CHAPTER II

LITERATURE REVIEW

2.1 The Role of Diet on Human Cancer

Epidemiologically, it has been clearly demonstrated that life style, especially foods eaten daily, greatly modulates the frequency of human cancers. This finding suggests that environmental carcinogens, especially those in the ordinary diet, play an important role in the development of human cancer (Ohgaki, Takayama and Sugimura, 1991). Diet plays an important role in cancer incidence patterns of various areas of the world. The shift of cancer occurrence to different organs was noticed among immigrants from one area to another. Cancer as a disease of DNA in somatic cells, DNA alterations are induced by genotoxic substances in food and the modulation of carcinogenesis by food components, leading to a postponement of the onset of cancer (Sugimura, 1997). The naturally occurring substances in food influence cancer initiation, promotion, progression, and demotion by a number of mechanisms, including (1) covalent binding to DNA of naturally occurring anticarcinogenic compounds to block the initiation of carcinogenesis; (2) induction of biotransforming enzymes such as cytochrome P450 and mixed-function oxidase (MFO) which can reduce carcinogenicity; (3) inhibition of tumor promotion by compounds such as retinol, tocopherol, and organosulfates found in garlics, onions, fruits, and vegetables; and (4) physical alteration of carcinogens by food constituents or by food preparation and handling so as to alter carcinogenicity (Lee Davis, 1989). Dietary habits are regarded as major determinants affecting cancer development in human such as excessive use of salt and salted, pickled foods, total fat, fried or broiled meats, and the relative low intake of fiber, protective fruits and vegetables (Weisburger, 1998). Preventive dietary advice often includes a reduction of alcohol, red meat

and animal fat, and increasing the intake of vegetables, fruits and fiber, and phytoestrogens from various sources (Hanf and Gonder, 2005).

2.2 Anticarcinogenic Components in Plants

Inhibitors of carcinogenesis, possibly present in fruits and vegetables may interfere at all stages of this multistage process which at least comprises initiation, promotion, and progression (Edenharder *et al.*, 1993; Edenharder *et al.*, 1994). Vegetables, fruits and their seeds are rich sources of Vitamins A, C, and E, or, perhaps, inhibitors of proteases as well, compounds which might protect the organism against cancer. Some plants may contain specific compounds which exhibit chemopreventive properties against neoplasms, such as ellipticine, indole derivatives, plant phenols, etc (Hocman, 1989). A high intake of fruits and vegetables seems to be protective with respect to the final outcome of hormone associated cancers such as breast and prostrate, and of smoking linked cancers such as lung and bladder (Armstrong and Doll, 1975; Miller *et al.*, 1980; Weisburger, 1991). In addition, consumption of cereals, vegetables, especially cruciferous vegetables, and fiber protects against colon cancer (Greenwald, 1992). A high consumption of fruits e.g. citrus fruits and many raw vegetables was found to reduce the risk of gastric cancer (Graham *et al.*, 1990). Cancer in the colon, breast, prostate and pancreas may be caused by heterocyclic amines (HAs) and can be inhibited by tea, beverages, fruits and vegetables (Edenharder *et al.*, 1999; Edenharder *et al.*, 2002). Weisburger, Dolan and Pittman (1998) found that black and green tea depressed the mutagenicity of PhIP in dose-related fashion. Oguri *et al.* (1998) reported that green tea catechins (epigallocatechin gallate) and two flavonoids (luteolin and quercetin) and caffeic acid were found to clearly suppress the formation of both MeIQx and PhIP. These phenolic antioxidants also reduced the total mutagenicity of HAs in cooked foods. Other antioxidants such as lycopene, the active antioxidant from tomatoes, and daidzein and genistein from soy products, also had a dose-related inhibition of the mutagenicity of PhIP. Using the Salmonella/microsome assay, the

eggplant fruit juice exhibited an antimutagenic activity against Trp-P-2. It is suggested that there are multiple components existing in the eggplant fruit such as lutein, pheophorbide or chlorophyllide, tannins and pheophytin a and b, as possible antimutagens (Yoshikawa *et al.*, 1996).

2.3 Mushrooms and Cancers

2.3.1 Button Mushroom (Family Agaricaceae)

Figure 1 Button mushroom

Button mushroom (Figure 1) is one of the most widely cultivated species of edible mushrooms. Button mushroom is terrestrial saprobes, and has cap that is not brightly colored. At maturity the gill is free or almost free from the stem, and is brown to chocolate brown. The stem breaks away cleanly from the cap, and has a partial veil which often forms a ring on the stem. The spore print is dark brown. The protein content of the mushrooms was at most 0.5% on fresh weight basis and 7% on dry weight basis (Braaksma and Schaap, 1996). The lipid extracted from button consists of a high degree of unsaturated fatty acids and the main constituent fatty acid was linoleic acid. Other fatty acids were also detected but were generally less than 7% of the total fatty acid content (Yilmaz *et al.*, 2006). The *Agaricus spp.* contains five known aromatic hydrazine derivatives (Toth, 1991), of

which the most abundant is agaritine, a phenylhydrazine derivative of glutamic acid. Most hydrazines are shown to have carcinogenic potential (Toth, 2000). Agaritine (L-glutamic acid, 5-(2-(4-(hydroxymethyl)phenyl)hydrazide)) is found in relatively high concentrations [up to 300 mg/kg fresh weight (Sharman *et al.*, 1990)] in button mushroom. Agaritine was identified and quantified in spores of button mushroom. A average of agaritine content was 0.304 ± 0.003% of the spores (Janak, Stormer and Koller, 2005). Agaritine is suspected of having a genotoxic potential because of its structural similarity to known carcinogenic hydrazines (Toth, 1980; Toth and Nagel, 1981). As a result, several genotoxicity, mutagenicity and carcinogenicity studies have been carried out with mushrooms, agaritine and/or its metabolites. Whereas agaritine was weakly mutagenic *in vitro* and *in vivo* (Friederich *et al.*, 1986; Shephard, Gunz and Schlatter, 1994). The mutagenicity of mushroom extracts *in vivo* and *in vitro* is controversial. Extract from button mushrom have been shown to elicit a positive but weak mutagenic response in mutagenicity systems, such as the Ames test, using a variety of *Salmonella typhimurium* strains (Friedrich *et al.*, 1986; Rogan *et al.*, 1982), but a more pronounced mutagnic response was evident when the TA104 bacterial strains was used (Papaparaskeva *et al.*, 1991). In contrast, button mushroom extracts failed to provoke a positive mutagenic response in other *in vitro* mutagenicity tests and *in vivo* tests such as the micronucleus test (Morales *et al.*, 1990; Pool-Zobel *et al.*, 1990). Shephard, Gunz and Schlatter (1994) suggested that there was no genotoxic activity other than that attributable to agaritine detected in button mushroom and the average button mushrooms consumption of 4 g/day would be expected to contribute a lifetime cumulative cancer risk of about two cases per 100,000 lives. On the other hand, Sterner *et al.* (1982) found that button mushroom was weakly mutagenic towards all three strains of *S. typhimurium* TA98, TA100 and TA2637. But, agarithine was weakly active towards TA2637 alone. This implies that this fungus might contain other mutagenic materials as well. In addition, Papaparaskeva, Ioannides and

Walker (1993) suggested that agaritine is not the principal mutagenic component in the mushroom. From the results that phenolic and quinonoid compounds, presumably through the generation of reactive oxygen species, may play a significant role in the mutagenicity of mushroom extracts. Toth and Erickson (1986) reported that excessive consumption of the edible mushroom button mushroom induced tumours at multiple sites in Swiss mice. In contrast, when button mushroom was tested in rats using a different protocol no carcinogenic response was evident. This finding raises the possibility that mushroom-induced tumors may be specific to mice (Matsumoto *et al.*,1991).

2.3.2 Shiitake mushroom (Family Tricholomataceae or Marasmiaceae or Omphalotaceae)

Figure 2 Shiitake mushrrom

Shiitake mushroom (Figure 2) also called forest mushroom and shiang-ku (fragrant mushroom), are traditional delicacies in Japan, Korea, Taiwan and China (Stamets, 1993). Shiitake mushroom is the second most popular edible mushroom in the global market. It grows on the trunks or stumps of trees. Several important bioactive compounds such as proteins, lipids, carbohydrates, fibers, minerals, vitamins (B1,B2 and C) and ergosterol have been isolated from this fungus (Ying *et al.*,1987;

et al.,1990). Protein contents of shiitake mushroom (23%) was high, but fat content was low (2%). Oleic and linoleic acids accounted for 72-77% of the total fat. Essential amino acid contents of shiitake were 39%. The chemical scores of shiitake mushroom was low compared to whole egg protein. Methionine was the limiting amino acid (Longvah and Deosthale, 1998). There are some evidences suggesting that consumption of shiitake mushroom promoted health in Austria (Volz, 1999) and in Japan (Borchers *et al.*, 1999). Studies have pointed out that shiitake and some of its active substances exert a protective effect against mutagenesis and carcinogenesis (Chiraha *et al.* 1970; Nanba and Kuroda, 1987; Hasegawa *et al.*, 1989). Shiitake musbhroom was observed to be effective in protecting against DNA damage, which can be responsible for the initiation of carcinogenesis (Ribeiro and Salvadori, 2003). Gu and Belury (2005) reported that ethanol-soluble extract of shiitake significantly decreased cell proliferation and induced apoptosis in time- and dose-dependent manners in carcinoma cells but had no effect in non-tumorigenic cell. Somes studies indicated that aqueous solutions extracted from different lineages of the mushroom, at different temperatures, exhibited protective activity against cyclophosphamide- and *N*-ethyl-*N*-nitrosourea-induced micronuclei in mice bone marrow and peripheral blood cells (Alves de Lima *et al.*, 2001; Sugui *et al.*, 2003).. Moreover, water crude extracts from shiitake mushroom showed the potent radical scavenging and antioxidant activities. Positive correlations were found between total phenolic content in the mushroom extracts and their antioxidant activities (Cheung *et al.*, 2003). As a result, shiitake mushroom had antioxidant activity against lipid peroxidation of rat brain homogenate. The antioxidant activities against lipid peroxidation in the above assays were found to correlate with the phenolic content of mushroom extracts (Cheung and Cheung, 2005). On the other hand, the antilipidemic effect of shiitake, inhibition of low density lyporotein (LDL) oxidation, is caused by eritadenin (Brandt and Piraino, 2000), a nucleotide derivative in mushroom (Tokuda *et al.*, 1974).

2.3.3 Pleurotus Mushrooms (Family Pleurotaceae)

Figure 3 Oyster mushroom

Figure 4 Abalone mushroom

Oyster (Figure 3) and abalone (Figure 4) mushrooms are commercially popular mushroom in Taiwan. Abalone mushroom also called summer oyster mushroom, tastes like its trivial name but is of vegetable nature (Stamets, 1993). Oyster mushroom also called hsiu-jen-ku (mini oyster mushroom), is apparently smaller and lighter than the abalone mushroom. Apart from the differences in their size and color (Yang, Lin and Mau, 2001), Oyster mushroom gests it's name from it's white, shell-like body rather than it's taste, which can vary from very mild to strong and sweet with the aroma of anise. It's taste ranges from soft to chewy. Oyster mushroom contains large quantities of essential fatty acids, including linoleic acid (Yilmaz *et al.*, 2006).

activities of oyster and abalone mushroom had moderate to high, reducing power and their scavenging effects are moderate to high. Total phenols are the major naturally occurring antioxidant components found. Overall, *P. ostreatus* is better in antioxidant activity, reducing power and scavenging abilities and higher in total phenol content (Yang, Lin and Mau, 2002). Cancer protective effects of oyster mushroom fruit bodies were demonstrated in rats (Zusman *et al.*, 1997). Besides, oyster mushroom diminishes the toxicity of cyclophosphamide in mice (Gerasimenya *et al.*, 2002). Antimutagenic effects were found for methanolic extracts of oyster mushroom (Lakshimi *et al.*, 2004). A dried oyster mushroom diet(5%) reduced pathological changes in dimethylhydrazine-induced colon cancer in rats but did not influence significantly the incidence of tumors. This effect is explained by the antioxidant properties of this mushroom and by its fiber content (Bobek, Galbavy and Ozdin, 1998). Additionally, a dimeric lectin was isolated from fresh fruiting bodies of the edible oyster mushroom. The lectin exerted potent antitumor activity in mice bearing sarcoma and hepatoma. Survival in these mice was prolonged and body weight increase reduced after lectin treatment (Wang, Gao and Ng‡, 2000).

A pronounced hypocholesteremic effect of oyster mushroom combined with inhibition of lipid peroxidation was shown in rats and rabbits. Oyster mushroom diet (10% dried fruiting bodies) significantly reduced the incidence and size of atherosclerotic plaques in rabbits (Bobek and Galbavy, 1999). The diet containing whole oyster mushroom strikingly reduced cholesterol content in the serum and liver. In addition, a shift in cholesterol distribution in lipoproteins (70% decrease in very low-density lipoprotein cholesterol and 50% increase in high-density lipoprotein cholesterol) was observed. Whole oyster mushroom reduced the levels of conjugated dienes in erythrocytes and in the liver, reduced the activity of catalase in erythrocytes and stimulated the activities of superoxide dismutase, catalase and glutathione peroxidase in liver (Bobek *et al.*, 1997). Moreover, Motomura *et al.* (2003) suggested that an extracellular enzyme from the edible oyster mushroom showed alfatoxin-

degradation activity. Fluorescence measurements indicated that the enzyme cleaves the lactone ring of aflatoxin.

2.4 Mutagens from Cooked Food

Human exposure to dietary chemicals appears to have a significant role in the initiation of cancer (Doll and Peto, 1981). On average, each human consumes roughly 15 tons of dry weight material in his or her life (Sugimura, 1978). Thus, foodstuffs may be one of the major potential sources of biologically active exogenous compounds. The diet contains various mutagens and carcinogens that can be classified into three groups: naturally occurring chemicals, synthetic compounds and compounds produced by cooking. The first group includes mycotoxins and plant alkaloids while the second is exemplified by food additives and pesticides. The third includes polycyclic aromatic hydrocarbons and heterocyclic amines (HCAs) (Nagao and Sugimura, 1993).

The body of epidemiological evidence available today clearly indicates that important differences in human cancer incidence of various organs, including colon, large intestine, prostate, liver and kidneys, depend on life style as well as dietary habits (Doll and Peto, 1981; Wynder and Gori, 1977). During heat processing of a typical western diet with its high meat and fat contents, food toxicants may be generated. To a large extent, human data now show that individuals who has a rule to eat their meat well done, especially with gravy, have an elevated risk of colon and breast cancers (Warzecha *et al.*, 2004; Weisburger *et al.*, 1998). There have been many reports concerning the production of mutagens in foods prepared by cooking methods such as frying, grilling, broiling or boiling. In most cases, these mutagens have been found in heated meat, fish and poultry products (Commoner *et al.*, 1978; Pariza *et al.*, 1979; Felton *et al.*, 1981; Bjeldanes *et al.*, 1982; Nagao *et al.*, 1977; Krone and Iwaoka, 1981).

During the 1960s and 1970s, much interest was focused on two classes of food toxicants producing tumors in long-term animal studies (1) polycyclic aromatic

hydrocarbons (PAHs) and (2) *N*-nitroso compounds (NOCs). These compounds are found in food as a result from food processing, e.g. curing, drying, smoking, roasting, refining, fermentation, and also from air pollution (Jagerstad and Skog, 2005). Among a multitude of PAHs known, formed through incomplete combustion of organic materials and also during heating of food, only some are tumorigenic. The significant human risk of PAHs has still to be evaluated individuals consuming a mixed-western diet are exposed to these genotoxic chemicals for nearly an entire lifetime. The amounts consumed depend strongly on food choices and cooking conditions (Rauscher, Edenharder and Platt, 1998). Furthermore, *N*-nitroso compounds may be formed endogenously. The most recently detected food toxicant produced by heat processing is acrylamide. Concern over acrylamide in foodstuffs arose in April 2002 when Swedish scientists reported unexpectedly high levels of this potentially carcinogenic compound in carbohydrate-rich foods heated to high temperatures such as crisps, French fries, bread and coffee, and begun to search for ways to reduce levels of the compound (Jagerstad and Skog, 2005).

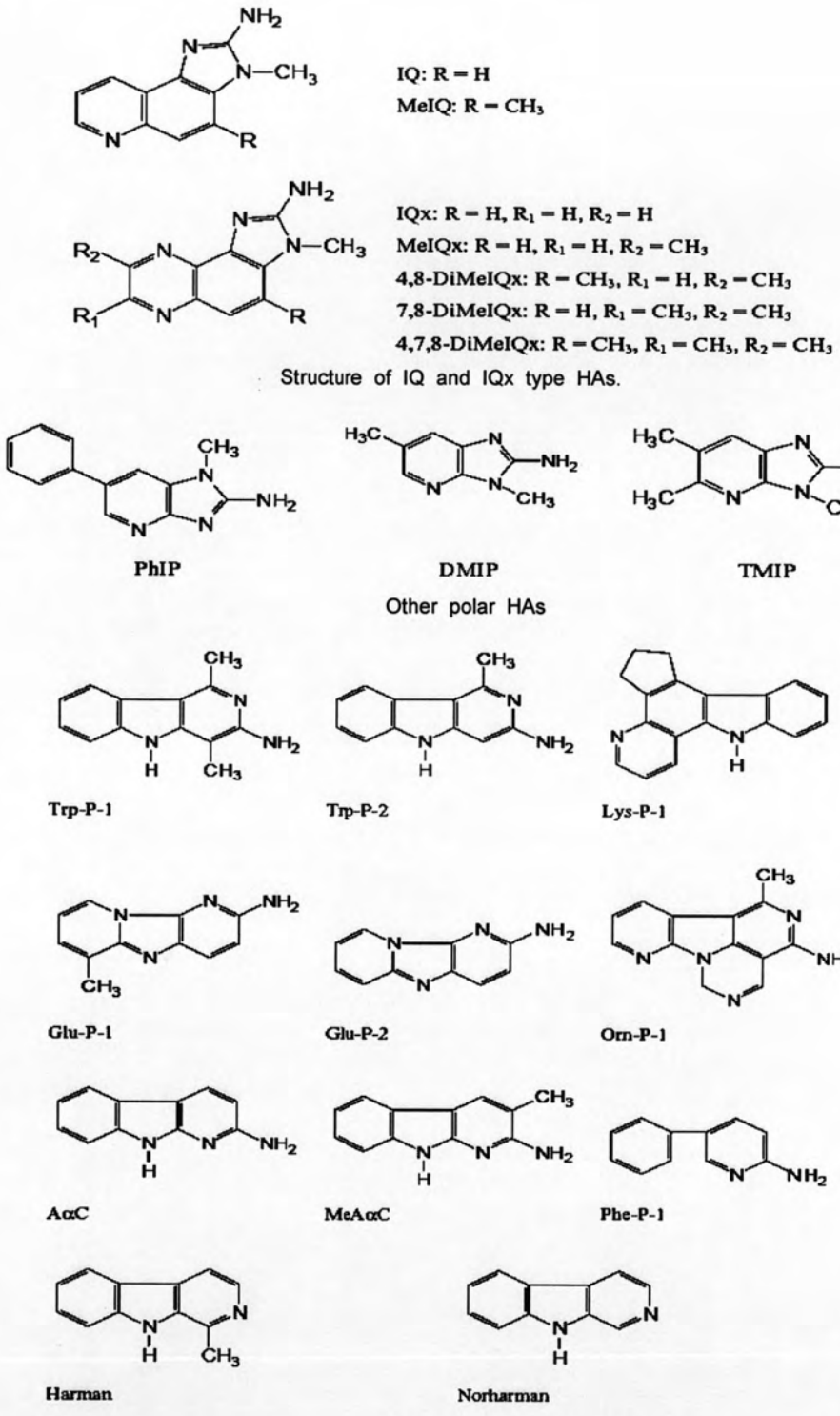
The browning of meat surface and the formation of typical flavours are considered appetising. By Maillard reactions not only flavors and colors but also hazardous substances are formed in the crust of cooked meat, because a strong mutagenic activity in the extracts of cooked meat was detected, not explainable by the content of PAHs (Nagao *et al.*, 1977). Japanese National Cancer Center Research Institute identified heterocyclic aromatic amines (HCAs) as pyrolysates of amino acids and the aminoimidazo quinoline and aminoimidazo quinoxaline derivatives as mutagenic principles in cooked meat (Spingarn *et al.*, 1980). Later, it was found that aminoimidazo pyridinederivative 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP) was accounted for about two-thirds of the HCA ingested with diets. These HCAs are formed at parts per billion levels in fried or grilled meat or fish (Skog *et al.*, 1998).

2.4.1 Heterocyclic Amines (HAs)

Heterocyclic amines (HAs) are substances with a high mutagenic and carcinogenic potentials (Sugimura, 1997). HAs represent a class of compounds possibly involved in the induction of tumors in human (Weisburger, 2000).

In general, HCAs consist of two or three rings with an exocyclic amino group attached to one of the rings. Based on their polarity, these HAs can be divided into two groups. One consists of a group of polar compounds which are mainly of the imidazoquinoline type (IQ), the imidazoquinoxaline type (IQx) as well as the imidazopyridine type, being trivially called the IQ-type (Figure 5). This substance group comprises compounds with a 2-aminoimidazole moiety in their molecules. The non-polar group, non-IQ type, has a 2-aminopyridine structure (Jagerstad *et al.*, 1991; Murkovic, 2004). In addition to their chemical stability to nitrite treatment under acidic conditions, the amino group of the IQ-type is not changed by treatment with 2 mM sodium nitrite, but the amino group of non-IQ type is converted to a hydroxyl group with 2 mM sodium nitrite. The amino group of the IQ-type is converted to a nitro group with 50 mM sodium nitrite and the resulting compound shows a similar mutagenicity to the original compound in the absence of S9 mix (Wakabayashi *et al.*, 1992).

Tar from cigarette smoke consists almost entirely of non-IQ type heterocyclic amines, but most of the mutagenicity of fried beef and broiled sardines is derived from IQ-type heterocyclic amines (Sugimura and Sato, 1983).



Structure of IQ and IQx type HAs.

Other polar HAs

Selection of non-polar HAs.

Figure 5 Structures of heterocyclic amines (Murkovic, 2004)

The formation of HCAs is mainly due to heat-induced nonenzymatic browning, known as the Maillard reaction. This reaction involves creatine, free amino acids and monosaccharides, which are present in meat and fish products and depend on physical parameters, such as cooking temperature and time, cooking technique and equipment, heat and mass transport, and on chemical parameters, especially the precursors to HCAs (Bordas *et al.*, 2004; Skog, Johansson and Jagerstad, 1998). During cooking, the temperature of the meat increases and HCAs precursors migrate to the meat surface. When critical temperatures at the meat surface are achieved and with dehydration of the meat, condensation and cyclization reactions occur resulting in heterocyclic quanidyl structures that produce various HAs compounds. Over 20 HAs compounds have been identified in cooked meats, however, the most frequently found HCAs are MeIQx, DiMeIQx, PhIP, IQ and A&C. HCAs levels increase as meats are cooked for longer periods or at higher temperatures (Keating, Layton and Felton, 1999). The IQ- type may be formed from creatine or creatinine, certain free amino acids and sugars via the Maillard reaction. These three groups of precursors are all present in uncooked meat and fish muscle, where free amino acids and sugars are supplied from muscle protein and glycogen respectively, whereas creatine is an energy metabolite only present in significant levels in muscle cells. Especially, creatinine is necessary since from it the imidazo moiety is formed. If it is not present, no HAs of the IQ-type are formed and the temperature that is needed for the formation of significant amounts is between 150 and 250 °C. At higher temperatures the non-IQ type are formed preferably. In general pan-frying and grilling produce high yield of HCAs at cooking temperatures from 200°C and above, boiling yields little, and deep-fat frying, roasting, and baking procedures give various yields (Jagerstad and Skog, 2005).

Ttryptophan is an important precursor of the nonmutagenic aminocarbolines harman and norharman, which were very easily formed at normal cooking temperature. The presence of water had a pronounced effect on the species of heterocyclic amines: dry heating favored the formation of PhIP and A&C, while aqueous

heating favored the formation of amino-b-carbolines and IQx compounds (Skog, Solyakov and Jagerstad, 2000). Moreover, enhancers and inhibitors are the factors that effect on HCAs formation (Vitaglione and Fogliano, 2004). The formation of HCAs during cooking can be decreased by synthetic and natural antioxidants, by tryptophan or proline, or by removing the essential creatine through brief microwave cooking prior to frying or broiling (Weisburger, 2002).

Antioxidants are considered beneficial in this respect, because free radical involvement in HCAs formation through the Maillard reaction would be expected. Antioxidants can act as inhibitors along the different pathways of the reaction, preventing the mutagens formation, through radical quenchers and free radical scavenging activity (Vitaglione and Fogliano, 2004). The addition of antioxidants Vitamin C, Vitamin E, BHT and BHA to beef were found to be effective towards HAs formation during cooking (Dashwood, 2002; Lan, Kao and Chen, 2004). Moreover, antioxidants (catechins, flavonoids, caffeic acid), organosulfur compounds (diallyl sulfide, dipropyl disulfide, diallyl disulfide), amino acids (l-tryptophan, l-proline), and Maillard reaction products have been reported to limit heterocyclic amine formation under various conditions (Jones and Weisburger, 1988; Oguri *et al.,* 1998). Fresh-made olive oil, containing a high amount of phenolic dihydroxyphenylethanol derivatives, inhibited heterocyclic amine formation *in vitro*. In addition, effect on the formation or inhibition of HAs of other compounds which are naturally present in foods, inclusion of soybean protein containing no creatin(in)e are effective for decreasing mutagen formation in fried beef hamburger. Carotenoids in food condiments based on tomato sauces or flavour spices or by marinating meats during cooking (Bordas *et al.*, 2004; Dashwood, 2002; Weisburger, 2000). On the other hand, Johansson and Jagerstad (1995) observed that hydroquinone combination with iron sulphate, increase in MeIQx, IQx and 7,8-DiMeIQx formation but beta-carotene and alpha-tocopherol were shown to inhibit the synergistic effect of hydroquinone and iron sulphate.

Heterocyclic amine (HCAs) compounds have been shown to be bacterial mutagens and animal carcinogens, and may be a risk factor for human cancer (Tran *et al.*, 2002). Epidemiological studies correlating meat intake and cooking practices have shown an increased risk of cancer for individuals that prefer well-done meat. These epidemiology studies identify breast, colon, stomach, pancrease, prostate, renal, lung and esophagus as the primary target organs (Anderson *et al.*, 2002; Felton *et al.*, 2004; Nowell *et al.*, 2002; Sinha, 2002). Using the Ames test the mutagenicity can be tested using sensitive bacterial test strains. HCAs show mutagenicity to *S. typhimurium* TA98 and TA100 with S9 mix. TA98, a detector of frameshift type mutation, proved more sensitive than TA100, a detector of basepair change type mutation. The carcinogenicity was shown in mouse and rat models. It is interesting in note that PhIP efficiently induces colon and prostate cancers in male rats and mammary gland cancers in female rats (Ito *et al.*, 1991; Shirai *et al.*, 1997). Urinary bladder cancers developed in rats fed diets containing Trp-P-2 (Takahashi *et al.*, 1993). In addition, MeA&C caused severe atrophy in the salivary glands and pancreas of rats (Takayama *et al.*, 1985). MeIQ, PhIP, and A&C were added to the diets of big blue mice, and their mutational spectra were analyzed in the colon, where high mutation frequencies were observed (Okonogi *et al.*, 1997). In the history of the monkey project, IQ has proven to be one of the most potent hepatocarcinogens, the time period induction was similar to that of N-nitrosodiethylamine (Adamson *et al.*, 1990; Wakabashi and Sugimura, 1998). The International Agency for Research on Cancer collated the studies on carcinogenicity and classified the IQ-type as probably carcinogenic to humans and MeIQ as well as MeIQx and PhIP, and the non-IQ type A&C, MeA&C, Glu-P-1, Glu-P-2, Trp-P-1, and Trp-P-2 as possibly carcinogenic to humans (Murkovic, 2004).

2.4.2 Heterocyclic Amines in Boiled Foods

Since a correlation between mutagenicity and carcinogenicity has been demonstrated (McCann *et al.*, 1975), it is possible that the strong mutagens in boiled pork may be among the factors responsible for diet-related cancers (Lin, Lee and Huang, 1982). The Ames test showed substances in boiled pork juice to be mutagenic. When the pork juice was heated under reflux at 102°C for 4 h, mutagens were formed, which were detected using *S. typhimurium* TA1538 and TA98 with S9 mix. The level of mutagenicity was dependent on the concentration of pork juice, the duration of boiling, and the pH (Lee and Lin, 1981; Lin *et al.*, 1982). Lee *et al.* (1994) have been found bacterial frameshift mutagens in boiled pork juice. The mutagenic fractions corresponding to the peaks of the standard mutagens MelQx, IQ and MelQ were confirmed by comparison of UV and mass spectra. One gram equivalent of original lean ground pork was estimated to contain 4.1 ng of MelQx, 3.7 ng of IQ, and 1.2 ng of MelQ, which accounted for 21.0%, 30.4%, and 38.1% respectively, of the total mutagenicity. The amount of IQ-type mutagens in boiled pork juice was about 4-fold higher than in broiled beef. Furthermore, five amino acids (glutamine, tyrosine, glycine, alanine, and threonine) and two monosaccharides (ribose and glucose) might participate in mutagen formation in boiled pork juice. The optimal conditions for mutagen formation in boiled pork juice were similar to the Maillard reaction. Jagerstad *et al.* (1983) suggested that the Maillard reaction pathway played an important role in the formation of mutagens. Taylor *et al.* (1986) has been studied on mutagen formation in foods during boiling. Taylor showed that maximal enhancement of mutagenicity was achieved by the addition of tryptophan and creatine phosphate to beef juice. The boiled beef juice contained IQ, Trp–P–2 and a minor amount of MeIQ. The Chinese in Taiwan usually cook ground pork with soy sauce for a relatively long time. A favorite Chinese dish, 'ru-thou fan', is made in this way.

When beef stock is concentrated by boiling, to a paste known commercially is beef extract. Münzner studied and used the nitrosated beef extract as a direct-acting standard mutagen for investigating the mechanism of modifying effect of vegetable juice. The beef extract requires metabolic activation for mutagenic expression. The products formed in the reaction between beef extract and nitrite under acid conditions showed direct-acting mutagenic response on the *Salmonella typhimurium* strains TA98, TA100 and TA1538 (Münzner and Wever, 1984; Münzner, 1986). The product (s) formed during the reaction of the beef extract and sodium nitrite in gastric-liked condition were proved to be several heterocyclic amine compounds such as IQ or MeIQ and were direct-acting mutagenic towards both strains TA98 and TA100 (Tsuda *et al.*, 1985). IQ and MeIQ are very reactive to nitrite and the mutagenicity of nitrosated MeIQ and IQ are even higher than that of the parent compounds (Lin, Cheung and Lin-Shiau, 1992).

2.5 Anticarcinogenesis by Food Components

Kitts (1994) defined bioactive compounds as "extranutritional" constituents that typically occur in small quantities in plant products and lipid-rich foods. They have also been variously called phytochemicals (from plants), zoochemicals (from animals) or nutriceuticals. Antimutagens can act along the pathway leading to mutagenesis. Ferguson, Philpott and Karunasingh (2004) identified classes of antimutagens by key steps of mutagenesis. Possible points of intervention and some examples of dietary antimutagens are identified in Table 1.

Table 1 Mechanisms by which dietary antimutagens could protect against mutation.

Mechanism against mutation	Examples of dietary antimutagens
(1) Extracellular mechanisms	
(1.1) Inhibition of mutagen uptake	Dietary fibers, probiotics
(1.2) Inhibition of endogenous formation	
(1.2.1) Inhibition of nitrosation	Vitamins (ascorbic acid, tocopherol), sulphur compounds (cysteine, glutathione, *N*-acetyl cysteine), phenols (cinnamic acid, chlorogenic acid, butylated hydroxyanisole)
(1.2.2) Modification of the intestinal flora	Prebiotics, probiotics
(1.3) Complexation and/or deactivation	Dietary fibres, hemin, chlorophyllin
(1.4) Favouring absorption of protective agents	Vitamin D3 and analogues
(2) Cellular mechanisms	
(2.1) Blocking or competition	
(2.1.1) Scavenging of reactive oxygen species	Provitamins and vitamins (beta-carotene, ascorbic acid, alpha-tocopherol), diterpenes (sarcophytol a), polyphenols including epigallocatechin gallate and various anthocyanins

Table 1 Mechanisms by which dietary antimutagens could protect against mutation (continued).

Mechanism against mutation	Examples of dietary antimutagens
(2.1.2) Protection of DNA nucleophilic sites	Ellagic acid, retinoids, polyamines
(2.2) Stimulation of trapping and detoxification in non-target cells	*N*-Acetyl cysteine
(2.3) Modification of transmembrane transport	Short chain fatty acids (caproate, caprylate), acylglycosylsterols, dietary calcium
(2.4) Modulation of xenobiotic metabolizing enzymes	
(2.4.1) Inhibition of promutagen activation	Isothiocyanates, monocyclic monoterpenoids (limonene, methol, carveol), retinoids, flavonoids, wheat bran
(2.4.2) Induction of detoxification pathways	Polyphenols, indoles, diterpene esters, riboflavin, 5′-phosphate, *S*-allyl-l-cysteine, allylic sulphides
(2.5) Modulation of DNA metabolism and repair	Cinnamaldehyde, vanillin, umbelliferone
(2.6) Enhancement of apoptosis	Retinoids, butyric acid, flavonoids
(2.7) Maintenance of genomic stability	Vitamins (folic acid, B12), minerals (selenium, zinc), polyphenols

2.6 Dietary Nitrosating Compounds: Nitrate and Nitrite in Foods

Carcinogen N-nitroso compounds are formed for nitrite and secondary amines under acidic condition (Mirvish, 1975) and also in vivo in experimental animals (Sander and Bfirkle, 1969). In man, ingested nitrite is mainly formed from nitrate by bacteria in the oral cavity (Spiegelhalder *et al.*, 1976). Ingestion of nitrate was suggested to be correlated with mortality of gastric cancer (Fine *et al.*, 1982). Nitrosamines are formed by the reaction of secondary amines and nitrite under acidic condition such as are found in the stomach (Tricker *et al.*, 1992; Zhukov, Maslova and Vasilevskaia, 1990). Nitrite is contained in leafy vegetables and is added to foods as a color-fixing agent. In addition, nitrite is available as a food additive in meats, fish and cheese. Sodium and potassium nitrate and nitrite are used to preserve meat products for the purpose of inhibiting growth and toxin formation by *Clostridium botulinum* (Olajos and Coulson, 1987). Nitrite is also present in saliva and is reduced from nitrate by bacteria in oral cavity and macrophages (McKnight *et al.*, 1997; Miwa *et al.*, 1987). It is possible that nitrite which humans are exposed to in daily foods may react with chemicals in the stomach, giving rise to mutagens or carcinogens. Several researchers have examined the mutagenic activity of nitrite-treated foods or chemicals. Direct-acting genotoxic compounds can be produced by reaction of their precursors with nitrite under acidic condition similar to gastric juice. Several research groups have reported the presence of nitrosatable precursors in foods, such as indoles, phenolics and carbolines (Wakabayashi *et al.*, 1989). At gastric pH, nitrite reacts actively with amines (Mirvish, 1975), amino acids (Ohta *et al.*, 1981, Ohta, Suzuki and Kurechi, 1983, Ohara *et al.*, 1986), mercaptans (Kubberod, 1974), and sorbic acid (Osawa and Namiki, 1982) and mutagens are frequently formed in these reactions. Wakabayashi *et al.* (1987) reported that various foodstuffs induced mutagenic activity after nitrite treatment. Epidemiological studies have shown a correlation between nitrite/nitrate ingestion and the incidence of gastric cancer (Mirvish, 1983; Correa, 1988). Tumor formation as a result of endogenous nitrosation has been demonstrated in rats given N-

methylbenzylamine in the diet and nitrite in the drinking water for 2 years (Tahira *et al.*, 1988). Endogenous nitrosation is also known to occur in humans: volunteers given a large dose of proline or thioproline and nitrate (the latter is converted to nitrite by oral cavity bacteria) excrete increased amounts of N-nitroso products in the urine (Bartsch *et al.*, 1989). Nitrosation can also take place in stimulated macrophages and endothelial cells, here arginine serves as precursor for the nitrosating agent (Leaf *et al.*, 1989). A good correlation between nitrate intake and gastric cancer mortality in various countries has been documented (Fine *et al.*, 1982).

2.7 The *Salmonella* Mutagenicity Test (Ames Test)

Ames *et al.* (1972) have developed a very sensitive and simple procedure for detecting mutagens using a group of histidine dependence (auxothrophy) strains on *Salmonella typhimurium* which revert to histidine independence (prototrophy) after the induction of specific kinds of genetic alteration in the DNA. In the assay, as originally described and usually performed, histidine auxotrophs are exposed to the test chemical on a petri dish, and the only measurement made is the number of revertant colonies on each plate. The compounds are tested on petri plates with several specially constructed mutants of *S. typhimurium* selected for sensitivity and specificity in being reverted from a histidine auxotrophs back to histidine prototrophs by a wide variety of mutagens. The active forms of most known carcinogens are mutagens, and there is substantial evidence that screening systems to detect mutagens can also serve to detect many potentially carcinogenic compounds. A high but not complete qualitative correlation was found between the results of this test and carcinogenicity studies *in vivo* when a broad spectrum of chemicals was tested.

The Salmonella test was first validated in a study of 300 chemicals, most of which were known carcinogens (McCann *et al.*, 1975; McCann and Ames, 1976; McCann and Ames, 1977). It was subsequently validated in studies by the Imperial

Chemical Industries (Purchase *et al.*, 1976), the National Cancer Center Research Institute in Tokyo (Sugimura *et al.*, 1976), and the International Agency for Research on Cancer (Bartsch *et al.*, 1980). Nearly 90% of the carcinogens tested were mutagenic in these studies, but there was considerable overlapping of chemicals tested. In a recent analysis, Rinkus and Legator (1979, 1981) concluded that the correlation between carcinogenicity and mutagenicity is lower than the earlier estimates. In a discussion of this analysis (Ames and McCann, 1981) estimated the correlation to be about 83%. All the validations show that the test fails to detect a few classes of carcinogens such as polychlorinated pesticides (Rinkus and Legator, 1979, 1981; Ames and McCann, 1981).

The *Salmonella* Tester Strains The reverse mutation system of *S. typhimurium* uses the genetically well-defined histidine requiring mutants developed by Ames and his colleagues (1973). The Salmonella strains that revert from histidine dependence (auxotroph) to histidine independence (prototroph). Maron and Ames (1983) had collected and characterized a large number of *S. typhimurium* strains containing mutations in different gene of the histidine operon. Maron and Ames (1983) had been developed the newly *Salmonella* tester strains to make them more effective in detecting mutagens that were not previously detected with the original strains. The newly standard tester strains contain other mutations that greatly increase their ability to detect mutagens such as :

rfa mutation, which causes partial loss of lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo[a]pyrene that do not penetrate the normal cell wall (Ames, Lee and Durston, 1973).

uvr B mutation is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (Ames, 1971).

R-factor plasmid (pKm101). The strains containing the plasmid show greatly enhanced response to chemical shown to be mutagenic and also give clear positive response to chemical described as weak, borderline or non mutagens with the original set of tester strains (McCann et al., 1975). Furthermore, MacPhee (1973a and 1973b) reported that pKM101 contains genes products associated with error-prone repair, which may be responsible for the enhance sensitivity seen in these strains.

Tester strains in bracket recommended for general mutagenesis testing indicates wild-types gene. The deletion through uvr B also includes the nitrate reductase (chl) and biotin (bio) genes, wheareas the gal strains and rfa/uvr B stains have a single deletion through gal chl bio uvr B.

Genotype of the *S. typhimurium* strains used for mutagenesis testing are shown in Table 2. The standard tester strains, TA97, TA98, TA100 and TA102 contain the R-factor plasmid, pKM 101. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. These standard tester strains are recommended for general mutagenesis testing. TA98 is derived from TA1538 by introduction of plasmid pKM101. It can detect mutagens that causes frameshift mutation with a DNA sequence (-CGCGCGCG-), which can be reverted to histidine independence by a variety of mutagens that act by adding or deleting base pairs. While TA100, the R-factor plasmid derivative of TA 1535, can detect mutagens that cause base-pair substitutions. The other Salmonella strains were related to these 4 strains but with different characteristics in terms of DNA-repair capacity cell permeability and the presence of plasmid pKM 101 also are available. However, some mutagens affect only one strain of frameshift mutation strains (TA1538 or TA98) or only base-pair substitution strains (TA1535 or TA100), imparting a degree of mutagen class specificity to the assay. Many or even most mutagens can affect both types of strains although the effective dose is often higher for one type of strain than for the other.

Table 2 Genotype of the TA strains used for mutagenesis testing.

Histidine mutation				LPS	Repair	R-factor
hisD6610 his01242 = TA88	hisD3052	hisG46	hisG428 (pAQ 1)			
TA90	TA1538	TA 1535	-	*rfa A*	*uvrB*	- R
[TA97]	[TA98]	[TA 100]	-	*rfa a*	*uvrB*	+ R
-	TA1978	TA1975	-	*rfa*	+	- R
TA 110	TA94	TA92	-	+	+	+ R
-	TA1534	TA1950	-	+	*uvrB*	- R
-	-	TA2410	-	+	*uvrB*	+ R
TA89	TA 1964	TA 1530	-	*gal*	*uvrB*	- R
-	TA2641	TA2631	-	*gal*	*uvrB*	+ R
-	-	-	[TA102]	*rfa*	+	+ *R*

Method used for Detecting Mutagens

Plate incorperating test. This test is the standard method that has been used for test the mutagenicity of chemicals. The test consists of combinding the test compound, the bacterial tester strain and S9 mix in soft agar which is poured onto a minimal agar plate. Positive and negative controls are also included in each assay. After incubation at 37^0C for 48 hours revertant colonies are counted. For initial screening, chemicals were tested in concentrations over a three-log does range in the presence and absence of S-9 mix. And positive or questionable result should be confirmed by demonstrating a dose-response relationship using a narrower range of concentrations. For most mutagens, there

is a concentration range that produces a linear dose-response curve and the number of revertants per plate reported for a mutagen should be taken from the region of the curve. However, a few mutagens such as 9-aminoacridine, MNNG, diethylsalfate and ethymethanesulfonate produce non linear dose-response curve. The compounds that produce negative result can be retested using the preincubation method.

Preincubation method Some mutagens, such as dimethyl and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahahi *et al.* (1975) in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen, S9 mix, and bacteria for 20-30 min at 37^0C and then added the top agar. The assay has also been used to detect the mutagenicity of 10 carcinogenic nitrosamines and several carcinogenic alkaloids. The mutagenicity activities of aflatoxin B1, benzidine, benzo(a)-pyrene, and methyl methanesulfonate have been determined using both plate incorporation and preincubation procedures and in all cases the sensitivity of the preincubation assay is equal or greater than that of the plate incorporation assay. The increased activity is attributed to the fact that the test compound, S-9 and bacteria are incubated at higher concentrations in the preincubation assay than in the standard plate incorporation test.

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay requires an extra step and therefore involves more works than the standard test but many laboratories use it for screening assays recommended by De Serres and Shelby (1979).