CHAPTER II

LITERATURE REVIEW

2.1 Black Rice

Rice (*Oryza sativa* L.) is the source of carbohydrate. The rice grain (Figure 1) consist the husk and the caryopsis. The brown rice is made by removal of the husk during milling. The remainder of the rice caryopsis is pericarp, aleurone plus seed coat and nucellus, embryo, and starchy endosperm. The aleurone layer varies from one to five cell layers and is thicker at the dorsal than at the ventral side and is also thicker in short-grain than in long-grain rice (del Rosario *et al.*,1968). Further milling

Figure1 A detailed structure of the rice grain (Blakeney, 1984)

to remove the pericarp, seed coat, aleurone layer and embryo to yield milled or white rice results in loss of lipid, protein, fiber, reducing sugars and total sugars, ash and minor components including vitamins, free amino acids and free fatty acids (Singh *et al.*, 1998; Park *et al.*,2001). Especially, the aleurone layers and the subaleurone layer are rich in protein, fiber, oil, minerals, vitamins, and other phytochemicals (Orthoefer and Eastman, 2004; Yokoyama, 2004). Carbohydrate, as starch, is the major component of milled rice (Table 1).

Table 1 Distribution of nutrients in milled rice

Nutrients		**Content per 100 g**
Energy (kcal)		344
Water (g)		12.9
Protein (g)		6.8
Lipids (g)		0.6
Carbohydrate (g)		77.8
Fiber (g)		1.4
Minerals (g)		0.5
Minerals	Sodium (mg)	6
	Potassium (mg)	105
	Magnesium (mg)	65
	Calcium (mg)	6
	Manganese (mg)	2
	Iron (μg)	600
	Copper (μg)	130
	Zinc (μg)	500
	Phosphorus (mg)	120
	Fluoride (μg)	50
	Iodine (μg)	2
	Selenium (μg)	10-70

หอสมุดกลาง สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 1 Distribution of nutrients in milled rice (continued)

Nutrients		Content per 100 g
Vitamins	Vitamin E (μg)	70
	Vitamin B_1 (μg)	60
	Vitamin B_2 (μg)	30
	Nicotinamide (μg)	1300
	Pantothenic acid (μg)	630
	Vitamin B_6 (μg)	150
	Biotin (μg)	3
	Folic acid (μg)	30
Amino Acids	Arginine (mg)	570
	Histidine (mg)	170
	Isoleucine (mg)	340
	Leucine (mg)	660
	Lysine (mg)	290
	Methionene (mg)	170
	Phenylalanine (mg)	390
	Threonine (mg)	280
	Thryptophan (mg)	90
	Tyrosine (mg)	260
	Valine (mg)	490
Carbohydrates	Sucrose (mg)	150
Lipids	Palmitic acid (mg)	110
	Stearic acid (mg)	12
	Oleic acid (mg)	220
	Linoleic acid (mg)	220
	Linolenic acid (mg)	12

From: Deutsche Forschungsanstalt für Lebensmittelchemie, Garching bei München (ed), Der kleine "Souci-Fachmann-Kraut" Lebensmitteltabelle für die Praxis, WVG, Stuttgart 1991

Black rice has more nutritive values than that of common rice, such as higher protein, total essential amino acids, vitamin B_1 (Ha *et al.*, 1999), and minerals (Fe, Zn, Mn, and P) (Zhang *et al.*, 2004). Black rice is colored because of anthocyanin found in the aleurone layer of the rice grains (Ryu *et al.*, 1998). Esscribano-Bailón *et al.* (2004) concluded that the principal anthocyanin in rice is cyanidin-3-glucoside and the minor proportion is cyanidin-3-gentiobioside, cyanidin-3-rhamnoside, cyanidin-3,5-diglucoside, cyanidin-3-rhamnoglucoside, malvidin-3-galactoside, peonidin-3-glucoside, peonidin-3-hamnoglucoside, and derivatives of delphinidin also have been described. Zhang *et al.* (2006) reported that the four active antioxidant components of the black rice extract were four anthocyanin compounds of malvidin, pelargonidin-3,5-diglucoside, cyanidin-3-glucoside and cyanidin-3,5-diglucoside and concluded that the anthocyanin compounds were the most important substantial foundations for antioxidation while Kaneda *et al.* (2006) indicated that both cyanidin-3-glucoside and cyanidin are the active components involved in the antioxidative activity of black rice bran extracts. The difference active components in each study might be due to the black rice materials and the different methods of extraction and separation used in each study. This high nutritional quality rice has been shown to possess bioactive properties, and rice bran contains high levels of several antioxidant compounds which will mentioned in next topic.

Hom Nil rice and black glutinous rice (Figure 2) are the black rice that consumed in Thailand. Hom Nil rice has been developed by Kasetsart University (อาทิตย์ กุคำอู และคณะ, 2550). It has been derived from the cross hybridization of Hom Mali rice and black rice from China. Hom Nil rice, Khao Hom Nil (ข้าวหอมนิล), or black fragrant rice possesses different aroma characteristics from common rice.

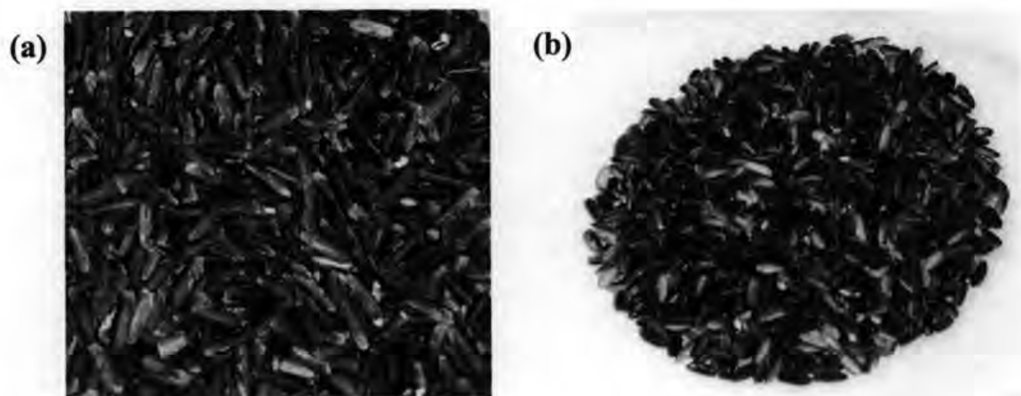


Figure 2 Hom Nil rice (a) and black glutinous rice (b)

It has to be mixed with white rice in cooking for better color and more appetizing. Black glutinous rice or black sticky rice, also called Khao-Kam (ข้าวก่ำ), is the most famous one that generally used as an ingredient in snacks and desserts. There are about 42 varieties of black glutinous rice grown in Thailand. Hom Nil rice and black glutinous rice also have high nutritive values (Table 2), including fiber, iron and anthocyanin, the antioxidant compound.

Table 2 The nutritive values of Hom Nil rice and black glutinous rice

Food composition per 100 g edible portion	Hom Nil rice[a]	Black glutinous rice[b]
Energy (Kcal)	-	361.00
Protein (g)	7.22	8.20
Fat (g)	-	3.00
Carbohydrate (g)	70.00	75.20
Fiber (g)	6.17	4.90
Calcium (mg)	11.93	26.00
Phosphorus (mg)	-	65.00
Iron (mg)	1.15	2.30
Vitamin A (mg)	-	3.00
Vitamin B_1 (mg)	0.41	0.55
Vitamin B_2 (mg)	0.02	0.29
Niacin (mg)	-	0.60
Zinc (mg)	2.90	-
Potassium (mg)	339.40	-
Copper (mg)	0.10	-
α-Tocopherol[c] (mg)	7.47	14.28
γ-Oryzanol[c] (mg)	345.04	357.30
Anthocyanin (mg)	41.17	77.71

[a] from evaluation of nutrition values in colored rice, 2007; [b] from Thai food composition table by Nutrition, Ministry of Public Health, Thailand, 2000; [c] from Lamaimaat Youngsuk, 2008; - = no reported

2.1.1 Rice Bran Oil

About 80% of the lipids of brown rice are in the bran and polish residue. Rice bran oil is the oil extracted from the germ and inner husk of rice. It is popular as cooking oil in several Asian countries. Rice bran oil contains a range of fatty acids, with 40% monounsaturated, 40% polyunsaturated, and 20% saturated fats. The fatty acid composition of rice bran oil is oleic 40.0-50.0%, linoleic 20.0-42.0%, linolenic 0.0-1.0%, palmitic 12.0-18.0%, palmitoleic 10.2-0.6%, stearic 1.0-3.0%, arachidic 0.0-1.0% and myristic 0.1-1.0% (Nicolosi *et al*., 1994). Rice bran oil is rich in vitamin E, γ-oryzanol, and phytosterols, which known as the antioxidants and may provide associated health benefits. Most studies of health benefits of rice bran oil indicated that rice bran oil possessed hypolipidemic action (Rukmini and Raghuram, 1991; Lichtenstein *et al*., 1994; Ausman *et al*., 2005; Wilson *et al*., 2007). In cholesterol-fed hamsters, significantly lower plasma and liver cholesterol and triglycerides were observed in those fed the full fat bran diet (Kahlon *et al*., 1992).

Gamma oryzanol (γ-oryzanol) (Figure 3) is an antioxidant that has many health benefits. Gamma oryzanol has immune system improvement efficiency (Teltathum *et al*., 2004) and might help prevent heart attacks (Cicero and Gaddi, 2001). After administration of γ-oryzanol, total cholesterol, triglyceride and HDL-cholesterol were improved in patient with hyperlipidemia (Ishihara, 1984). Gamma oryzanol also could reduce serum total cholesterol in rats (Seetharamaiah and Chandrasekhara, 1989; Sugano and Tsuji, 1997).

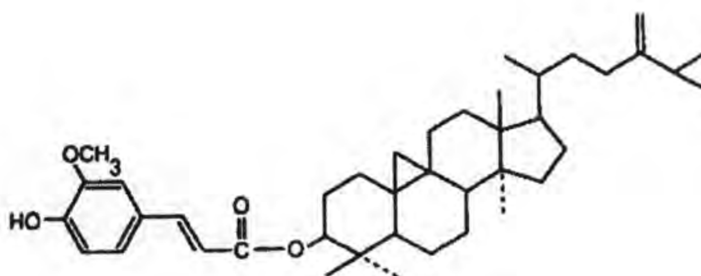
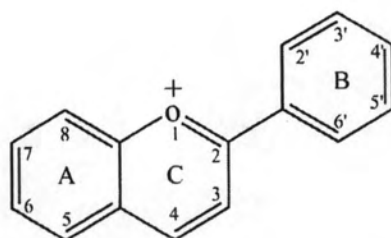


Figure 3 The chemical structure of the oryzanol (24-methylene-cycloartanol ester of ferulic acid)

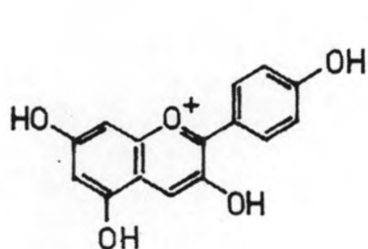
2.1.2 Anthocyanin

Anthocyanin is the colored pigments responsible for the red, purple and blue colors of many fruits, vegetables, flowers, leaves, roots and other storage organisms of plants, including black rice (Markakis, P. 1982). It is found in nature in the form of polyhydroxylated and/or methoxylated heterosides which derive from the flavylium ion or 2-phenylbenzopyrilium (Figure 4). The de-glycosylated or aglycone forms of anthocyanin are known as anthocyanidin and the six most common anthocyanidin skeletons are cyanidin, delphinidin, pelarogonidin, malvidin, petunidin, and peonidin (Figure 4). However cyanidins is considered the widest spread anthocyanin in the plant kingdom (Galvano *et al.*, 2004). Anthocyanidin is found together with one or several sugars and usually conjugated to the anthocyanidin skeleton via the C3 hydroxyl group in ring C, which, in turn, can be acylated with different organic acids. The presence of these hydroxyl groups on the structure makes these compounds quite soluble in water, ethanol, and methanol (Delgado-Vargas *et al.*, 2000). Anthocyanin is stable at low pH which predominant form, the flavylium cation, is red and change into quinonoidal forms with blue color when pH increased. Finally, it will turn into pseudobases and chalcones at high pH with colourless and yellow respectively (Brouillard and Dangles, 1993). Anthocyanin is relatively unstable and easily oxidized by many factors, such as pH, temperature and UV radiation (Markakis, 1982).

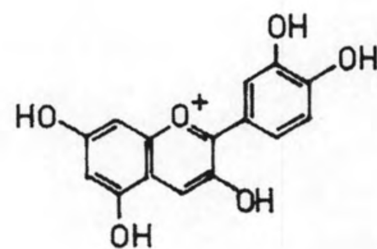
The phenolic structure of anthocyanin is possessed for their antioxidant activity; i.e., ability to scavenge reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), peroxide (ROO^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Wang and Jiao, 2000). The interest in anthocyanin pigments has increased because of the health benefits as dietary antioxidant, protect human RBCs from oxidative stress (Tedesco *et al.*, 2001), antithrombotic properties (Rechner and Kroner, 2005), and anti-inflammation (Park *et al.*, 2007). Anthocyanin has been shown to inhibit the development of cancer in carcinogen-treated animals and in animals with a hereditary predisposition to cancer, such as esophageal cancer (Stoner *et al.*, 2007), colon cancer (LaLa *et al.*, 2006), skin cancer (Afaq *et al.*, 2005), and lung cancer (Ding *et al.*, 2006).



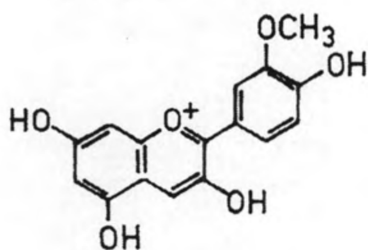
Flavylium ion



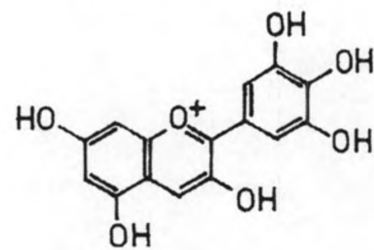
Pelarogonidin



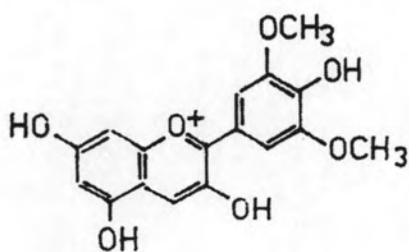
Cyanidin



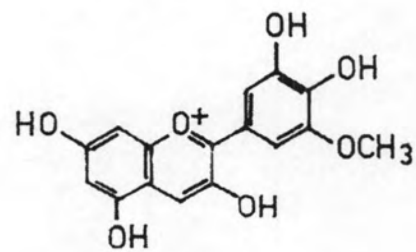
Peonidin



Delphinidin



Malvidin



Petunidin

Figure 4 The chemical structure of the flavylium ion or 2-phenylbenzoryrilium and anthocyanidins

Galvano *et al.* (2004) have been concluded the biological properties of cyanidins and its glycosides as follow:

1. Antimutagenicity in bacterial model (*Salmonella typhimurium*)
2. Inhibition of the epidermal growth-factor receptor (EGFR) of the human vulva carcinoma cell line A431
3. Suppression of the incidence and multiplicity of colorectal adenomas and carcinomas
4. Gastric protective effects
5. Improvement of dark adaptation and transient alteration of vision
6. Decrease in LDL susceptibility to lipid peroxidation
7. Protection against oxidative damage in human erythrocytes
8. Protection against oxidative stress caused by hepatic ischemia/reperfusion (I/R) injury in rats
9. Protection against oxidative stress caused by heart ischemia/reperfusion (I/R) injury in rats
10. Improvement of plasma antioxidant capacity in rats
11. Protection against oxidative damage in red blood cells
12. Protection towards calf thymus DNA against oxidative damage
13. Reduction of DNA cleavage
14. Suppression of the toxicity in macrophage cells by reduction of NO content
15. Prevention of damage induced by UV light in liposome
16. Prevention of inflammation
17. Protection against human ocular diseases

2.1.3 Antioxidant activity

There were several studies that revealed the antioxidant activity and phenolic compounds of the black rice (Ichikawa *et al.*, 2001; Hu *et al.*, 2003; Kong and Lee, 2010). Black rice contains rich anthocyanin compounds and also contains active components such as unsaturated fatty acids, vitamins and microelements which all these substances might have the antioxidation functions and free-radical scavenging effects. Kong and Lee (2009) reported that the black rice bran possessed the antioxidant compounds, including γ-oryzanol, vitamin E homologues, and anthocyanin.

Nam *et al.* (2006) suggested that the pigmented rice extracts possess components acting as electron donors and acted as pro-oxidants in the linoleic peroxidation assay. They also reported that pigmented rice extracts scavenged superoxide anions more effectively than hydroxyl radicals. Zhang *et al.* (2006) proposed the mechanism for antioxidation of anthocyanidin extracted from black rice. On one hand, due to conjugated effect, unpaired electrons in oxygen of anthocyanin were not fixed to oxygen atom, but close to benzene ring, hydroxyl bond was weakened as a result, hydrogen activity of hydroxyl group was increased, and hydrogen could be transferred and proton concerted electron transferred (Zhang and Chen 2000; Zhang and Wang 2004). Otherwise, the antioxidation of anthocyanidin extracted from black rice might relate to multi-hydroxyl substitution structure of the mother nucleus-2-benzopyran. The mechanism should be due to balance phenol and quinone and steady free-radical formation, the main form of dehydrogenation reaction of cyanidin-3-glucoside, for example, is shown in Figure 5. It possessed the stability of phenol and quinone balance formed by polyphenol substance, and it had ortho-hydroxyl group and the number was quite significant. On one hand, it had the capacity of providing hydrogen atom several times; on the other hand, phenoxyl free radical produced by ortho-hydroxyl benzene derivant could form hydrogen bond inside the molecule so that stability could be secured. It could also form a stable compound (quinone) through electron transfer, thus further improving its activity. The radical scavenging activity of anthocyanin is due to the presence of hydroxyl groups in position 3 of ring C and also in the 3′, 4′ and 5′ positions in ring B of the molecule. In general, the radical scavenging activity of the anthocyanidins, aglycones, is superior to their respective anthocyanin and it decreases as the number of sugar moieties increase (Wang and Stoner, 2008).

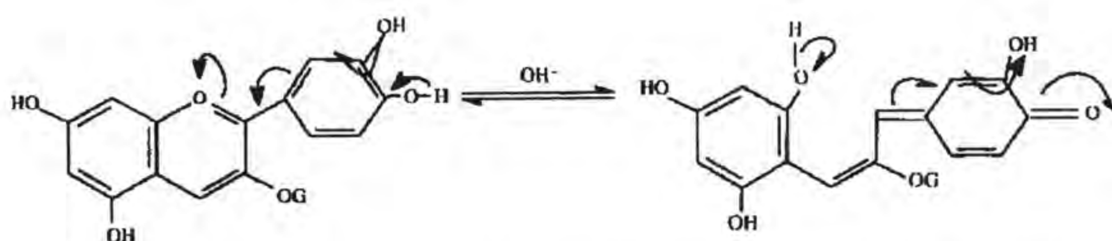


Figure 5 The main form of dehydrogenation reaction of cyanidin-3-glucoside

2.1.4 Biological properties

Black rice possess many biological properties beyond antioxidation property which mostly from anthocyanin. Toyokuni *et al.* (2001) showed the antioxidant effect of black rice against oxidative renal injury caused by ferric nitrilotriacetate, the toxicity of which was due to Fenton-like reaction occurring in the lumina of renal proximal tubules. Ling *et al.* (2001) suggested that black rice consumption reduced the progression of atherosclerotic plaque development induced by dietary cholesterol and black rice possessed the antiatherogenic effect by enhanced serum HDL cholesterol and apo A-I concentrations, and the increased antioxidant and decreased oxidative status. Hu *et al.* (2002) reported that black rice extract significantly suppressed the oxidation of human LDL induced by cupric ions. Hu *et al.* (2003) concluded that black rice and specific anthocyanin components present in black rice contributed to marked antioxidant activities in preventing DNA damage and LDL deterioration *in vitro*, and also suppressed the production of nitric oxide in the activated macrophage without introducing cytotoxicity. Chen *et al.* (2006) indicated that anthocyanin extract from black rice exerted an inhibitory effect of cell invasion on various cancer cells (SKHep-1cells, SCC-4, Huh-7, and HeLa) associated with a reduced expression of matrixmetalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA). Kaneda *et al.* (2006) found that black rice bran extract possessed both strong ROS-scavenging activities and suppressed cell-damaging effects of UVB.

2.2 Gastric Cancer

Gastric cancer is one of the major health concerns. It holds the position of fourth most common cancer and second most common cause of cancer death in the world because of its combination of high incidence and poor survival (Paking *et al.*, 2005; Brenner *et al.*, 2009). Food and nutrition play an important role in prevention and causation of stomach cancer. Recently, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) (WCRF and AICR, 2007) in their extensive report on the scientific literature on diet, physical activity and prevention of cancer, have concluded that stomach cancer is mostly preventable by appropriate diets and associated factors as the following conclusions (Catalano *et al.*, 2009):

1. There was strong evidence that non-starchy vegetables, including specifically allium vegetables, as well as fruits could protect against stomach cancer.
2. There was also strong evidence that salt, and also salt-preserved foods, were causes of this cancer.
3. There was limited evidence suggesting that pulses (legumes), including soya and soya products, and also foods containing selenium could protect against stomach cancer.
4. There was also limited evidence suggesting that chilli, processed meat, smoked foods, and grilled (broiled) and barbecued (char broiled) animal foods were causes of stomach cancer.

The other aetiological factors of gastric cancer might be cigarette smoking (Gonzalez *et al.*, 2003) and familiar gastric cancer. Gastric cancers arise in individuals with a family history of the condition (Barber *et al.*, 2006) and the risk of stomach cancer was increased in first-degree relatives of patients with the disease by approximately two to three fold (Dhillon *et al.*, 2001). There were some evidences showed that meat consumption may correlate with gastric cancer, such as people who consumed large amounts of red meat and processed meat might be at increased risk of developing of non-cardia gastric cancer and the risk was higher if they were also infected with *Helicobacter pylori* (Fuccio *et al.*, 2007). Furthermore, Tsugane and Sasazuki (2007) suggested that infection with *H. pylori* is a strong and established risk factor of gastric cancer but was not a sufficient cause for its development. They also indicated that processed meat and *N*-nitroso compounds might be positively associated with the risk of gastric cancer.

2.3 Dietary Mutagens

2.3.1 Nitrosating and Nitrating Species

Nitrate is a naturally occurring compound that present in high concentrations in green leafy vegetables, beet roots and food components in the Mediterranean diet (green salad and spinach) (Lundberg *et al.*, 2008). Raat *et al.* (2009) described the mechanism of changing of nitrate into nitrite as follow, 1) mouth commensal bacteria are able to convert approximately 20% of salivary nitrate into nitrite, 2) the salivary glands of humans and various other mammals are able to concentrate and secrete plasma nitrate into saliva which this mechanism allows nitrate to come into repetitive

contact with mouth bacteria realizing a more efficient conversion of nitrate to nitrite over time. After that, acidification of nitrite in the stomach produces nitrosative species, which can form potentially carcinogenic *N*-nitroso compounds (Combet *et al.*, 2007) by the reaction of nitrite and nitrogen oxides (NO) with secondary amines and *N*-alkylamides (Walker, 1990; Lijinsky, 1999).

Nitrite is added to meat and fish for curing and it reacts with deoxymyoglobin in the meat to form NO, which inhibits bacterial growth and in particular the growth of *Clostridium botulinum* that produces a potentially fatal toxin (Gladwin, 2004). Therefore processed food sources, that have nitrite as food additives, are the main sources of direct dietary nitrite intake while vegetables are a major source of nitrate providing over 85% of the average daily human dietary intake (Gangolli *et al.*, 1994). Nitrosamines found in foods such as bacon, cured meats, beer, cheese, sausage (Table 3). The safety of nitrite and nitrate has been in considered since it was first reported in the 1970s that carcinogenic *N*-nitroso compounds such as nitrosamines could be formed in meat products preserved with nitrite or nitrate (Hotchkiss, 1988). However, nitrites are not directly mutagenic in higher organisms but they can become dangerous through interactive mutagenicity with other substance (Couch and Friedman, 1975; Zimmermann, 1977).

Table 3 Nitrosamines found in foods (Lijinsky, 1999)

Nitrosamine	Food and highest concentration recorded (μg per kg)
N-Nitroso-	
Dimethylamine	beer (8), bacon (17), cheese (5), cured meats (22), sausage (12), Thai fish (25), smoked pickled fish (32), dried milk (4.5), broiled squid Japan (300), salted meat Russia (54), etc.
Diethylamine	corn bread, seafood China (4.8), sausage (10), cheese (20)
Pyrrolidine	fried bacon (100), sausage China and Germany (45), broiled squid Japan (10), smoked meat (10), ham (36)
Piperidine	bologna, sausage (50), spiced smoked meat (9), Chinese pickle (14), Thai fish (23), Thai pork (6), Tunis stew base (43)
Methylbenzylamine	corn bread China (>100)
Thiazolidine	smoked pork (5), sausage (5), smoked oyster (109), fried bacon (240), cured meat (27), smoked fish (2)
Proline	fried bacon (68), cured meat (400), smoked pork (2100) sausage (940), smoked oyster (167)
Thiazolidine	fried bacon (14000), cured meat (3900)
Carboxylicacid	smoked fish (1600)

Under acidic conditions (e.g. in stomach), nitrite is in nitrous acid (HNO_2) conformation which is readily decomposed to give a range of nitrosating species, including dinitrogen trioxide (N_2O_3) and nitrosonium ion (NO^+). A reaction of two HNO_2 molecules will produce dinitrogen trioxide (N_2O_3) which is also a potent nitrosating agent (Lu *et al.*, 2007). *N*-nitrosamines arise from the reaction of nitrite sources with amines, amides, and other proteins (R_2NH), such as primary, secondary and tertiary amines. Typically this reaction proceeds via the attack of the nitrosonium ion on an amine: $R_2NH + NO^+ \rightarrow R_2N\text{-}NO + H^+$. Figure 6 shows the reaction of *N*-nitrosamines. Aniline, the aromatic amine, reacted with nitrosonium ion to form *N*-phenyl-nitrosamine. *N*-nitrosamines formation can occur in acid conditions like in the human stomach (Xu and Reed, 1993).

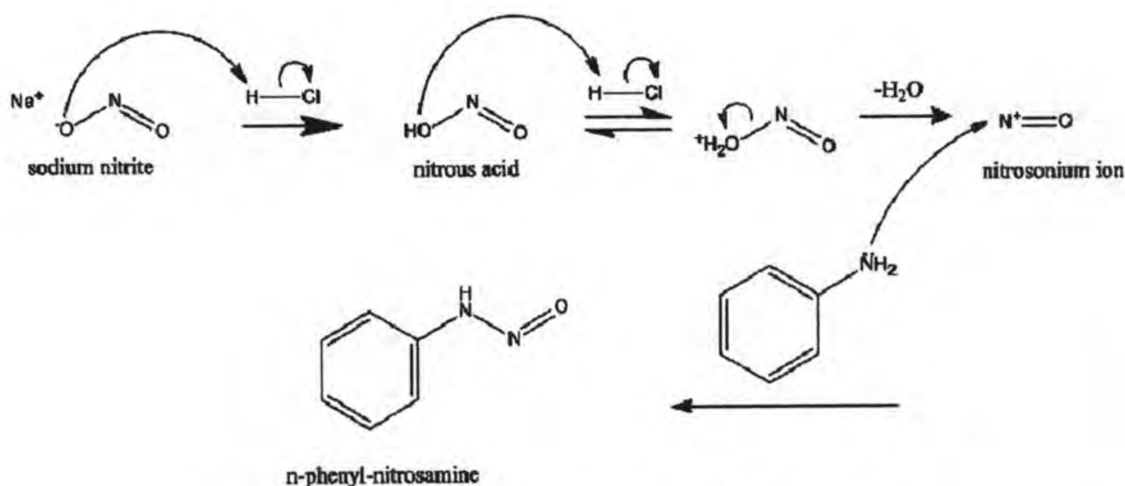


Figure 6 Formation of *N*-nitrosamines (Wikipedia, 2009)

Many studies have shown that a number of *N*-nitrosamines are carcinogenic in animal species, with a wide range of potencies, although the evidence on whether dietary *N*-nitrosamines cause cancer in humans has long been considered unclear. For example, IARC concluded that nitrite in food is associated with an increasing of the incidence of gastric cancer but classified nitrite in foods as having limited evidence of carcinogenicity to humans (Grosse *et al.*, 2006). The available evidence supports a positive association between nitrite and nitrosamine intake and gastric cancer, between meat and processed meat intake and gastric cancer, and between preserved fish, vegetable and smoked food intake and gastric cancer, but is not conclusive (Jakszyn and Gonzalez, 2006). Palli (1996) indicated that salted/smoked and pickled/preserved food, which is rich in salt, nitrites and preformed nitroso

compounds, were associated with an increased risk of gastric cancer. Nitrites and nitrates are permitted for use as preservatives for meat products, certain cheeses and pickled fish in the EU but there have been repeated recommendations to use the minimum amount of nitrate/nitrite necessary to ensure microbiological safety accordingly controlled by EU legislation (Barlow and Schlatter, 2009).

2.3.2 Nitrite as a Converter for Direct Mutagen

For many years, there has been interest in nitrite as a potential pre-carcinogen for gastric cancer. Several investigators suggested that direct-acting mutagens formed from nitrite and the precursors of mutagens in the acid condition of stomach were possible candidates for the causation of human gastric cancer (Marquardt *et al.*, 1977; Piacek-Llanes and Tannenbaum, 1982; Wakabayashi *et al.*, 1984). For instance, various foodstuffs have mutagenic activity when treated with nitrite. Some vegetables were found to be mutagenic upon nitrite treatment in acid condition which determined using the *Salmonella typhimurium* assay (Wakabayashi *et al.*, 1984; Wakabayashi *et al.*, 1985; Tiedink *et al.*, 1988). Smoked fish and meat products, a frequent consumed food item that was associated with increased risk of gastric cancer, were shown to produce potent direct-acting genotoxic activity when treated with nitrite under acidic conditions by *in vitro* study (Ohshima *et al.*, 1989). The products formed by the reaction of beef extract with nitrite were mutagenic toward *Salmonella typhimurium* strain TA1538, TA98 and TA100 (Munzner and Wever, 1984). The polycyclic aromatic hydrocarbons (PAHs) containing fractions of smoked and charcoal-broiled foods, i.e., Sheat fish, Mimrow, Freshwater catfish, chicken wings, rice pork sausage and pork, in addition to naphthalene, acenaphthene, anthracene, phenanthrene, naphthacene, benz[*a*]anthracene, dibenz[*ah*]anthracene, 10-dimethyl-1,2-benzanthracene, pyrene, benzo[*a*]pyrene, benzo[*e*]pyrene and coronene showed mutagenicity to *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation after being treated with nitrite at pH 3.0-3.5 and 37°C (Kangsadalampai *et al.*, 1996). Broiled chicken, pork, mutton, beef and sun-dried sardine were found to yield direct-acting mutagenicity after treated with 50 mM sodium nitrite at pH 3.0 for 1 h at 37 °C on *Salmonella typhimurium* strains TA98 and TA100 (Yano *et al.*, 1988) (Table 4).

Table 4 Mutagenicity of cooked meats after nitrite treatment (Yano *et al.*, 1988)

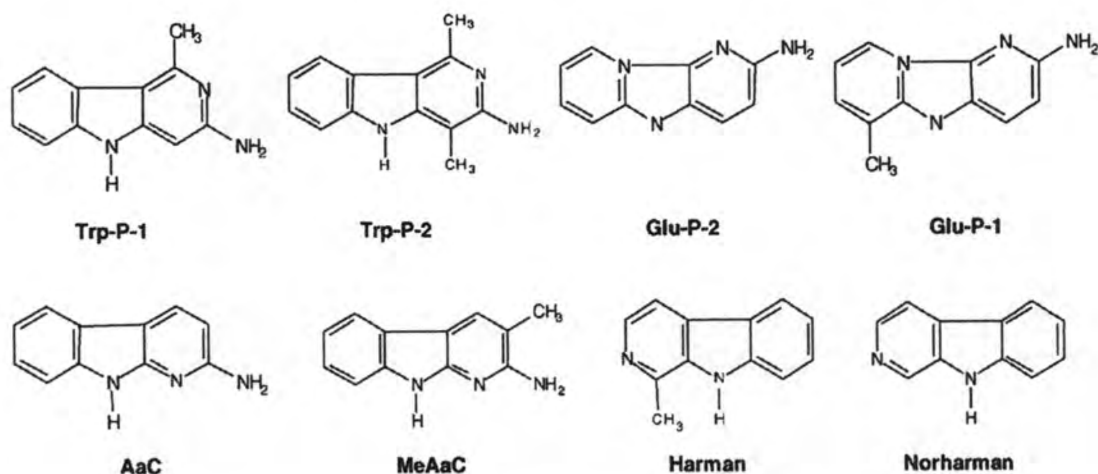
Food	Revertants/g original material	
	TA98	TA100
Chicken	33,300	12,800
Beef	22,600	7,400
Mutton	43,600	5,700
Pork	15,000	3,800
Sun-dried sardine	20,200	17,900

2.4 Mutagenicity Models for Antimutagenicity

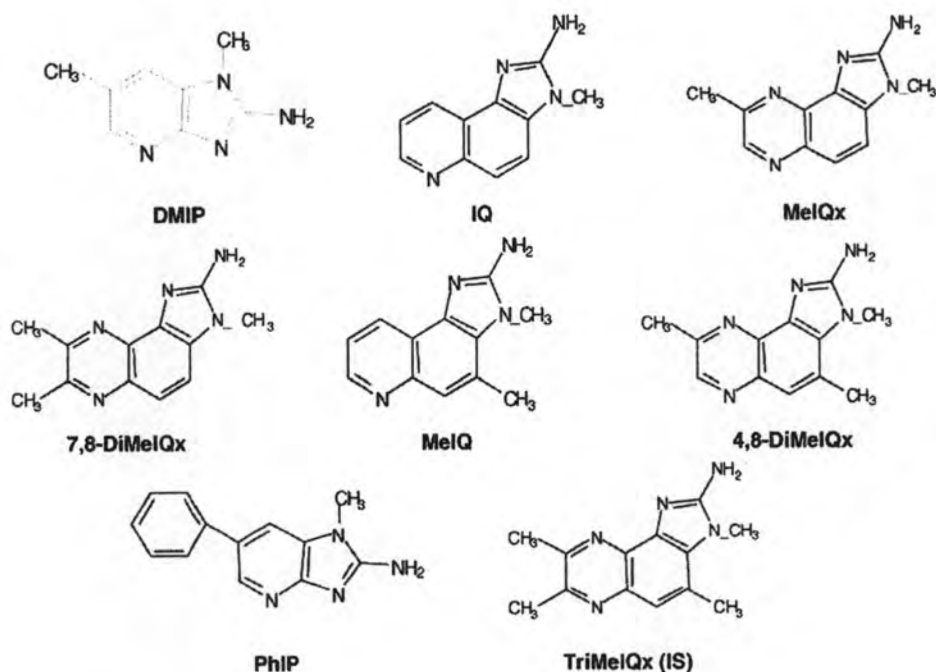
Meat is a primary source of protein, providing all essential amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine and valine), as well as good amounts of various micronutrients (National Health and Medical Research Council, 2006). Commonly eaten meat products prepared from beef, pork, mutton, fish and chicken all show some levels of mutagenic activity after cooking. Both heterocyclic amines (HAs; Layton *et al.*, 1995) and polycyclic aromatic hydrocarbons (PAHs; Phillips, 1999) are formed during the high-temperature cooking of meat and fish.

Heterocyclic amines are potent mutagens formed during heat-processing of proteinaceous food. HAs can be divided by their chemical structure into five groups. Pyrolytic mutagens (group I), aminocarbolines, mean high-temperature mutagens (Figures 7) whereas thermic mutagens (group II-V), aminoimidazoazaarenes, are mutagens formed at temperatures below 300°C (Figures 8). Pyrido-imidazoles or -indoles (group I) are formed at temperatures which too high for normal domestic cooking temperatures. A number of heterocyclic amine mutagens have been identified by pyrolysing single amino acids, tryptophan, glutamic acid, phenylalanine, lysine or ornithine (Felton and Knize, 1990). For example, 3-amino-l,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-l-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 2-amino-5-phenyipyridine (Phe-P-1) have been identified in broiled sardine (Yamaizumi *et al.*, 1980) and 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole in broiled cuttlefish (Yamaguchi *et al.*, 1980a). In addition, Trp-P-1 has been isolated from fried beef (Yamaguchi *et al.*, 1980b) and Trp-P-2 in beef extract (Taylor *et al.*, 1985).

Two α-carbolines, 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), were isolated from soya bean globulin pyrolysates (Yoshida *et al.*, 1978). AαC and MeAαC have been found in grilled beef, chicken, mushroom (Matsumoto *et al.*, 1981) and also in grilled fish (Gross and Grüter, 1992), while 9*H*-pyrido-[3,4-*b*]indole (norharman) and 1-methyl-9*H*-pyrido-[4,3-*b*]indole (harman) are formed when proteinous food is heat-prepared (Pfau and Skog, 2004; Khan *et al.*, 2009). Quinolines (group II), the derivatives of imidazoquinolines, are commonly called IQ compounds. IQ compounds have been found in many cooked meat such as beef, pork, and fish. However, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) has also been identified in model systems using creatin(in)e (denotes creatine or creatinine) and any of the amino acids proline, phenylalanine or serine and also using creatinine, glycine and fructose or a combination of creatin(in)e, phenylalanine and glucose, while 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) has also been produced in model systems from creatine, fructose and alanine (Skog, 1993). Quinoxalines (group III), the derivatives of imidazoquinoxalines, are commonly called IQx compounds. IQx compounds have been found in many cooked meat such as beef, pork, mutton, chicken and fish. 2-amino-3-methylimi-dazo[4,5-*f*]quinoxaline (IQx) has been isolated from model systems heated at 180°C, containing creatinine, glucose and either glycine or threonine (Skog and Jägerstad, 1993), while 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was isolated from model systems with creatin(in)e, and either glycine, alanine, lysine, phenylalanine or threonine in combination with glucose, fructose or ribose (Skog, 1993) and also been produced from creatin(in)e and either serine, alanine or threonine heated without sugar (Övervik *et al.*, 1989). 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) are found in chicken meat (Bermudo *et al.*, 2005). Pyridines (group IV), the imidazopyridines, has been found in many cooked beef, pork, mutton, chicken and fish, especially in fried and broiled meat. Model systems using creatin(in)e, phenylalanine or leucine, with or without glucose, have produced 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Övervik *et al.*, 1989; Skog and Jägerstad, 1991). Furopyridines (group V) has been found in fried ground beef and pork. Methylimidazofuropyridine (MeIFP) has been isolated from fried ground beef with added milk and creatinine (Skog, 1993).



Figures 7 Chemical structures of pyrolytic mutagens (aminocarbolines)



Figures 8 Chemical structures of thermic mutagens (aminoimidazoazaarenes)

The PAHs enter the environment from a variety of sources, including diesel and gasoline engine emissions, cigarette smoke condensate, carbon blacks, wood burning stoves and coalfired power plant emissions (Hites *et a*l., 1980; Jacob *et al.*, 1986; Tokiwa and Ohnishi, 1986). PAHs become a constituent of foodstuffs by formation during cooking or processing, by contamination from PAHs-containing smoke typically associated with cooking, or by introduction through contact with other sources of environmental PAHs, such as water or soil. PAHs on the surface of

หอสมุดกลาง สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

well-done charcoal-broiled steaks apparently formed through pyrolysis of fat dripping into flames and being absorbed by the food (Lijinsky and Shubik, 1964). For example, 1) in meat products, PAHs were typically found at low levels but in wood smoked and grilled meat products much higher levels could be found, such as in a sausage product, 2) in non-smoked fish products, PAHs were low but much higher in smoking fish, 3) in vegetables, PAHs were found at low levels but the levels were higher in vegetables grown near highways than those grown in isolation (Benford *et al.*, 2010). The chemical structures of some PAHs are shown in Figure 9. Benzo[a]pyrene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, dibenzo [a,l]pyrene, cyclopenta[cd]pyrene, indeno[1,2,3-cd]pyrene, benzo[ghi]pyrene, benz[a] anthracene, dibenz[a,h]anthracene, benzo[b]fluoranthene, benzo[j]fluoran-thene, benzo[k]fluoranthene, chrysene and 5-methylchrysene have shown clear genotoxicity in standard assays *in vitro* and *in vivo*. Anthracene, benzo[a]fluorene, naphthalene and pyrene seem to lack genotoxicity activity. The data for the remaining PAHs were limited or equivocal (FAO/WHO, 2006). Many nitro-PAHs are mutagenic in bacteria (Campbell *et al.*, 1981; Mermelstein *et al.*, 1981; Ohgaki *et al.*, 1982; El-Bayoumy and Hecht 1983; Howard *et al.*, 1983b; Ball *et al.*, 1984; King *et al.*, 1984; Heflich *et al.*, 1985), genotoxic in prokaryotic and eukaryotic cell lines (Campbell *et al.*, 1981; DiPaolo *et al.*, 1983; Howard *et al.*, 1983b; Rosenkranz and Mermelstein 1983; King *et al.*, 1984) and carcinogenic in experimental animals (Hirose *et al.*, 1982; Ohgaki *et al.*, 1982; El-Bayoumy *et al.*, 1984; Stanton *et al.*, 1985).

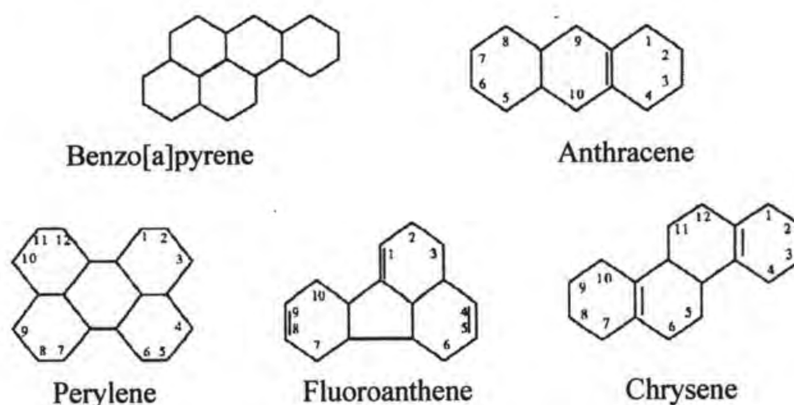


Figure 9 Chemical structures of PAHs

2.4.1 Nitrite-Treated Chicken Extract Model

Cooking procedures such as broiling, frying, barbecuing, heat processing and pyrolysis of protein-rich foods, such as chicken, induce the formation of potent mutagenic and carcinogenic compounds called HAs which appears to be influenced primarily by cooking temperature and time (Felton and Knize, 1991). Bjeldanes *et al.* (1982) reported that white chicken meat formed more mutagen than red chicken meat cooked under the same condition. HAs that might be found in cooked chicken are AαC, MeAαC, MeIQ, MeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), PhIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 9*H*-pyrido-[3,4-*b*]indole (norharman), 1-methyl-9*H*-pyrido-[4,3-*b*]indole (harman) (Matsumoto *et al.*, 1981; Skog, 1993; Brockstedt and Pfau, 1998; Chen and Yang, 1998; Chiu *et al.*, 1998; Pais *et al.*, 1999; Totsuka *et al.*, 1999; Bång *et al.*, 2002; Solyakov and Skog, 2002; Bermudo *et al.*, 2005).

The mutagenic activity in *Salmonella typhimurium* has been reported in cooked chicken (Felton and Knize, 1991). The nitrosated products of chicken extracts were speculated to contain direct mutagens such as nitro compounds. IQ was converted to 3-methyl-2-nitroimidazo[4,5-*f*]quinoline after sodium nitrite treatment at pH 3.0 showed mutagenicity toward *Salmonella typhimurium* strains TA98 and TA100 without metabolic activation (Kangsadalampai and Peerawong, 1997). Sasagawa *et al.* (1988) also reported that IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP were converted into the direct acting mutagenic products towards *Salmonella typhimurium* strains TA98 and TA100 without S9 mix after treated with sufficient concentration of nitrite. They also suggested that a new-type mutagen formed by treatment of IQ with nitrite was isolated and identified as 2-nitro-3-methyl-imidazo[4,5-*f*]quinoline (NO_2-IQ). Lin *et al.* (1992) demonstrated that the food mutagens IQ and MeIQ treated with nitrite at pH 3.0 for 1 h at 37°C were converted to extremely strong mutagenic derivatives in both the presence and the absence of rat liver S9 mix towards *Salmonella typhimurium* TA97, TA98, TA100 and TA1535. Thus, the mutagenicity of nitrite-treated chicken extract in acid condition has been chosen as a model for antimutagenicity evaluation in this study.

2.4.2 Nitrite-Treated 1-Aminopyrene Model

1-Aminopyrene is a derivative of 1-nitropyrene in human gastrointestinal tract which human intestinal microflora reduced 1-nitropyrene to 1-aminopyrene (El-Bayoumy *et al.*, 1983; Howard *et al.*, 1983a; Cerniglia *et al.*, 1984). 1-Nitropyrene, a prevalent environmental pollutant, is the most studied of the nitro-PAHs. Nitro-PAHs formed via reactions of nitrogen oxides with PAHs as a result of incomplete combustion or pyrolysis of fat in meat produced pyrene and nitrogen oxides from burning of cooking gas (Honda *et al.*, 1983; Rosenkranz and Mermelstein, 1983; Tokiwa *et al.*, 1985; Kinouchi *et al.*, 1986; Edenharder *et al.*, 1993).

1-Aminopyrene, non-mutagenic PAHs derivative, was known to be mutagenic or carcinogenic after metabolic activation (Kinouchi *et al.*, 1986) and found to be transformed into direct-acting mutagen when treated with sodium nitrite in acid condition. Kato *et al.* (1991) demonstrated that 1-aminopyrene treated with sodium nitrite at pH 3.0 and 37°C for 4 hours showed mutagenicity towards *Salmonella typhimurium* strains TA98 (frameshift mutation) and TA100 (base-pair substitution mutation) without metabolic activation. Kangsadalampai *et al.* (1996) also showed that treatment of 1-aminopyrene with sodium nitrite for 4 hours at 37°C and in acid solution pH 3.0-3.5 exhibited strong mutagenicity on *Salmonella typhimurium* both strains TA98 and TA100 without metabolic activation. The mutation might be due to presence of nitroreductase (IARC, 1989a) and *O*-acetyltransferase (Mermelstein *et al.*, 1981), the activating systems, presented in bacterial cells for nitrite treated 1-aminopyrene (supposed to be 1-nitropyrene). 1-Nitropyrene metabolized by those two enzymes to be arylhydroxylamine which can interact with DNA. There has been a special emphasis on investigations concerning the mammalian metabolic activation, genotoxicity and carcinogenicity of 1-nitropyrene (Hirose *et al.*, 1982; Ohgaki *et al.*, 1982; El-Bayoumy and Hecht 1983, 1984; Ball *et al.*, 1984; Kinouchi *et al.*, 1987). Thus, the mutagenicity of nitrite-treated 1-aminopyrene in acid condition has been established as a model for antimutagenicity study of some substances concerning the phenomenon occurred during gastric digestion.

2.5 Ames Test

There are various methods for detecting carcinogens and mutagens. One of the popular methods is *Salmonella typhimurium*/microsome assay, also known as *Salmonella* test or Ames test. As cancer is often linked to DNA damage, the test also serves as a quick assay to estimate the carcinogenic potential of a compound since it is difficult to determine whether standard carcinogen assays on rodents were successful (McCann *et al.*, 1975a; Sugimura *et al.*, 1976; Zeiger, 1985; Zeiger *et al.*, 1990). The procedure is described in a series of papers from the early 1970s by Bruce Ames and his group at the University of California, Berkeley.

Ames test is the test used to determine the mutagenic potential of a substance that leads to gene mutations based on the mutation rate of bacteria that are exposed to the substance. The test uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis, so that they require histidine for growth and form colonies. The studies performed to identify and map the genes responsible for histidine biosynthesis produced a large number of spontaneous, radiation and chemical-induced histidine mutants of *Salmonella typhimurium* LT-2 (Whitfield *et al.*, 1966; Hartman *et al.*, 1971). Some mutants contained additions or deletions of one or more bases (frameshift mutants), and others contained single base changes (base-pair substitution mutants). The variable being tested is the mutagen's ability to cause a reversion that can restore the gene's function and allow cell to growth on a histidine-free medium. Thus, the test can refer to as a "reversion assay". The tester strains are specially constructed to have different mutations in various genes required to synthesize histidine, which allows for the detection of mutagens acting via different mechanisms. Some compounds are quite specific, causing reversions in just one or two strains (Chapla *et al.*, 1972).

2.5.1 Development of the Plate Incorporation

In 1966, Ames and Whitfield proposed the histidine mutant strains for screening chemicals for mutagens using a spot test procedure (Ames and Whitfield, 1966). The spot test consists of applying a small amount of the test chemical directly to the center of a selective agar medium plate seeded with the test organism. As the chemical diffuses into the agar, a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied. In 1973, Ames *et al.* developed the plate incorporation assay procedure that is more quantitative and sensitive than the spot test (Ames *et al*, 1973 a and b). The procedure consists of adding the buffer or S-9 mix, the histidine dependent bacteria and test chemical to 2 ml top agar containing biotin and a trace amount of histidine (0.05 mM each). When histidine dependent bacteria are grown on glucose minimal agar plate containing a trace amount of histidine, only the cells that revert to histidine independence are able to form colonies. The number of spontaneously induced revertant colonies is relatively constant for each strain.

2.5.2 Metabolic Activation Systems

Some carcinogenic substances, such as aromatic amines or PAHs, are not mutagenic themselves but their metabolic products are. The cytochrome P450 metabolic oxidation system, which is present mainly in the liver and to a lesser extent in the lung and kidneys of human and lower animals, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophillic forms. Some of the intermediate metabolites are potent mutagens in the Ames test. Because bacteria are unable to metabolize chemicals via cytochrome P450, as in mammals and other vertebrates, rat liver extract is optionally added to simulate the effect of metabolism (Smith, 1966; Malling, 1971; Miller and Miller, 1971; Garner *et al.*, 1972; Ames *et al.*, 1973a). The metabolic activation system usually consists of a 9,000 g supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactors for NADPH-support oxidation (S-9 mix) (Maron and Ames, 1983). In addition, the animals are pretreated with the mixed-function oxidase inducer Aroclor, phenobarbital or b-naphthoflavone to increase the level of metabolizing enzyme.

2.5.3 The Salmonella Tester Strains

The genotypes of the commonly used *Salmonella typhimurium* tester strains are show in Table 5. All strains are histidine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations that have made the tester strains more sensitive to chemical mutagens are:

Table 5 Genotype of the most commonly used Salmonella tester strains

Mutation (strain)	*bio chlD uvrB gal*	LPS defect	Plasmid	Mutational event
hisG46				
TA1535	Deletion mutation	*rfa*	No plasmid	base-pair substitution
TA100	Deletion mutation	*rfa*	pKM101	base-pair substitution
hisD3052				
TA1538	Deletion mutation	*rfa*	No plasmid	frameshift
TA98	Deletion mutation	*rfa*	pKM101	frameshift
hisC3076				
TA1537	Deletion mutation	*rfa*	No plasmid	frameshift
hisD6610				
hisO1242				
TA97	Deletion mutation	*rfa*	pKM101	frameshift
hisG428				
TA104	Deletion mutation	*rfa*	No plasmid	base-pair substitution
TA102	Wild type	*rfa*	pKM101	base-pair substitution

Modified from Maron and Ames, 1983

1. A deletion mutation via the *uvrB-bio* genes in all strains, except TA102. The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by the error-prone DNA repair mechanism. However, the deletion via the biotin gene makes the bacteria biotin dependent (Ames *et al.*, 1973 a).

2. A mutation (*rfa*) in all strains leads to a defective lipolysaccharide (LPS) layer that coats the bacterial surface. It's making the bacteria more permeable to bulky substances (Ames *et al.*, 1973 a).

3. Introduction of plasmid pKM101 in strains TA1535 and TA1538 resulting in the corresponding isogenic strains TA100 and TA98 (Ames *et al.*, 1975). Plasmid pKM101 enhances chemicals and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway (McCann *et al.*, 1975b; Walker and Dobson, 1979; Shanabruch and Walker, 1980). The plasmid confers ampicillin resistance which is a convenient marker to detect the presence of the plasmid.

4. Insertion of mutation *hisG428* on the multicopy plasmid pAQ1 which was introduced in strain TA102 for amplifying the number of target sites. To enhance the ability of this strain to detect DNA cross-link agents, the *uvrB* gene was retained making the bacterium DNA repair proficient (Levin *et al.*, 1982).

2.5.4 Testing of Chemicals in a reduced oxygen atmosphere

Anaerobic environments, such as anaerobic chambers, have been used to study mutagenicity of chemicals and fecal samples under reduced oxygen levels (Bruyninckx *et al.*, 1978; Venitt and Bosworth, 1983; Hartman *et al.*, 1984; MacPhee and Jolly, 1985; Venitt and Bosworth, 1988; Mortelmans and Cox, 1992). Highly reduced levels of oxygen may lead to a reduction in both spontaneous and induced revertant colonies and may interfere with the interpretation of the results. A complete absence of revertant colonies is observed when a strict anaerobic environment and reproduced and an aerobic sterilized media are used (Mortelmans and Cox, 1992).

2.5.5 Validation Studies

Many validation studies have been performed to determine the reproducibility of test results on an intra- and inter-laboratory level (Purchase *et al.*, 1978; Venitt and Crofton-Sleigh, 1981; Dunkel *et al.*, 1984; Margolin *et al.*, 1984; Dunkel *et al.*, 1985; Zeiger, 1985; Piegorsch and Zeiger, 1991). Furthermore, many studies have been performed to determine the sensitivity and correlation of the Ames test with animal carcinogenicity studies, which has been shown that Ames test is a high predictive for positive mutagenic response on rodent carcinogenicity (Tennant *et al.*, 1987; McCann *et al.*, 1975a; Ames and MaCann, 1976; Sugimura *et al.*, 1976; Zeiger, 1997). Therefore, the test is useful as a first screen to determine the mutagenic potential of the new chemicals and drugs.

2.5.6 Spontaneous Control Values

Each tester strain has characteristic spontaneous mutant frequency, which is usually some day-to-day and laboratory-to-laboratory variation in the number of the spontaneous revertant colonies. The solvent that used in each test may also affect the spontaneous mutant frequency (Maron *et al.*, 1981). The range of spontaneous histidine revertant (negative solvent) control values per plate with and without metabolic activation is show in Table 6.

Table 6 Spontaneous revertant control value per plate

Strain	Number of revertants	
	Without S-9	**With S-9**
TA97	75-200	100-200
TA98	20-50	20-50
TA100	75-200	75-200
TA102	100-300	200-400
TA104	200-300	300-400
TA1535	5-20	5-20
TA1537	5-20	5-20
TA1538	5-20	5-20

Modified from Ames *et al.*, 1975; Maron and Ames, 1983; Kier *et al.*, 1986

2.5.7 Experimental Design

Assay procedure The choice of assay for liquid chemicals is the pre-incubation assay because of these chemicals may be volatile. In addition, some researchers believe that the pre-incubation assay is more selective in detecting the mutagens than the plate incorporation assay because the short-lived mutagenic metabolites have a better chance to react with the tester strains in the small volume of the preincubation mixture. For general screening purpose the laboratory should adopt one testing procedure that will help in developing historical database for the negative control and also the solvent and positive control chemicals. The modified (Kado *et al.*, 1983) *Salmonella* microsuspension assay is recommended for testing samples that are available in only small amounts (i.e. mg) or complex mixture such as urine.

The reductive metabolism assay is recommended for chemicals that require reduction for their activation, such as azo dyes, while the desiccator assays are recommended for volatile liquids and for gaseous substances.

The pre-incubation assay The tester strains are exposed to the chemical for a short period (20 to 30 minutes) in a small volume (0.5 ml) of either S-9mix or buffer, before plating on glucose agar minimal medium supplemented with a trace amount of histidine. The other advantage of the pre-incubation assay is the effective concentration of S-9mix in the pre-incubation volume is higher than in the plate incorporation assay (Zeiger *et al.*, 1987 and 1988).

The Kado microsuspension assay for testing small sample volumes This procedure was used to detect mutagenic metabolites in urine samples that obtained from test chemicals-treated animals (Kado *et al.*, 1983). The overnight cultures are centrifuged to obtain a ten-fold higher than normal density for use in the pre-incubation assay. The exposure of a higher number to the urine samples is believed to enhance the detection of mutagenic metabolites in them. Thus, this procedure is suitable for testing small quantities (about 20 mg) of samples where the sample quantities are limited, and for testing complex mixtures other than urine samples.

Solvents The solvent of choice is sterile distilled water, but the chemicals that cannot dissolve in water should be dissolved in dimethyl sulfoxide (DMSO). Other solvents that may be used are acetone, ethyl alcohol (95%), tetrahydrofuran, dimethylformamide and methyl ethyl ketone, which high concentrations of these solvents may be toxic to the bacteria. Therefore, these solvents should be determined in preliminary toxicity assay to find the maximum concentration that can be used without interfere the growth and survival of bacteria.

Positive control chemicals Diagnostic mutagens, positive control chemicals, must be included in each test to confirm the specificity and reversion properties of each tester strain, and efficacy of the metabolic activation system. The list of positive control chemicals with their respective concentration per plate that have been routinely used in the laboratories is showed in Table 7. The positive control of choice in each experimental is the chemicals that are member of a specific chemical class, chemical with a similar structure, or require similar metabolism.

Table 7 Positive control chemicals with concentration per plate

Strain	Control chemical (mg/plate)[a]	
	Without activation	With activation
TA97	9-Aminoacridine (50)	2-Aminoanthracene (1-5)
TA98	4-Nitro-o-phenylenediamine (2.5)	2-Aminoanthracene (1-5)
TA100	Sodium azide (5)	2-Aminoanthracene (1-5)
TA102	Mitomycin C (0.5)	2-Aminoanthracene (5-10)
TA104	Methyl methane sulfonate (250)	2-Aminoanthracene (5-10)
TA1535	Sodium azide (5)	2-Aminoanthracene (2-10)
TA1537	9-Aminoacridine (50)	2-Aminoanthracene (2-10)
TA1538	4-Nitro-o-phenylenediamine (2.5)	2-Aminoanthracene (2-10)

Modified from Ames *et al.*, 1975; Maron and Ames, 1983; Kier *et al.*, 1986

[a] Concentration based on 100 × 15-mm petri plate containing 20-25 ml of glucose minimal agar.

Data reporting The results are generally reported as mean revertant colonies per plate, the S.D. or S.E.M. for the test chemical and each control. The concentration of the chemical is presented as mg or mg per plate. Information regarding toxicity and/or precipitation of the test chemical should be included. The result can also be expressed as the fold-increase/decrease from the solvent control value.

Data evaluation There is a non-statistical procedure used to evaluate the results of *Salmonella* experiments (Zeiger *et al.*, 1992) which has the criteria to interpret the results as follow:

1. *Positive*: A compound is considered a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more stains. A compound is considered a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains but the number of revertant colonies is not double the background number of colonies.

2. *Negative*: A compound is considered a nonmutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments. For toxic compounds, ideally only the highest dose level should exhibit evidence of toxicity (e.g. thinning or complete absence of background lawn with or without the presence of pinpoint colonies.

3. *Inconclusive*: If a compound cannot be identified clearly as a mutagen or non mutagen, the results are classified as inconclusive (e.g. if there is a scattering of slightly elevated revertant counts or one elevated count). Regardless of whether a statistical or non-statistical approach is used for data evaluation, it should be adequately described and consistent across strains and activation procedures.

Response that are judged to be weakly positive or equivocal should be considered for additional testing, using a modification of the exposure protocol, or a change in the dose range tested or S-9 mix concentration used. Because control ranges vary among laboratories, each laboratory should establish its own solvent and positive control ranges for each tester strain, with and without S-9 mix. This range should be used to develop acceptance criteria for individual experiments, and experiments should be rejected if solvent controls are outside the acceptable range, or if positive control chemicals do not respond appropriately.

2.6 Antioxidation Assays

Antioxidants are considered important substances for many health benefits (Droge, 2002; Lee *et al*., 2004; Valko *et al*., 2007). The requirement of a standard assay is very important in order to compare the results of the study. Therefore many methods for determination of antioxidant activity in food samples have been proposed during the past decade. Both *in vivo* and *in vitro* assays were used which each method also has its own advantage and limitation.

2.6.1 DPPH Assay for Free Radical Scavenging Activity

The determination of free radical scavenging activity mainly based on reaction between a chromogen compound and an antioxidant. After reaction, the residual concentration of chromogen compound is determined spectrophotometrically or color-imetrically (Miller *et al*., 1993; Rice-Evans and Miller, 1994). The most often used chromogen compound is DPPH because they react directly and rapidly with antioxidant compounds (Sharma and Bhat, 2009). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), also known as 2,2-diphenyl-1-picrylhydrazyl or R,R-diphenyl-*â*-picrylhydrazyl, is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund *et al*., 2005). DPPH was reacted with Trolox, a water-soluble vitamin E analogue, and the results determined spectrophotometrically at 515 nm. Thus, the degree of discoloration of the solution indicates the scavenging

efficiency of the added substance compared with respect to that of Trolox (Pietta *et al.*, 1998; Milardovic *et al.*, 2006).

2.6.2 Ferric Reducing Antioxidation Power (FRAP) Assay

The FRAP assay is the most widely used to measures the total antioxidant capacity of the sample, because of its simple procedure, inexpensive reagents and equipment and a speedy reaction applicable over a wide concentration range (Prior and Cao, 1999; Nilsson *et al.*, 2005). In the presence of the chromogenic ligand like tripyridyltriazine (TPTZ) that is rather selective for ferrous (Fe^{2+}) and ferric (Fe^{3+}) acts as an oxidant toward the antioxidants in the sample. At low pH, when a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form, an intense blue color with an absorption maximum at 600 nm. The reaction is nonspecific, and any half-reaction which as a less-positive redox potential, under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half-reaction will drive Fe^{3+}-TPTZ reduction. Test conditions favor reduction of the complex and, thereby, color development, provided that the antioxidant is present. In the FRAP assay, excess Fe^{3+} is used, and the rate-limiting factor of Fe^{2+}-TPTZ, and hence color, formation is the reducing ability of the sample (Benzie and Strain, 1996).

2.6.3 Total Phenolic Content Assay

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups, and generally are categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Liu, 2004). Phenolics compounds are mainly exist as glycosides linked to various sugar moieties or as other complexes linked to organic acids, amines, lipids, carbohydrates, and other phenols. Total polyphenols content were assayed using the Folin–Ciocalteau method as described previously with slightly modification (Amarowicz *et al.*, 2005; Georgé *et al.*, 2005). The level of total phenolic compounds determined according to the Folin–Ciocalteu method are not absolute measurements of the amounts of phenolic compounds but are in fact based on their chemical reducing capacity, relative to an equivalent reducing capacity of gallic acid (Katalinić *et al.*, 2004).