu et al. (1996)

Although peroxynitrite has been widely studied, very little evidence exists for its production *in vivo*. It is a generally accepted principle that peroxynitrite is formed where nitration of tyrosine can be demonstrated (Halliwell, 1997). A study by Linares et al. (2001) has taken steps to remedy this by comparing mouse strains which are resistant or susceptible to *Lieshmania amazonensis* infection. In the presence of low and unchanged levels of peroxidase, control of the parasite required induction of nitric oxide synthase and nitration of parasite proteins was observed. Although nitrogen dioxide could conceivably act to nitrate tyrosine residues in these experiments, the rise in tyrosine nitration was palleled by an increase in tyrosine hydroxylation suggestive of the mixed reaction mechanisms of peroxynitrite. Nitration of tyrosine is not a specific marker of peroxynitrite (Halliwell, 1997). Many species are capable of nitrating tyrosine and other targets.

Nitrite, an oxidation product of nitric oxide, is present at low to sub-micromolar levels in biological fluids and is quite unreactive unless it becomes protonated to form nitrous acid

(HNO_2). Nitrous acid is able to nitrate phenolic structures and is used in organic synthesis for this purpose. Therefore, nitrous acid could potentially contribute to nitration *in vivo*. However, the requirement for acidic conditions perhaps eliminates this species as a contributor to overall nitration of endogenous targets since sufficiently low pH levels are unlikely *in vivo* (Halliwell, 1997).

2.4 Meat Curing

Historically, meat curing was a practice primarily done to preserve meat. As meat curing progressed, the definition was understood as the addition of salt, sugar, spices and nitrate or nitrite for aiding in flavor and preservation properties. As time passed, various spices and flavorings were added to achieve distinctive product and brand flavor characteristics. Today, meat curing is utilized to achieve consumer demands for products that have unique sensory characteristics and convenience attributes associated with cured meats. Meat curing has traditionally been associated with processed meats for the purpose of altering the color, texture, flavor, safety and shelf life characteristics which makes these products unique from other meat products (Pegg and Shahidi, 2000).

The curing process is a dynamic, complex and still not fully understood system of reactions, meat pigment changes, chemical state alterations as well as an entire host of secondary reactions. Meat curing results in a vast variety of processed meat products that are available to consumers. Variations in raw materials, formulations and processing technologies and techniques lead to the immense amount of different cured products that are manufactured and available to consumers today. For whole muscle type products, curing can be accomplished by various methods such as submersion of cure in brines or pickles, injection of cure containing brine or direct addition of dry cure for dry cured type products. Curing comminuted products is most commonly accomplished by direct addition of cure during the grinding, mixing, chopping or by other comminuting processes.

2.4.1 The curing reaction

Although nitrates were first discovered as curing agents, research findings have demonstrated that the role of nitrate is to serve as a source of nitrite for curing reactions Pegg and Shahidi (2000).

The chemistry of nitrite curing is indeed complex and in many cases not clearly understood. The term nitrite is generically used to describe both the anion, nitrite and the neutral nitrous acid. Nitrite curing has been often associated with the production of potentially dangerous

compounds formed from nitrosating species of nitrite. In examining the chemistry of meat curing, Pegg and Shahidi (2000) revealed that nitrite itself is not the primary nitrosating species or reactive compound. It was further revealed by Pegg and Shahidi (2000) and Sebranek and Fox (1985) that one of the derivatives, nitrous acid (HNO_2), actually can form nitrosating (*N*-nitroso producing) compounds which are the compounds involved in potential nitrosamine formation. These nitrosating compounds enter into a number of complex reactions which, in the end, yield nitrosylmyochromagen. The pK_a of a compound represents the acid dissocation constant, the strength of the acid and the ability of the compound to donate protons for affecting reactions. Nitrite has a pK_a of 3.36. These authors further explained that since the pH of meat (approximately 5.5 to 6.5) is clearly above the pK_a of HNO_2, the concentration of HNO_2 in cured meat is therefore extremely low. It is believed that the main reactive species of HNO_2 in meat is the anhydride of HNO_2 which is dinitrogen trioxide (N_2O_3). The dinitrogen trioxide reacts with reductants that are naturally found in muscle as well as any ones added such as ascorbates. This compound can readily form nitroso compounds.

Reducing reactions can increase the nitric oxide production which can then form coordinate-covalent complexes of nitric oxide with the heme pigments of meat. These complexes, nitrosylmyoglobin, nitroyslhaemoglobin and dinitrosylhaemochrome, form the red and pink colors of cured meats. The amount of nitrous oxide produced during curing is dependent on pH, temperature and time.

Beyond the reactions explained, there are also many other complex reactions that can and do occur. A lower pH will increase the conversion of nitrous acid to nitric oxide. When nitrite is added to comminuted meat, a browning effect occurs due to properties of nitrite acting as a strong heme oxidant. Myoglobin and oxymyoglobin are oxidized to metmyoglobin by nitrite. Through the series of already mentioned, complex reactions involving the reduction of nitrite to nitrous acid, the intermediate pigment, nitrosylmetmyoglobin, is formed. Nitrosylmetmyoglobin is not a stable pigment and therefore autoreduces in the presence of both endogenous and exogenous reductants to form a more stable nitric oxide myoglobin or nitrosylmyoglobin. Upon thermal processing, the globin portion denatures and detaches from the iron atom. The resulting pigment formed from the thermal processing is the stable nitrosylmyochromogen or nitrosylhemochrome. For the use of nitrate in a curing system, an additional step of the conversion of nitrate to nitrite is necessary. This step is normally accomplished by the bacterial reduction of nitrate to nitrite (Pinotti et al., 2001). Bacterial reduction can be accomplished by microorganisms found in the

natural flora of meat or by intentional addition of microorganisms with nitrate reducing properties (Sanz et al., 1997, 1998).

Pegg and Shahidi (2000) examine the importance of salt or sodium chloride in meat curing summarized that chloride ions could actually help catalyze nitrosation reactions. Additionally, salt levels used in the curing process are generally not high enough to provide complete preservation but at levels typically used in conjunction with nitrite, salt does offer preservative effects.

2.4.2 Residual nitrite

When nitrite is added to meat systems, it reacts chemically or is bound to components such as protein. Heat during thermal processing serves to speed up these reactions. After normal manufacturing processes, the amount of detectable nitrite is usually only approximately 10 to 20 % of the initial added amount when analytically measured (Reis et al., 2009; Pariza, 1997). These levels of nitrite or also called residual nitrite decline over the storage life of cured meat products until they are often non detectable.

In a comprehensive study of nitrite and cure accelerator levels for cooked sausages and dry and semi-dry sausages, the nitrite safety reported “highly variable” residual nitrite and cure accelerator levels not only within product categories but also between specific types of sausages (beef, pork and poultry) (Keeton et al., 2009). They reported that generally, 25 to 50 % of added nitrite remained in the product during 24 to 48 hours after processing. Purchasing and analyzing local retail commercial cured bacon, sliced ham, and wieners and analyzing for nitrite revealed residual nitrite in bacon to range between 1 and 15 ppm, sliced ham to range between 3 and 9 ppm (Cassens, 1997)

2.4.3 Factors affecting levels of residual nitrite

There are several factors that can affect the amount of residual nitrite found in cured, cooked meat products. Those factors can include non-meat ingredients added in conjunction with nitrite or nitrate such as cure accelerators, physical processing procedures. The factors can result in physical nitrite loss during processing, packaging characteristics such as packaging type and method, and storage parameters such as storage time and storage temperature. The use of cure accelerators such as erythorbates and ascorbates are the largest factor that contribute to the levels of residual nitrite found in cured meat products.

In a review by Pegg and Shahidi (2000), the importance of pH on the residual nitrite levels was discussed. A small pH decrease, as low as 0.2 pH units, during product manufacture

can result in a doubling in the rate of color formation due to more favorable nitrite-myoglobin interactions. Due to these more favorable reactive conditions, subsequent lower residual nitrite concentrations were found. Research by Kilic et al. (2001) supported these statements. In their investigations on the influence turkey meat had on residual nitrite in cured meat products, the authors reported treatments with higher pH values to also had higher residual nitrite levels.

Different raw materials and non-meat ingredients can also contribute to pH and the effects pH has on residual nitrite levels. Kilic et al. (2002) found a relationship between residual nitrite and pH values. Including poultry meat in treatments was found to alter overall product pH and thus affect residual nitrite levels. The addition of phosphates that altered product pH was also found to affect residual nitrite levels. Kilic et al. (2001) conversely found that heating temperature during thermal processing affected residual nitrite concentration by lowering levels as temperatures increased. Pérez-Rodríguez et al. (1996) indicated that the length of thermal processing can cause different depletion rates of residual nitrite.

Storage time at 14 days resulted in approximately a 90 % reduction of the ingoing nitrite levels that ranged between 30 and 300 ppm nitrite. Levels of nitrate remained comparable to ingoing levels up to approximately 21 days when frankfurters were stored at 7 ºC. At 27 ºC storage temperatures, approximately one-half of initial ingoing nitrate levels were present at 14 days storage. This was believed to be due to the microbial activity at higher storage temperatures on the conversion of nitrate to nitrite. Ahn et al., (2000) support the storage time effects but also noted packaging effects. Sausage samples stored in vacuum packages had lower residual nitrite values than samples stored in aerobic conditions. The authors believed this phenomenon was caused by the environment being in the reduced state thus allowing the conversion of nitrite to nitric oxide and the lower residual nitrite levels found.

2.5 Oxidation and antioxidant strategies

2.5.1 Lipid oxidation

Regardless of microbial spoilage, lipid oxidation is the main factor reducing the quality of meat and meat products. Though lipid oxidative reactions contribute to certain desirable quality attributes such as the development of a pleasant flavors in cooked meats (Kanner, 1994), the overall effect of lipid oxidation is negative leading to adverse effects on sensory traits, nutritional value and healthiness of muscle foods (Morrissey et al., 1998).

Mechanism of lipid oxidation. The overall mechanism of fatty acid oxidation is generally a free radicals process including initiation, propagation and termination stages. The first

step of lipid oxidation involves the removal of a hydrogen atom from a methylene carbon in a fatty acid (RH) to generate fatty acyl (R^-) and peroxyl radicals (RO_2^-). The initiation step needs to be catalysed and the role of iron in promoting the generation of species capable to abstract a hydrogen atom from an unsaturated fatty acid has been described (Gray et al., 1996). The radicals previously generated propagate the chain reaction to other fatty acids which are consequently oxidized (Figure 2.5). The reaction between fatty acids and peroxyl radicals lead to the formation of new peroxyl radicals and lipid hydroperoxides $(ROOH)$. Radicals and hydroperoxides (primary lipid oxidation products) are finally decomposed to generate stable molecules with small molecular weight (secondary lipid oxidation products) such as hydrocarbons, aldehydes, ketones, acids, esters, lactones and a large variety of nitrogen and sulphur containing compounds (Morrissey et al., 1998).

Initiation Fatty acyl (R^-) and radicals formation (RO_2^-)

Propagation
$$\begin{cases} R^\bullet + O_2 \rightarrow RO_2^\bullet \\ RO_2^- + RH \rightarrow ROOH + R^\bullet \end{cases}$$

Termination
$$\left.\begin{cases} 2R^\bullet \\ R^\bullet + RO_2^\bullet \\ 2RO_2^\bullet \end{cases}\right\} \quad \text{(Stable products)}$$

Figure 2.5 Mechanism of lipid oxidation

Source: Morrissey et al. (1998)

As far as muscle foods contain unsaturated fatty acids and pro-oxidant components are prone to suffer oxidative reactions. Between the muscle lipid fractions, the higher sensitivity of the polar lipid fraction to oxidation is mainly explained by the facts that polar lipids from cellular membranes contain a higher proportion of unsaturated fatty acids and are in close relationship with oxidation promoters located in the aqueous phase of the muscular cell (Gandemer, 2002). As opposed to the neutral lipid fraction, the polar fraction is primarily responsible for lipid oxidation in muscle foods. Though muscle tissues have endogenous antioxidant mechanisms to control lipid oxidation in vivo such as antioxidant enzymes and lipid-soluble tocopherols and β-carotenes (Chan and Decker, 1994), their effectiveness is largely diminished with increasing time post-mortem. In fact, the third phase of lipid oxidation in muscle foods frequently occurs during handling, processing, storage and cooking processes.

Factors influencing lipid oxidation. As partially described above, there are several aspects affecting the occurrence and intensity of lipid oxidative reactions in muscle foods. The amount and characteristics of the muscle lipids and the presence of pro-oxidant (*i.e*. iron, sodium chloride) and antioxidant (*i.e*. tocopherols, sodium ascorbate and nitrite) factors are known to be largely influential (Gray et al., 1996; Morrissey et al., 1998).

The amount and composition of muscle lipids largely determine the oxidative stability of a muscle food. Sasaki et al. (2001) reported significant positive correlations between fat content and lipid oxidation suggesting that the higher amount of total lipids, the higher substrate to undergo oxidative reactions. The sensitivity of fatty acids to oxidative reactions increases with the number of double bounds and therefore, poly-unsaturated fatty acid (PUFA) is more prone to be oxidized than mono-unsaturated fatty acid (MUFA) or saturated fatty acid (SFA) (Morrissey et al., 1998). High levels of PUFA in muscle foods have been previously associated with high oxidative instability during meat cooking and subsequent storage (Cortinas et al., 2005). As described above, amongst the meat components, the role of iron and heme pigments in the promotion of lipid peroxidation has been well established (Kanner, 1994). Iron, free and protein bound, heme and non-heme, oxidized or reduced has the ability to promote the oxidation of unsaturated fatty acids in meat but the relative contribution of each chemical form has not been assigned (Gray et al., 1996). The mechanisms by which iron could promote the initiation of lipid oxidative reactions can be classified in three types of reactions:

i) a direct initiation by higher valence state iron $\left(Fe^{3+}\right)$ or by reactive oxygen species (ROS) produced by a metal autoxidation process

ii) an indirect initiation by hypervalent iron complexes such as those in heme protein and porphyrin compounds and

iii) an indirect initiation-propagation of lipid oxidation through the decomposition of preformed hydroperoxides into peroxyl radicals

In addition to the intrinsic pro- and antioxidant components of the muscle itself, a number of extrinsic factors influence lipid oxidation in muscle foods. The manufacture of meat products involves the addition of non-meat ingredients and the application of technological processes that can modify the oxidation status of the muscle foods.

Meat cooking enhances the development of oxidative reactions since the reaction between molecular oxygen and muscle lipids increases with increasing temperatures (Monahan, 2000). In fact, cooking process leads to a increase lipid oxidation in muscle foods and the

development of the ‘warmed-over’ flavor of refrigerated cooked meats. The acceleration of lipid oxidation following cooking has been attributed to heat-induced changes in muscle components including disruption of cellular compartmentalization and exposure of membrane lipids to a pro-oxidative environment, thermal activation or release of catalytic free iron from myoglobin and thermal inactivation of antioxidant enzymes. The intensity of lipid oxidation is dependent on the cooking temperature since the formation of maillard reaction products with antioxidant activity at temperatures above 100 °C, would inhibit the development of oxidative reactions to some extent. The manufacture of meat products includes a number of technological processes such as cutting or mincing which involves some physical disruption which leads to the exposure of muscle lipids to the pro-oxidative environment (Monahan, 2000). The vacuum packaging and modified atmosphere packaging successful strategies to minimize lipid oxidation in raw and cooked meats (Kingston et al., 1998).

The use of certain additives enhances the susceptibility of muscle lipids to oxidative reactions. Sodium chloride is commonly used in meat products to reduce water activity and inhibit microbial spoilage and contributes to saltiness. Sodium chloride can promote lipid oxidation possibly through displacement of iron from heme proteins. On the other hand, sodium nitrite exhibit antioxidant effect in cured meats and several mechanisms have been proposed including:

i) formation of stable complex with heme pigments

ii) chelation of free iron released from heme pigments following heating or

iii) stabilization of unsaturated fatty acids

Phosphates are widely used in comminuted cooked meat products to increase water binding capacity and also act as antioxidants through metal chelating.

Other antioxidant strategies are also deliberately used to inhibit the adverse effect of lipid oxidation in muscle foods including the modification of the muscle lipid characteristics through dietary means and the direct addition of synthetic and natural antioxidants.

2.5.2 Protein oxidation

The major concerns regarding the occurrence of oxidative processes in muscle foods are related to the adverse effect of those on certain quality traits. Whereas the undesirable oxidative changes in muscle foods have been extensively studied, the precise mechanisms of alteration have not been accurately identified (Xiong, 2000). For instance, color, flavor and texture changes during refrigerated storage of meat coincide with the development of lipid oxidation and in many

cases, the oxidation of unsaturated fatty acids have been highlighted as the main cause of these adverse changes. Some other changes in muscles such as those related to the loss of texture-forming ability and water holding capacity have been associated to a loss of protein functionality though no exact mechanism has been elucidated yet (Xiong, 2000).

Though recent studies of protein oxidation in biomedical sciences have been shown on the mechanisms by which extra-cellular and membrane proteins can be affected by ROS leading to adverse biological effects (Stadtman, 2001), hardly any work devoted to the study of protein oxidation in muscle foods has been carried out. Recent studies on model and food systems have pointed out that the oxidative damage of proteins leads to alterations in gelation, emulsification, viscosity and solubility (Wang and Xiong, 1998). However, about protein oxidation in muscle foods concerning the precise chemical mechanism of protein oxidation, the characterization of the protein oxidation products, the adverse effects on meat quality and the effectiveness of different antioxidant strategies against protein oxidation.

Mechanism of protein oxidation. In muscle foods, the occurrence of protein oxidation can be linked to any of the pro-oxidant factors traditionally associated to lipid oxidation. Therefore, proteins can be oxidized by similar oxidation promoters capable to oxidize unsaturated fatty acids such as heme pigments, transition metal ions and various oxidative enzymes (Xiong, 2000). In the presence of oxidizing lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation, including initiation, propagation and termination stages (Figure 2.6). Theoretically, all amino acids are susceptible to be oxidized by free radicals and non-radical ROS but, actually, some of them are particularly vulnerable, with the cysteine and other amino acids with reactive side chains (sulphydryl, amino group, imidazole or indole ring) being the most susceptible to undergo oxidative reactions (Xiong, 2000).

The attack of ROS on muscle proteins leads to the loss of sulphydryl groups and the generation of carbonyl compounds. The generation of carbonyls (aldehydes and ketones) has been highlighted as one of the most relevant changes in oxidized proteins. Protein carbonyls can be generated via four possible pathways (Xiong, 2000):

i) direct oxidation of amino acid side chains

ii) fragmentation of the peptide backbone

iii) reactions with reducing sugars and

iv) binding non-protein carbonyl compounds

Nevertheless, the deamination reaction is considered the most common way of protein carbonyls formation.

Initiation	$RH \rightarrow R^{\bullet}$
Propagation	$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet}$
Hydrogen abstraction	$RO_2^{\bullet} + P \rightarrow ROOH + P^{\bullet}(-H)$
Addition	$RO_2^{\bullet} + P \rightarrow ROOP^{\bullet}$
Complex	$ROOP^{\bullet} + P + O_2 \rightarrow RO_2PO_2P^{\bullet}$
Polymerization	$P-P^{\bullet} + P^{\bullet} + P \rightarrow P-P-P^{\bullet} + P-P-P$

Figure 2.6 Mechanism of protein oxidation (RH: fatty acid; P: Protein)

Source: Xiong (2000)

Howell et al. (2001) have demonstrated that ROS can cause meat protein polymerization by formation of protein-protein cross-linked derivatives by the following mechanisms:

i) by the oxidation of cysteine sulphydryl groups to form disulphide linkages

ii) by the complexing of two oxidised tyrosine residues

iii) by the interactions of an aldehydes group in one protein with the amine group of a lysine residue in another protein

iv) by the crosslinking of two amine groups (lysine residues) in two different proteins through a dialdehyde (i.e. malondialdehyde) and

v) by the condensation of protein free radicals

Finally, peptide scission can take place concurrent with formation of polymers. According to Stadtman (2001) free radicals can abstract a hydrogen atom from the α-carbon of a polypeptide backbone leading to the generation of new radicals which finally undergo the peptide bond scission. The protein degradation products include those that contain the reactive carbonyl groups described above.

2.6 Nanotechnology

2.6.1 Introduction

The starting point must be to identify what is meant by the nanoscale. One widely used definition is a size range with a lower limit of approximately 1 and an upper limit of 100 nanometres. In respect of impacts on human health and/or the environment, there is no good scientific evidence in favor of either the lower or upper limit. Based on their origin, three types of

nanoscale materials (natural, by-products of human activity, engineered or manufactured) can be distinguished. Since the nanotechnologies are only concerned with the third category, further attention will be confined to this type. An engineered or manufactured 'nanomaterial' is a categorization of a material by the size of its constituting parts. It may be considered to include biological materials that are commonly used and processed and thus can be considered to be "engineered" or "manufactured" in the food and pharmaceuticals industry. Therefore, a modification of the definition might be necessary for regulatory purposes for sector uses such as food/feed and pharmaceuticals. Development of a more suitable definition depends on an understanding of the key physico-chemical and biological properties that influence the adverse effects of nanomaterials.

2.6.2 Relevant physicochemical properties

Size. This aspect has not surprisingly had the greatest attention to date. There is sufficient evidence that reduction of size at the nanoscale results in changes in some properties of the material as a consequence for example of the increase in surface-to-volume ratio. These nano specific properties raise concerns on their potential for harm to humans and the environment. Based on the likelihood of exposure and uptake by biological organisms a particular focus of attention from a risk assessment view point is required for those nanomaterials that either exist as, or may be converted to nanoparticles (3 dimensions in the nanoscale) or nanofibres, nanorods or nanotubes (two dimensions in the nanoscale).

Surface properties and chemical reactivity. The chemical reactivity increases with increasing surface area. This property may or may not be associated with an increase in biological activity or toxicity. The design of nanomaterials often includes the application of coatings and other means of modifying surface properties. Nanoparticles have the potential to generate free radicals and active oxygen. This is an important property in view of the favored theory regarding the toxicity of nanoparticles that they mediate at least some of their effect through the generation of active oxygen.

Solubilisation and other changes. Like other particulate matter, nanomaterials can:

i) be solubilized or degraded chemically

ii) form agglomerates or stable dispersions depending on solvent chemistry and their surface coating and/or

iii) have the ability to react with proteins (Linse et al., 2007).

Behaviour in biological systems. It is too early in the development of the nanotechnologies to identify general rules that can confidently be applied to predicting the risk from individual products other than the focus of concern should nanomaterials that have two or three dimensions in the nanoscale. Consequently all aspects of the life cycle from the production phase to the waste treatment at the end of the life cycle of nanomaterial products need to be considered.

Exposure and toxicokinetic aspects. It appears that for some types of nanoparticles size may be a limiting factor for absorption across the intestinal wall whereas for others similar absorption occurs up to 500 nanometres. From studies using metal particles it appears that there is increasing distribution among body organs with diminishing particle size following oral administration to rodents. Inhalation studies indicate that there is also the potential for uptake across the lung. So far it has not been possible to identify the key characteristics of nanoparticles that influence the extent of uptake nor those that facilitate persistence.

Hazard aspects. It cannot be assumed that a nanomaterial will necessarily have different hazard properties compared to its constituents, nor is it the case that nanoparticles of comparable size will have similar toxicity. Rather some may be virtually not toxic while others are clearly toxic. Although most of the existing toxicological and ecotoxicological methods for hazard identification are likely to be appropriate, they may not be sufficient to address all the hazards of nanomaterials. A particular concern with some in vitro techniques for example is whether they are able to take up the nanoparticles.

2.6.3 Main applications and potential benefits

Recent advances in nanosciences and nanotechnologies have led to a lot of interest in the control and manipulation of material properties at the nano-scale. The new materials, products and applications derived from nanotechnologies are anticipated to bring lots of improvements to the food and related sectors, impacting agriculture and food production, food processing, packaging, distribution, storage and developments of innovative products. A number of recent reports have identified the current and short-term projected applications of nanotechnologies for food and related sectors (Chaudhry et al., 2008). The main driving principle behind these developments seems to be aimed at enhancing uptake and bioavailability of nano-sized nutrients and supplements, and improving taste, consistency, stability and texture of food products (Chaudhry et al., 2008). A major area for current nanotechnology applications in the food sector is for food packaging. The new nanoparticle-polymer composites can offer a number of

improvements in mechanical performance as well as certain functional properties, such as antimicrobial activity to protect the packaged foodstuffs. Food packaging applications of nanotechnologies are estimated to make up the largest share of the current and short-term predicted nano-food market (Chaudhry et al., 2010).

Other main applications relate to health-food sector, where nano-sized supplements and nutraceuticals have been developed to enhance nutrition, and to improve health and well-being. Compared to this, most applications relating to the mainstream food and beverage areas are at the R&D stage, and only a few products are currently available. These applications include development of nano-structured (also termed as nano-textured) food materials. This relates to processing foodstuffs to develop nano-structures and stable emulsions to improve consistency, taste and texture attributes. Nano-textured foodstuffs can also enable a reduction in the use of fat. A typical product of this technology would be a nano-textured ice cream, mayonnaise or spread, which is low-fat but as "creamy" as the full-fat alternative. Such products would offer 'healthy' but still tasteful food products to the consumer. Examples include ongoing research and development (R&D) in Taiwan and Japan on development of micronized starch, cellulose, wheat and rice flour, and a range of spices and herbs for herbal medicine and food applications (FAO, 2010).

Another area of application relates to the use of nano-sized additives in food products. The main claimed benefits include better dispersibility of water-insoluble additives (colors, flavors, preservatives and supplements) in food products without the use of additional fat or surfactants. This is also claimed to enhance taste and flavor due to the enlarged surface areas of the nano-sized additives, and enhance absorption and bioavailability in the body compared with conventional bulk forms. Currently available examples include vitamins, antioxidants, colors, flavors, and preservatives. Also developed for use in food products are nano-sized carrier systems for nutrients and supplements. These are based on nanoencapsulation of the substances in the form of liposomes, micelles, or protein based carriers. These nano-carrier systems are used to mask the undesirable taste of certain additives and supplements, or to protect of from degradation during processing. The nano-encapsulated nutrients and supplements are also claimed for enhanced bioavailability, antimicrobial activity, and other health benefits. An example application, currently under research and development, is that of a mayonnaise which is composed of an emulsion that contains nano-droplets of water inside. The mayonnaise would

offer taste and texture attributes similar to the full fat equivalent, but with a substantial reduction in the fat intake of the consumer (Chaudhry et al., 2010; FAO, 2010).

Certain inorganic nano-sized additives are also finding applications in health food area. Example of these include transition metals (silver, iron), alkaline earth metals (calcium, magnesium), and non metals (selenium, silica). The use of inorganic nano-additives is claimed for enhanced tastes and flavors due to enlarged surface areas. An example is nano-salt, the use of which would give more salt particles on a product (chips/crisps) and allow the consumer to taste the salt more when added at a lower level. Food supplements in this category are also claimed for enhanced absorption and improved bioavailability compared with conventional equivalents (Chaudhry et al., 2010; FAO, 2010).