

**MUTAGENICITY MODIFICATION OF OOLONG TEA ON
NITRITE TREATED FILTRATE OF FIVE-SPICE SEASONED
PORK: STUDIES ON PRECURSOR FORMED DURING
COOKING AND ON MUTAGENIC PRODUCT IN
ACID SOLUTION USING AMES TEST**

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จาก

บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล

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It was found that the filtrate from “five-spice seasoned pork” (a popular Thai dish consisting of pork boiled for a long period of time) did not have a mutagenicity when tested with the Ames test using *Salmonella typhimurium* TA 100 (without S-9 mix). However, when the filtrate from five-spice seasoned pork reacted with nitrite in an acidic circumstance (pH 3-3.5), it showed a direct mutagenicity towards the bacteria. The same result was obtained when only the spices were used without the pork. It was also found that cooking five-spice seasoned pork using a conventional method for four hours at 100°C reduced the mutagenicity from the reaction of food sample and nitrite when compared with pork cooked using an air-limited method.

The study also showed that oolong tea itself had no mutagenicity when it was treated using the same methods that were used to cooked the pork or when it was brewed for 5 minutes and for 30 minutes. But oolong tea showed direct mutagenicity on the bacteria when it was pre-exposed to react with nitrite.

The study also showed that oolong tea can cause antimutagenicity. If a lot of tea leaves were boiled with the ingredients used to cook five-spice seasoned pork then the tea could reduce the formation of the precursor which could further react with nitrite to produce a direct mutagenic product which would react against the bacterial tester strain. However, a large amount of tea leaves may change the flavor and taste of the food. This study also showed that the aqueous extracts from oolong tea leaves (brewed for 5 or for 30 minutes) could inhibit the mutagen formation which occurred during the reaction between filtrate from five-spice seasoned pork and nitrite. Interestingly it also inhibited the mutagenic product which formed from this reaction. The findings from this study suggest that tea should be drunk after eating a meal to minimize the risk of exposure to a mutagen precursor formed during the normal cooking process.

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ลินนา ทองยงค์ : การปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของน้ำหมูพะโล้ที่ทำปฏิกิริยากับไนไตรท์ด้วยชาอูหลง : ศึกษาการเกิดสารตั้งต้นในน้ำหมูพะโล้ระหว่างการปรุงและผลต่อสารก่อกลายพันธุ์ที่เกิดขึ้นภายหลังทำปฏิกิริยากับไนไตรท์ในสภาวะกรดโดยใช้การทดสอบเอมส์ (MUTAGENICITY MODIFICATION OF OOLONG TEA ON NITRITE TREATED FILTRATE OF FIVE-SPICE SEASONED PORK: STUDIES ON PRECURSOR FORMED DURING COOKING AND ON MUTAGENIC PRODUCT IN ACID SOLUTION USING AMES TEST). คณะกรรมการควบคุมวิทยานิพนธ์ : แก้ว กังสดาลอำไพ , Ph.D., วิสิฐ จະวะสิต , Ph.D., อาณัติ นิธิธรรมยง, Ph.D. 161 หน้า. ISBN 974-665-949-9

จากการศึกษาพบว่า น้ำหมูพะโล้ ซึ่งเป็นอาหารประเภทต้มเนื้อเป็นเวลานาน ๆ และเป็นอาหารที่คนไทยนิยมบริโภคไม่มีฤทธิ์ก่อกลายพันธุ์ เมื่อทดสอบโดยใช้วิธีเอมส์ โดยใช้เชื้อ *Salmonella typhimurium* สายพันธุ์ TA100 ในสถานะที่ไม่มีสารออกฤทธิ์กระตุ้นการก่อกลายพันธุ์ แต่เมื่อนำน้ำหมูพะโล้ไปทำปฏิกิริยากับไนไตรท์ในสภาวะที่เป็นกรด (ความเป็นกรดเป็นค่า 3 - 3.5) พบว่าแสดงฤทธิ์ก่อกลายพันธุ์โดยตรงต่อแบคทีเรีย ซึ่งเมื่อทดสอบฤทธิ์ก่อกลายพันธุ์ในสารสกัดจากเครื่องเทศที่ใช้เป็นส่วนประกอบในการปรุงหมูพะโล้ก็ให้ผลในทำนองเดียวกัน นอกจากนั้นยังพบว่าการปรุงหมูพะโล้โดยวิธีดั้งเดิมคือต้มแบบเปิดฝา เป็นเวลา 4 ชั่วโมง ที่อุณหภูมิ 100 องศาเซลเซียส ช่วยลดฤทธิ์การก่อกลายพันธุ์ที่เกิดขึ้นจากการทำปฏิกิริยากับไนไตรท์ได้ดีกว่าการต้มแบบจำกัดอากาศ

จากการศึกษาฤทธิ์ก่อกลายพันธุ์ของสารสกัดจากใบชาอูหลง ซึ่งผ่านการสกัดโดยวิธีต่าง ๆ ได้แก่ สกัดโดยใช้วิธีเดียวกับการปรุงหมูพะโล้ (แบบดั้งเดิมและแบบจำกัดอากาศ) หรือสกัดโดยแช่น้ำต้มเดือด 5 และ 30 นาที พบว่าทุกตัวอย่างไม่มีฤทธิ์ก่อกลายพันธุ์โดยตัวของมันเอง แต่จะแสดงฤทธิ์ก่อกลายพันธุ์โดยตรงต่อแบคทีเรียเมื่อทำปฏิกิริยากับไนไตรท์ นอกจากนั้นยังได้ทำการศึกษาดังกล่าวในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ที่เกิดขึ้นในหมูพะโล้ พบว่าถ้าต้มใบชา (ปริมาณมาก) พร้อมกันไปกับส่วนประกอบอื่น ๆ ของหมูพะโล้ จะช่วยลดการเกิดสารตั้งต้นของสารก่อกลายพันธุ์ที่เกิดขึ้นระหว่างการปรุงหมูพะโล้ ซึ่งสามารถทำปฏิกิริยากับไนไตรท์ต่อไปได้เป็นสารก่อกลายพันธุ์โดยตรง แต่วิธีนี้อาจทำให้รสชาติของอาหารเปลี่ยนแปลงไป และการศึกษาข้างชี้ให้เห็นว่า สารสกัดด้วยน้ำจากใบชา (สกัดเป็นเวลา 5 หรือ 30 นาที) สามารถยับยั้งการก่อตัวของสารก่อกลายพันธุ์ที่เกิดขึ้นจากปฏิกิริยาระหว่างน้ำหมูพะโล้และไนไตรท์ ที่น่าสนใจก็คือสามารถยับยั้งฤทธิ์ก่อกลายพันธุ์ของผลิตภัณฑ์สุดท้ายที่เกิดจากปฏิกิริยาดังกล่าวข้างต้นได้ จากการศึกษา นำไปสู่การประยุกต์ใช้ในชีวิตประจำวัน เพื่อลดความเสี่ยงในการสัมผัสกับสารตั้งต้นของสารก่อกลายพันธุ์ที่อาจเกิดขึ้นในระหว่างการปรุงอาหาร

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LIST OF ABBREVIATIONS

BC	Before Christ
cm	centimetre
° C	degree celsius
<i>et al.</i>	<i>et alii</i> (and other)
etc	et-cet-era (and other similar things)
g	gram
HAs	Heterocyclic amines
h	hour
ibid	ibidem (in the same article)
μl	microlitre
mg	milligram
mM	millimolar
Min	minute
M	molar
ng	nanogram
Sec	second
viz	videlicet (often read out as namely)

CHAPTER 1

INTRODUCTION

Cancer is a major public health problem today. Many laboratory studies and human epidemiological data suggest that most cancer deaths are attributable to life style, including tobacco smoking, occupational hazard, a variety of toxic insults and environmental pollutants, and diet (Alcantara and Specman, 1976).

In view of the important of diet, dietary chemicals have been shown to play a significant role in the initiation of cancer (Doll and Peter, 1981). Many epidemiological studies have shown a relationship between occurrence of various forms of cancers and ingestion of certain food components or food consumption patterns, such as the increased occurrence of gastric cancer and the intake of foods with high concentration of sodium chloride (Hirohata and Kono,1997; De Stefani *et al.*, 1998). Exposure to nitrate and nitrite, precursors of carcinogenic N- nitroso compounds, has also been correlated with stomach cancer mortality (Mirvish, 1983; Weisburger and Horn, 1984). Laboratory studies, using long term animal tests according to the maximum tolerable dose (MTD) principle, have demonstrated the existence of various carcinogens in food components which include food contaminants, food additives, and chemical substances produced during the cooking processes of food (Sugimura, 1985, 1986).

During the cooking process, a number of genotoxic compounds are formed from endogenous constituents of meat and among these are various heterocyclic amines

(Felton *et al.*, 1986a). These substances are very potent mutagens in the Ames/*Salmonella* assay and are also carcinogens in laboratory animals (Takayama *et al.*, 1985; Ito *et al.*, 1991). Because heterocyclic amines are found in a variety of cooked meats and fish that contribute a major part of our diet, they represent a potential risk factor for public health in the etiology of human cancer. A number of these mutagens in processed food are always present at certain amounts and they cannot be removed completely from our daily foods. The most urgent and important task is, therefore to eliminate such potential mutagens from our daily life as much as possible.

On the other hand, in many epidemiological studies investigating relationship between diet and cancer, a protective effect of the consumption of vegetables and fruits on various types of cancer has been found (Wattenberg, 1983, 1985; Weisburger, 1991). It has been known for decade that there are substances in commonly consumed foods that reduce the incidence of chemically induced carcinogenesis in laboratory rodents. Further studies elucidated many active “chemopreventive” substances in plant foods, including dietary fiber, vitamin C, vitamin E, beta carotene and phenolic compounds (Ames, 1983; Williams and Wynder, 1996). Of particular interest as chemopreventive agents are phenolic compounds derived from fruits, vegetables and beverages, which may be consumed in large quantities (up to 1-2 grams per day) in some human diets (Newmark, 1996).

The most important single group of phenolics in food is flavonoids. Hertog *et al.*, (1993) reported that the average intake of flavonoids was 23 mg/day, and the most important source of flavonoids was tea (48% of total intake). The effects of tea consumption on cancer is an area of great scientific and public interest, and this topic was reviewed in 1993 in an article entitled “Tea and Cancer”(Yang and Wang, 1993).

Since then more than one hundred new researches articles related to this topic have been published.

Although many investigations have been done in intact animals to determine the effect of tea and its components on carcinogenesis (Katiya *et al.*, 1992; Xu *et al.*, 1992; Shi *et al.*, 1994; Yamane *et al.*, 1995), comparatively little is known about the modification effect of tea on mutagens formation during processing of food. More research is needed to elucidate the interaction between tea components and mutagens existing in normally prepared foods.

The objectives of this study were to determine the mutagenicity of a popular Thai food (five-spice seasoned pork) prepared by different cooking conditions on treatment with and without nitrite and also the mutagenicity of some spices used in preparing this food. Moreover, in view of the interest arising for the possible protective role of tea against cancers, this study was designed to investigate the modification effect of oolong tea leaves on the formation of mutagen precursor during cooking of five-spice seasoned pork. In addition, it is also of interest to know whether extracts from tea can exert protective effect against the formation of direct mutagen occurred during the reaction between nitrite and filtrate of five-spice seasoned pork or against mutagenic product of this reaction.

CHAPTER II

LITERATURE REVIEW

Diet is generally accepted as one of the important factors in human cancer development. The effect of diet have an impact on human cancer development similar to cigarette smoking. This is proven by carefully conducted epidemiological studies (Wynder and Gori,1977; Doll and Peter,1981). Concern about the role of diet in human cancer has prompted the search for compounds in common foods that may act as carcinogens or mutagens. The presence of carcinogenic substances in food was first reported by a Swedish scientist, E.M.P. Widmark, at the University of Lund, in 1939 that extracts of roasted horse meat caused carcinogenic effects, when applied to mouse skin (Widmark,1939). More research on toxin formation in cooked meats was reported in 1964, when Lijinsky and Shubik demonstrated polycyclic aromatic hydrocarbons on the surface of well-done charcoal-broiled steaks, apparently formed through pyrolysis of fat dripping into flames and being absorbed by the food. Ames *et al.* in 1975 developed a short-term mutagenicity assay which was utilized by Sugimura and his group to demonstrate the presence of mutagenic activity in the charred surface of beef and fish, broiled over a naked flame or charcoal (Nagao *et al.*,1977; Sugimura *et al.*,1977a). Soon after this discovery, Commoner *et al.* (1978) showed that mutagenic activity was formed in meat also under normal domestic cooking conditions and even by boiling beef extracts. Several of this mutagens have now been identified as heterocyclic amines (Felton and Knize,1991a). Since then,

around 20 highly mutagenic heterocyclic amines have been isolated and identified from pyrolysates of amino acids or proteins and from various cooked foods, mainly protein-rich foods of animal origin (Felton and Knize,1990;Wakabayashi *et al.*,1992)

2.1 Heterocyclic Amines (HAs)

2.1.1 Chemistry of heterocyclic amines

The mutagens identified in cooked foods are heterocyclic amines. On the basis of their chemical structure, food mutagens can be divided into five groups (Skog,1993). Mutagens formed at temperatures below 300°C are often referred to as “thermic mutagens”, whereas high-temperature mutagens are referred to as “pyrolytic mutagens”. The pyrolytic mutagens (group I) have an amino group attached to a pyridine ring, whereas the thermic mutagens have an amino group attached to an imidazole ring. By treating with 2 mM nitrite under acid conditions, the pyrolytic mutagens can be deaminated and no longer show any mutagenic activity, in contrast to the thermic mutagens which are unaffected by acid nitrite treatment (Tsuda *et al.*, 1985). Treatment of thermic mutagens with a much higher concentration of nitrite (50mM) resulted in conversion of amino groups to nitro groups (Sasagawa,1988). The nitro derivatives of imidazoquinoline and imidazoquinoxaline showed almost the same mutagenicity in the absence and presence of S9 mix as parent amino derivatives. Figure 1 shows the chemical structure of thermic mutagens identified in cooked food or model systems.

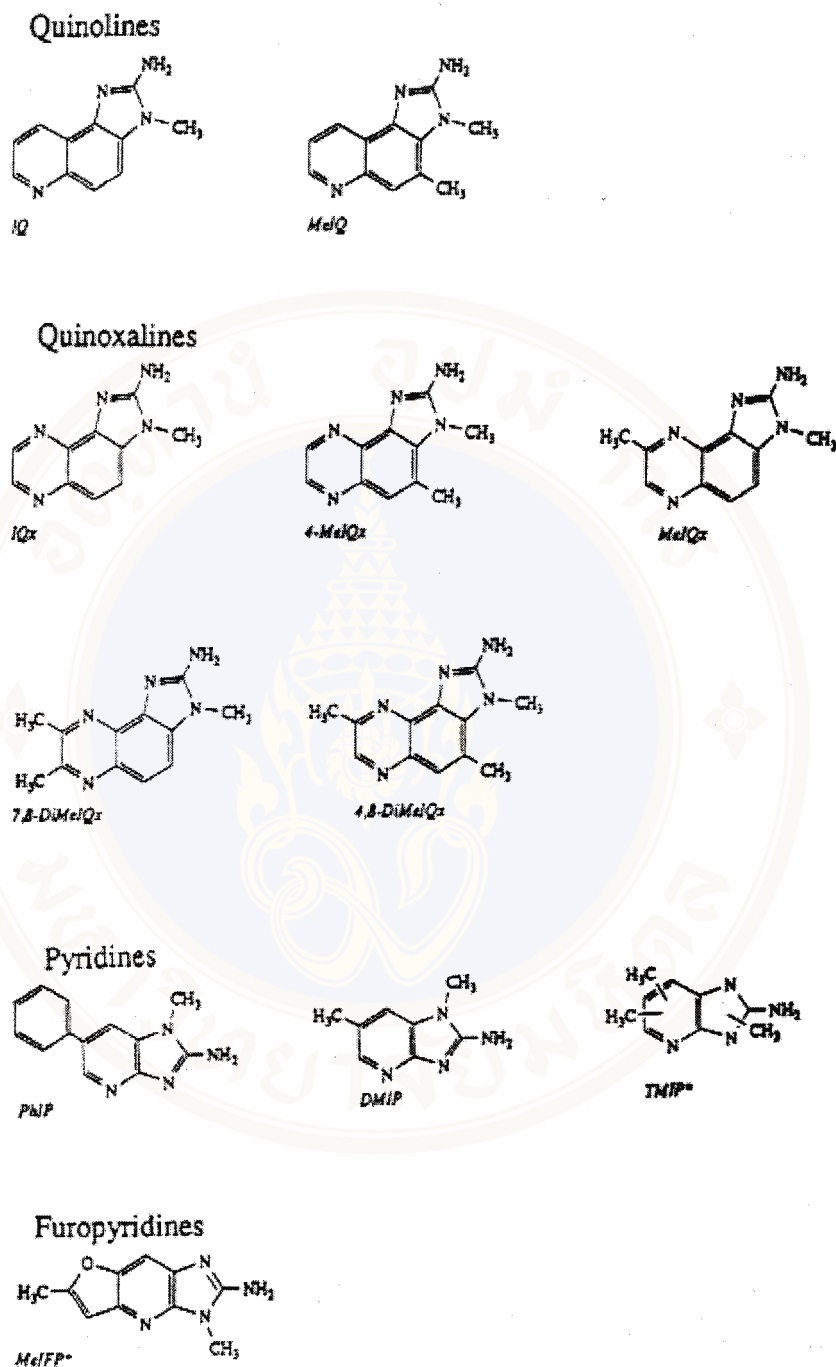


Figure 1 Chemical structures and common names for thermic mutagens found in cooked foods or model systems Quinolines (*IQ* = 2-amino 3-methylimidazo [4,5- *f*] quinoline; *MeIQ* = 2-amino -3,4-dimethylimidazo [4,5- *f*] quinoline) ; quinoxalines (*IQx*=2-amino 3-methylimidazo [4,5 *f*] quinoxaline ; 4-*MeIQx*= 2-amino 3,4-dimethylimidazo [4,5 *f*]quinoxaline ; *MeIQx*=2-amino 3,8-dimethylimidazo [4,5- *f*] quinoxaline ; 7,8-*DiMeIQx*=2 amino -3,7,8-trimethylimidazo [4-5- *f*]quinoxaline ; 4,8-*DiMeIQx*=2-amino-3,4,8-trimethylimidazo [4,5- *f*]quinoxaline) ; , pyridines (*PhIP* = 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine ; *DMIP* = 2-amino-1,6-dimethylimidazopyridine; *TMIP* = 2-amino-*n,n,n*-trimethylimidazopyridine); , furopyridines (*MeIFP*= methylimidazofuropyridine).* Suggested structure.

Group I Pyrido-imidazoles or - indoles

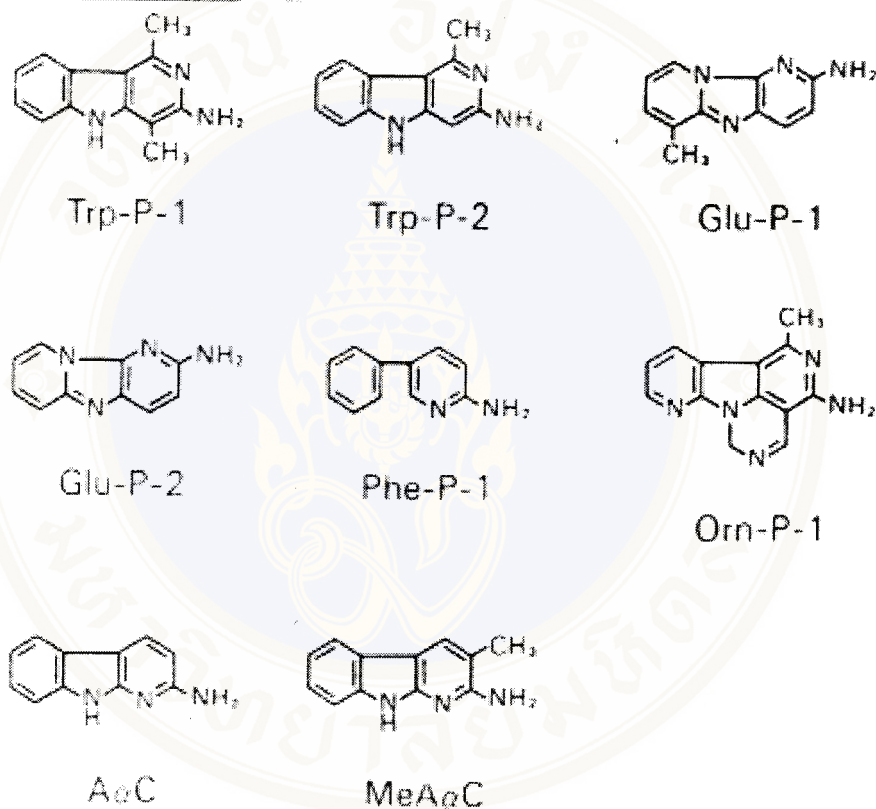
The mutagens in this group are formed at temperatures above 300°C, far higher than normal domestic cooking temperatures, and they are not commonly found in the western diet. A number of heterocyclic amine mutagens have been identified by pyrolysing single amino acids, tryptophan, glutamic acid, phenylalanine, lysine or ornithine (Sugimura and Sato,1983).

Sugimura *et al.* (1977b) isolated Trp-P-1 and Trp-P-2 from smoke condensates of tryptophan. From the glutamic acid pyrolysate, Glu-P-1 and Glu-P-2 were isolated and identified (Yamamoto *et al.*,1978). From phenylalanine pyrolysate, Phe-P-1 was isolated (Sugimura *et al.*,1977b). Orn-P-1 was isolated and identified as heterocyclic amine (Yakota *et al.*,1981). It was reported by Yoshida *et al.* (1978) that the pyrolysis of soybean globulin yields α - carbolines (A α C and Me A α C) The structure of these chemical are given in Figure 2. All these chemicals are heterocyclic amines. They all required metabolic activation to exert mutagenic activity. *Salmonella typhimurium* TA98 is more susceptible than TA100 (Sugimura and Sato,1983).

Group II Quinolines

The mutagens in groups II and III are derivatives of imidazoquinolines or imidazoquinoxalines and are commonly called IQ compounds. From the crust of broiled sardines, Kasai *et al.*, in1980 isolated 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ) and 2-amino-3,4-dimethylimidazo [4,5-*f*] -quinoline (MeIQ) (Kasai *et al.*,1980a;1980b). IQ has been found in fried ground beef, in broiled fish and in beef extract (Felton *et al.*, 1984; Taylor *et al.*,1985;Yamaizumi *et al.*,1986). 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. (Skog, 1993) MeIQ has been identified in fried ground beef and in broiled fish (Felton *et al.*, 1984; Yamaizumi *et al.*, 1986) and has also been produced in model systems from creatine, fructose and alanine (Skog, 1993).



- Figure 2 Chemical structures and mutagens isolated from pyrolysates of amino acids and a protein ; Trp-P-1=3-3-Amino-1,4-dimethyl-5H-pyrido [4,3-b] indole ; Trp-P-2 = 3-Amino-1-methyl-5H-pyrido [4,3-b] indole ; Glu-P-1 = 2-Amino-6-methyldipyrido[1,2-a : 3,2-d] imidazole; Glu-P-2 = 2-Aminodipyrido [1,2-a : 3',2'-d] imidazole ; Phe-P-1 = 2-Amino-5-phenylpyridine ; Orn-P-1 = 4-Amino-6-methyl-1H-2,5,10 , 10 b-tetraaza-fluoranthene; AαC = 2-Amino-9H-pyrido [2,3-b] indole ; Me AαC = 2-Amino-3-methyl-9H- pyrido[2,3b]' indole.

Group III Quinoxalines

From the crust of fried ground beef, Kasai *et al.* (1981) identified 2-amino-3,8-dimethylimidazo-[4,5-*f*] quinoxaline (MeIQx). Later, MeIQx was also 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). MeIQx has been identified in fried ground beef, fried and smoked fish, and in food-grade beef extract. (Kasai *et al.*,1981; Felton *et al.*,1984; Kato *et al.*,1986; Zhang *et al.*,1988; Gross,1990) An isomer, 4-MeIQx, has been reported to be present in fried pork (Vahl *et al.*,1988). 4,8-DiMeIQx has been identified in fried beef, fried and smoked fish and in food grade beef extract (Kato *et al.*,1986; Zhang *et al.*,1988; Gross,1990).

After reflux boiling of creatinine, glycine and glucose, 2-amino-3,7,8-trimethylimidazo[4,5-*f*] quinoxaline (7,8-DiMeIQx) was isolated (Negishi *et al.*,1984), and later also identified in fried beef (Turesky *et al.*,1988). This is an example of a new mutagen first found in a model system and later isolated from cooked food, demonstrating that model systems are useful tools in studying mutagen formation. A few years ago another mutagenic quinoxaline, 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQx), was isolated from a fried-meat emulsion fortified with creatine (Becher *et al.*,1988)

Group IV Pyridines

A new group of heterocyclic amines mutagens, the imidazopyridines, was identified. 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) was first isolated from the crust of fried ground beef by Felton *et al.* (1986b), and later from a fried meat product with added creatinine (Becher *et al.*,1988). Two other mutagenic pyridines, 2-amino-n,n,n-trimethylimidazopyridine (TMIP) and 2-amino-1,6-

dimethylimidazopyridine (DMIP), have been identified in fried meat products (Felton *et al.*,1984; Becher *et al.*,1988). However, the exact structure of TMIP has not yet been determined.

Group V Furopyridines

New heterocyclic amine mutagens are still being discovered. Although their structures have not been fully elucidated, two mutagens of molecular weight (MW) 202 and 216, respectively, have been isolated from fried ground beef and pork and from a fried meat emulsion (Felton *et al.*,1986b; Becher *et al.*,1988). Recently, a methylimidazo-fuopyrine (MeIFP) containing oxygen (MW 202) has been isolated from fried ground beef with added milk and creatinine. Evidence suggests that this mutagen is related to the food mutagen with MW 216 (Knize *et al.*, 1990).

Some authors classified HAs into two major classes; aminoimidazoazaarenes (IQ type) and carboline (non-IQ type) (Robbana-Barnet *et al.*, 1996). The aminoimidazoazaarene (AIAs) compounds have a 2-aminoimidazo group fused to a quinoline (IQ and MeIQ), a quinoxaline (MeIQx), or a pyridine (PhIP) ring. The carbolines and its analogues of the type “non-IQ” contrary to the AIAs (type "IQ"), include the aminopyridoindoles (Trp-P-1, Trp-P-2, A α C, Me α C), the aminopyridoimidazoles (Glu-P-1, Glu-P-2, Lys-P-1, Orn-P-1), and an aminophenyl-pyridine (Phe-P-1).

2.1.2 Formation of mutagenic heterocyclic amines

Pyrolysing of dry material is performed at high temperatures, usually well above 300°C . The identification of a new class of mutagenic heterocyclic amines constituting imidazoquinoline (IQ ; MeIQ) and imidazoquinoxaline (MeIQx)

in the early eighties (Kasai, 1980b) drew attention to mutagen formation during moderate cooking condition between 100°C and 300°C . These food mutagens were isolated from the crust of broiled or fried meat and fish cooked under normal heating condition (100-300°C), and also from meat extracts manufactured by long term boiling until all the water had evaporated. Non-enzymatic browning (Maillard reaction) was suggested to be involved in their formation (Wei *et al.*, 1981; Jagerstad *et al.*, 1983a).

The Maillard reaction takes place in foods through the reaction of carbonyl compounds (aldehydes and ketones), notably reducing sugars, such as glucose, fructose, etc., with compounds possessing free amino groups, such as amino acids, peptides, and proteins. The reaction is of great importance for the development of flavors, texture, and brown pigments during heat treatment of foodstuffs, thereby contributing to the palatability of cooked foods. The number of different compounds that are formed during the reaction is overwhelming. After an initial condensation between reducing sugar and amino groups, rearrangements produce Amadori compounds. These react further by various pathways (enolization, dehydration, retro-aldol condensation, Strecker degradation) to produce hundreds of reaction products, viz., furans, pyrazines, imidazoles, pyridines, etc. A tentative pathway for the formation of IQ compounds, by the Maillard reaction, was suggested by Jagerstad *et al.* (1983a) and it is illustrated in Figure 3. Three precursors, all naturally occurring in muscle meat, were assumed to participate, namely creatine, certain amino acids and sugars. Creatinine was postulated to form the 2-aminoimidazo part by cyclization and water elimination, a reaction that takes place spontaneously when the temperature is raised above 100°C . The 2-aminoimidazo part is a common moiety of all IQ compounds. It is also responsible for the mutagenicity of the IQ compounds.

The quinoline and quinoxaline part of the molecule was postulated to arise through condensation reaction between pyrazines or pyridines and aldehydes.

This hypothesis was verified using a model system where creatin (in) e, glycine or alanine, and glucose, dissolved in diethylene glycol (DEG) containing 14% water, were boiled under reflux at 130°C for 2 h. The mixtures showed high mutagenic activity, whereas heating the reactants two by two produced only weak, if any, mutagenic activity. The addition of synthetic pyridines or pyrazines to the reaction mixture increased the mutagenic activity by about 50% (Jagerstad *et al.*,1983b). In a collaborative study between groups in Sweden and Japan, two mutagenic compounds, MeIQx and 7,8-DiMeIQx, were identified after reflux boiling a mixture of creatine, glycine and glucose at 130°C for 2 h (Neigishi *et al.*,1984). Substituting the amino acid threonine for glycine produced MeIQx and 4,8-DeMeIQx (Neigishi *et al.*,1985).

Over 20 HAs compounds have been identified in cooked meats, however, the most frequently found HAs are 2-amino-3,8-dimethylimidazo [4,5- *f*] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo [4,5- *f*] quinoxaline (DiMeIQ), 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), 2-amino-3-methylimidazo [4,5- *f*] quinoline (IQ) and 2-amino-pH-pyrido [2,3-*b*] indole (AαC). MeIQx, DiMeIQx and PhIP are the predominant HAs found in cooked meats, both in the number of meats in which they occur and in concentration (Table 1) (Keating *et al.*,1999).

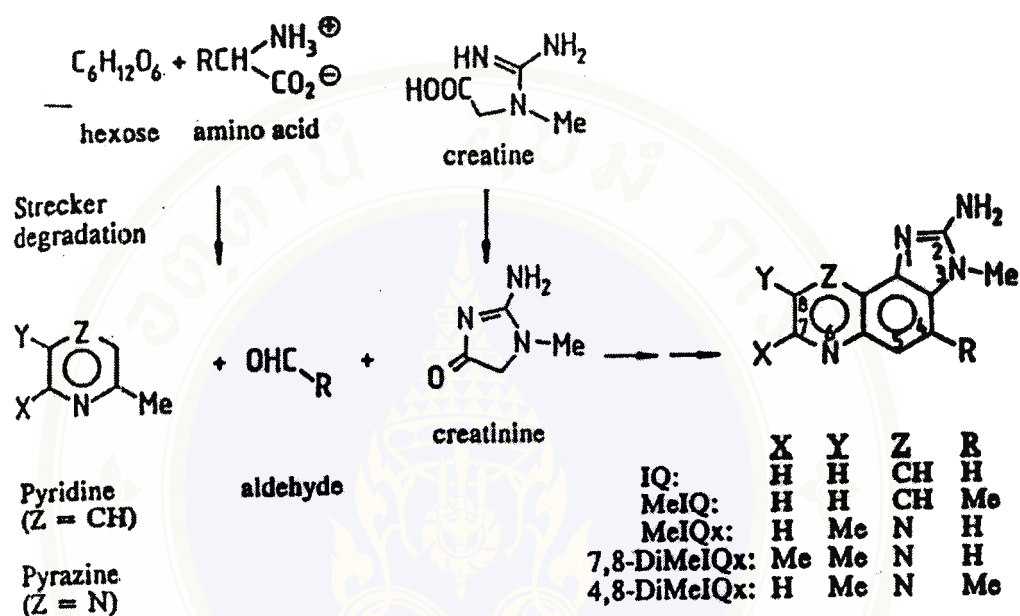


Fig. 3. Suggested pathway for the formation of imidazoquinolines and quinoxalines (Jagerstad *et al.*,1983b).

Table 1. Heterocyclic amines concentrations in meats prepared under variable cooking conditions (Keating *et al.*,1999).

Meat type	HAs concentration (ng /g) ^a			Reference
	MeIQx	DiMeIQx	PhIP	
Pan-fried				
Steak-sirloin	13 - 8.2	-	1.9 - 23.2	(Sinha <i>et al.</i> ,1998a)
	0.02 - 1.6	0.02 - 0.06	0.06 - 1.8	(Skog <i>et al.</i> ,1995)
Steak-minute	n.d. - 6.2	n.d. - 2.7	n.d. - 12.7	(Skog <i>et al.</i> ,1995)
Hamburger	0.34 - 4.3	-	n.d. - 2.3	(Sinha <i>et al.</i> ,1998a)
	n.d. - 7.3	n.d. - 1.6	n.d. - 32	(Knize <i>et al.</i> ,1994)
	n.d. - 2.2	n.d. - 0.8	0.01 - 1.1	(Skog <i>et al.</i> ,1995)
Chicken	0.4 - 0.5	0.2 - 0.5	0.5 - 10	(Skog <i>et al.</i> ,1997)
	1.0 - 3.0	1.0 - 4.0	12 - 70	(Sinha <i>et al.</i> ,1995)
Bacon	0.4 - 4.3	-	n.d. - 4.8	(Sinha <i>et al.</i> ,1998b)
	n.d. - 23.7	n.d. - 1.4	n.d. - 4.5	(Skog <i>et al.</i> ,1995)
	2.8 - 5.9	1.7 - 3.4	0.2 - 1.0	(Johansson & Jaggerstad,1994)
Sausage	n.d.	n.d. - 0.1	n.d. - 0.1	(Skog <i>et al.</i> ,1995)
	n.d. - 1.3	-	n.d. - 0.1	(Sinha <i>et al.</i> ,1998b)
Pork-chop	n.d. - 2.6	n.d. - 1.1	n.d. - 4.8	(Skog <i>et al.</i> ,1995)
	n.d. - 5.4	-	n.d. - 2.3	(Sinha <i>et al.</i> ,1998b)
Pork-fillet	n.d. - 4.6	n.d. - 3.3	n.d. - 13.4	(Skog <i>et al.</i> ,1997)
Broiled				
Steak	n.d. - 1.7	-	2.1 - 7.1	(Sinha <i>et al.</i> ,1998a)
Hamburger	n.d. - 1.6	-	n.d.	(Sinha <i>et al.</i> ,1998a)
Chicken	n.d. - 3.0	n.d.	6.0 - 150	(Sinha <i>et al.</i> ,1995)
Bacon	n.d. - 4.0	-	1.4 - 30.3	(Sinha <i>et al.</i> ,1998b)
Grilled				
Steak	0.2 - 2.7	-	2.5 - 30.0	(Sinha <i>et al.</i> ,1998a)
Hamburger	n.d. - 4.6	-	n.d. - 16.8	(Sinha <i>et al.</i> ,1998a)
Chicken	n.d. - 9	n.d. - 2	27 - 480	(Sinha <i>et al.</i> ,1995)
Bacon	n.d. - 1.5	-	n.d. - 3.1	(Sinha <i>et al.</i> ,1998b)

^a Values represent the concentration ranges in ng of HAs per g of cooked meat.

n.d. = not detected.

2.1.3 Factors affecting the yields of heterocyclic amines

Condition that affect the yield of mutagenic heterocyclic amines are of great interest because they might contribute to the understanding of the reaction mechanism and become of practical importance as a way to limit their formation.

Temperature and time

Studies in both model systems and cooking show the great influence of time and temperature on formation of mutagenic heterocyclic amines. Generally the mutagenicity as measured in the Ames test increases with increasing temperature. The time effect is linear within limited time intervals, but then reaches a plateau. The onset of mutagenicity in meat and meat extracts was found at temperature of 100°C (Dolara *et al.*,1979). The authors did not identify the mutagenic compounds. The mutagenic compounds began to form in meats and model systems at temperatures of 150 °C or higher (Skog and Jagerstad,1990; Jackson and Hargraves,1995) and the concentration of the HAs increased with processing temperature (Skog and Jagerstad,1990; Knize *et al.*,1994; Jackson and Hargraves,1995)

At all processing temperatures, there was a time lag before HAs could be detected (Jackson and Hargraves,1995). This lag could be related to the time required for the mixture or meat surface to reach 100°C - 150°C.

Mutagenic activity or HAs formation in muscle products or in model systems increased with processing time at 150°C - 175°C. However, at higher temperature (190°-250°C), the concentrations of HAs increased during an initial time of processing and then decreased or plateaued (Skog and Jagerstad,1990;Knize *et al.*,1994).

The precursor of HAs

Creatine

It is well understood that the basis for the mutagenic activity of muscle meats is the presence of creatine stored in high concentration in these tissues. During heating, creatine is transformed into creatinine from which the imidazole part of HAs is formed. Higher processing temperatures lead to a more rapid decrease in creatine and increase in creatinine. Most of this conversion seems to occur in the first 40 min of processing (Jackson and Hargraves, 1995).

It is therefore an essential precursor. There are many arguments to back up this hypothesis: adding creatine or creatinine to meat or fish during heat treatment leads to a net increase in the formation of mutagenic agents of the IQ type (Jagerstad *et al.*, 1983a; Felton and Knize, 1991a); protein-rich foods containing little or no creatine (cheese, tofu, beans, liver, kidney, shrimp, and plants) produce very few mutagenic agents (Chen *et al.*, 1990); models systems containing creatine or creatinine generate HAs (Laser- Reutersward *et al.*, 1987); creatine has been demonstrated to form a part of the PhIP molecule by using radiolabeled creatine (Felton and Knize, 1991a); and beef flavors with high creatine (1.5 mg/g) or creatinine (2 mg/g) levels exhibited higher mutagenic activities than did flavor with low levels of these compounds (Jackson *et al.*, 1994).

Amino acids.

Studies carried out on model systems show that amino acids or short chain peptides are absolutely necessary for the formation of aminoimidazoazaarene (AIA) (Jagerstad *et al.*, 1983a). Using different amino acids results in considerably different values of total mutagenicity. One possible explanation is that each amino

acid might affect not only the concentration of each mutagenic heterocyclic amine produced, but also the relative proportion between them. Because the mutagenic heterocyclic amines differ markedly in specific mutagenicity, various amounts as well as proportions have a great influence on the total mutagenicity.

There are also reports that certain amino acids enhance or inhibit the mutagenicity when added to model systems or meat before frying. Proline, for instance, produced IQ when dry-heated together with creatine (Yoshida *et al.*, 1984). Accordingly, Ashoor *et al.*(1980) found that proline, but none of the other amino acids, added to ground-beef patties before frying enhanced mutagen formation. Taylor *et al.* (1986) upon adding tryptophan to their meat extract system observed an increase in the formation of IQ. Overvik *et al.* (1989) reported dramatically enhanced mutagenicity in the crust and pan-residue after adding 1% of each amino acid to beef patties, especially after adding threonine, serine, proline and alanine. On the other hand, Jones and Weisburger (1988) have reported that both proline and tryptophan act as inhibitors in model systems. The inhibitory effect of tryptophan was also studied in a meat-cooking experiment, whereas tryptophan was found to be more inhibitory when spread directly on the surface of the beef patty compared with blending with the other ingredients before frying or broiling.

Sugars.

The effect of various monosaccharides and disaccharides has been investigated in the model system. Muramatsu and Matsushima (1985) found that ribose produces more mutagenicity than glucose, and according to data presented by Skog and Jagerstad(1990), fructose is more effective than glucose, and sucrose is more effective than lactose. Monosaccharides and disaccharides show an optimum effect on the

mutagenicity yield when present in amounts around half that of creatin (in) e or amino acid. By increasing the concentration of glucose or fructose, the formation of mutagenic compounds decreased. Heating mixtures of creatin (in) e and glycine or phenylalanine with increasing amounts of glucose or fructose resulted in the formation of 3-4 different peaks corresponding to mutagenic products. All the peaks decreased when the monosaccharides were added in increasing amounts. At equimolar levels or in excess of those of the other reactants, the formation of mutagenic compounds was almost completely inhibited (Skog and Jagerstad,1990).

The mechanism behind the inhibitory effect of hexoses is unknown. It can only be speculated that by increasing the concentration of reducing sugar the Maillard reaction might favor other Maillard reaction products, which might interfere or compete with the reaction route resulting in the formation of mutagenic heterocyclic amines. By monitoring the recovery of creatinine during this reaction, Skog and Jagerstad (1990) found more than 90% unreacted creatine when glucose was added in half the molar concentration of creatine. With increasing amounts of glucose added, the recovery of creatin(in)e decreased. The decrease in the creatin(in)e recovery was seen only in the presence of amino acid , which indicates that the Maillard reaction was involved and that some Maillard reaction product might have reacted with creatin (in) e to non-mutagenic products.

Role of water and lipid.

Under certain conditions the HAs may form in the total absence of water (Yoshida and Fukuhara,1982; Overvick *et al.*,1989). During cooking water soluble mutagenic agent precursors, migrate with the water towards the surface of the food where they are then exposed to the temperature required for the reactions leading to

the formation of HAs (Overvick *et al.*,1989). The external surfaces of grilled meat show greater levels of mutagenic activity than the inner part where normally the center temperature is lower (Dolara *et al.*,1979). Above an initial optimal level of water in the food, such as 40% for beef (Bjeldane *et al.*,1982), mutagenic activity is reduced, probably because of a dilution effect on the precursors.

The role played by intrinsic lipids in the development of mutagenic activity is not clear. However, they do seem to play an important role in the formation of HAs. Most authors agree that there exists an optimum level of intrinsic lipids necessary for a maximum formation of mutagenic agents (Spingarn *et al.*, 1981). In the case of minced meat, this level is 10%. Not long after this, Barnes and Weisburger (1983) found that in beef patties containing 11% or 25% of lipids after cooking, the amount of IQ produced is 4 times greater for the higher level of lipids. The differences found for the optimum level of lipids is probably related to the differences in the experimental procedures used. Most studies investigating the influence of lipids on the formation of mutagens in meat systems have used the Ames test. The effects of fats might have been underestimated, since the Ames test is inhibited by long-chain fatty acids (Barnes and Weisburger, 1983).

A number of hypothesis (Robbana-Barnet *et al.*,1996) have been put forward in order to explain the role of lipids : some researchers believe that increasing the level of lipids up to the optimal value may favor the transfer of heat. Above this value, the reduction in the formation of mutagenic agents could be due either to a reduction in cooking time, or a dilution effect on the precursors. The addition of lipids to model systems increases the mutagenic activity and the yield of HAs. For example, corn oil or olive oil added to model system containing creatine, glycine, and glucose dissolved

in water, heated at 180°C for 30 min, almost doubled the yield of MeIQx compared to the yield without fat (Johansson *et al.*,1993).

2.1.4 Modifying factors of mutagenic activity and HAs formation

Free radical reactions (Skog *et al.*,1995)

As the Maillard reaction is known to involve free radical reactions and free radicals have been suggested to be involved in the formation of HAs.. The enhancing effect of fats on the HAs yield might be due to free radicals produced during thermally induced for oxidation.

The addition of iron (Fe^{2+} and Fe^{3+}) to a creatinine, glycine, glucose model system increased the formation of IQx, MeIQx and DiMeIQx about two fold. Iron is a well-known catalyst of lipid oxidation at lower temperatures, and it was suggested that the increase in the formation of MeIQx by iron could be due to free radicals formed via iron-catalyzed lipid oxidation. In contrast, copper had no enhancing effect on the MeIQx yield (Johansson and Jagerstad,1996). Similarly, hydroquinone (known to initiate free-radical reactions) did not affect the formation of MeIQx or IQx in the model system, whereas the amount of 7,8-DiMeIQx increase (Johansson and Jagerstad,1996). However, when both hydroquinone and iron sulfate were added to the model system, an increase (seven to 14-fold) in IQx, MeIQx and 7,8-DiMeIQx was observed. Hydroquinone was probably oxidized to a radical by iron, and may have increased the formation of these HAs through a free radical mediated reaction mechanism.

Surprisingly, and in contrast to the view that a free-radical-dependent mechanism lies behind HAs formation, most antioxidants assumed to scavenge or quench free

radicals, increased the formation of MeIQx in a model system (Johansson and Jagerstad,1996). The addition of tert-butylhydroquinone (TBHQ) increased the amount of MeIQx about twofold, and the addition of α - or γ -tocopherols, and butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) also increased the amount of MeIQx formed. However, when β -carotene or γ -tocopherol was added to model mixtures containing hydroquinone and iron sulfate, the formation of MeIQx, IQx and 7,8-DiMeIQx decreased (Johansson and Jagerstad,1996). The free-radical scavengers β -carotene and γ -tocopherol, were probably able to scavenge the hydroquinone radicals thus inhibiting HAs formation (Skog *et al*,1995). Furthermore, in a model system, ascorbic acid in high concentrations (1000 ppm) decreased the amount of MeIQx formed, while lower concentrations showed no effect (Johansson and Jagerstad,1996).

Type of cooking and preparation of the food

Generally speaking, the types of cooking that involve temperatures of around 100°C boiling in water, steaming or stewing without previous browning, lead to a production of mutagenic agents that is too low to be quantifiable (Bjeldanes *et al*,1982). Microwave ovens (not using a grill that may be added) do not lead to the formation of these mutagenic agents (Nader *et al*,1981; Felton *et al*,1992). Microwave treatment for 1 minute to remove the juice containing creatin (in)e before normal cooking is reported to reduce HAs formation (Felton *et al*,1994). As a practical measure, preventing direct contact of meat and fish with a naked gas or a charcoal flames reduced HAs formation. Cooking procedures that heat foods by radiative and conductive process (grilling, frying) lead to an increase mutagenicity activity (Bjeldanes *et al*,1982). Cooking method such as roasting and baking, which

heat food by indirect convection, produce low or intermediate levels of mutagenicity activity in most protein-rich food (Bjeldanes *et al.*,1982; Doolittle *et al.*,1989). Wrapping meat and fish in aluminum foil before putting them in an oven is also an effective way to reduce HAs formation. Re-heating or keeping food warm does not alter the amount of mutagenic agents present (Berg *et al.*,1990). On the other hand, pre-treatments such as freezing or steaming in preserved foods may lead to an increased formation during further preparations. These preliminary treatments bring about the destruction of cell walls, which could lead to the liberation of precursors (Robbana-Barnet *et al.*,1996). High levels of mutagenic agents formed are found in the crust, cooking juices, residues left in the frying pan and the vapors given off in cooking (Overvick *et al.*,1987; 1989).

Using fats (butter, margarine, or oils) in cooking greatly increases in the amounts of mutagenic agents formed when the temperatures are high (more than 200°C) (Nilson *et al.*,1986). Coating foods with breadcrumbs before frying may also reduce the formation of HAs due to the insulating effect of the coating. However, HAs were found in the crust of fried fish coated with meal probably due to the very thin layer of coating (Skog *et al.*,1997).

Other modifying factors

A number of other food constituents are involved in the formation of HAs. In beef grilled in the presence of soya protein, chlorogenic acid, or cotton grain flour, a reduced mutagenic activity is seen (Wang *et al.*,1982). The inhibitory effect of the soya protein might be related to the presence of phenolic compounds, or to its water binding capacity, leading to a higher water content in the cooked product with less transport of the precursors to the surface (Skog,1993).

Certain phenolic compounds more or less complex (tannic acid, quercetol, rutin, catechin, and propyl gallate) seem to reduce the amount of 2-amino-9*H*-pyrido[2,3-*b*]indole (A α C) formed during albumine pyrolysis (Fukuhara *et al.*,1981). Recently, Weisburger and coworkers (1994) showed that tea polyphenols may represent another approach to lower HAs formation.

In addition, flavones reduce the formation of mutagenic agents. This property may be explained by the reduced formation of certain products of the Maillard reaction that are precursors of HAs formation (Robbana-Barnet *et al.*,1996). Flavones or flavonols that contain C5, C7 and C4 hydroxyl groups are potent inhibitors of P-450 enzyme activities induced by Aroclor 1254 (P450IA1 and P450IA2), and may potentially be useful as chemopreventive agents against HAs-induced mutagenesis or carcinogenesis. Other products of the Maillard reaction are inhibitors of HAs formation. The possible mechanisms of the antimutagenic effect of Maillard reaction products prepared from xylose and lysine to IQ has been suggested to be due to the interaction of this Maillard product with proximate metabolites of IQ to form inactive adducts and not to inhibit the activity of hepatic microsomal enzymes, direct reaction with intact IQ or interaction with DNA (Yen and Hsieh,1994).

2.1.5 Mutagenicity of HAs

The most frequently used in vitro procedure for mutagenicity test of HAs has been the Ames Salmonella / microsomal assay (Wagabayashi *et al.*,1992; Felton and Knize,1991b). These compounds show higher mutagenicity in *S.typhimurium* TA98, a frameshift mutation detector, than in *S. typhimurium* TA100, a base-pair change mutation detector. They need metabolic activation to exert their mutagenicity,

Table 2 (Sugimura *et al.*,1993; Wakabayashi and Sugimura,1998) shows the mutagenic potential of heterocyclic amines towards *S. typhimurium* TA98 and TA100 with S9 mix, the S9 fraction of which was prepared from the liver of rats treated with polychlorinated biphenyls.

Table 2 Mutagenicities of heterocyclic amines and typical carcinogens in *Salmonella typhimurium* TA98 and TA100 (Sugimura *et al.*,1993; Wagabayashi and Sugimura,1998)

Compound	Revertants / µg	
	TA98	TA100
Trp-P-1	39000	1700
Trp-P-2	104200	1800
Glu-P-1	49000	3200
Glu-P-2	1900	1200
Phe-P-1	41	23
Orn-P-1	56800	
AαC	300	20
MeAαC	200	120
IQ	433000	7000
MeIQ	661000	30000
IQx	75000	1500
MeIQx	145000	14000
4,8-DiMeIQx	183000	8000
7,8-DiMeIQx	163000	9900
PhIP	1800	120
4'-OH-PhIP	2	0
Cre-P-1	19000	400
Aflatoxin B ₁	6000	28000
4-Nitroquinoline 1-oxide	970	9900
Benzo [a] pyrene	320	660

The mutagenicities towards TA98 range from 2 to 661000 revertants per μg as determined by the method of preincubation. Some heterocyclic amines show higher mutagenicity than typical mutagens/carcinogens such as aflatoxin B₁, 4-nitroquinoline 1-oxide and benzo [a] pyrene (Table 2).

2.1.6 Carcinogenicity of HAs in experimental animal

Most carcinogenesis experiments using HAs have been performed employing F344 rats and CDF₁ mice of both sexes under standard *in vivo* conditions, with chronic feeding of commercial pellet diet containing HAs at concentrations of 100 to 800 ppm. (Ito *et al.*,1991; Sugimura,1992; Wakabayashi *et al.*,1992; Takahashi *et al.*,1993; Ohgaki *et al.*,1995; Shirai *et al.*,1997). The results are summarized in Table 3. Target organs in rats include the liver, urinary bladder, small and large intestines, Zymbal gland, clitoral gland, skin, oral cavity, mammary gland, and prostate. Target organs in mice are the liver, blood vessels, forestomach, lung, hematopoietic system, and lymphoid tissue. It is interesting to note that PhIP efficiently induces colon cancers in male rats and mammary gland cancers in female rats. (Ito *et al.*,1991) Furthermore, recent investigations revealed PhIP to be a carcinogen for the ventral lobe of the rat prostate (Shirai *et al.*,1997). Urinary bladder cancers developed in rats fed diets containing Trp-P-2 (Takahashi *et al.*,1993). In addition, MeA α C caused severe atrophy in the salivary glands and pancreas of rats (Takayama *et al.*,1985).

IQ induced hepatocellular carcinomas in cynomolgus monkeys (Adamson *et al.*,1990). The time period for liver cancer induction was similar to that of N-nitrosodiethylamine in cynomolgus monkeys.

Table 3 Carcinogenicities of heterocyclic amines in rats and mice (Wakabayashi and Sugimura,1998).

Chemical	Species	Concentration in diet (%)	Duration of feeding (weeks)	Target organs
Trp-P-1	Rats	0.015	52	Liver
	Mice	0.02	89	Liver
Trp-P-2	Rats	0.01	112	Liver, urinary bladder
	Mice	0.02	89	Liver
Glu-P-1	Rats	0.05	67	Liver,small and large intestines, Zymbal gland, clitoral gland,
	Mice	0.05	68	Liver, blood vessels
Glu-P-2	Rats	0.05	104	Liver, small and large intestines, Zymbal gland, Clitoral gland
	Mice	0.05	84	Liver, blood vessels
AαC	Mice	0.08	98	Liver, blood vessels
MeAαC	Rats	0.02,0.01	100	Liver
	Mice	0.08	84	Liver, blood vessels
IQ	Rats	0.03	72	Liver, small and large intestines, Zymbal gland, Clitoral gland ,skin
	Mice	0.03	96	Liver, forestomach, lung
MeIQ	Rats	0.03	40	Liver, intestine, Zymbal gland, Skin, oral cavity, mammary gland
	Mice	0.04,0.01	91	Liver, forestomach
MeIQx	Rats	0.04	61	Liver, Zymbal gland, clitoral gland, skin
	Mice	0.06	84	Liver, lung, hematopoietic system
PhIP	Rats	0.04	52	Large intestine, mammary gland, prostate
	Mice	0.04	82	Lymphoid tissue

2.1.7 Metabolism of HAs and their modification of DNA (Wakabayashi *et al.*,1992; Wagabayashi and Sugimura,1998)

Heterocyclic amines are metabolically activated by cyto-chrome P450s with conversion of an amino group to a hydroxyamino group. The principal cytochrome P450 isozyme responsible for this conversion is induced in rat liver by 3-methylcholanthrene. Recent investigations using cloned complementary DNAs for various molecular species of cytochrome P450s driven by vaccinia virus expression vector indicated that cytochrome P450IA2 is the most effective for metabolic activation of heterocyclic amines. Hydroxyamino derivatives of heterocyclic amines are further activated by forming esters with acetic acid, sulfuric acid, and proline. These esters may be the ultimate forms producing DNA adducts IQ, MeIQ, MeIQx, PhIP, Trp-P-2, Glu-P-1, and MeA α C have been reported to form mainly adducts at the C-8 position of the guanine base in DNA. These DNA adducts should be responsible for the genetic alteration leading to carcinogenesis. In DNA adduct formation by carcinogenic heterocyclic amines was detected by the ^{32}P -postlabeling method (Synderwine *et al.*,1988). All heterocyclic amines examined except PhIP produced higher levels of DNA adducts in the liver than in other organs : with PhIP, which is not a hepatocarcinogen, the adduct level in the liver was lower than in the other organs, including the lung, pancreas, and heart (Robbana-Barnet *et al.*,1996). Furthermore, the DNA adduct levels of various heterocyclic amines in rat liver correlated well with the carcinogenic potencies of these compounds in the liver. Thus, the levels of DNA adducts formed by carcinogenic doses of heterocyclic amines in the liver are probably reliable indicators of their hepatocarcinogenicities.

The pharmacokinetics of heterocyclic amines are important. Ingested heterocyclic amines are absorbed from the intestine, and their metabolic activation and inactivation occur mainly in the liver. Hydroxylation of ring carbons followed by conjugation leads to their inactivation, and detoxified forms of heterocyclic amines are excreted in the urine and feces. However, the pattern of *in vivo* metabolism differs for different heterocyclic amines. PhIP is absorbed from the intestine and most of it is excreted as PhIP in the bile and feces in rats. However, in mice treated with polychlorinated biphenyls, little unmetabolized PhIP is detected in the feces.

2.1.8 Quantification of HAs in cooked food and human urine

In general, HAs are present in cooked foods at minute levels. There are now many clean-up methods available to determine precisely their content in specific food (Wakabayashi and Sugimura, 1998). The procedures consist of blue-cotton treatment, HCl-methylene dichloride partition, and conventional and high-performance liquid chromatography. An electrochemical method is effective for detecting imidazoquinoline and imidazoquinoxaline derivatives.

Analytical data, measured by a combination of blue cotton treatment and HPLC, are available for nine HAs in broiled beef, chicken, and mutton, fried ground beef and fish, and food-grade beef extract (Wakabayashi *et al.*, 1993). Among the HAs detected, PhIP was the most abundant, present at levels of 0.56 to 69.2 ng/g. The level of MeIQx was second highest, at 0.64 to 6.44 ng/g, and the other HAs were found at levels of 0.03 to 2.50 ng/g.

Using a similar quantification method, levels of unmetabolized HAs in human urine have been determined. Four HAs—MeIQx, PhIP, Trp-P-1, and Trp-P-2—were

detected in 24 urine samples from ten healthy volunteers living in Tokyo and eating an ordinary diet (Ushiyama *et al.*,1991). However, they were not detected in urine samples of inpatients receiving parenteral alimentation. Because 1.2 to 4.3 % of an oral dose of MeIQx is reported to be excreted unchanged in the urine (Lynch *et al.*,1992), the daily exposure of the volunteers was estimated to be 0.3 to 3.9 µg per person. This is in the same range that has been calculated for other carcinogens such as N-nitrosodimethylamine and benzo[a] pyrene (Wakabayashi *et al.*,1993). Urinary levels of MeIQx also are reported to be positively associated with frequencies of cooked meat intake in residents of Los Angeles (Ji *et al.*,1994). These observations strongly suggest that humans are continuously exposed to HAs derived from foods in their daily life.

2.1.9 Factors to be considered for risk evaluation (Wakabayashi and Sugimura,1998)

Although it is evident that, in comparison with the dose necessary to induce tumors in rodents in long-term feeding experiments, the magnitude of HAs exposure in humans may not be sufficient to itself produce cancers, we need to pay attention to the presence of somatic cells that already have mutations due to various other genotoxic xenobiotics and autubiotics. Human carinogenesis is understood to be a multi-step process that involves multiple genetic alterations, additional mutations could be critical to complete the conversion of normal to malignant cells. Mutations causing genomic instability because of impaired control of DNA replication and DNA repair are particularly important in this respect, because an explosive increase in genetic alterations may result. Cancer cells arise from stem cell populations and the

number of these cells is proportional to the total number of cells in the body. Human weighs 250 times more than a rat, therefore the target cell number size of a human is 250 times greater than that of a rat, meaning that the chance of being targeted by genotoxic agents is 250 times greater in humans than in rats (Sugimura *et al.*,1994). A long life span also increases the chance of a single somatic cell acquiring a sufficient number of mutations. From these observation, HAs clearly cannot be assumed to be without significance based on the ratio of the HAs intake dose to the HAs carcinogenic dose extrapolating from animal experiments.

2.1.10 Epidemiology

Epidemiologic surveys have indicated positive links between consumption of cooked meat and fish and an elevated risk of various kinds of cancers. Consumption of well-browned meat was found to be associated with an increased risk of colorectal cancers (Schiffman and Felton,1990; Gerhardsson *et al.*,1991). Ward *et al.* (1997) reported a link between high intake of cooked meat and risk of stomach cancer. They indicated that length of cooking time is an important factor in cancer risk. There have been four other studies that showed a positive relationship between intake of cooked meat and increased risk of pancreatic, urothelial, mammary, and esophageal neoplasms (Norell *et al.*,1986; Steineck *et al.*,1990;De Stafani *et al.*,1997; Ward *et al.*,1997) However, one study showed no correlation between HAs intake and cancer development (Augustsson *et al.*,1995).

2.2 Nitrite and Gastric Cancer

2.2.1 Risk factors of gastric cancer

Life style, particularly diet and nutrition are relevant to neoplastic disease in the gastrointestinal tract. Comparison of high and low risk dietary factors responsible for large bowel, stomach and esophagus are summarized (Table 4). Data from several types of epidemiological studies, such as case-control, ecological, migration, and family studies, have revealed various etiological factors for stomach cancer, including a diet rich in salted or smoked food and low in vitamin C. Exposure to nitrate and nitrite, precursors of carcinogenic N-nitroso compounds, has also been correlated with gastric cancer mortality (Mirvish,1975). N-nitroso compounds are among the most potent chemical carcinogens in laboratory animals and certain N-nitrosamides have been shown to induce adenocarcinomas of the glandular stomach resembling human gastric cancer (Sugimura and Fujimura,1967).

2.2.2 N - nitroso compounds

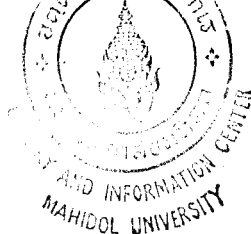
The N - nitroso compounds represent a major class of important chemical carcinogens and mutagen that have been implicated as hazardous substances in the environment. These compounds are produced by the reaction of nitrite with certain nitrogen compounds (Mirvish,1975) , such as secondary or tertiary amines in an acidic medium. Nitrosation can also occur in the mammalian stomach if nitrite and amine (or other precursors) are present simultaneously. Nitrites are added to some cured meat and fish products for the purposes of enhancing color, improving flavor and preventing growth and toxin formation by *Clostridium botulinum*. The last function is the most important and no satisfactory substitute for nitrites is available. Nitrate may

also play a role, as they can be converted into nitrite by bacteria in the oral cavity. Recently, nitrite and nitrate have been found to be produced from arginine in mammalian cells, and immunostimulants are known to enhance the synthesis of nitrite and nitrate (Kikugawa and Nagao.1991).

Table 4 Comparison of high and low risk dietary factors for cancer in specific organs (Weisburger and Horn,1984)

Organ	Population Lower Risk	Dietary Factors Lower Risk	Population High Risk	Dietary Factors High Risk
Esophagus	USA. Utah : Rural Norway	Less alcohol and tobacco use	France. Calvados Normandy USA lower socio-Economic groups Eastern Iran	Extensive alcohol and tobacco use Alcohol, smoking Low intake Vitamins C, A ; ? Opium use ? Dietary carcinogen Low vitaminC and E
			Central China	
Stomach	USA	Fresh fruit, salads. Vitamins C and E	Japan, Chile, Colombia	Salted, pickled food nitrate in soil and water; low vitamins C, E
Colon	Japan	Low fat	USA. Western Europe. New Zealand. Australia Scandinavia	High fat, low fiber, fried food
	Mormons Seventh Day Adventists	Higher fiber Low or no fried food, higher Fiber	USA. In general USA in generalIbid.	Ibid.
	Finland	Higher fiber Lower fried food	USA in general. Denmark	Ibid.

N - nitroso compounds are divided into the nitrosamines derived from dialkyl, alkaryl, diaryl or cyclic secondary amines : and the nitrosamides, derived from N - alkylureas, N - alkyl carbamates and simple N - alkylamides. Most tested nitrosamines and nitrosamides have proved to be strong carcinogen (Mirvish,1975). The N - nitroso compounds produce reactive electrophiles, which can bind to cellular



nucleophiles, among them DNA (Shepard *et al.*,1987). The carcinogenic potency of these compounds is thought to rest primarily on their ability to form promutagenic DNA adduct (Shepard *et al.*,1987). Direct-acting genotoxic N - nitrosamidines and nitrosamides such as N-alkyl-N'- nitro-N-nitrosoguanidine and N-alkyl-N-nitrosoarea have been known to induce cancer in the glandular stomach of experimental animals (Kikugawa and Nagao.1991). These types of direct-acting genotoxic compounds can be produced by reaction of their precursors if present in foods with nitrite under acidic conditions similar to those of gastric juice. Recent hypotheses for the development of gastric cancer suggest that exposure in the stomach to direct-acting genotoxic N-nitroso compound, formed endogenously, may be involved (Mirvish,1983).

Human exposure to carcinogen N - nitroso compounds may result from ingestion or inhalation of performed compounds in the environmental or from nitrosation of amino precursors in the body (Katiya *et al.*,1992). It has been suggested that the endogenous formation of N - nitroso compounds from ingested precursor is probably the largest single sources of exposure to these compounds for the general population (Ohshima and Bartsch.1981).

2.2.3 Nitrosamide as possible etiologic agents for gastric cancer

The N - nitrosamide formed in the human stomach by interaction between nitrite and suitable amine precursors have therefore been suspected of playing an important role in gastric carcinogenesis (Shepard *et al.*,1987). Several amines, which show direct acting genotoxicity, after nitrosation in vitro, have been identified in food frequently consumed in high-risk areas for stomach cancer. They include 4-chloro-6-methoxyindole in fava beans consumed in Columbia (Yang *et al.*,1984) , several

indole derivatives such as tyramine and β -carboline derivatives in Japanese soy sauce (Wakabayashi *et al.*,1987). The stomach is the preferred *in vivo* site because amide nitrosation is catalyzed by acid, proceeding ten times faster for each one - unit drop in pH (Mirvish,1975). Nitrosamides are unstable and hence might act on the stomach if they were produced there.

2.2.4 Nitrosamide precursors in foods

If the nitrosamide theory is correct then gastric cancer should be associated with the consumption not only of nitrate and nitrite but also of food containing compounds that react with nitrite to yield nitrosamide, i.e., nitrosamide precursors.

Several research groups looked for the presence of nitrosatable mutagen precursors in foods, to produce direct-acting mutagens by reaction with nitrite. Fava beans commonly eaten in Colombia (Yang *et al.*,1984) where stomach cancer incidence is high showed direct-acting mutagenicity towards *Salmonella* strains after nitrite treatment. Japan also has a high incidence of stomach cancer, and Japanese fish, soy sauce, bean paste and fish sauce which are favorites of the Japanese showed direct-acting mutagenicity on *S. typhimurium* TA100 after treatment with nitrite (Marquardt *et al.*,1977; Wakabayashi *et al.*,1983). Wakabayashi *et al.*(1984) have made extensive studies on the appearance of direct-acting mutagenicity of various foodstuffs produced in Japan and Southeast Asia on nitrite treatment (Wakabayashi *et al.*,1984). After nitrite treatment, various kinds of pickled vegetables and sun-dried fishes produced in Japan showed direct-acting mutagenicity on *S. typhimurium* TA100, inducing 1,900 to 18,000 revertants/g. Kimchis, sundried fishes, sun-dried squid, soy sauces, fish sauces, bean pastes, and shrimp paste produced in Korea, the

Philippines, and Thailand also showed direct-acting mutagenicity after nitrite treatment. They also found that various broiled meats and fish contained nitrosatable mutagen precursors (Yano *et al.*, 1988). Broiled chicken, pork, mutton, beef, and sun-dried sardine were found to yield direct-acting mutagenicity after nitrite treatment. In contrast, raw meat showed no mutagenicity after nitrite treatment. Treatment of broiled chicken with 0.5 to 3 mM nitrite, which is a physiologically feasible concentration in the human stomach under some conditions, induced direct-acting mutagenicity (Yano *et al.*, 1988).

In a recent study, Kagnsadalampai *et al.* (1996) found that nitrite-treated products of extracts from smoked and charcoal-broiled fish, chicken and pork demonstrated the mutagenicity towards *Salmonella typhimurium* strain TA98 and TA100 in the absence of metabolic activation. Shibamoto (1983) found that soy sauce treated with 2000 ppm nitrite was mutagenic and suggested that N-nitrosodimethylamine, N-nitrosodiethylamine and N - nitrosoproline as possible mutagenic products in nitrite treated soy sauce. Namaki *et al.* (1984) tested the mutagenicity of spices after treatment with nitrite. Among several spices treated with nitrite, pepper exhibited the strongest mutagenic activity by the Ames method. No mutagenicity was observed for spices alone. Mutagen production was observed between pH 2 and 6, with the maximum between pH 3 and 3.5 and the reaction was very fast at 40°C or above, even at the low levels of nitrite permitted by legal regulations. The mutagenicities of spice-nitrite reaction products were completely inactivated by S9 mix, but the activity of pepper-nitrite products toward TA100 remained unchanged.

Aminoimidazoquinoline, aminoimidazoquinoxaline, and aminoimidazopyridine compounds, which are found in cooked foods and are mutagens to bacteria with S9 mix before nitrite treatment, became mutagenic to TA98, TA100, and *E. coli* WP2 uvrA without S9 mix following treatment with 50 mM nitrite (Sasagawa *et al.*, 1988). Formation of a nitro derivative by nitrite treatment was observed with mutagenic and carcinogenic 2-amino-3-methylimidazo [4,5-*f*]-quinoline (IQ). IQ was converted to 3-methyl-2-nitroimidazo [4,5-*f*]-quinoline, showing mutagenicity towards *Salmonella* strains without S9 mix, after 50 mM nitrite treatment at pH 3. In contrast, treatment of IQ with a much lower amount of nitrite (2 mM) produced no effect (Sasagawa *et al.*, 1988). The other aminoimidazoquinoline, aminoimidazoquinoxaline, and aminoimidazopyridine compounds must react with nitrite in a similar manner to IQ.

2.2.5 Prevention of nitrosation

Many compounds including food constituents and food additives are known to inhibit the formation of nitrosamines and N-nitroso compounds. Since the mechanisms for the formation of N-nitroso compounds and the active nitrosating agents are different depending on the reaction conditions and the nitrosatable amines or amides, the inhibitory effect of each compound is not uniform. Inorganic salts, alcohols, and monophenolics are inhibitory and stimulatory depending on the nitrosatable compounds and reaction condition. Sorbic acid and certain monophenolics produce hazardous mutagenic compounds by inhibiting nitrosamine formation. Many dietary constituents, such as vitamin C and E, polyphenol and complex mixture as fruit juices, may inhibit the nitrosation reaction (Bartsch *et al.*, 1988). Epidemiological studies showed a protective effect of vitamin C on gastric

cancer ; all seven studies evaluating the effect of vitamin C on cancer of the stomach found a statistically significant protection of approximately two fold (Block,1991). Another constituent of the human diet that has been reported to inhibit nitrosation is green tea. Tanaka *et al.*(1998) found that green tea extracts show an inhibitory effect on nitrosamine formation . In contrast Japanese green tea extracts show an enhancing effect on nitrosamine formation (Nakamura and Kawabata,1981). Results from epidemiology studies concerning the relation between tea consumption and various human cancers are inconclusive (Yang and Wang,1993).

2.3 Tea

Tea originated in Southern China and is consumed by over two third of the world's population. The Chinese used tea as a medicinal drink as early as 3000 BC. and by the end of the sixth century as a beverage. Since tea has not been only an effective pharmaceutical activity, but also an excellent beverage, its cultivation area has gradually expanded in the world, especially in tropical countries. An estimated 2.5 million metric tons of dried tea are manufactured annually. Of this amount, about 20% is green tea, mainly consumed in Asian countries where tea is a major beverage; about 78% is black tea, mainly consumed in the Western nations and some Asian countries ; and about 2% is Oolong tea, mainly produced and consumed in southeastern China (Yang and Wang,1993; Kuroda and Hara,1999).

2.3.1 Tea production (Harold and Graham,1992)

Tea is the plant, leaf, or beverage originating from what is now considered

a single species-*Camellia sinensis* (L.) O. Kuntze. Two major varieties are recognized-*sinensis* and *assamica*. In the field the obvious difference observed between the major varieties is leaf size. *Var. sinensis* is small-leaved (5-12 cm). *Var. assamica* may have leaves up to 20 cm in length.

The term green tea refers to the product manufactured from fresh leaf while preventing oxidation of the polyphenolic components. Black tea manufacture is carried out so as to ensure a high degree of enzymatically catalyzed aerobic oxidation of the leaf polyphenols followed by a series of chemical condensations. Oolong tea is partially oxidized.

Manufacture of green tea

The primary goal in the manufacture of green tea is the preservation of the leaf catechins. The steps include plucking, rapid enzyme inactivation by steaming or pan firing, rolling, and high temperature air-drying. Glycosides of aromatic and terpene alcohols that found in the growing leaves are rapidly hydrolyzed after plucking to form the free volatile alcohols.

In Japan enzyme inactivation is generally carried out by steaming in large rotating cylinders for 20-50 sec. followed by a series of twisting and drying steps designed to produce a desirable appearance and reduce moisture gradually to about 3%. Tea so produced is called sencha and is graded into several quality categories.

Most Chinese green tea is produced by rapid pan firing or roasting followed by twisting and drying. In India a China-style green tea is manufactured by using long, rotating heated cylinders for the inactivation step. Dwell time may be 7-10 min.

The several styles of green tea manufacture must result in compositional differences. It would be highly desirable for all reports of feeding studies or in vitro experimentation to specify clearly the tea processing techniques, which were utilized.

The rolling process imparts a twist, which improves appearance. During the final drying step many new aromatic compounds are formed which impart important characteristics of green tea flavor. Green tea composition is similar to that of the fresh leaf with regard to the major components.

Manufacture of black tea

Most of the world's tea is consumed as black tea beverage. Basic steps in black tea production are plucking, withering, maceration (rolling), and drying. Plucking is predominantly a hand operation but is being supplanted in some areas with mechanical harvesters, usually with the inclusion of more coarse leaf, resulting in decreased catechin levels. Withering lowers the moisture level and renders the leaf more workable in preparation for the maceration step. It is that latter step which disrupts cell structure and initiates oxidation. The processing equipment is designed to ensure intimate contact among the polyphenol oxidase, the polyphenols, and the atmospheric oxygen.

Oxidation is allowed to proceed for 45-90 min, depending on ambient temperature, the nature of the leaf, and the style of tea being produced. Temperatures between 20 and 30°C with high humidity and ample exposure of the macerated leaf to air represent desirable oxidation conditions. During this period leaf color and aroma change markedly.

The enzymatic oxidation step results in the formation of quinones and is the key reaction that initiates all of the subsequent changes, which occur. Until the early part of the century the process was believed to be a microbial fermentation.

Manufacture of oolong tea

The manufacture of oolong teas allows for a short period of oxidation. The process is carried out in several different ways and products vary with respect to the degree of catechin oxidation, which is effected. Pouchong tea is considered to be about 1/3 fermented compared with black tea ; normal oolong tea is considered to be about 1/2 fermented. Oolong tea extracts contain catechins at a level of 8-20% of the total dry matter. Oolong tea composition would be expected to be intermediate between green and black teas.

2.3.2 The chemistry of tea

The varietal differences, environmental effects, methods of processing and modes of propagation all suggest that the chemical composition of tea leaves should also vary. Data shown in Table 5 reflect a representative tea but variation may be considerable (Harold and Graham,1992).

Table 5 Chemical component of unprocessed tea (Harold and Graham,1992)

Chemicals	% of dry weight	Chemicals	% of dry weight
Polyphenols	36	Carbohydrates	25
Methyl xanthines	3.5	Protein	15
Amino acids	4	Lignin	6.5
Organic acids	1.5	Lipids	2
Carotenoids	<0.1	Chlorophyll, etc.	0.5
Volatiles	<0.1	Ash	5

The most important chemicals present in tea, which are of considerable pharmacological significance, are the polyphenols and caffeine. The amounts of polyphenols depend on the genetic makeup and environmental factors such as climate, light, rainfall, temperature, nutrient availability, and leaf age.

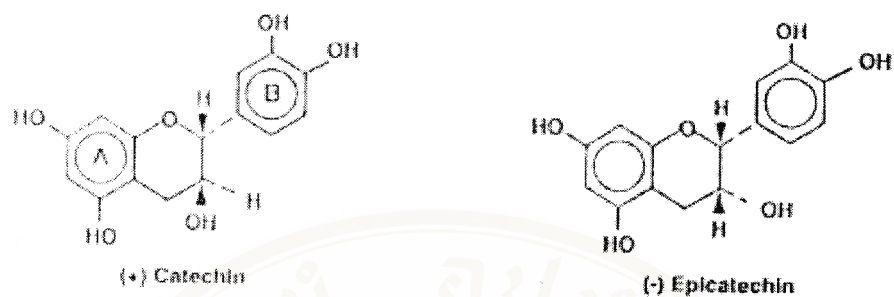
Commercial green tea is made by steaming or drying fresh tea leaves at elevated temperatures. Its chemical composition is similar to that of fresh tea leaves. Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids. Most of the green tea polyphenols are flavanols, commonly known as catechins. Simple catechin structures are shown in Figure 4(A). Two configurations are possible but most of the catechin mass is in the so – called “epi-” form.

When three hydroxyl groups are present on the “B” ring the substances are known as gallocatechins as shown in Fig. 4(B). Another variation results from the esterification of the OH group on the pyran ring with gallic acid. The catechin gallates are illustrated in Fig. 4(C). The principal catechins are present at high concentrations in young tea leaf.

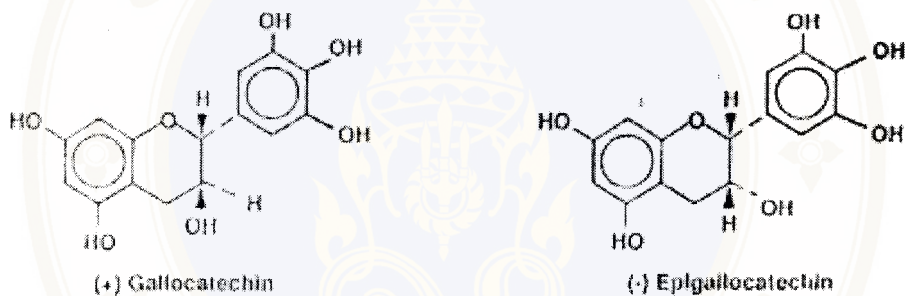
Catechin concentration is highly dependent on leaf age. The leaf bud and the first leaf are richest in epigallocatechin gallate (Harold and Graham,1992). Catechin levels also vary greatly with varietal differences. Green tea is often manufactured from tea leaf with somewhat lower catechin levels than leaf used for black tea manufacture.

Catechins are the predominant group of substances in green tea and they are the most significant of all tea components. They play the most important role in the series of oxidations and condensations, which occur during the production of black tea.

A



B



C

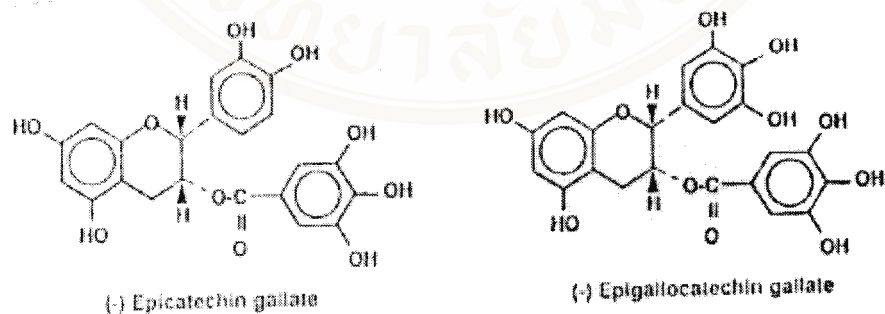


Figure 4 Structure of principal fresh tea leaf catechins (Harold and Graham,1992)

The catechins are colorless, astringent, water-soluble compounds. They are readily oxidizable, although their oxidation potentials vary. This property has been exploited through their use as food antioxidants. They retard rancidity in fats and oils by quenching free radical peroxide activity brought about by aerobic oxidation. Based on the concentrations required for 50% inhibition of oxidation, the inhibitory effect of some tea leaf catechins on the aerobic oxidation of linoleic acid is similar to that of BHA (Tanizawa *et al.*,1984).

In biological systems oxygen is an important acceptor of electrons, leading to the formation of active oxygen and hydroxyl free radicals. Scavenging effects of green tea extracts and green tea polyphenol fractions are superior to those of ascorbic acid (vitamin C) and tocopherol (vitamin E) with respect to some active oxygen radicals but are less pronounced with hydroxyl free radicals (Zhao *et al.*,1989). Effects depend on the particular free radical system under investigation. The galocatechin and the catechin gallates exhibit the strongest radical quenching properties (Uchida *et al.*,1987).

In the manufacture of black tea, the quinone derived from a simple catechin or its gallate may react with a quinone derived from a galocatechin or its gallate to form bisflavanol, theaflavin, thearubigins, and other oligomers in a process commonly known as “fermentation.” Theaflavins (about 1%-2% of the total dry matter of black tea) possess benzotropolone rings with dihydroxy or trihydroxy substitution systems, which give the characteristic color and taste of black tea. About 10%-20% of the dry weight of black tea is due to thearubigens, which are even more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well

characterized. The structures of theaflavins and thearubigins are shown in Figure 5 (Yang and Wang, 1993).

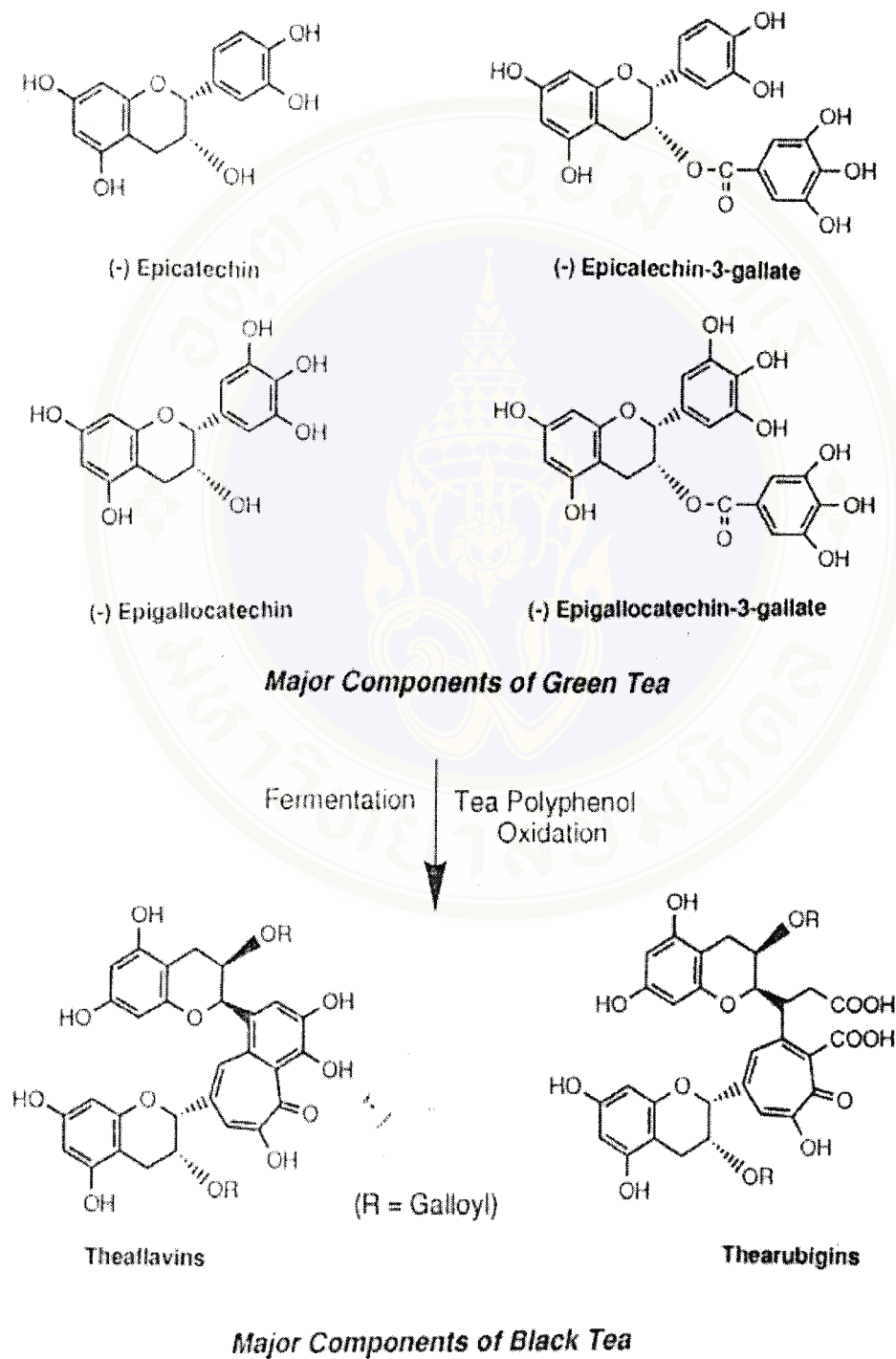


Figure 5 Structures of major tea polyphenols in green tea and black tea

Oolong tea, a partially fermented tea, contains monomeric catechins, theaflavins, and thearubigins. Some characteristic components, such as epigallocatechin esters, theasinensins, dimeric catechins, and dimeric proanthocyanidins, are also found in oolong tea (Yang and Wang, 1993; Harold and Graham, 1992)

The flavanols are easily oxidized to the corresponding *O*-quinones. These flavanols and quinones can function as either hydrogen acceptors or hydrogen donors. In addition, tea polyphenols effectively interact with reactive oxygen species. In the flavanol structure, the 5- and 7-dihydroxy groups and 1-oxygen make the carbons at positions six and eight strongly nucleophilic. During enzyme oxidation or non-enzyme oxidation, including autooxidation or coupled oxidation, tea flavanols may undergo oxidative condensation via either C-O or C-C bond formation in oxidative polymerization reactions. Tea polyphenols also have high complexation affinity to metals, alkaloids, and biologic macromolecules such as lipids, carbohydrates, proteins, and nucleic acids.

The term “tannins” has been used by many to describe certain tea constituents. In industrial and botanic literatures, tannins are characterized as plant materials that give a blue color with ferric salts and produce leather from hide. Thus, tannins are a group of chemicals, usually with large molecular weights and diverse structures. Monomeric flavanols, the major components in green tea, are precursors of condensed tannins. It would be more appropriate to use the term “tea polyphenols” or “tea flavanols” because they are quite distinct from commercial tannins and tannic acid. “Herbal teas,” which contain various known or unknown components, are quite different from tea (*Camellia sinensis*). The constituents of the polyphenols in extracts of green tea and black tea are shown in Table 6 (Kuroda and Hara, 1999).

EGCG is the highest component of the epicatechins in green tea leaves and is followed by EGC, EC and ECG. In black tea, theaflavins such as TF, TFA, TFB and TFDG are contained in high amounts. A cup (200 ml) of green tea (Gun Powder, Hangzhou, China) contains about 142 mg EGCG, 65 mg EGC, 28 mg ECG, 17 mg EC, and 76 mg caffeine (Yang and Wang,1993).

Table 6 Catechins in green tea and black tea (Kuroda and Hara,1999)

Catechins	Green tea (µg/ml)	Black tea (µg/ml)
Catechins	1064	300
(+)Catechin (C)	21	20
(+)Gallocatechin (GC)		
Epicatechins		
(-)-Epicatechin (EC)	98	37
(-)-Epicatechin-3-gallate (ECG)	90	73
(-)-Epigallocatechin (EGC)	411	42
(-)-Epigallocatechin-3-gallate (EGC)	444	128
Theaflavins	0	64
Theaflavin (TF)	0	22
Theaflavin gallate A(TFA)	0	20
Theaflavin gallate B(TFB)	0	13
Theaflavin digallate (TFDG)	0	9
Thearubigins		
Procyanidine (PC)	3	
Procyanidin gallate (PCG)		
Proderfinidine (PD)		
Proderfinidine gallate (PDG)		

2.3.3 Epidemiological studies of tea and cancer

The Committee on Diet and Health of the National Research Council and a monograph produced by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO, 1991) indicate that epidemiology study on tea and cancer so far have yield inconsistent results. The relationships between tea

consumption and cancer incidence in epidemiological and experimental studies was reviewed (Yang and Wang,1993; Kuroda and Hara,1999). A summary of case-control studies on the relationships between tea consumption and human cancer is shown in Table 7 (Yang *et al.*,1996).

Table 7. Case-control studies on the association between tea consumption and human cancers (Yang *et al.*,1996)

Organs	Number of reports		
	Negative	Positive	No relationship
Bladder and urinary tract	-	-	17
Breast	-	-	6
Colon and rectum	4	1	6
Esophagus	1	7 *	6
Kidney	-	1	5
Liver	-	-	1
Lung	-	1	1
Nasopharynx	-	-	3
Oral and tongue	1	-	1
Pancreas	2	1	8
Prostate	-	-	1
Stomach	4	1	8

*6 studies were with hot tea.

Tea consumption and esophageal cancer in humans

It has been suggested previously that excessive consumption of tea may be a causative factor for esophageal cancer (Kapadia *et al.*,1983). Several case-control studies, however, showed that there was no association between drinking of tea at normal temperature (35-47⁰ C) and esophageal cancer, but ingestion of very hot tea (55-67⁰ C) was associated with a two- to three-fold increase in risk for esophageal

cancer(Kaufman *et al.*,1965; Bashirov *et al.*,1968; Cook-Mozaffari *et al.*,1979;Victora *et al.*,1987; La Vecchia *et al.*,1992;). The high temperature of hot tea, rather than the chemicals in tea, was suggested to be an important etiological factor in human esophageal cancer (WHO,1991). A recent small case-control study conducted in northern China. (Hu *et al.*,1994), reported that high temperature of meals and drinks had a strong association with esophageal cancer risk, but after adjustment of the temperature, the tea consumption was still a risk factor. On the other hand, a recent report by Gao *et al.* (1994) indicated that, in a case-control study on 902 patients and 1552 controls in Shanghai, frequent consumption of green tea was associated with a lower incidence of esophageal cancer. After adjustment for age and other possible confounding factors, a protective effect of green tea drinking on esophageal cancer was observed among women: odds ratio, 0.50; 95% confidence interval, 0.30-0.83, and this risk decreased (p for trend < 0.01) as the quantity of tea consumed increased. Among men, the protective effect was not statistically significant. Among nonsmokers and non-alcohol-drinkers, statistically significant decreases in risk were observed among tea drinkers for both men (69 cases and 192 controls) and women (194 cases and 564 controls).

Tea consumption and stomach cancer in humans

Early correlative and cohort studies indicated that tea consumption was either negatively or positively associated with stomach cancer (Stocks,1970; Kinlen and McPherson,1984; Oguni *et al.*,1992). Many case-control studies on stomach cancer and tea consumption conducted in Buffalo (Graham *et al.*,1967), Kansas City (Higginson,1966), Nagoya (Japan) (Tajima and Tominaga,1985), Piraeus (Greece), (Trichopoulos *et al.*,1985), Milan (Italy) (La Vecchia *et al.*,1992), Spain,(Agudo *et*

al.,1992), and Turkey (Demirer *et al.*,1990) all indicated that there was no significant association between tea consumption and cancer of the stomach. A very small case-control study in Taiwan (10 cases and 14 controls) suggested that green tea consumption was a risk factor for gastric cancer (Lee *et al.*,1990). On the other hand, some recent case-control studies suggested that tea consumption reduced the risk of stomach cancer in Kyushu (Japan) (Kono,1992), Shanghai (China) (Yu and Hsie,1991), northwestern Turkey (Memik *et al.*,1992) and central Sweden (Hansson *et al.*,1993). For example, in Kynshu (139 cases and 2574 controls), individuals consuming green tea more frequently or in larger quantities (10 or more cups a day) tended to have a lower risk for gastric cancer (Kono,1992). In a population-based case-control study (669 cases and 880 controls) in central Sweden, tea (mostly black tea) consumption was associated with lower incidence of gastric cancer (Hansson *et al.*,1993).

Different etiological factors and molecular mechanisms may be involved even in cancers of the same organ site. Therefore, it is not surprising that a clear-cut conclusion on the relationship between tea consumption and human cancer has not been found. Many confounding factors, such as cigarette smoking, alcohol drinking, and the temperature of tea, have been adjusted in many newer studies, and these results tend to be more informative. If we assume that N-nitroso compounds are major causative factor for gastric cancer in Kyushu and Shanghai-a hypothesis remaining to be substantiated then the protective effect of tea may be due to its ability to inhibit the endogenous formation of these nitroso compounds via nitrosation reactions. (Yang and Wang,1993; Kohlmeier *et al.*,1997).

2.3.4 Laboratory studies on tea and cancer

Both mutagenic and antimutagenic activities for tea and tea constituents have been reported. Enzymatic hydrolyzates of the constituents in green tea and black tea infusions were found to be mutagenic in the Ames test, either in the presence or absence of a microsomal activation system, and the mutagens were later found to be common flavonoids: kaempferol, quercetin, and myricetin (Nagao *et al.*,1979; Uyeta *et al.*,1981). Freshly brewed tea was also reported to cause mutation in bacteria in the absence of a microsomal activation system (Alejandre-Duran *et al.*,1987; Ariza *et al.*,1988). This mutagenicity was inhibited by catalase. The hydrogen peroxide formed in the assay mixture is believed to play an essential role in the mutagenicity observed.

2.3.4.1 Antimutagenic activity

Antimutagenic activity of tea polyphenols has been extensively investigated, mostly in microbial systems and in mammalian in vitro and in vivo systems. These studies, reviewed by Kuroda and Hara (1999), are shown in Table 8.

2.3.4.2 Anticarcinogenic activity

Anticarcinogenic activity of tea polyphenols was also reported against various tumors induced by carcinogens given to rats and mice. These studies, reviewed by Kuroda and Hara (1999), are shown in Table 9.

Table 8 Studies on antimutagenicity of tea polyphenols (Kuroda and Hara, 1999)

Tea polyphenols	Mutagens	Test systems
ECG, EGCG	Trp-P-2, <i>N</i> -OH-Trp-P-2	<i>S. typhimurium</i> TA98, TA100
EGCG	B[a]P diol oxide	<i>S. typhimurium</i> TA98, TA100
EGCG	Spontaneous mutations	<i>B. subtilis</i> NIG1125
Tannic acid	UV, 4NQO, γ -rays, MNNG	<i>E. coli</i> B/rWP2 WP2s
GC, ECG, EGC, EGCG	UV, 4NQO, MNNG	<i>E. coli</i> B/rWP2 WP2s ZA159
Green and black tea extracts	MNNG	<i>E. coli</i> B/rWP2 <i>S. typhimurium</i> TA100
Black and green tea extracts	PhIP	<i>S. typhimurium</i> TA98
Polyphenols 60, 100, B	Aryl- and heterocyclic amines, AFB ₁ , B[a]P, DBE, 2-NP	<i>S. typhimurium</i> TA98 TA100, TA1535
Gallic acid, methyl gallate, Catechins, TF, tannic acid	PhIP	<i>S. typhimurium</i> TA98
Black tea TF, TF2A, TFDG	H ₂ O ₂	<i>S. typhimurium</i> TA104
Black tea extract	IQ, B[a]P, AFB ₁ , Trp-P-1, Glu-P-1	<i>S. typhimurium</i> TA98 TA100
EC, EGCG	4NQO, EMS	Chinese hamster V79 cells, 6TG ^r mutation
Green tea extract	AFB ₁	Rat bone marrow cells, chromosome aberrations
Green tea extracts	IQ, Glu-P-1, B[a]P, DMBA, 2AF, 2AA, 9AA, MNNG	<i>S. typhimurium</i> TA98

Abbreviations: EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin gallate; EGCG, (-)-epigallocatechin-3-gallate; GC, (+)-gallocatechin; TF, theaflavin; TFA, theaflavin gallate; TFDG, theaflavin digallate; 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine; 2AF, 2-aminofluorene; AFB₁, aflatoxin B₁; B[a]P, benzo[a]pyrene; DBE, 1,2-dibromoethane; DMBA, 7,12-dimethylbenzanthracene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 2-NP, 2-nitropropane; 4NQO, 4-nitroquinoline

Table 9 Studies on anticarcinogenicity of tea polyphenols (Kuroda and Hara, 1999)

Tea polyphenols	Carcinogens	Animals	Organs
EGCG, crude extract	20 MC	Mouse	Sarcoma 180, Ehrlich solid tumors
Chinese tea	N-nitroso methyl Benzyl amine	Rat Rat	Solid tumors Esophageal tumors
Decaffeinated green and black teas	NMBA	Rat	Esophageal tumors
EGCG	MNNG	Mouse	Duodenum tumors
Green tea polyphenol	Azoxymethane	Rat	Colon cancer
EGCG	MNNG	Rat	Glandular stomach cancer
Green tea polyphenols		Rat	Gastrointestinal carcinoma
Green tea infusion	NDEA	Rat	Forestomach tumors
Green tea infusion	NDEA	Mouse	Forestomach tumors
Green tea catechin and EGCG		Human	Stomach cancer cells, KATO III
Green tea catechins	DMBA	Rat	Mammary gland
Green tea catechins	PhIP	Rat	Mammary gland tumors
Tea	DMBA	Rat	Mammary gland tumors
Green tea catechin and EGCG	DMH	Mouse	Large intestinal tumors
Tea, tea and milk	AOM	Rat	Colon crypts
EGCG	Spontaneous	Mouse Human	Hepatoma Hepatoma cell line
EC, EGC, ECG, EGCG, black tea extract, oolong tea extract	DEN	Rat	Spontaneous hepatoma
Green tea	AFB ₁	Human Rat	Hepatoma Glutathions-S-transferase positive foci in hepatocytes
Green tea	NDEA, B[a]P	Mouse	Lung tumor
Green tea catechin	DEN, DMH, MNU BBN, DHPN	Rat	Small intestinal tumors
EGC, EGCG		Human	Lymphoid leukemia cells
EGCG	Telocidine	Mouse	Skin tumors
Green tea	DMBA	Mouse	Skin tumors
TF, TFDG, EGCG	Metalloproteinase (type IV collagenase)	Mouse	Lung tumor cells LL2-Lu3
Green tea infusion		Mouse	Lung nodules,
EGCG	Urokinase	Mouse	Tumor

Abbreviations: AOM, azoxymethane; AFB₁, aflatoxin B₁; BBN, N-butyl-n-(4-hydroxy-butyl) nitrosamine; B[a]P, benzo[a]pyrene; DBE, 1,2-dibromoethane; DEN, N-diethyl-nitrosamine; DHPN, 2,2'-dihydroxy-di-n-propyl-nitrosaminebenzanthracene; DMBA, 7,12-dimethyl benzanthrane; DMH, 1,2-dimethylhydrazine; MC, mitomycin; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MNU, N-methylnitrosourea; NDEA, N-nitrosodiethylamine; NMBA, N-nitrosomethylbenzylamine; 2-NP, 2-nitropropane; 4NQO, 4-nitroquinoline

2.3.5 Biochemical mechanisms for inhibition of mutagenesis and carcinogenesis.

The aforementioned inhibitory effects of tea against mutagenesis and carcinogenesis have been attributed to the biologic activities of the polyphenols in tea. However, this concept remains to be further substantiated. The most noteworthy properties of tea polyphenols that may affect carcinogenesis are their antioxidative activities, inhibitory action against nitrosation reactions, modulation of carcinogen-metabolizing enzymes, trapping of ultimate carcinogens, and inhibition of cell proliferation-related activities (Yang and Wang,1993). The major green tea polyphenols-EGCG, EGC, ECG, EC, and (+)-catechin-are believed to have these activities, although they may vary quantitatively. In black tea, the levels of these flavanols are much lower than in green tea. It is thought that theaflavins, thearubigens, and other black tea polyphenols are also active in the mechanisms discussed here, although further study is required to confirm that hypothesis.

2.3.5.1 Antioxidative function of tea polyphenols

Tea polyphenols possess strong antioxidative activity via three mechanisms: (Yang and Wang,1993).

1) Because of the presence of the “catechol” structure, most tea polyphenols are strong metal ion chelators. They can bind and thus decrease the level of free cellular ferric and ferrous ions, which are required for the generation of reactive oxygen radicals by the Fenton and Haber-Weiss reactions.

2) Tea polyphenols such as EGCG, EGC, and ECG are strong scavengers against superoxide anion radicals and hydroxyl radicals-two major reactive oxygen species

that can damage DNA and other cellular molecules and can initiate lipid peroxidation reactions.

3) Tea flavanols can react with peroxy radicals and thus terminate lipid peroxidation chain reactions. Reactive oxygen species may play important roles in carcinogenesis through damaging DNA, altering gene expression, or affecting cell growth and differentiation (Cerutti,1989).

The concept that tea components may inhibit carcinogenesis through their antioxidative activities is supported by the recent findings that (+)-catechin inhibited NNK-induced DNA single-strand breaks in rat hepatocytes (Liu and Castonguay,1991). Oral administration of green tea inhibited the formation of 8-hydroxydeoxyguanosine in mice (Adamson *et al.*,1990), and topically-applied green tea polyphenols inhibited TPA-induced hydrogen peroxide formation in mouse epidermis (Laskin *et al.*,1992).

2.3.5.2 Inhibition of nitrosation reactions

N-Nitroso compounds, many of which are formed by the endogenous nitrosation of nitrogenous compounds, have been implicated as etiological factors for gastric, esophageal, and other cancers in many areas of the world (Bartsch and Montesano,1984; Yang and Lu,1990). Tea components have been demonstrated to react with the nitrosating species and thus inhibit nitrosation (Nakamura and Kawabata,1981; Tanaka *et al.*,1998). Oral feeding of tea infusion markedly inhibited esophageal tumorigenesis caused by nitrosoamine precursors in rats or mice (Xu and Han,1990; Oguni *et al.*,1992). The inhibition of N-nitrosoproline formation by green tea and black tea has also been demonstrated in human volunteers (Stich,1992; Wang

and Wu,1990). It was estimated that consumption of 3-5 g of tea per day may effectively block nitrosation reactions in humans. It should also be mentioned that at low concentrations, tea polyphenols can be nitrosated to form C-nitroso derivatives, which can nitrosate other compounds through transnitrosation reactions, thus serving as potential catalysts of nitrosation reactions (Yang and Wang,1993). This two-edged situation is similar to that with many antioxidants, including tea polyphenols, which may behave as pro-oxidants at low concentrations. The inhibition of nitrosation by tea components may be an important factor in affecting cancers caused by endogenously formed N-nitroso compounds.

2.3.5.3 Modulation of xenobiotic metabolizing enzymes

Most carcinogens require metabolic activation by cytochromes P450 or other enzymes in order to convert to electrophiles before exerting their carcinogenic effects. Green tea polyphenols (EC, ECG, EGC, and EGCG) were found to inhibit rat liver microsomal mono-oxygenase activities, including those of aryl hydrocarbon hydroxylase, -7-ethoxyresorufin O-deethylase, and 7-ethoxycoumarin O-deethylase (Wang *et al*,1988). Oral feeding of Chinese tea also inhibited NMBzA (N-nitroso methyl benzylamine)-induced rat esophageal DNA methylation (Chen, 1992). Oral administration of green tea polyphenols to mice for 4 weeks has been reported to cause moderate enhancement (a) in glutathione peroxidase, catalase, and NADPH-quinone oxidoreductase activities in small bowel, lung, and liver and (b) in glutathione S-transferase activity in small bowel and liver (Khan *et al.*, 1992). Elevation of these conjugating and antioxidative enzymes may be expected to have protective functions against carcinogenesis.

2.3.5.4 Trapping of activated forms of carcinogens

The structure of flavanols provides exceptionally strong nucleophilic centers at positions six and eight. This property enables flavanols to react with electrophilic carcinogenic species to form flavanol-carcinogen adducts and thus prevent tumorigenesis. The interaction of tea polyphenols with BPDE {(±)7β, 8α-dihydroxy-9α,10α-epoxy-7, 8, 9, 10-tetrahydrobenzo (a) pyrene], an ultimate carcinogen, and MNNG, a direct acting carcinogen, has been suggested (Stich *et al.*, 1982). Topical application of green tea polyphenols prior to BPDE treatment resulted in inhibition of skin tumor initiation in Sencar mice (Khan *et al.*,1988).

2.3.5.5 Inhibition of activities related to tumor promotion and cell proliferation

The induction of ornithine decarboxylase and protein kinase C activities by 12-tetra decanoylphorbol –13-acetate (TPA) is believed to be closely related to the tumor promotion activity of this compound. Topical applications of a green tea polyphenol fraction or EGCG resulted in a statistically significant inhibition of TPA-induced epidermal ornithine decarboxylase activity in different strains of mice (Wang *et al.*,1990; Huang *et al.*,1992). Inhibition of TPA-induced protein kinase C activity (Yoshiawa *et al.*,1992; Shirai *et al.*,1997) and inhibition of intercellular communication by green tea polyphenols have been reported (Ruch *et al.*,1989). It is proposed that EGCG interrupted the interaction of tumor promoters with their receptors on the cell membrane (Yoshiawa *et al.*,1992). The cyclo-oxygenase and lipooxygenase pathways of arachidonic acid metabolism may play a role in carcinogenesis, especially in the promotion of cell proliferation. Inhibitory effects of

green tea polyphenols on TPA-induced mouse epidermal lipo-oxygenase and cyclo-oxygenase activities in vivo have been observed (Katiya *et al.*,1992). Administration of black tea polyphenols inhibited the metabolism of arachidonic acid and decreased the level of plasma thromboxane-B2 formation in patients with hyperlipidemia accompanied by hyperfibrinogenemia (Lou,1987).

2.4 Aminopyrene-Nitrite Mutagenicity Model

Aminopyrene is a natural compound produced in human feces or in anaerobe incubation of 1-nitropyrene with fecal bacterial (Nagahara *et al.*,1986). 1-Nitro pyrene can be metabolized by human, rat and mouse intestinal microflora to 1-aminopyrene and others. The predominant metabolite produced by human, rat or mouse intestinal microflora following 12 h incubation with 1-nitropyrene is aminopyrene. Aminopyrene was known to be non-mutagenic when it was tested without metabolic activation (Kinouchi *et al.*,1982). Kato *et al.* (1991). demonstrated that aminopyrene treated with 4 equivalent amounts of nitrite at pH 3 and 37°C showed mutagenicity to *Salmonella typhimurium* TA98 and TA100 strain without metabolic activation. The results did agree with the work of Kangsadalampai *et al.* (1996) which stated that nitrite treated aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene towards *S. typhimurium*, both strains TA98 and 100 , in the absence of metabolic activation. Thus, the mutagenicity of aminopyrene and nitrite in acid condition has been established as a model for antimutagenicity studies of some chemical concerning the phenomenon occurred during stomach digestion.

2.5 The *Salmonella* Mutagenic Assay (Ames Test)

Genetic toxicology has become an integral part of the overall risk assessment process and has gained in recent times as a reliable predictor for carcinogenic activity. Genetic toxicity assays used most commonly today have been selected not only because of their large database, relatively low cost, and ease of performance, but because they have been shown to detect many rodent and, presumptively, human carcinogens. Consequently, genetic toxicity assays are used to predict the potential carcinogenicity of agents (Stellman *et al.*,1998). Bacterial mutagenicity assays have been widely used in genotoxicology research for two decades (Josephy *et al.*,1997). In particular, the Ames, an assay for point mutation, remains the most widely used in vitro genotoxicity assay (Kangsadalampai *et al.*,1996). The simplicity, sensitivity, and accuracy of this method for screening large number of environmental sources of potential carcinogens has resulted in its current use in over 1,000 government, industrial, and academic laboratories throughout the world. The potential of this method for use as a bioassay for the development of safe, useful chemicals raised many questions about the extend to which this kind of approach should be used in a program aimed at cancer prevention. The *Salmonella* test was first validated in a study of 300 chemicals, most of which were known carcinogens (McCann *et al.*,1975a; McCann and Ames,1976). 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).

The Ames test measures back mutation to histidine independence of histidine mutants of *Salmonella typhimurium* and can be conducted with strains that are also

repair deficient, possess abnormalities in the cell wall to make them permeable to carcinogens, and carry an R factor enhancing mutagenesis, or that are nitroreductase-deficient or responsive to oxidative mutagens. Hence these organisms are highly susceptible to mutagenesis, making them sensitive indicators. Tester strains TA98 and TA100 are recommended for general mutagenesis testing. TA98 was derived from TA 1538 by which plasmid pKM 101 was introduced. It can detect mutagens that cause 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 pair (Ames, 1971; Hartman *et al.*, 1971; Isono and Yourno, 1974; Ames *et al.*, 1975; Mc Cann *et al.*, 1975b). While TA100, the R-factor plasmid derivative TA1535, can detect mutagens that cause base-pair substitutions. The others *Salmonella* strains 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 and have been described (Levin *et al.*, 1982).

However, some mutagens affect only one strain of frameshift mutation strains (TA1538 or TA98) or only base-pair substitution strains (TA1535 or TA100), thus imparting a degree of mutagen class specificity to the assay. But, many or even most mutagens can affect both types of strains although the effective dose will often be higher for one type of strain than the other (Stellman *et al.*, 1998).

Studies in the mid 1980s indicated that the use of only two strains of the *Salmonella* system (TA98 and TA100) were sufficient to detect approximately 90% of the known *Salmonella* mutagens. Thus these two strains are used for most screening purposes (Stellman *et al.*, 1998). These assays can be performed in a variety of ways, but the pre-incubation method gave the best result. Mc Cann and Ames, (1977)

discussed several aspects of the experimental basis for their current assessment of the value of the test as a useful predictive tools :

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weakness of the test at this stage in its development.
2. Current applications of the test method to problems that were not approachable using conventional animal test methods.
3. Some of the environmental chemicals that have already been pinpointed as potential carcinogens by the test and the current status of carcinogenicity tests of these chemicals in animals.
4. The evidence that the correlation between carcinogenicity and mutagenicity in the *Salmonella* test reflected more than a useful coincidence and fitted into a compelling collection of evidence supporting a central role for somatic mutation in the initiation of human cancer.

2.6 Genotoxic Heterocyclic Amines in Cooked Food and Ames Test

Measuring the amount of HAs in food is both difficult and delicate. Up until 1990, it was not possible to simultaneously quantify the different compounds present. This explains why most of the previous work studying the formation and the presence of these substances in foods has been conducted in an indirect manner, by measuring the amount of bacterial mutagenicity in so called basic fractions (that is, fractions of samples containing HAs). The species of bacteria used are relatively specific to HAs, but the mutagenicity may be modified by other food constituents present within the test fractions (Robbana-Barnet *et al.*, 1996). The respective roles played by the HAs of

the type IQ and those of type non-IQ in the overall mutagenicity observed may be determined by treating the fractions with nitrites in an acid medium. This selective treatment inactivates the compounds of the type non-IQ (Tsuda *et al.*,1985).

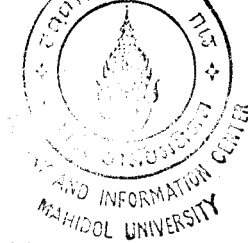
The results obtained so far by studying mutagenicity may not be considered as definitive, especially as the most abundant amines found in terms of mass such as PhIP and 4'-OH PhIP in fried beef (Robbana-Barnet *et al.*,1996), show weak mutagenic activity in bacteria. However, quantitative assays on specific amines, and more recently on the totality of these genetically toxic substances found within specific foodstuffs have not, up until now, shown to be contradictory to previous results obtained by the observation of mutagenicity (Robbana-Barnet *et al.*,1996).

The most frequently used *in vitro* procedure for mutagenicity test of HAs has been the Ames *Salmonella* / microsomal assay (Felton and Knize,1991b; Wakabayashi *et al.*,1992).

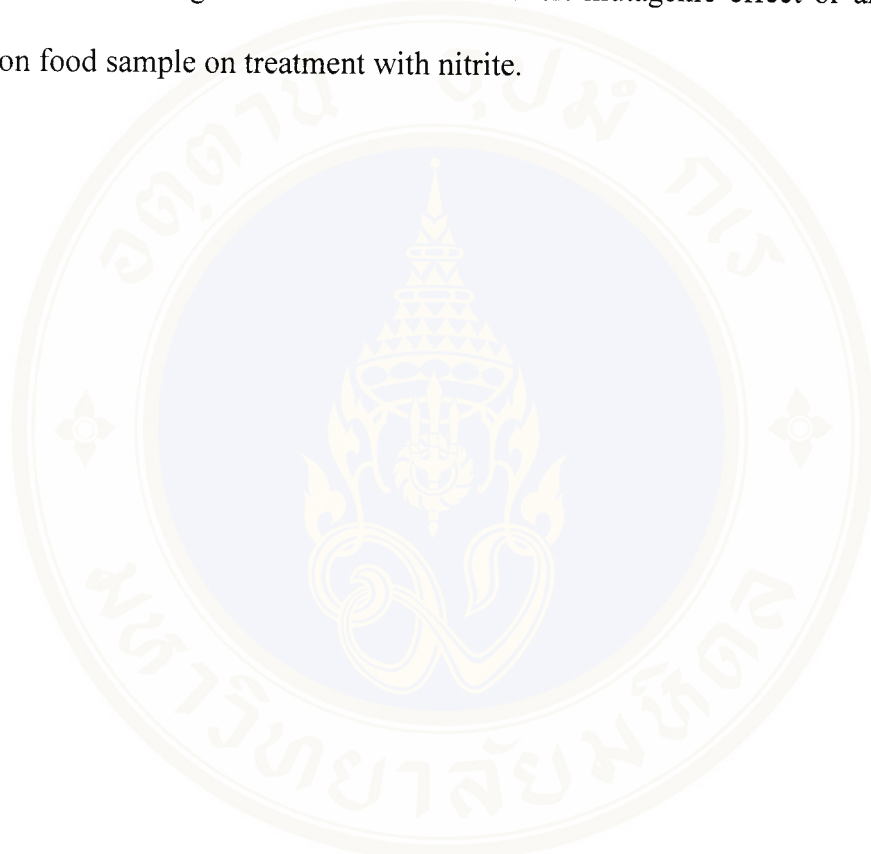
Statement of the thesis problems

In our daily life, it is impossible to avoid several food mutagens, because they are present in our daily-consumed diet, especially the mutagenic-carcinogenic heterocyclic amines in cooked meat. These mutagens can be produced even under normal cooking condition, such as boiling. It is therefore important to find different ways to minimize the amount of such mutagens in our daily diet especially those prepared from meat. In addition, it is well known that sodium nitrite and secondary amines are precursor of N-nitroso compounds. Such precursors are contained in many food, including fish and meat meal and can react under the acidic conditions of the stomach to form nitrosamine or related carcinogens (Shephard *et al.*,1987; Mirvish,1983), and thus constitute an important risk for human gastric cancer. The level legally of nitrite permitted by most food administration and control agencies ranged from 50 to 100 mg/kg of meat (as NO²) (Namiki *et al.*,1984). Nitrite may also be derived from nitrates which are abundant in vegetables. Thus, the total amount of nitrites contained in ingested food can be sometimes considerably higher than the above levels. Most Thai people has daily experience in consuming nitrite, since it has been widely used as coloring agent and preservative for meat. to inhibit *Clostridium botulinum*.

Five-spice seasoned pork (Moo-tom-pa-lo) is one of the most popular food in Thailand that used pork in its recipe and cooked by boiling for at least 2-4 h. Nevertheless, there is no available data on the direct mutagenic potential of this type of Thai food (boiled pork or meat) interact with nitrite.



On the other hand, tea which is consumed by over two-third of the world's population as a beverage was shown to exert antimutagenicity. More research demonstrated this effect of green tea and black tea but few was available for oolong tea, which preferred to consume by Thai people. Therefore it is of great interest to know whether oolong tea or its extract will exert mutagenic effect or antimutagenic effect on food sample on treatment with nitrite.



General Objectives

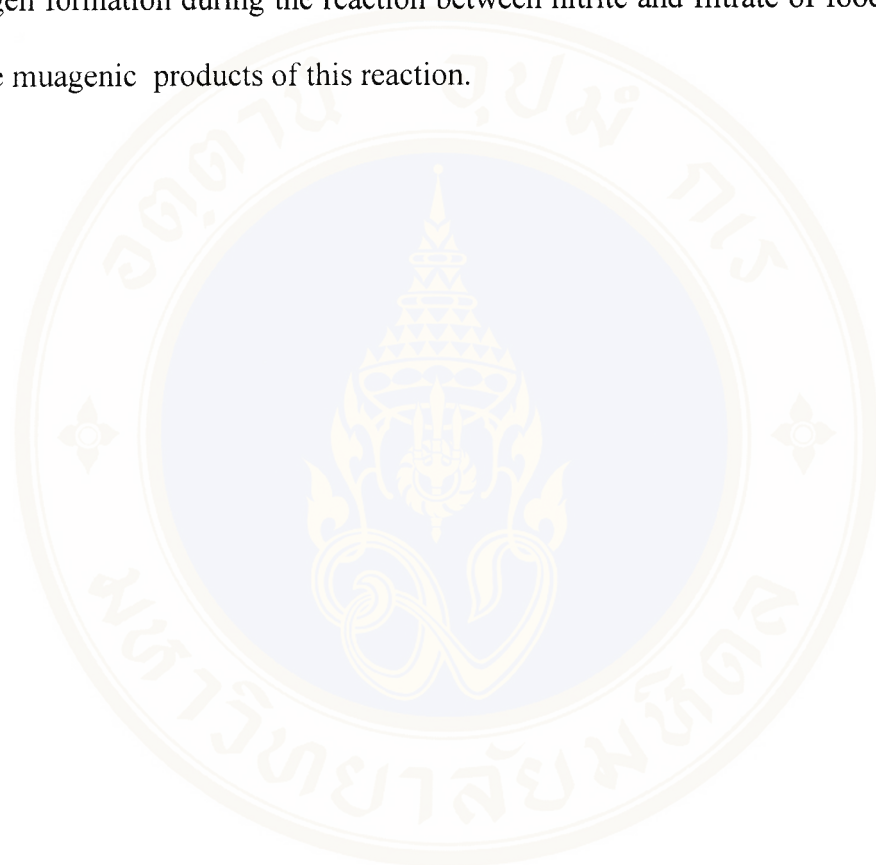
The main objective of this study was to investigate the mutagenic potential of the Thai food (five-spice seasoned pork), with and without nitrite treatment in Ames test in the absence of microsome preparation. Some spices that used in the recipe of this food were also examined for their mutagenicity. The influence of cooking method on mutagenicity of food samples was determined. In addition, the modification effect of oolong tea leaves on the formation of mutagen precursor during cooking of five-spice seasoned pork was determined as well as the modification effect of extracts from oolong tea leaves on the mutagen formation during the reaction between nitrite and filtrate of food sample and on the mutagenic product of this reaction.. This present study was conducted in order to find the best way to minimize or eliminate the mutagen or mutagen precursor that may be occurred in our daily food especially those prepared from meat.

Specific objectives

1. To investigate the direct mutagenicity of five-spice seasoned pork prepared by two different domestic cooking conditions in the Ames test
2. To determine the direct mutagenic activity of extracts from spices that used in preparing five-spice seasoned pork and also their possible mutagenicity after nitrite treatment in the Ames test
3. To determine the mutagenicity of extracts from oolong tea leaves prepared by different methods in the presence and absence of nitrite in the Ames test

4. To investigate the modification effect of various amounts of oolong tea leaves added before cooking on the formation of mutagen precursors during cooking of five-spice seasoned pork

5. To investigate the modification effect of extracts from oolong tea leaves on the mutagen formation during the reaction between nitrite and filtrate of food sample and on the muagenic products of this reaction.



CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

3.1.1 Chemical for Ames test

Aminopyrene (Aldrich, St Louis, USA) and sodium nitrite (Sigma Chemical, Co. St Louis, MO, USA) were used as precursors of standard mutagen in this assay. Ammonium sulfamate ($\text{NH}_2\text{SO}_3\text{NH}_4$), d-biotin, and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma Chemical, Co. (St. Louis, USA). Magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), histidine monohydrochloride, hydrochloric acid, potassium chloride (KCl), sodium chloride (NaCl), and crystal violet indicator were supplied by E. Merck (Darmstadt, Germany). Sodium ammonium hydrogen phosphate tetrahydrate GR ($\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$), dipotassium hydrogen phosphate anhydrous (K_2HPO_4), D(+)-Glucose monohydrate and disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were furnished by Fluka (Biochemica, Switzerland). Citric acid was obtained from BDH Chemicals Ltd.; (Pool, England) Ampicillin sodium salt was purchased from General Drugs House (Bangkok, Thailand). Bacto agar was a product of Difco laboratories (Michigan, USA). Oxoid nutrient broth No. 2 was supplied by Oxoid Ltd. (Basingstoke, Hants, England). Acetonitrile was purchased from J.T.Baker Inc. (Phillipsburg, USA). Other chemicals were of laboratory grade.

3.1.2 Food sample and tea leaves

The ingredients for a popular Thai food called "five-spice seasoned pork"(Moo-tom- pa-lo) dish were lean pork, soy sauces (black and light) and five spice powder (a mixture of five different spices: cassia, cloves, fennel, Sichuan, pepper and star anise) (Hutton and Cassio,1998). They were purchased from a local market in Bangkok, Thailand as well as oolong tea.

3.2 Experimental Design

The method to determine the mutagenicity modification of oolong tea on filtrate of five-spice seasoned pork was carried out according to the experiment protocol shown in Figure 1.

Firstly, the mutagenicity of the filtrates from five-spice seasoned pork which is a very popular food in Thailand prepared by two different cooking conditions namely, a conventional method and an air-limited method were assayed with and without nitrite treatment using the standard Ames test in the absence of metabolic activation. The mutagenicity of extracts from spices using in the preparation of this Thai food was also determined.

Secondly, oolong tea leaves were added to the food sample before cooking in order to answer the question; "Do tea leaves have any modification effect on the formation of mutagen precursor occurred during the cooking process of "five-spice seasoned pork". And the direct mutagenicity of extracts from oolong tea leaves alone prepared under both conditions as were also done with the food sample (a conventional method and an air limited method) was also assayed with and without nitrite treatment using the Ames test.

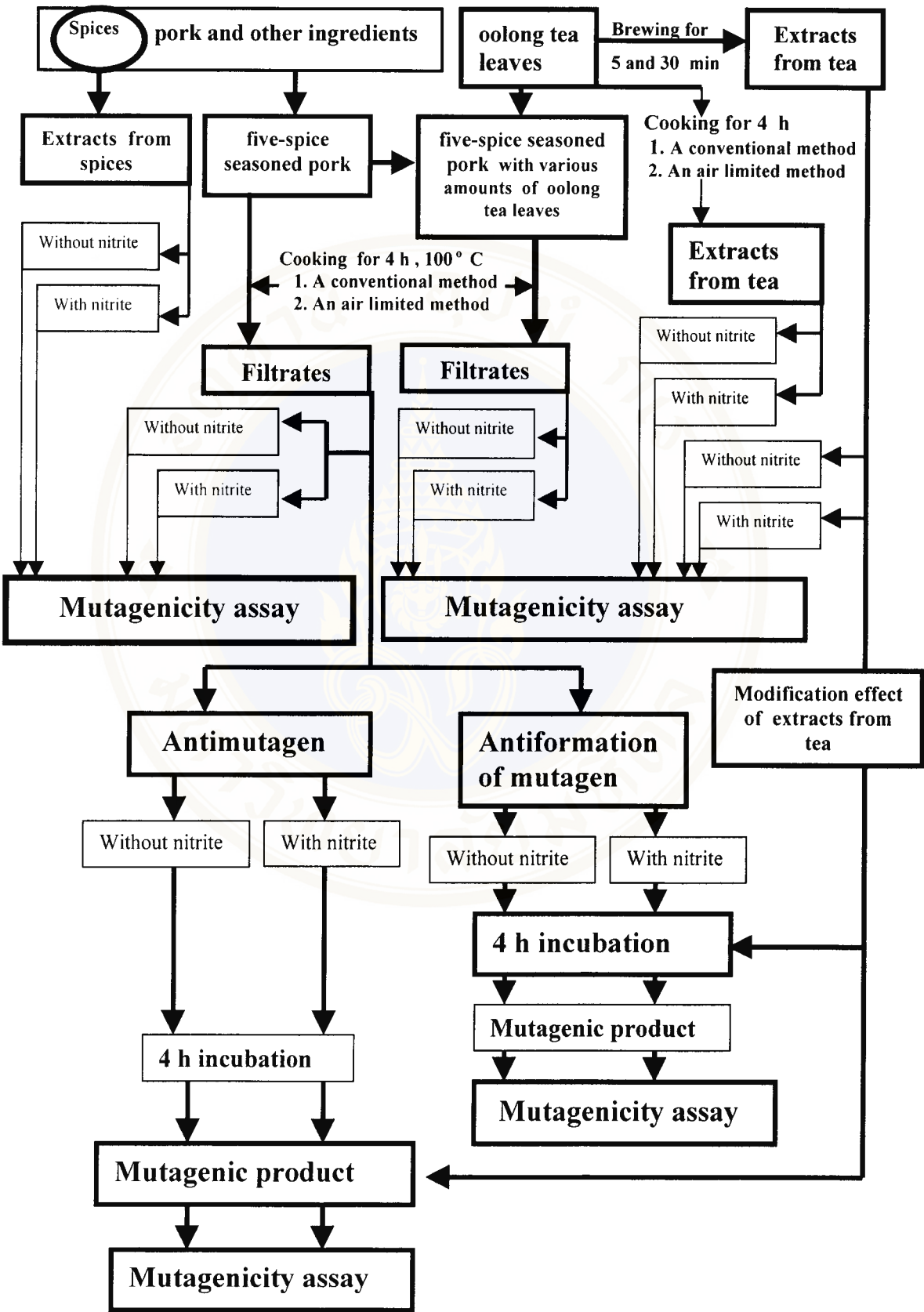


Figure 6 Experimental design

The remaining experiments were conducted to find out whether extracts from oolong tea leaves have any modification effect on the mutagen formation during the reaction between nitrite and the filtrate from food sample and also on the mutagenic product of this reaction. Lastly, the direct mutagenicity of nitrite treated extracts from tea leaves brewed for 5 and 30 min with boiled water was also evaluated.

3.3 Ames Test

Ames test, the screening test for checking mutagenicity of a chemical or a mixture of chemicals was used in this study to check the mutagenicity of filtrates of five-spice seasoned pork, extracts from spices, extracts from oolong tea alone, their mutagenic product and the mutagenicity of food sample modified by tea leaves and extracts from tea.

Modified method of Yahagi *et al.* (1975), including pre-incubation of the test sample with *Salmonella typhimurium* was used throughout this study.

Two strains of *Salmonella typhimurium* TA98 and TA100, which required histidine as growth factor, were used. They were provided by Assoc. Prof. Dr. Kaew Kangsadalampai of the Institute of Nutrition, Mahidol University. Overnight culture of each strain was prepared in Oxoid nutrient broth No. 2 at 37°C with shaking. Manipulation of the culture was done as suggested by Maron and Ames (1983) (Appendix).

Minimal agar plate, containing 30 ml of minimal glucose agar medium consisting of 1.5% Bacto-Difco agar and 2% glucose in Vogel-Boner medium E and top agar, containing 0.6% agar and 0.5% NaCl, were prepared as described by Maron and Ames (1983) (Appendix A) . Ten ml of a sterile solution of 0.5 mM

L-histidine / biotin was added to each 100 ml of the top agar and mixed thoroughly by swirling before use (Appendix).

3.4 Effect of Different Cooking Conditions on the Mutagenicity of Five-spice Seasoned Pork

This experiment was performed to determine the influence of two different cooking conditions namely; a conventional method and an air limited method on mutagen precursor formation during the cooking process of five-spice seasoned pork. The design of the experiment is illustrated in Figure 7.

3.4.1 Preparation of filtrate from five-spice seasoned pork

Five-spice seasoned pork

Ingredients

Lean meat	100	g
Five-spice powder	0.1	g
Star anise	0.1	g
Cinnamon	0.1	g
Black soy sauce	1	ml
Light soy sauce	4	ml

Five-spice seasoned pork was prepared by mixing all ingredients indicated above with 70 ml water and each mixture was cooked with two different conditions namely, boiling all ingredients at 100⁰C in a container without cover as a conventional method and in air limited condition was represented by heating at 100 ° C under reflux.

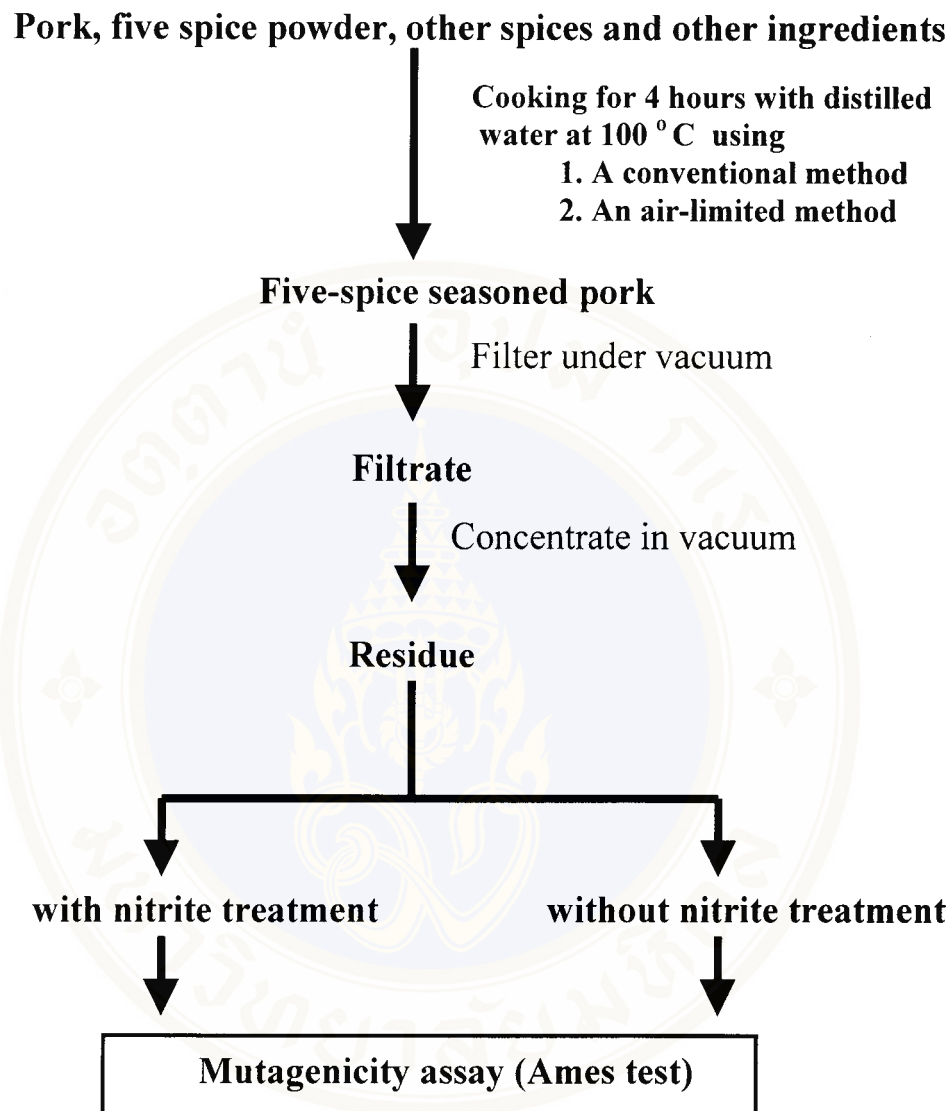


Figure 7 Steps to investigate the mutagenicity of five-spice seasoned pork prepared by two different conditions (a conventional method and an air-limited method)

After cooking for 4 hours each mixture was vacuum filtered using Whatman filter paper No.1 and the filtrate was evaporated down to about 10% of its original volume under reduce pressure. Filtering the residue through a sterile 0.2-micron filter. This filtrate was subjected to the mutagenicity assay.

3.4.2 Mutagenicity of filtrates from five-spice seasoned pork

The filtrate from each food sample in various amounts (5, 10, 20 or 50 μl) was introduced into the tube containing appropriate volume of distilled water to obtain the final volume of 100 μl , dilute HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable. The distilled water was added to obtain the final volume of 1000 μl . Then the tube with stopper was shaken at 37°C for 4 h and stopped by placing the tube in an ice bath for 1 min. Distilled water was added to the reaction mixture to obtain the final volume of 1250 μl , and the reaction tube was immersed in an ice bath for another 10-min.

Direct mutagenicity of the reaction mixture was assayed according to the pre-incubation (at 37°C for 20 min) method of Yahagi *et al.* (1975), using *Salmonella typhimurium* in the absence of S-9 mix.

Mutagenic assay

To a tube containing 0.1 ml of the reaction mixture was added 0.5 ml of $\text{Na}_3\text{PO}_4\text{-KCl}$ buffer (pH 7.4), and then 0.1 ml of fresh overnight nutrient broth culture of the tester strain ($1\text{-}2 \times 10^8$ cells). The tube was incubated at 37°C in a shaking water bath for 20 min. After incubation, 2 ml of molten top agar (45°C) containing 0.5 mM L-histidine / biotin was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C in the dark for 2 days. The procedure, step by step, of the test is shown in Figure 8.

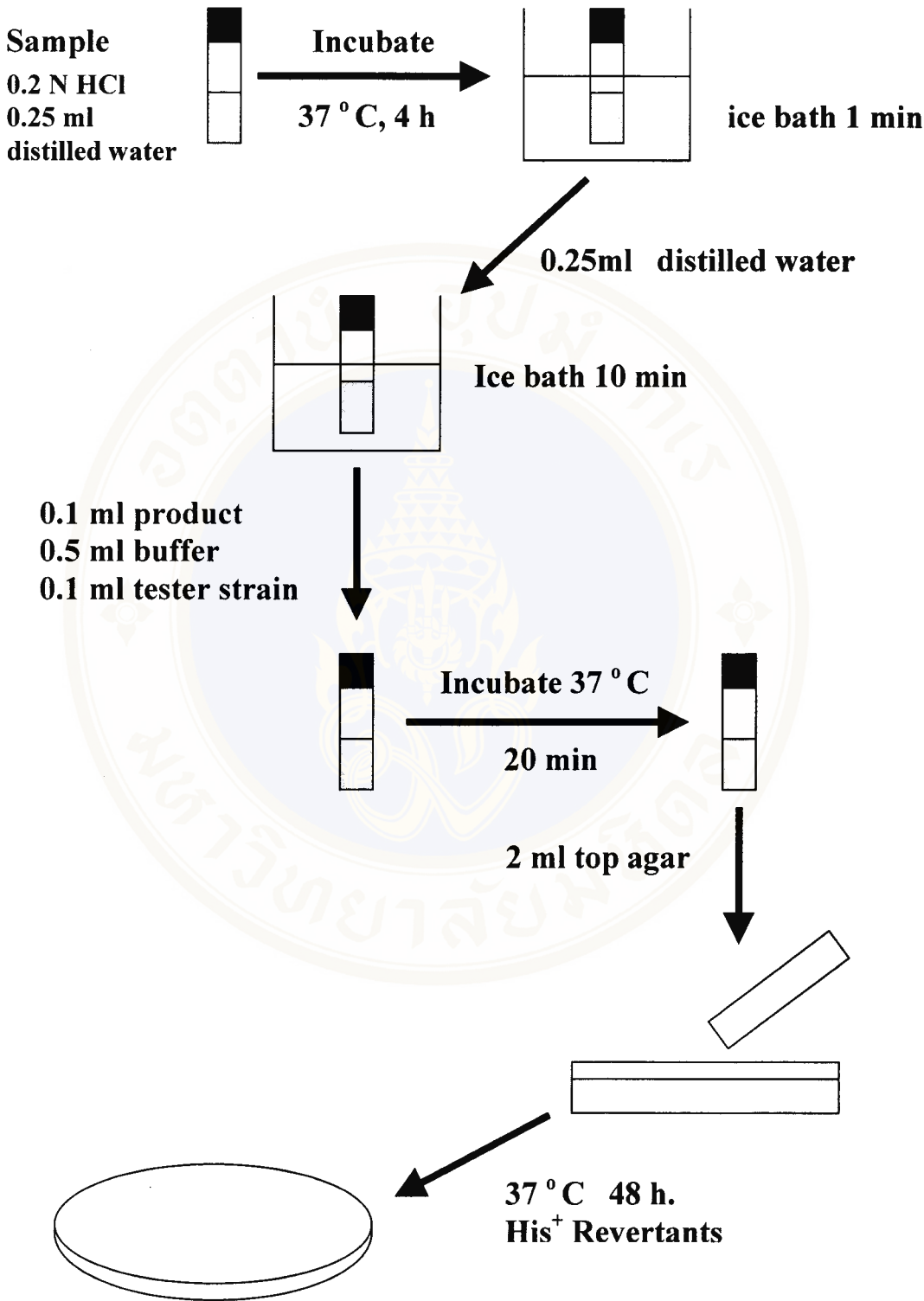


Figure 8. Direct mutagenicity evaluation of sample using the Ames test (pre-incubation modification)

Induced his⁺ revertant colonies were counted and compared to the established spontaneous mutation. Duplicate plates were carried out for each sample and all experiments were performed at least twice.

For each experiment, positive control and negative control plates were included in the assay. Negative control plate containing the bacteria and distilled water was used to establish the spontaneous mutation of each tester strain.

Aminopyrene treated with nitrite in acid solution was used as a positive control. Briefly explain, appropriate volume (20 µl for testing on *Salmonella typhimurium* TA98 and 40 µl for TA 100) of aminopyrene (0.075 mg/ml) in a tube fitted with a plastic stopper was mixed with 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 0.25 ml of 2 M sodium nitrite. The volume was adjusted to be 1,000 µl giving the final concentration of 500 mM.nitrite.

3.4.3 Mutagenicity of nitrite treated filtrates from five-spice seasoned pork

To determine the mutagenicity of filtrates from five-spice seasoned pork treated with nitrite, an aliquot of each filtrate 5,10, 20 and 50 µl was introduced into the tube containing 95, 90, 80 and 50 µl of distilled water respectively. Dilute HCl, which contain sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added. Adding 0.25 ml of 2M sodium nitrite started the reaction. The final volume was 1000 µl. Then the tube was incubated at 37 °C with shaking for 4 h. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Ammonium sulfamate (2M, 0.25 ml) was added to the reaction mixture to decompose the residue nitrite and the reaction tube was immersed in an ice bath for another 10 min.

Finally, the mixture was determined for its mutagenicity by Ames test as described in Figure 9.

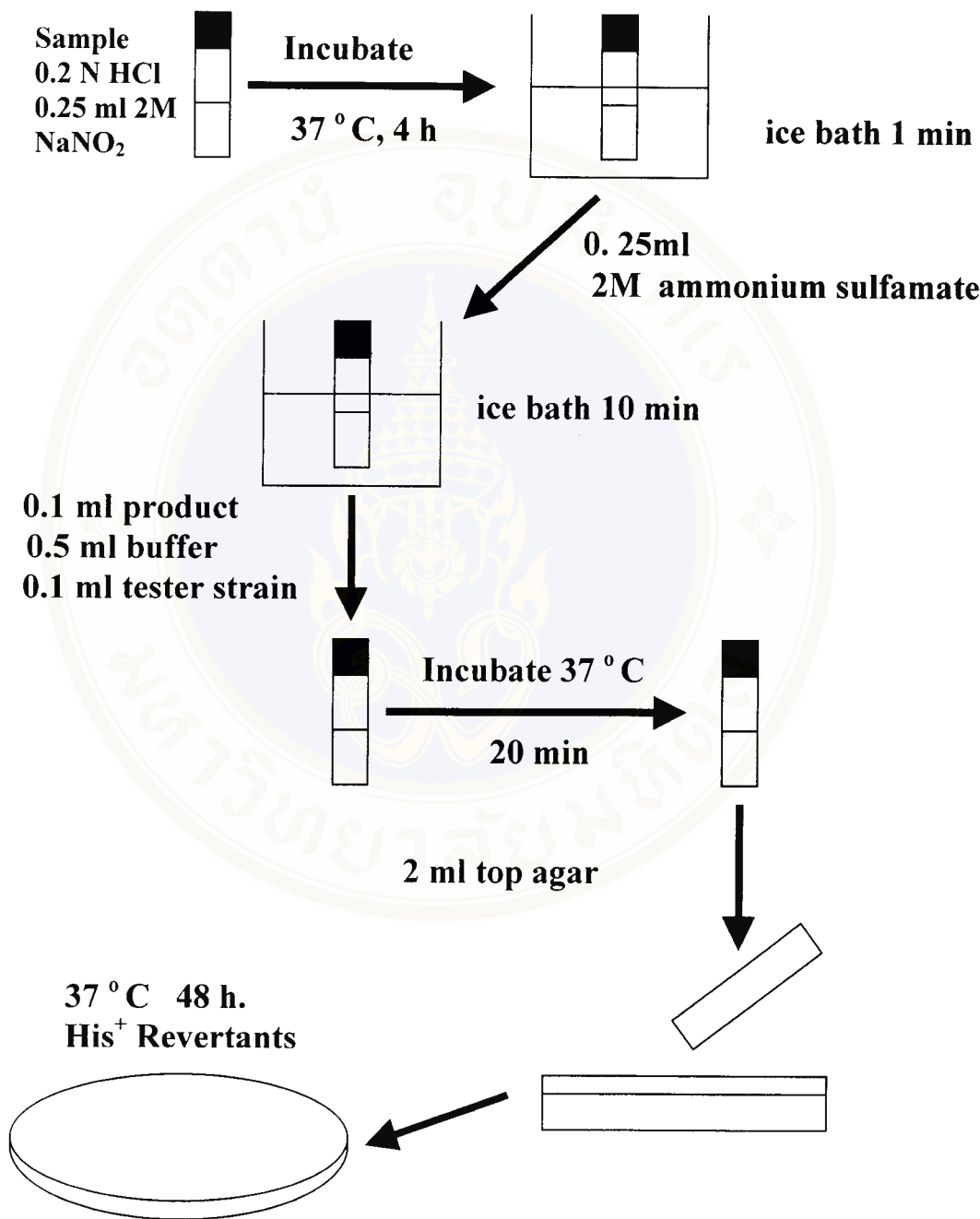


Figure 9. Steps to determine the mutagenicity of nitrite treated food sample using the Ames mutagenicity test (pre-incubation modification) in the absence of S-9 mix

3.5 Mutagenic Activity of Extracts from Spices

3.5.1 Preparation of extracts from spices

All spices including, star anise, cinnamon and five-spice powder were purchased from a local market in Bangkok, Thailand. Twenty grams of each sample was refluxed at 100°C with 100 ml of distilled water for 4 h, then the extract was filtered and the filtrate obtained was centrifuged at 5000 g for 60 min. The supernatant fraction was decanted and concentrated in vacuum at 30-35°C to one - tenth of the original volume. The residue was filtered through sterile 0.2 micron filter and the filtrate was subjected to the mutagenicity assay. The scheme for preparation of extracts from spice is shown in Figure 10.

3.5.2 Mutagenicity of extracts from spices treated with and without nitrite

Aliquot of each spice extract (50, 100 and 200 µl) was introduced into the tube containing 150, 100 or 0 µl of distilled water respectively to adjust the volume to 200 µl. Then the experiment was conducted followed the procedures of Figure 8 for without nitrite treatment and Figure 9 for with nitrite treatment. Duplicate plates were used for each of four different concentrations of the extracts and all experiments were performed at least twice.

Star anise, cinnamon and five-spice powder,

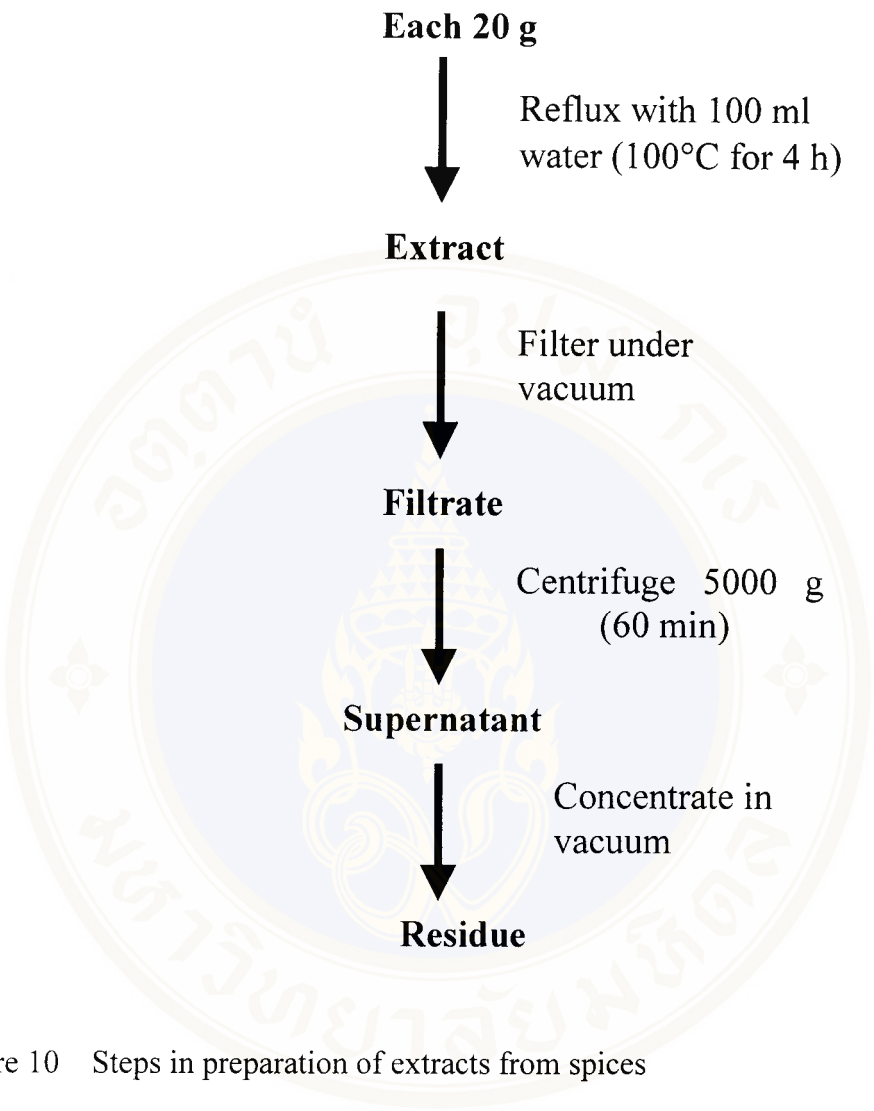


Figure 10 Steps in preparation of extracts from spices

3.6 Mutagenicity of Extracts from Oolong Tea Leaves

This experiment was performed to evaluate the mutagenicity background of extracts from tea leaves alone prepared by two different cooking conditions that used in preparing food sample. The mutagenicity background of extracts from tea leaves extracted for 5 and 30 min was also determined. The scheme of the preparation is shown in Figure 11.

3.6.1 Preparation of extracts from tea leaves

Firstly, tea leaves in various amounts (0.01, 0.1, 1 and 5 g) in 70 ml water were prepared with two different conditions; a conventional method and an air limited method. After boiling for 4 h, each mixture was filtered to obtain an aqueous extract of tea which was then concentrated under reduced pressure to one-tenth of original volume.

The residue was filter-sterilized and tested either immediately for mutagenicity assay or if it is not impossible to do so, then it was stored at 4°C and tested in the same manner within 24 hours.

Secondly, each 10 g of tea leaves was dispersed in 100 ml of boiling water. After being extracted for 5 and for 30 min, each mixture was filtered and concentrated as described above. Then, the residue was separated into two portions, one was used as the extract from tea in the experiment 3.8.2 and 3.8.3 (for testing its mutagenicity modification on filtrate of food sample) and the other was filter-sterilized and tested for its own mutagenicity.

3.6.2 Mutagenicity of extracts of tea leaves obtained from a conventional method and an air-limited method treated with and without nitrite.

An aliquot of 50 µl of each extract from tea was adjusted to 100 µl by distilled water and the mutagenicity of the extracts with and without nitrite treatment was conducted follow the procedures shown in Figure 9 and 8 respectively.

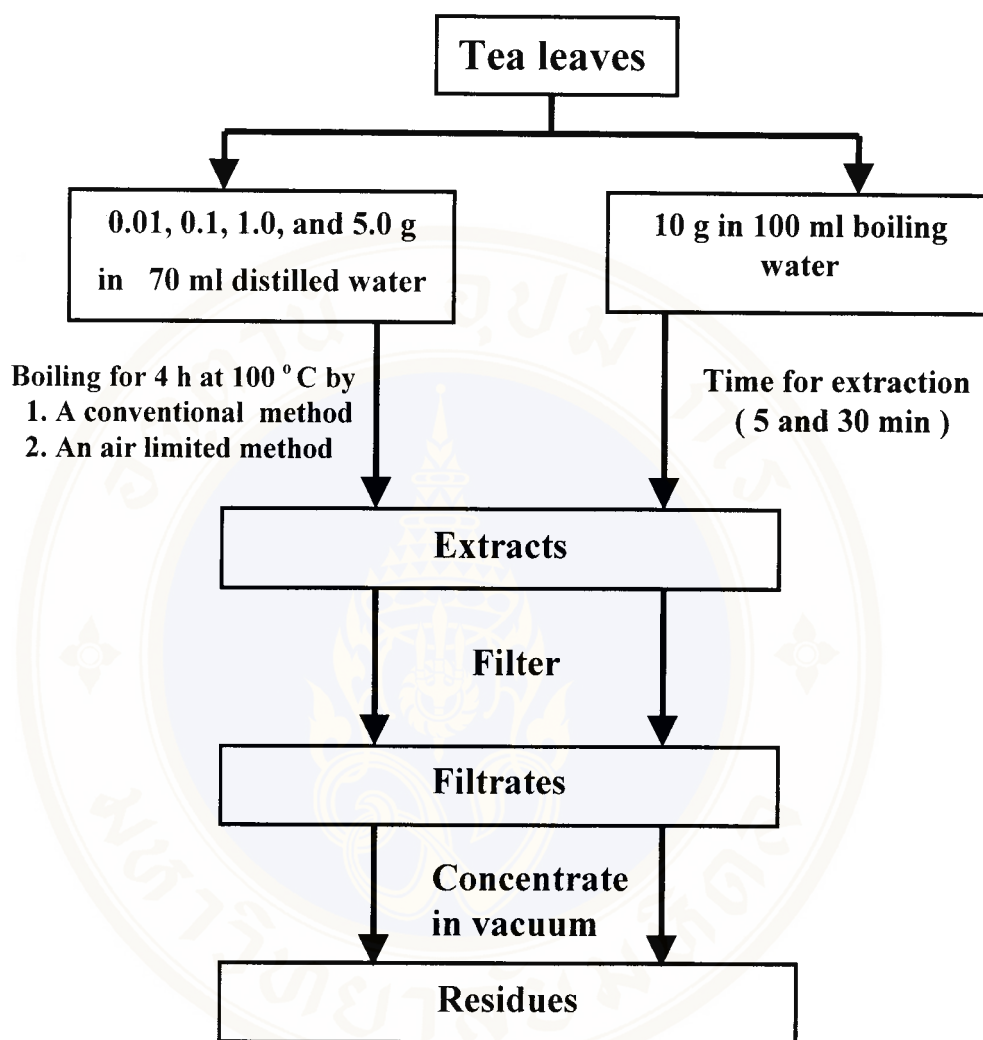


Figure 11 Steps to prepare the extracts from tea

3.6.3 Mutagenicity of extracted from oolong tea leaves extracted for 5 and 30 min treated with and without nitrite

Aliquot of each extract from tea (10, 50, 100 or 200 μ l) was adjusted to 200 μ l by distilled water and the mutagenicity of extract from tea treated with and without nitrite was determined according to the former experiment.

3.7 Mutagenicity of Filtrate of Five-spice Seasoned Pork Prepared in the Presence of Oolong Tea Leaves

This assay was performed to determine whether oolong tea leaves have a modification effect on the formation of mutagen precursor occurred during cooking of five-spice seasoned pork. The food samples were prepared using two different cooking conditions (a conventional method and an air-limited method). The design of the experiment is shown in Figure 12.

3.7.1 Preparation of extracts of five-spice seasoned pork with tea leaves

Oolong tea leaves in various amounts (0, 0.01, 0.1, 1 or 5 g) were added to each food sample (five-spice seasoned pork) before cooking. Then the entire mixture was cooked by two different 4-h cooking conditions namely, a conventional method and an air limited method. After cooking for 4 hours each mixture was filtered through a filter paper and the filtrate was evaporated down to about 10% of the original volume under reduce pressure. This concentrated extracted was then sterile at 121°C for 15 min and subjected to the mutagenicity test.

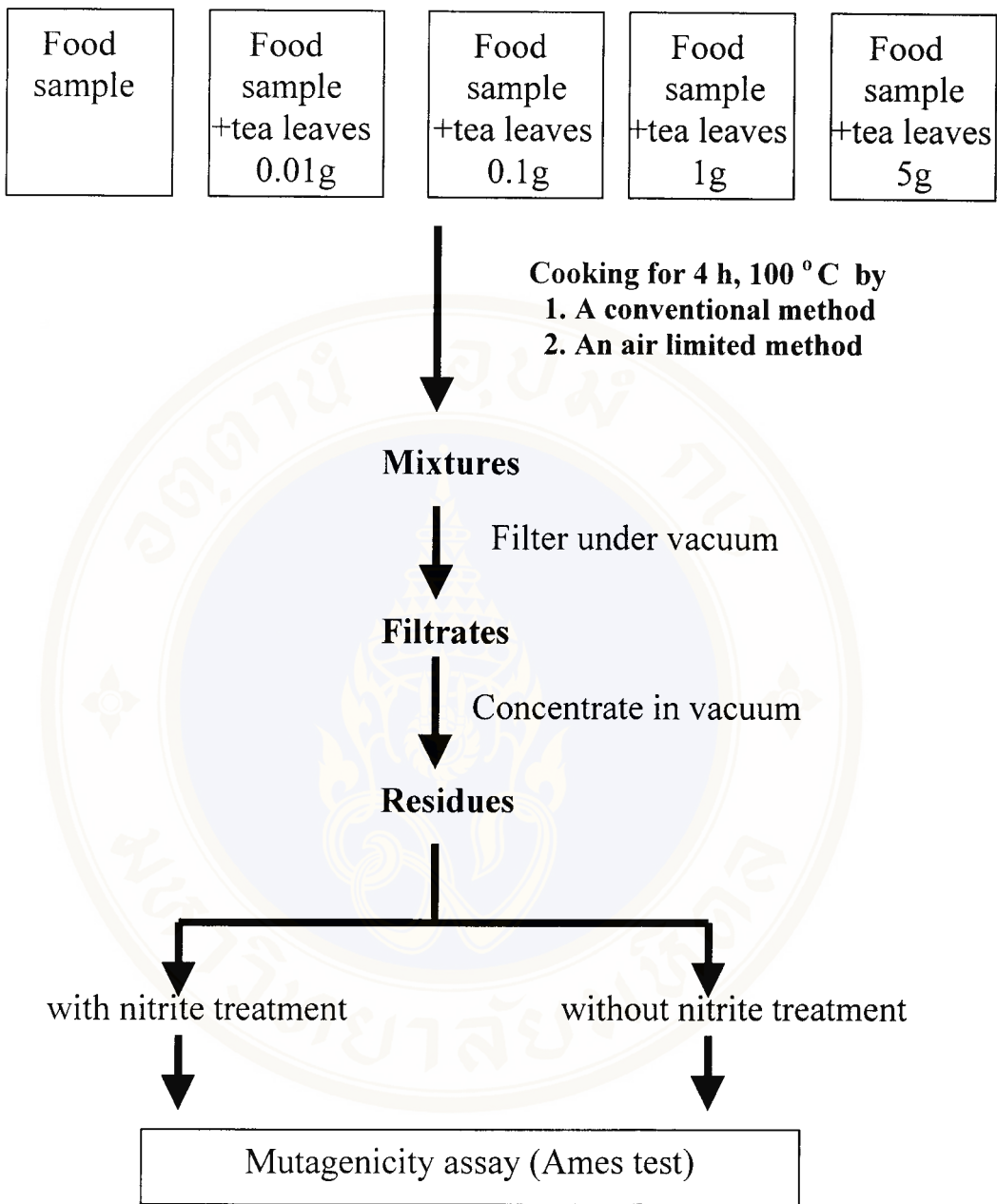


Figure 12 Steps to determine the mutagenicity of five-spice seasoned pork prepared in the presence of oolong tea leaves

3.7.2 Mutagenicity of filtrates of five-spice seasoned pork prepared in the presence of various amounts of oolong tea leaves treated with and without nitrite

Each sample of filtrate of the mixture (five-spice seasoned pork cooked with tea leaves) from experiment 3.7.1 in various amount (5, 10, 20 and 50 μ l) was added to the tube containing appropriate amounts of distilled water to obtain the final volume of 100 μ l. The experiment procedures to obtain the mutagenicity of non-nitrite and nitrite treated of filtrates of these mixtures were carried out following the diagrams as shown in Figure 8 and 9 respectively.

3.8 Mutagenicity Modification of Extracts from Oolong Tea Leaves on Filtrate of Five-spice Seasoned Pork

This experiment was performed to find out; firstly, the modification effect of extracts from oolong tea leaves on the formation of mutagen occurred during the reaction between filtrate of five-spice seasoned pork and nitrite in an acid medium. (Antiformation of mutagen). Secondly, the mutagenicity modification effect of extracts from oolong tea on the mutagenic product from this reaction was also investigated (Antimutagen). Five-spice seasoned pork was prepared by Two different cooking conditions (a conventional method and an air-limited method). The influence of time for extraction on the mutagenicity modification of tea was also examined. The design of the experiment is illustrated in Figure 13.

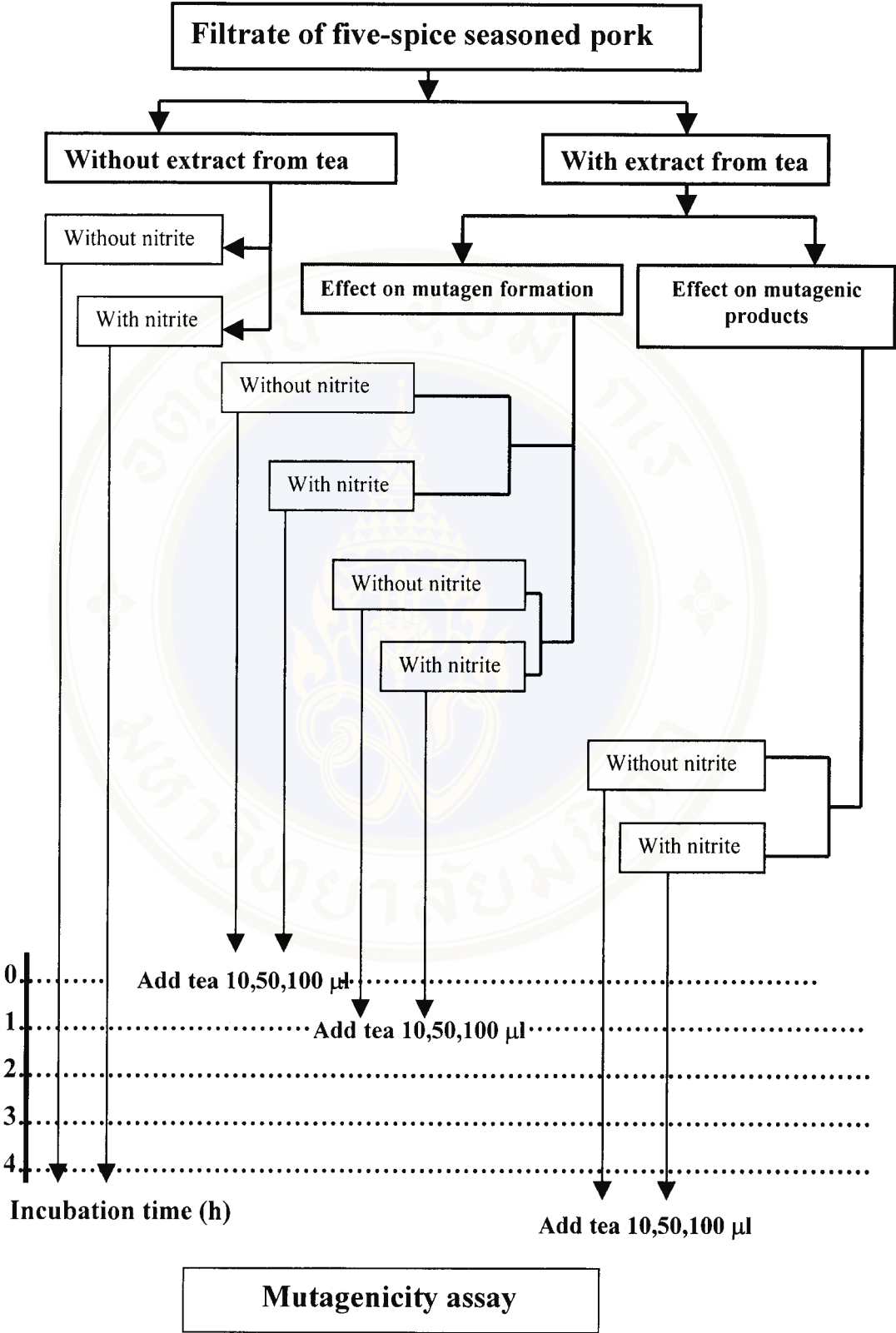


Figure 13 Steps to investigate the mutagenicity modification of extract from oolong tea leaves on filtrate of five-spice seasoned pork

3.8.1 Mutagenicity of filtrate of five-spice seasoned pork (without added extract from tea)

This experiment was conducted to evaluate the mutagenicity background of non-nitrite and nitrite treated filtrate of five-spice seasoned pork cooked in a regular method and cooked under air limited method.

Twenty μl of each filtrate of food sample was adjusted to 100 μl with distilled water. The experimental procedures to obtain the mutagenicity of non-nitrite and nitrite treated filtrates of Five-spice seasoned pork were carried out following the diagrams as shown in Figure 8 and 9 respectively.

3.8.2 Mutagenicity modification of extract from oolong tea leaves on mutagen formation

3.8.2.1 Incubation without nitrite

A 20 μl aliquot of each filtrate of five spice seasoned pork obtained from two different cooking conditions (a conventional method and an air limited method) was introduced into the tube containing 80 μl of distilled water. The pH was adjusted (3.0-3.5) with dilute HCl and 0.25 ml of distilled water was added to the reaction mixture. Before incubation, aliquot (10, 50 and 100 μl) of each extract from tea was added to the reaction mixture (duration of interaction = 4 h). Then the mixture was incubated at 37°C in shaking water bath for 4 h. The remaining experiment was conducted as shown in Figure 8

In the other set of experiment, an aliquot of 20 μl of each filtrate from Five - spice seasoned pork was adjusted to 100 μl with distilled water. Before incubation,

the mixture was added with dilute HCl and 0.25 ml of distilled water. Then the tube was incubated at 37°C in shaking water bath. After incubation for 1 h, aliquot (10, 50 and 100 µl) of each extract from tea was added and the tube was incubated for another 3 h (duration of interaction = 3 h). After incubation for total 4 h the mixture was subjected to mutagenicity assay following the steps in Figure 8.

3.8.2.2 Incubation with nitrite

Two sets of experiments was conducted in this study as well as the former one (3.8.2.1), but each aliquot of filtrate from five spice seasoned pork was interacted with 0.25 ml 2 M sodium nitrite instead of 0.25 ml of distilled water.

3.8.3 Mutagenicity modification of extract from oolong tea leaves on mutagenic product

3.8.3.1 Incubation without nitrite

An aliquot of 20 µl of filtrate of food sample was used in this study and the experiment was performed following the steps in Figure 8. After 4 h of incubation period, aliquot (10, 50 and 100 µl) of each extract from tea was added (duration of interaction = 0 h). Then each food sample - nitrite-tea mixture was determined for its mutagenicity by Ames test as described previously (Experiment 3.4.2).

3.8.3.2 Incubation with nitrite

The reaction between an aliquot of 20 µl of filtrate of food sample and 0.25 ml 2 M sodium nitrite was conducted in this study following the step in Figure 9.

Before the reaction mixture was subjected to the mutagenicity test, it was added with aliquot (10, 50 and 100 μ l) of each extract from tea.

3.9 Data Manipulation

The mutagenicity of each sample was presented as number of histidine revertants per plate. The data were reported as means with standard deviation of four plates from two different experiments. And for easier to compare the degree of mutagenicity, the results are also expressed as mutagenicity index (MI) which was calculated from the average value of number of histidine revertants per plate of sample divided by average value of spontaneous revertants. Sample expressed its mutagenicity higher than 2 times of spontaneous revertants with a dose-response relationship was evaluated mutagenic.

CHAPTER IV

RESULTS

4.1 Mutagenicity of Filtrate of Five-spice seasoned Pork

Table 10 shows the mutagenicity of the filtrate of five-spice seasoned pork prepared with two different 4-h cooking conditions namely, a conventional method and an air-limited method. None of the sample exhibited direct mutagenicity on *S. typhimurium* TA 100. Being treated with nitrite in a mild acid solution, they became mutagenic with a dose-response relationship manner through only one tester strain. No appreciable mutagenicity was observed on TA 98 (data not shown); therefore, only TA 100 was used through this study. There was no obvious difference on the mutagenicity of the nitrite treated samples that prepared from different methods when lower amounts of filtrates of samples (calculated 0.4-1.6 $\mu\text{l}/\text{plate}$) were assayed. It was noted that with 8 $\mu\text{l}/\text{plate}$ of both samples presented their killing effect to TA 100. Overall results suggested that both preparations of five-spice seasoned pork produced some compounds readily to interact with nitrite in acid solution (pH 3-3.5) to produce direct mutagen capable of base-pair substitutions mutation in the tester strain. However, it was revealed that the sample from air-limited method of cooking express higher mutagenicity than that of conventional method one after nitrite treatment when the highest amount of filtrates (4.0 $\mu\text{l}/\text{plate}$) were examined. The mutagenicity indices (MIs) were 9.30 and 5.01, respectively.

Table 10 Mutagenicity of the filtrate of five-spice seasoned pork prepared with two different 4-h cooking conditions namely, a conventional method and an air-limited method after nitrite treatment ^a on *Salmonella typhimurium* TA100 without metabolic activation

Method of cooking	Amount of sample (µl/plate)	No. of revertants/plate ^b		MI ^c	
		without nitrite	with nitrite	without nitrite	with nitrite
Conventional method	spontaneous	187±9	187±9	1.00	1.00
	0.4	278±23	358±40	1.49	1.91
	0.8	298±38	489±62	1.59	2.61
	1.6	317±36	572±52	1.70	3.06
	4.0	327±19	944±155	1.75	5.01
	8.0	killing effect	killing effect	-	-
Air-limited method	spontaneous	157±20	157±20	1.00	1.00
	0.4	183±6	244±15	1.16	1.55
	0.8	214±22	405±31	1.36	2.58
	1.6	220±14	606±31	1.41	3.86
	4.0	209±23	1461±104	1.33	9.30
	8.0	killing effect	killing effect	-	-

^a The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and selected doses of samples.

^b Data are expressed as mean and standard deviation of four plates from two experiments.

^c Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold number indicate positive mutagenic response.

4.2 Mutagenicity of Extracts from Spices

The mutagenicity of the aqueous extracts from spices using in five-spice seasoned pork namely star anise, cinnamon and five-spice powder on *Salmonella typhimurium* TA 98 and TA 100 is shown in Tables 11 and 12, respectively.

After being treated with sodium nitrite in a dilute acid solution, all treated samples were found mutagenic on both tester strains. Almost all sample treated with nitrite expressed their mutagenicity with a dose-response manner on both strains. The exception was found on the extract from star anise that the mutagenicity on *S. typhimurium* TA 100 decreased when the concentration of sample tested was the highest (16 μ l). Mutagenicity index (MI) obtained from this study suggested that star anise exhibited the strongest index after nitrite treatment.

4.3 Mutagenicity of Extracts from Oolong Tea Leaves

Table 13 shows the mutagenicity of extracts from oolong tea leaves boiled in a conventional method and an air-limited method at 100 °C for 4 h. The mutagenicity with a dose response relationship occurred after the extracts were treated with nitrite. There was no difference on the mutagenicity of the treated extracts from different preparations.

The mutagenicity of nitrite treated extracts from oolong tea leaves brewed for 5 and 30 min was shown in Table 14 with a dose-response relationship. There were no obvious difference in mutagenicity between the extract from 5 and 30 min brewing. It was suggested that at the highest volume of plate incorporation (16 μ l/plate) of both preparations, toxic sign was expressed as the number of revertants was lower than that of the lower concentration.

Table 11. Mutagenicity of extracts from spices after nitrite treatment ^a on *Salmonella typhimurium* TA 98 without metabolic activation

Sample	Amount of sample (μl/plate)	No. of revertants/plate ^b		MI ^c	
		without nitrite	with nitrite	without nitrite	with nitrite
Star anise	spontaneous	18±4	18±4	1.00	1.00
	4	21±4	226±4	1.17	12.56
	8	20±3	316±8	1.11	17.56
	16	24±4	430±48	1.33	23.89
Cinnamon	spontaneous	16±3	16±3	1.00	1.00
	4	17±2	43±2	1.06	2.69
	8	16±2	66±20	1.00	4.12
	16	17±3	94±7	1.06	5.88
Five-spice powder	spontaneous	20±1	20±1	1.00	1.00
	4	20±1	38±9	1.00	1.90
	8	22±5	47±3	1.10	2.35
	16	21±4	85±11	1.10	4.25

^a The reaction with nitrite was done in a mild acid solution containing 250 μl of 0.5 M sodium nitrite and each selected dose of sample.

^b Data are expressed as means and standard deviations of four plates from two experiments.

^c Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold figures indicate positive mutagenic response.

Table 12 Mutagenicity of extracts from spices after nitrite treatment ^a on *Salmonella typhimurium* TA 100 without metabolic activation.

Sample	Amount of sample (µl/plate)	No. of revertants/plate ^b		MI ^c	
		without nitrite	with nitrite	without nitrite	with nitrite
Star anise	spontaneous	140±16	140±16	1.00	1.00
	4	150±14	917±64	1.07	6.55
	8	146±18	1092±75	1.04	7.80
	16	144±24	877±105	1.03	6.25
Cinnamon	spontaneous	108±8	108±8	1.00	1.00
	4	115±12	280±38	1.06	2.59
	8	110±5	367±13	1.02	3.40
	16	104±8	503±55	0.96	4.66
Five-spice powder	spontaneous	117±14	117±14	1.00	1.00
	4	170±30	442±58	1.45	3.78
	8	182±38	506±52	1.56	4.32
	16	152±20	583±40	1.30	4.59

^a The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and each selected dose of sample.

^b Data are expressed as means and standard deviations of four plates from two experiments.

^c Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold figures indicate positive mutagenic response.

Table 13. Mutagenicity of extracts from oolong tea leaves prepared in a conventional method and an air-limited method after nitrite treatment^a on *Salmonella typhimurium* TA 100 without metabolic activation.

Concentrated residues of .g tea in 70 ml water ^b	No. of revertants/plate ^c		MI ^d	
	without nitrite	with nitrite	without nitrite	with nitrite
A conventional method				
Spontaneous	102±16	102±16	1.00	1.00
0.01	96±12	98± 9	0.94	0.96
0.1	104±11	102±17	1.02	1.00
1.0	107±34	207±20	1.05	2.03
5.0	87±18	601±64	0.85	5.89
An air limited method				
Spontaneous	102±16	102±16	1.00	1.00
0.01	104±15	110±15	1.02	1.08
0.1	102±17	112±25	1.00	1.00
1.0	113±23	219±21	1.11	2.15
5.0	83±10	647±31	0.81	6.34

^a The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and 50 µl of extract from Oolong tea leaves.

^b Four calculated µl/plate of each concentrated residue of extract from tea was used.

^c Data are expressed as mean and standard deviation of four plates from two experiments.

^d Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold figures indicate positive mutagenic response.

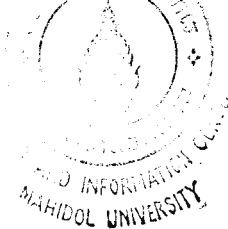


Table 14 Mutagenicity of extracts from oolong tea leaves brewed for 5 or 30 min after nitrite treatment^a on *Salmonella typhimurium* TA 100 without metabolic activation.

Time for extraction	Amount of extracts from tea (μl/plate)	No. of revertants/plate ^b		MI ^c	
		without nitrite	with nitrite	without nitrite	with nitrite
5 min	spontaneous	83±10	83±10	1.00	1.00
	0.8	76±10	228±19	0.92	2.75
	4.0	62±6	713±23	0.75	8.59
	8.0	65±2	953±10	0.78	11.48
	16.0	65±12	754±55	0.78	9.08
30 min	spontaneous	81±21	81±21	1.00	1.00
	0.8	79±14	222±14	0.98	2.74
	4.0	70±22	596±80	0.86	7.36
	8.0	70±6	1030±133	0.86	12.72
	16.0	78±8	676±54	0.96	8.34

^a The reaction with nitrite was done in a mild acid solution containing 250 μl of 0.5 M sodium nitrite and selected doses of samples.

^b Data are expressed as mean and standard deviation of four plates from two experiments.

^c Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold number indicate positive mutagenic response.

4.4 Mutagenicity of Filtrates of Five-spice Seasoned Pork Prepared in the Presence of Oolong Tea Leaves

The mutagenic potential expressed as revertants of *S. typhimurium* TA 100 of filtrates from five-spice seasoned pork cooked in a regular method in the presence of tea leaves is shown in Table 15. In addition, Table 17 shows the mutagenicity of filtrates obtained from five-spice seasoned pork cooked under air-limited method in the presence of tea for 4 h on *S. typhimurium* TA 100. None of the samples exhibited mutagenic to *S. typhimurium* TA 100. However, after being treated with nitrite the mutagenicity of almost all samples increased in a dose-response relationship manner. The mutagenicity among the nitrite treated samples (filtrate from five-spice seasoned pork cooked in the presence of 0, 0.01, 0.1, 1.0 and 5.0 g tea) represented by number of revertants/plate was not much different for all samples between 0.4-1.6 $\mu\text{l}/\text{plate}$. It was noted that the mutagenicity of the filtrate (4 $\mu\text{l}/\text{plate}$) of pork cooked in the air-limited method in the presence of 0.1 g tea was revealed to be the highest. However, number of revertants was decreased in the presence of higher amount of tea leaves during cooking. This observation suggested that there was inhibitory effect on the formation of precursor of mutagen after nitrite treatment of filtrate from five-spice seasoned pork cooked with air-limited method.

Considering the number of revertants per plate of the different nitrite treated filtrate, it was shown that the addition of tea leaves seemed to increase the mutagenicity of each filtrate. Thus, the mutagenicity index (MI = dividing number of revertants per plate by concurrent running spontaneous revertants) was introduced to clarify the role of tea leaves. Tables 16 and 18 shows the MI of each filtrate as calculated MI (summation of MI of filtrate from Five-spice seasoned pork and that of

tea leaves with equal weight introduced in cooking preparation) and observed MI. It was found that MI obtained from the determination has a tendency to be less than calculated ones. The MI of the nitrite treated filtrate from pork cooked under air-limited method in the presence of 0.1 g and 1.0 g tea leaves was higher than those of calculated ones. However, the MI of nitrite treated filtrate cooked in the presence of 5.0 g tea leaves expressed only one-fifth of the calculated MI ; thus, it was indicated an inhibition on formation of precursor for nitrite treatment during air limited method cooking of five-spice seasoned pork. The reduction of MI of nitrite treated filtrate from conventional cooking method was also revealed with, however, no relationship with respect to the amount of tea leaves added to the cooking process.

Table 15 Mutagenicity of filtrates of five-spice seasoned pork cooked in a **conventional or regular method** in the presence of oolong tea leaves after nitrite treatment on *Salmonella typhimurium* TA 100 without metabolic activation .

Sample	Amount of sample (µl/plate)	No. of revertants/plate ^a		MI ^c	
		without nitrite	with nitrite ^b	without nitrite	with nitrite
1) Filtrate of food sample	spontaneous	187±9	187±9	1.00	1.00
	0.4	278±23	358±40	1.49	1.91
	0.8	298±38	489±62	1.59	2.61
	1.6	317±36	572±52	1.70	3.06
	4.0	327±19	944±155	1.75	5.05
2) Filtrate of food sample with tea 0.01 g	spontaneous	187±9	187±9	1.00	1.00
	0.4	297±17	403±39	1.59	2.16
	0.8	283±66	476±47	1.51	2.54
	1.6	335±7	539±92	1.79	2.88
	4.0	350±7	1069±226	1.87	5.72
3) Filtrate of food sample with tea 0.1 g	spontaneous	187±9	187±9	1.00	1.00
	0.4	293±41	336±31	1.57	1.80
	0.8	246±14	503±74	1.32	2.70
	1.6	312±48	616±70	1.67	3.29
	4.0	350±7	1126±119	1.87	6.02
4) Filtrate of food sample with tea 1.0 g	spontaneous	187±9	187±9	1.00	1.00
	0.4	246±14	380±11	1.32	2.03
	0.8	277±67	561±39	1.48	3.00
	1.6	333±52	562±85	1.78	3.00
	4.0	348±75	1231±141	1.86	6.58

Table 15 (Cont) Mutagenicity of filtrates of five-spice seasoned pork cooked in a **conventional or regular method** in the presence of oolong tea leaves after nitrite treatment on *Salmonella typhimurium* TA 100 without metabolic activation .

Sample	Amount of sample (µl/plate)	No. of revertants/plate ^a		MI ^c	
		without nitrite	with nitrite ^b	without nitrite	with nitrite
5) Filtrate of	spontaneous	198±18	198±18	1.00	1.00
food sample	0.4	254±31	364±28	1.28	1.84
with tea	0.8	278±50	502±734	1.40	2.54
5.0 g	1.6	331±13	926±46	1.58	4.68
	4.0	310±49	1157±184	1.56	5.84

^a Data are expressed as means and standard deviations of four plates from two experiments.

^b The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and selected doses of samples.

^c Mutagenicity index is calculated by dividing the average value of number of histidine revertants/plate of sample mixture with by that of spontaneous.

Bold figures indicate positive mutagenic response.

Table 16 Comparison between the calculated mutagenicity index obtained by summing the mutagenicity index of all nitrite treated components^a and of observed ones of nitrite treated filtrate of five-spice seasoned pork cooked in **a conventional method** in the presence of tea leaves.

Concentrated residues of..g tea in 70 ml water	Mutagenicity index ^b			Observed ^c
	A ^c MI of nitrite treated extract from tea	B ^c Nitrite treated filtrate of food sample	Summation of A and B	
0.01	0.96	5.05	6.01	5.72
0.10	1.00	5.05	6.05	6.02
1.0	2.03	5.05	7.08	6.58
5.0	5.89	5.05	10.94	5.84

^a Five-spice seasoned pork, tea leaves and simultaneous cooking of five-spice seasoned pork with various amounts of tea leaves were prepared by the same condition (**a conventional method**).

^b Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^c Four calculated µl /plate of each sample was used.

Table 17 Mutagenicity of filtrates of five-spice seasoned pork cooked under **air-limited method** in the presence of oolong tea leaves on *Salmonella typhimurium* TA 100 without metabolic activation

Sample	Amount of sample (μ l/plate)	No. of revertants/plate ^a		MI ^c	
		without nitrite	with nitrite ^b	without nitrite	with nitrite
1) Filtrate of food sample	Spontaneous	157 $\pm$ 20	157 $\pm$ 20	1.00	1.00
	0.4	183 $\pm$ 6	244 $\pm$ 15	1.17	1.55
	0.8	214 $\pm$ 22	405$\pm$31	1.36	2.58
	1.6	220 $\pm$ 14	606$\pm$31	1.40	3.86
	4.0	209 $\pm$ 23	1461$\pm$64	1.33	9.30
2) Filtrate of food sample with tea 0.01g	Spontaneous	157 $\pm$ 20	157 $\pm$ 20	1.00	1.00
	0.4	186 $\pm$ 29	272 $\pm$ 25	1.18	1.73
	0.8	209 $\pm$ 29	427$\pm$38	1.33	2.72
	1.6	264 $\pm$ 34	461$\pm$90	1.68	2.94
	4.0	269 $\pm$ 21	1756$\pm$804	1.71	11.18
3) Filtrate of food sample with tea 0.1g	Spontaneous	157 $\pm$ 20	157 $\pm$ 20	1.00	1.00
	0.4	176 $\pm$ 40	263 $\pm$ 42	1.12	1.68
	0.8	218 $\pm$ 42	489$\pm$38	1.39	3.11
	1.6	258 $\pm$ 36	590$\pm$117	1.64	3.76
	4.0	222 $\pm$ 16	2458$\pm$275	1.41	15.66
4) Filtrate of food sample with tea 1g	Spontaneous	157 $\pm$ 20	157 $\pm$ 20	1.00	1.00
	0.4	190 $\pm$ 17	256 $\pm$ 19	1.21	1.63
	0.8	241 $\pm$ 27	410$\pm$17	1.54	2.61
	1.6	256 $\pm$ 20	563$\pm$41	1.63	3.59
	4.0	266 $\pm$ 38	1936$\pm$374	1.69	12.33

Table 17 (Cont) Mutagenicity of filtrates of five-spice seasoned pork cooked under **air-limited method** in the presence of oolong tea leaves on *Salmonella typhimurium* TA 100 without metabolic activation

Sample	Amount of sample (µl/plate)	No. of revertants/plate ^a		MI ^c	
		without nitrite	with nitrite ^b	without nitrite	with nitrite
5) Filtrate of food sample with tea 5g	Spontaneous	145±12	145±12	1.00	1.00
	0.4	219±14	285±39	1.51	1.96
	0.8	266±61	400±53	1.83	2.76
	1.6	240±14	552±80	1.66	3.81
	4.0	237±43	472±44	1.63	3.26

^a Data are expressed as mean and standard deviation of four plates from two experiments.

^b The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and selected doses of samples.

^c Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

Bold number indicate positive metabolic response.

Table 18 Comparison between the calculated mutagenicity index obtained by summing the mutagenicity index of all nitrite treated components^a and of observed ones of nitrite treated filtrate of five-spice seasoned pork cooked in **an air-limited method** in the presence of tea leaves.

Concentrated residues of ..g tea in 70 ml water	Mutagenicity index ^b			Observed ^c
	A ^c MI of nitrite treated extract from tea	B ^c nitrite treated filtrate of food sample	Summation of A and B	
0.01 g	1.08	9.30	10.38	11.18
0.10 g	1.10	9.30	10.40	15.66
1.0 g	2.15	9.30	11.45	12.33
5.0 g	6.34	9.30	15.64	3.26

^a Five-spice seasoned pork, tea leaves and simultaneous cooking of five-spice seasoned pork with various amounts of tea leaves were prepared by the same condition (**an air limited method**).

^b Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^c Four µl /plate of each sample was used.

4.5 Effect of Extracts from Oolong Tea Leaves on Mutagenicity of Nitrite Treated Filtrate of Five-spice Seasoned Pork

This experiment was performed to find out the effect of extracts from tea leaves brewed for 5 and 30 min on nitrite treated filtrates of five-spice seasoned pork and nitrite. Each extract from tea was present in the reaction mixture of filtrate of food sample and nitrite for 4, 3 or 0 h. Five-spice seasoned pork prepared with a conventional method and an air limited method were used in this study and all data are shown in Tables 19 and 21, respectively. Almost all untreated samples exhibited no detectable mutagenicity, except some showed higher number of histidine revertants per plate than two times of spontaneous revertants. This phenomenon might be due to trace amount of histidine existed in the extract from cooked meat. After treatment with nitrite in mild acid solution, all samples became mutagenic toward *Salmonella typhimurium* TA100 without activation. The mutagenicity of nitrite treated sample was still dose-dependently increased when each extract from either 5 or 30 min brewed tea was added to the filtrate of food sample before the reaction with nitrite was started (duration of presence was 4 h) or when the reaction was already started for 1 h (duration of presence was 3 h). On the other hand, the mutagenicity of nitrite treated filtrate of food sample was dose dependently decreased by the addition of each extract from tea after the reaction with nitrite was ended. (duration of interaction = 0 h). This suggested that the extract from tea had a stimulating function on formation of convertible mutagen in the reaction mixture. The overall mutagenicity of nitrite treated filtrate of food sample prepared under air limited method in the presence of 30 min brewed tea was higher than that of sample in the presence of 5 min brewed tea.

Alteration on mutagenicity of nitrite treated filtrate of five-spice seasoned pork by extracts from both 5 and 30 min brewed tea are expressed as mutagenicity index (MI). Comparison between the predicted (calculated) and experimental (observed) MI of the nitrite treated filtrate of food sample prepared with a conventional method and an air-limited method are shown in Tables 20 and 22, respectively. It was found that the addition of each extract from tea at the beginning of the reaction (Duration of interaction = 4 h) could decrease the overall observed MI of food sample-nitrite-tea mixture. Although the observed MIs were lower than calculated values in almost all concentration of each tea extract, this inhibitory effect of extract from tea added in this manner may not be evaluated as beneficial for practical use because the overall mutagenicity of the mixtures were still quite higher than that of nitrite treated filtrate of food sample alone. The lower observed MIs were obtained when each extract from tea was added after the reaction was stopped. The strongest inhibition was shown at the highest concentration (8 μ l of extract from tea). Therefore, from the present experiment, it is revealed that extracts from both 5 and 30 min brewed tea seem to have inhibitory effect on the mutagenic product of the reaction between nitrite and filtrate of food sample (antimutagen) rather than anti-forming of mutagen.

Table 19 Effect of duration of brewing of tea and duration of presence (4, 3 and 0 h) of oolong tea on the mutagenicity of nitrite treated filtrate of five-spice seasoned pork prepared with **a conventional method** on *Salmonella typhimurium* TA100 without metabolic activation

Sample	Duration of presence (h)	Amount of extracts from tea (µl/plate)	No. of revertants/plate ^a		MI ^c	
			without nitrite	with nitrite ^b	without nitrite	with nitrite
5 min brewed tea	4	spontaneous	126±25	126±25	1.00	1.00
		0.8	182±31	569±48	1.44	4.52
		4.0	226±42	1209±124	1.79	9.59
		8.0	242±16	1358±153	1.92	10.78
	3	0.8	231±23	702±4	1.83	5.57
		4.0	237±29	1048±60	1.88	8.32
		8.0	220±30	1251±38	1.75	9.93
	0	0.8	231±18	640±25	1.83	5.08
		4.0	242±12	507±76	1.92	4.02
		8.0	202±19	450±24	1.60	3.57
	control ^d	0	250±24	503±94	1.98	3.99
30 min brewed tea	4	spontaneous	134±34	134±34	1.00	1.00
		0.8	213±34	682±47	1.59	5.09
		4.0	252±14	1296±107	1.88	9.67
		8.0	212±10	1506±52	1.58	11.24
	3	0.8	206±24	694±69	1.54	5.18
		4.0	226±26	1044±39	1.69	7.79
		8.0	230±28	1002±124	1.72	7.47

Table 19 (Cont) Effect of duration of brewing of tea and duration of presence (4, 3 and 0 h) of oolong tea on the mutagenicity of nitrite treated filtrate of five-spice seasoned pork prepared with **a conventional method** on *Salmonella typhimurium* TA100 without metabolic activation

Sample	Duration of presence (h)	Amount of extracts from tea (µl/plate)	No. of revertants/plate ^a		MI ^c	
			without nitrite	with nitrite ^b	without nitrite	with nitrite
30 min brewed tea	0	spontaneous	134±34	134±34	1.00	1.00
		0.8	208±51	534±38	1.55	3.99
		4.0	228±36	456±30	1.70	3.40
		8.0	222±39	308±28	1.66	2.30
control ^d		0	237±48	530±50	1.77	3.96

^a Data are expressed as mean and standard deviation of four plates from two experiments.

^b The reaction with nitrite was done in a mild acid solution containing 250 µl of 0.5 M sodium nitrite and 20 µl of filtrate of five-spice seasoned pork prepared with **a conventional method**.

^c Mutagenicity index is calculated by the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^d An average value of number of histidine revertants per plate of 20 µl of filtrate of Five-spice seasoned pork-nitrite mixture incubated for 4 h in the absence of extract from tea.

Bold figures indicate positive metabolic response.

Table 20 Comparison of the calculated mutagenicity index obtained by summing the mutagenicity index of individual components (nitrite treated extract from tea; 5 and 30 min brewing and nitrite treated filtrate of five-spice seasoned pork) and observed values of nitrite treated filtrate of five-spice seasoned pork prepared with a **conventional method** in the presence of extract from tea leaves. by varying the interaction time between tea and the reaction mixture.

Sample	Duration of interaction (h)	Amount of extract from tea (µl/plate)	MI ^a			
			A ^b nitrite treated extract from tea	B ^b nitrite treated filtrate of food sample	Summation of A and B (Calculated)	Observed ^b
5 min brewed tea	4	0.8	2.75	3.99	6.74	4.52
		4.0	8.59	3.99	12.58	9.59
		8.0	11.48	3.99	15.47	10.78
	0	0.8	0.92	3.99	4.91	5.08
		4.0	0.75	3.99	4.74	4.02
		8.0	0.78	3.99	4.77	3.57
30 min brewed tea	4	0.8	2.85	3.96	6.81	5.09
		4.0	7.64	3.96	11.60	9.67
		8.0	13.20	3.96	17.16	11.24
	0	0.8	1.01	3.96	4.97	3.99
		0.4	0.90	3.96	4.86	3.40
		8.0	0.90	3.96	4.86	2.30

^a Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^b Four calculated µl/plate of nitrite treated filtrate of five-spice seasoned pork prepared with a **conventional method** was used in the assay.

Table 21 Effect of duration of brewing of tea and duration of presence (4, 3 and 0 h) of oolong tea on the mutagenicity of nitrite treated filtrate of five-spice seasoned pork prepared with **an air-limited method** on *Salmonella typhimurium* TA100 without metabolic activation

Sample	Duration of presence (h)	Amount of extracts from tea (μl/plate)	No. of revertants/plate ^a		MI ^c	
			without nitrite	with nitrite ^b	without nitrite	with nitrite
5 min brewed tea		spontaneous	155±26	155±26	1.00	1.00
	4	0.8	163±17	592±61	1.05	3.82
		4.0	165±48	1214±66	1.06	7.83
		8.0	222±15	1498±98	1.43	9.66
	3	0.8	231±32	588±111	1.49	3.79
		4.0	210±31	1028±34	1.35	6.63
		8.0	225±26	1288±38	1.45	8.31
	0	0.8	238±12	463±58	1.54	2.99
		4.0	234±27	397±58	1.51	2.56
		8.0	232±34	278±18	1.50	1.79
control ^d		0	292±52	485±63	1.88	3.13
30 min brewed tea		spontaneous	104±5	104±5	1.00	1.00
	4	0.8	216±14	656±45	2.08	6.31
		4.0	216±20	1292±117	2.08	12.42
		8.0	196±33	1478±246	1.88	14.21
	3	0.8	194±26	700±97	1.86	6.73
		4.0	213±13	1094±142	2.05	10.52
		8.0	209±24	1170±81	2.01	11.25

Table 21 (Cont) Effect of duration of brewing of tea and duration of presence (4, 3 and 0 h) of oolong tea on the mutagenicity of nitrite treated filtrate of five-spice seasoned pork prepared with **an air-limited method** on *Salmonella typhimurium* TA100 without metabolic activation

Sample	Duration of presence (h)	Amount of extracts from tea (µl/plate)	No. of revertants/plate ^a		MI ^c	
			without nitrite	with nitrite ^b	without nitrite	with nitrite
30 min brewed tea	0	spontaneous	104±5	104±5	1.00	1.00
		0.8	216±24	514±78	2.08	4.94
		4.0	194±39	368±79	1.86	3.53
		8.0	189±18	344±45	1.82	3.30
control ^d		0	204±22	469±69	1.96	4.51

^a Data are expressed as mean and standard deviation of four plates from two experiments.

^b The reaction with nitrite was done in a mild acid solution containing 250 µl 0.5 M sodium nitrite and 20 µl of filtrate of five-spice seasoned pork prepared under air-limited method.

^c Mutagenicity index is calculated by the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^d An average value of number of histidine revertants per plate of 20 µl of filtrate of five-spice seasoned pork-nitrite mixture incubated for 4 h in the absence of extract from tea.

Bold number indicate positive metabolic response.

Table 22 Comparison of the calculated mutagenicity index obtained by summing the mutagenicity index of individual components (nitrite treated extract from tea; 5 and 30 min brewing and nitrite treated filtrate of five-spice seasoned pork) and observed values of nitrite treated filtrate of five-spice seasoned pork prepared under **air-limited method** in the presence of extract from tea leaves. by varying the interaction time between tea and the reaction mixture.

Sample	Duration of interaction (h)	Amount of extract from tea µl/plate	MI ^a			
			A ^b nitrite treated extract from tea	B ^b nitrite treated filtrate of food sample	Summation of A and B (Calculated)	Observed ^b
5 min brewed tea	4	0.8	2.75	3.13	5.88	3.82
		4.0	8.59	3.13	11.72	7.83
		8.0	11.48	3.13	14.61	9.66
	0	0.8	0.92	3.13	4.05	2.99
		4.0	0.75	3.13	3.88	2.56
		8.0	0.78	3.13	3.91	1.79
30 min brewed tea	4	0.8	2.85	4.51	7.36	6.31
		4.0	7.64	4.51	12.15	12.42
		8.0	13.20	4.51	17.71	14.21
	0	0.8	1.01	4.51	5.52	4.94
		4.0	0.90	4.51	5.41	3.53
		8.0	0.90	4.51	5.41	3.30

^a Mutagenicity index is calculated from the average value of number of histidine revertants/plate of sample mixture divided by that of spontaneous.

^b Four µl/plate of nitrite treated filtrate of five-spice seasoned pork prepared under **air-limited method** was used in the assay.

CHAPTER V

DISCUSSION

5.1 Mutagenicity of Filtrate of Five-spice Seasoned Pork

Food mutagens have been a major focus of concerns during the past two decades. Much knowledge had been accumulated about mutagens in food, especially about those produced by cooking proteinaceous food (Peerawong and Kangsadalampai, 1998). In this study “five-spice seasoned pork” one of the most popular food in Thailand which prepared by boiling pork with some spices and soy sauces for at least 3-4 hour in order to obtain a good flavor, palatable taste and soft texture of meat was chosen to represent the proteinaceous food. Two different cooking conditions were performed to simulate the actual condition of cooking namely, an air-limited method (cooking in a pot with lid) and a conventional method (cooking in a pot without lid).

The consumption of nitrite and dietary nitrogen containing compounds might be an etiological factor in the development of some cancers, particularly of the gastrointestinal tract, a possibility reviewed by Mirvish (1983). Many investigators (Marquardt *et al.*, 1977; Piacek-Llanes and Tannenbaum, 1982; Wakabayashi *et al.*, 1984) suggested that direct-acting mutagens/carcinogens formed from nitrite and the precursors of mutagens in the acid conditions of the stomach were possible candidates for the causation of human gastric cancer. In this study, attempts were made to simulate the events, which may occur during consumption of a meal consisting of cooked meat and nitrite. An aqueous filtrate of five-spice seasoned pork was treated with nitrite at pH 3-3.5 to simulate the acid condition of the stomach.

Moreover, in order to determine the direct mutagenicity of the filtrate of food sample towards *Salmonella typhimurium* the metabolic activation system or S9 mix was excluded.

The finding obtained from this study demonstrated that none of the filtrates of five-spice seasoned pork prepared either in a conventional method or an air-limited method had direct mutagen that could cause frame shift mutation. In addition, the findings of direct mutagenic activity in all nitrite treated filtrates of five-spice seasoned pork suggested that they might contain some convertible indirect mutagens which were also found in beef extract (Commoner *et al.*,1978; Dorala *et al.*,1979). The mutagens in meat extracts formed during normal cooking were identified as heterocyclic amines or HAs (Starvic,1994). HAs formed at temperature below 300°C are often referred to as thermic mutagens (Skog, 1993) or IQ type. This type includes a quinolines (IQ and MeIQ), a quinoxalines (MeIQx and DiMeIQx) and pyridines (PhIP). Sugimura and Wagabayashi (1990) reported that IQ and MeIQ were mutagenically active compounds in the *Salmonella* microsomal bioassay. The mode of action of HAs indicate that they require metabolic activation through the cytochrome P450 system (Ohgaki *et al.*,1995). Thus, it is not surprising that why filtrates of five-spice season pork showed no mutagenicity on *Salmonella typhimurium* TA 100 in the absence of S9 mix. Treatment of IQ type heterocyclic amines with a much higher concentration of nitrite (50 mM.) resulted in conversion of amino groups to nitro groups (Sasagawa *et al.*,1988). The nitro derivative of imidazoquinoline and imidazoquinoxaline showed almost the same mutagenicity in the absence and presence of metabolic activation as parent amino derivative. In this study, the mutagenic product obtained from the reaction between nitrite and filtrates of five-spice seasoned

pork that caused direct mutagenicity towards *Salmonella typhimurium* TA100 were speculated to contain direct mutagens such as nitro compounds. These substances might be derived from the reaction between IQ type heterocyclic amines which occurred during cooking of proteinaceous food (five-spice seasoned pork) and nitrite as proposed by the previous study (Sasagawa *et al.*, 1988; Peerawong and Kangsadalampai, 1998).

As shown in this study, the mutagenicity of nitrite treated filtrates of five-spice season pork is affected by the method of preparation. Cooking under air-limited condition increased mutagenicity nearly two fold compared with that with a conventional condition (mutagenicity indices were 9.30 and 5.01 respectively). Several parameters, such as temperature, heating time and type of cooking have been reported to affect the variety and amounts of HAs formed during cooking (Chiu and Chen, 2000). However, knowledge about influence of cooking under air limited condition on the formation of mutagen in proteinaceous food is unavailable. The mechanism behind this catalytic effect of an air-limited condition is unknown. It can only be speculated that some convertible indirect mutagen could be easily formed in the air-limited condition. Finding from this study indicated that cooking meat with a conventional method is the preferable method to minimize convertible indirect mutagens which occurred during cooking of meat meal.

5.2 Mutagenicity of Extracts from Spices

Five-spice seasoned pork contains not only lean meat (pork) but also the added spices and seasoning which were intended to create flavor and aroma. These spices include star anise, cinnamon and five-spice powder (pa-lo powder). The results from

this study suggest that only nitrite treated extracts from all kinds of spices used in food preparation exerted the direct mutagenicity towards *Salmonella typhimurium* both strain TA98 and TA100. This is supported by the study of Namiki *et al.*(1984) and Osawa *et al.*(1984).

Among three kinds of spices in this study, star anise exhibited the strongest mutagenic activity with number of revertants per plate decreased at the highest concentration suggesting that this concentration was the lethal dose on *Salmonella typhimurium* TA100. However this effect was not obtained towards *Salmonella typhimurium* TA98. Although all spices examined in this study exhibited direct mutagenicity on both strains of *Salmonella typhimurium* (TA98 and TA100) after nitrite treatment, only 0.1 % (compared with 100 g of lean meat) of each spice was used in the recipe of five-spice seasoned pork. This lower amount of spices appeared to be regardless to play an essential role in the expression of mutagenicity of nitrite treated filtrates of five-spice seasoned pork.

5.3 Mutagenicity of Extracts from Oolong Tea Leaves

It is evident in this study that all extracts from oolong tea leaves prepared with different methods showed no mutagenicity on *Salmonella typhimurium* TA100 without metabolic activation. After being treated with nitrite, all extracts from tea leaves showed similar profiles of mutagenicity against *Salmonella typhimurium* TA100 without metabolic activation. This event was supported by Yang and Wang, (1993) who stated that on treatment with nitrite tea polyphenols could be nitrosated to form C-nitroso derivatives which could further nitrosate other compounds through

transamination reaction. This phenomenon explained the potential catalysis of nitrosation reactions by tea components

5.4 Mutagenicity of Filtrate of Five-spice Seasoned Pork Prepared in the Presence of Oolong Tea Leaves

Although the results from the preceding study (5.1) showed that the filtrate from five-spice seasoned pork had no direct mutagenicity on *Salmonella typhimurium* TA100, it was found that it contained some convertible compounds that could further react with nitrite to produce direct mutagenic product on the bacterial tester strain. The occurrence of such questionable compounds brought up a concern about possible risk for human's health. Therefore, it was of interest to find out how to reduce the formation of such compounds.

Oolong tea leaves were added to "five-spice seasoned pork" before cooking for the sake of preventing the formation of convertible indirect mutagen during cooking. Comparing between the calculated mutagenicity index (MI) and observed MI of each nitrite treated food sample, it was suggested that the addition of lower amounts of tea leaves (0.01-1.0 g) had no inhibitory effect but rather had slight enhancing effect especially on the mutagenicity of nitrite treated sample that cooked under air-limited method. The present results corresponded to the study of Nakamura and Kawabata (1981) who found that green tea extract enhanced the nitrosation of secondary amine under acid condition. Interestingly it was found that when the highest amount of tea leaves (5 g) were added as an ingredient, the mutagenicity of nitrite treated filtrates decreased markedly. This strong mutagenicity inhibition of tea leaves was clearly

demonstrated, especially in the sample cooked under air-limited method in the presence of 5 g tea leaves.

Possible mechanism of this two edged situation of tea (catalytic effect at low amount and inhibitory effect at high amount) was elucidated by the study of Pignatelli *et al.* (1982). They proposed that, at low concentration, C-nitroso derivatives which may be formed from the reaction between tea polyphenol and nitrite during an initial step, further react with the nitrosating species to generate a more powerful nitrosating agent (thought to be a nitroso quinone oxime derivatives). This postulated mechanism implies that assay systems containing increasing amounts of a catalytically active phenolic compound lead to increasing concentration of the C-nitroso intermediate, and consequently to a reduced concentration of the nitrosating species. This mechanism would explain why a large excess of phenolic compound could inhibit nitrosation. Because the nitrosating agent would be completely used up in forming the catalyst and none would be left to continue the catalytic reaction. The finding from this study was also confirmed by the previous study of Wu *et al* (1993).who demonstrated an *in vitro* inhibitory effect of high concentration of green tea on formation of N-nitrosomorpholine, while low concentrations of tea promoted N-nitrosation.

The results from this study demonstrated that the inhibition effect of tea in the model of five-spice seasoned pork cooked with a conventional method was found to be lesser than that in the model of food cooked under air-limited method. Since the condition of a conventional cooking enable food sample to expose directly to oxygen in the air. Therefore, oxidation of tea polyphenol would occur resulting the lesser amount of this ingredient in stimulating the conversion of indirect mutagens to direct ones.

Although the addition of high amounts of oolong tea leaves before cooking could diminish the overall direct mutagenicity of five-spice seasoned pork treated with nitrite. This attempt may not be an appropriate one and may not be beneficial to consumers in daily life since the overall mutagenicity of the mixture of food sample, tea and nitrite was still higher than that of nitrite treated food sample alone. Moreover, a large amount of tea leaves may change the flavor and taste of the food. For this reason, the idea of cooking five-spice seasoned pork with tea leaves in order to minimize the mutagenicity of food sample on treatment with nitrite may not be accepted by the consumer. Therefore, the alternative way to diminish the mutagenicity of nitrite treated filtrate of food samples was investigated.

5.5 Effect of of Extracts from Oolong Tea Leaves on Mutagenicity of Nitrite Treated Filtrates of Five-spice Seasoned Pork

This experiment was designed to investigate the practical way of drinking oolong tea as antimutagen. . Because time for extraction might influence their inhibitory activity; therefore, tea was extracted with two different brewing time (5 and 30 min). The experiment was conducted to simulate the situation that may happen in our daily life. Adding the extract from tea at time 0 h (duration that tea was present in the nitrite treatment was 4 h) or after 1 h of incubation (duration of presence of tea was 3 h), and at the end of the nitrite treatment (duration of presence of tea was 0 h) to the mixture of food sample and nitrite are similar to tea consumption behavior namely; before, during and after meal respectively.

Adding the extracts from tea (both 5 and 30 min brewing) either at the begin (0 h) or the end of the reaction (4 h) between nitrite and filtrate of five-spice seasoned pork

could inhibit overall mutagenicity of nitrite treated filtrate of food sample. This was elaborated by considering the observed MIs that were lower than calculated MIs. A similar experiment was conducted by Kigashimoto *et al.* (2000). They found that the mutagenicity of nitrite-treated MTCCA (1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid), a major mutagen precursor in soy sauce on *Salmonella typhimurium* TA100 in the presence of ethanol, was dose dependently decreased by the addition of the infused green and oolong teas or catechins to the reaction mixture during or after nitrosation.

However, the results from this study demonstrated that when extract from tea was added to the food sample mixture at the beginning or during the reaction, the observed MIs of nitrite treated filtrate of food sample in the presence of tea extracts were still higher than those of nitrite treated filtrate of food sample only.

In contrary, when the addition of extracts from tea was made at the end of the reaction, the observed MIs of the mixtures were quite lower than those of the calculated values. This event clearly demonstrated that extract from tea would give the best antimutagenic activity when it was added to the mixtures after the reaction was stopped.

The fact that extracts from tea had considerable antimutagenicity when it was added after nitrite treatment suggested that the antimutagenicity of these extracts may resulted from a desmutagenic action such as an adsorption of the mutagen(s) (Kigashimoto *et al.*, 2000). The mechanism of desmutagenic action of tea polyphenols and extracts from tea was supported by Jain *et al.* (1989). They indicated that the extracts from green tea and black tea decreased the mutagenic activity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *E. Coli* WP 2 in a desmutagenic manner by

interactions with intact MNNG or its active form. Since the extracts from oolong tea also showed antimutagenic activity when it was added during nitrite treatment; hence, it may inhibit the formation of mutagenic compounds. The mechanism of antimutagenicity of tea extract during nitrosation was proposed by Tanaka *et al* (1998). They stated that the inhibition of nitrosation by catechins and tea extracts may be a result of competition between the nitrosatable precursor and catechins to utilize nitrite in the reaction mixture.

The influence of time used for extraction of tea was also investigated in this study. Although the results showed that 5 min brewed tea seem to exert much higher antimutagenic activity than 30 min brewed one when an air-limited method food sample was studied, this phenomenon was not exhibited in a conventional method food sample model. However, both types of extracts from tea effectively decreased mutagenicity of nitrite treated food samples when they were added after the nitrite treatment was over. Hermaez *et al.* (1998) reported that most of the antimutagens were released from the tea leaves within 1-2 min of extraction. Fractionation of tea with HPLC showed that various catechins, including EGCG, EGC and ECG were present in the tea extract brewed for 1-2 min and accounted for most of the antimutagenic activity (Hermaez, 1998). Other components of tea, such as caffeine and tannins, were released in larger quantities after prolong brewing (3,5 or 10 min), but these provided no additional inhibitory activity.

In conclusion, the results from this study suggested that mutagenicity of nitrite treated filtrates of five-spice seasoned pork prepared with two different cooking conditions namely; a conventional method and an air-limited method may be

effectively decreased by the addition of extracts from tea after the reaction between nitrite and filtrates of food sample was stopped.

5.6 Implication to the Consumer

Since several mutagens may be formed during ordinary cooking procedure and may be presented in a variety of cooked food especially those produced by boiling meat for a long time. Moreover, the mutagenicity of cooked meat is also markedly affected by cooking method. According to the results obtained from this study, it was found that meat should be cooked with a conventional method. Cooking in a pot without a cover could diminish the convertible indirect mutagen occurred during the cooking process that voluntary to react with nitrite in an acid medium to form the direct mutagen.

In addition, findings from this experiment also suggested that brewing tea at higher concentration (10 g tea in 100 ml water) but less than 5 min as well as drinking it about 4 h after meal may provide the best protection against direct mutagen formed from the reaction between nitrite and some convertible indirect mutagen existed in cooked meat in an acid medium. The present results, together with the fact that tea has a long history of human use suggest a beneficial role of tea drinking against nitrite derived health hazards in man.

CHAPTER VI

CONCLUSION

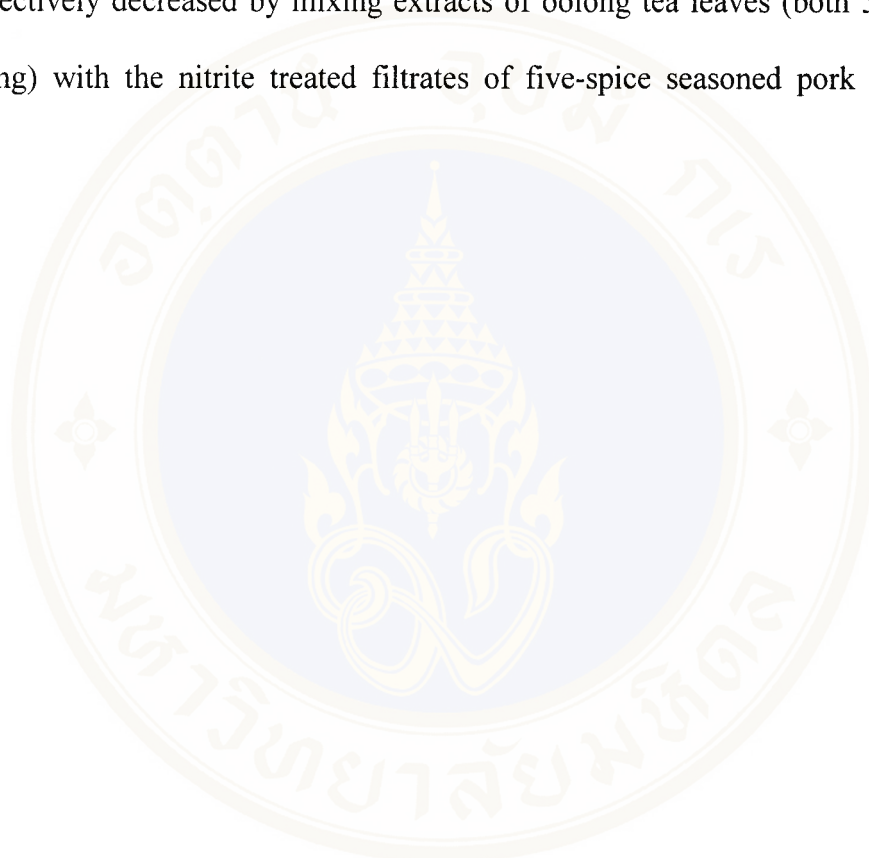
The effect of cooking conditions on the *in vitro* mutagenicity of five-spice seasoned pork containing lean pork, some spices and some sauces was examined using Ames bacterial strain TA100 without S-9 metabolic activation. Two methods of food preparation; a conventional method and an air-limited method for 4 h at 100°C were compared. The filtrate of food sample cooked with a conventional method resulted in lower *in vitro* mutagenicity than that of the other condition.

The mutagenicity of spices used in preparing five-spice seasoned pork including five-spice powder, star anise and cinnamon were also determined. The aqueous extracts of all spices showed no mutagenicity towards *Salmonella typhimurium* TA98 and TA100 in the absence of metabolic activation, but after treatment with nitrite they exerted the mutagenicity. Similar results also obtained when aqueous extracts from tea prepared with different methods (boiling for 4 h at 100°C in a container without cover and in a reflux system and brewing for 5 and for 30 min) were assayed on *Salmonella typhimurium* TA100 without metabolic activation.

The addition of high amount of oolong tea leaves before cooking of five-spice seasoned pork might decrease the formation of convertible indirect mutagens occurred during the cooking process because the overall mutagenicity of each nitrite treated filtrate of five-spice seasoned pork with tea leaves was less than that without tea

leaves. However, this may not be beneficial for practical use, since it may change the flavor and taste of such food.

Moreover, it is suggested that mutagenicity of nitrite treated filtrate of five-spice seasoned pork on *Salmonella typhimurium* TA100 without metabolic activation may be effectively decreased by mixing extracts of oolong tea leaves (both 5 and 30 min brewing) with the nitrite treated filtrates of five-spice seasoned pork in the Ames assay.



BIBLIOGRAPHY

Adamson RH, Thorgeirsson UP, Synderwine EG, Thorgeirsson SS, Reeves J.

Dalgand DW, et al. Carcinogenicity of 2-amino-3-methylimidazo [4,5-f]-quinoline in nonhuman primates. Induction of tumors in three Macaques.

Jpn J Cancer Res 1990; 81:10-4.

Agudo A, Gonzalez CA, Marcos G, Sanz M, Saigi E, Verge J, et al.

Consumption of alcohol, coffee, and tobacco and gastric cancer in Spain.

Cancer Causes Control 1992; 3(2):137-43.

Alcantara EN, Speckman EW. Diet, nutrition and cancer. Am J Clin Nutr 1976;

29: 1035-47.

Alejandro – Duran E, Alonso – Moraga A, Pueyo C. Implication of active

oxygen species in the direct acting mutagenicity of tea. Mutat Res 1987;

188: 251-7.

Ames BN. The detection of chemical mutagens with enteric bacteria. In : .

Hollaender A, editor. Chemical mutagens, principles and methods for

their detection. New York: Plenum Press;1971. p. 267-82.

Ames BN. Dietary Carcinogens and anticarcinogens. Science 1983;221:1256-64.

Ames BN , McCann J, Yamasaki E. Methods for detecting carcinogens and

mutagens with the *Salmonella* / mammalian - microsomal mutagenicity test.

Mutat Res 1975 ; 31 : 347-64.

- Ariza RR, Dorado G, Barbancho M. Study of the causes of direct – acting mutagenicity in coffee and tea using the Ara test in *Salmonella typhimurium*. Mutat Res 1988;201: 89-96.
- Ashoor S, Dietrich R, Chu F, Pariza M. Proline enhances mutagen formation in ground beef during frying. Life Sci 1980 ; 26: 1801-5.
- Augustsson K, Skog K, Jagerstad M, Overvik E, Steineck G. Cancer risks after intake of heterocyclic amines and mutagenic activity in a population-based study in Stockholm. Abstract presented at the 6th International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds. Monterey, CA, USA. 1995 ; November 4-8 . p. 89.
- Barnes WS, Weisburger JH. Lipid content and mutagen formation in the cooking of beef. Proc. Am Assoc Cancer Res 1983 ; 24: 65.
- Bartsch H, Malaveille C, Camus AM, Martel-Planche G, Brun G,Hautefeuille A, *et al.* Validation and comparative studies on 180 chemicals with *S. typhimurium* strains and V79 Chinese hamster cells in the presence of various metabolizing system. Mutat Res 1980;76: 1-50.
- Bartsch H, Montesano R. Relevance of nitrosamines to human cancer. Carcinogenesis 1984; 5: 1381-93.
- Bartsch H, Ohshima H, Pignatelli B. Inhibitors of endogenous nitrosation. Mechanism and implications in human cancer prevention. Mutat Res1988; 202: 307-24.
- Bashirov MS, Nugmanov SN, Kolycheva NI. Epidemiological study on esophageal cancer in the Akhtubinsk region of the Kazakh Socialist Republic (Russ). Vopr Onkol 1968;14: 3-7.

- Becher G, Knize MG, Nes IF, Felton JS. Isolation and identification of mutagens from a fried Norwegian meat product. *Carcinogenesis* 1988; 9: 247-53.
- Berg I, Overvick E, Gustafsson JA. Effect of cooking time on mutagen formation in smoke crust and pan residue from pan-broiled pork. *Food Chem Toxicol* 1990; 28: 421- 6.
- Bjeldanes LF, Morris MM, Felton JS, Hatch S. Mutagens from the cooking of food. II survey by Ames/*Salmonella* test of mutagen formation in the major protein rich foods of the American diet. *Food Chem Toxicol* 1982 ; 20 : 357-63.
- Block G. Vitamin C and cancer preventive : the epidemiologic evidence. *Am J Clin Nutr* 1991 ; 53: 270s-282s.
- Cerutti PA. Mechanisms of action of oxidant carcinogens. *Cancer Detect Prev* 1989; 14: 281-4.
- Chen C, Pearson AM, Gray JJ. Meat mutagens. *Adv Food Nutr Res* 1990; 34: 387-449.
- Chen JS. The effect of Chinese tea on the occurrence of esophageal tumors induced by N-nitrosomethyl benzylamine in rats. *Prev Med* 1992; 21: 385-91.
- Chiu CP, Chen BH. Stability of heterocyclic amines during heating. *Food Chem Toxicol* 2000; 68: 267-72.
- Commoner B , Vithayathil AJ, Dolara P, Nairs, Madyastha P, Cuca GC. Formation of mutagen in beef and beef extract during cooking. *Science* 1978 ; 201: 913- 6.

- Cook – Mozaffari PJ, Azordegan F, Day NE, Ressicaud A, Sabai C, Aramesh B. Esophageal cancer studies in the Caspian Littoral of Iran: results of a case-control study. *Br J Cancer* 1979;39: 293-309.
- Demirer T, Icli F, Uzunlimoglu O, Kucuk O. Diet and stomach cancer incidence. A case control study in Turkey. *Cancer* 1990; 65(10): 2344-8.
- De Stefani E, Boffetta P, Mendilaharsu M, Carzoglio J, Deneo-Pellegrini H. Dietary nitrosamines, heterocyclic amines, and risk of gastric cancer: A case control study in Uruguay. *Nutr Cancer* 1998;30(2): 158-62.
- De Stefani E, Ronco A, Mendilaharsu M, Guidobono M, Deneo-Pellegrini H. Meat intake, heterocyclic amines, and risk of breast cancer : A case control study in Uruguay. *Cancer Epidemiol Biomarker Prev* 1997; 6:573-81.
- Dolara P, Commoner B, Vithayathil A, Cuca G, Tuley E, Madyastha P, *et al.* The effect of temperature on the formation of mutagens in heated beef stock and cooked ground beef. *Mutat Res* 1979 : 60: 231-7.
- Doll R, Peto R. The causes of cancer : Quantitative estimates of avoidable risk of cancer in United States today. *J Natl Cancer Inst* 1981; 66: 1191-308.
- Doolittle DJ, Rahn CA, Burger CT, Lee CK, Reed B, Riccio E. Effect of cooking methods on the mutagenicity of food and on urinary mutagenicity of human consumer. *Food Chem Toxicol* 1989; 27: 657-66.
- Felton JS, Fultz E, Dolbeare FA, Knize MG. Effect of microwave pretreatment on heterocyclic aromatic amine mutagens carcinogens in fried beef patties. *Food Chem Toxicol* 1994 ; 32 : 897-903.

- Felton JS, Knize MG. New mutagen from cooked food. In : Pariza MW, Felton JS, Aeschbacher HU, Sato S, editors. Mutagen and carcinogen in the diet. New York : A John Wiley & Sons ; 1990. p. 19 -38.
- Felton JS, Knize MG. Mutagen formation in muscle meats and model heating systems. In: Hayatsu H. editor, Mutagen in food : detection and prevention. Florida : FL-CRC Press; 1991a.p. 58-66.
- Felton JS, Knize MG. Occurrence, identification and bacterial mutagenicity of heterocyclic amines in cooked food. *Mutat Res* 1991b;259: 205-17.
- Felton JS, Knize MG, Bjeldanes LF, Kimble BJ, Healy SK, Stuermer DH, *et al.* Isolation and characterization of new mutagen from fried ground beef. *Carcinogenesis* 1984 ; 5 : 95-102.
- Felton JS, Knize MG, Roper M, Fultz E, Shen NH, Turteltaub KW. Chemical analysis prevention and low-level dosimetry of heterocyclic amines from cooked food. *Cancer Res* 1992; (supple) 52 : 2103s-07s.
- Felton JS, Knize MG, Shen NH, Andresen BD, Bjeldanes LF, Hatch FT. Identification of the mutagens in cooked beef. *Environ Health Perspect* 1986b; 67:17-24.
- Felton JS, Knize MG, Shen NH, Lewis PR, Andresen BD, Happe J, Hatch FT. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP) . *Carcinogenesis* 1986 a ; 7: 1081-6
- Fukuhara Y, Yoshida D, Goto F. Reduction of mutagenic products in the presence of polyphenols during pyrolysis of protein. *Agric Biol Chem* 1981; 45: 1061-6.

- Gao YT, McLaughlin JK, Blot WJ, Ji B, Dai Q, Fraumeni JJ. Reduced risk of esophageal cancer associated with green tea consumption. *J Natl Cancer Inst* 1994; 86: 855-8.
- Gerhardsson de Verdier M, Hagman U, Peters RK, Steineck G, Overvik E. Meat, cooking methods and colorectal Cancer : A case-referent study in Stockholm. *Int. J. Cancer* 1991; 49: 520 - 5.
- Graham S, Lilienfeld AM, Tiding JF. Dietary and purgation : factors in the epidemiology of gastric cancer. *Cancer* 1967; 20: 2224-34
- Gross GA. Simple method for quantifying mutagenic heterocyclic aromatic amine in food products. *Carcinogenesis* 1990 ; 11: 1597-603.
- Hansson LE, Nyren O, Bergstrom R, Wolk A, Lindgren A, Baron J, Adami HO. Diet and risk of gastric cancer. A population based case control study in Sweden. *Int J Cancer* 1993;55: 181-9.
- Harold N, Graham D. Green tea composition, consumption, and polyphenol chemistry. *Prev Med* 1992; 21: 334-50.
- Hartman PPE, Leviene K, Hartmen Z, Berger H. Classification and mapping of spontaneous and induced mutations in the histidine operon of *Salmonella*. *Adv Genet* 1971;16: 1-34.
- Hermaez J, Xu M, Dashwood RH. Antimutagenic activity of tea towards 2-hydroxyamino-3-methylimidazo [4,5-f] quinoline : Effect of tea consumption and brew time on electrophile scavenging . *Mutat Res* 1998 ; 402: 299-306.

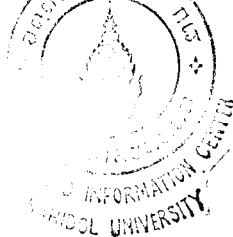
- Hertog MG, Hollman PCH, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 1993;20: 21-9.
- Higginson J. Etiological factors in gastrointestinal cancer in man. *J Natl Cancer Inst* 1966; 37: 527-45.
- Hirohata T, Kono S. Diet/nutrition and stomach cancer in Japan. *Int J Cancer* 1997; suppl10: 34-6.
- Hu J, Nyven O, Wolk A, Bergstrom R, Yuen J, Adami HO, *et al.* Risk factors for esophageal cancer in northern China. *Int J Cancer* 1994;57(1): 38-46.
- Huang MT, Ho CT, Wang ZY. Inhibitory effect of topical application of a green tea polyphenol fraction on tumor initiation and promotion in mouse skin. *Carcinogenesis* 1992; 13: 947-54.
- Hutton W, Cassio A. Tropical herbs & spices of Thailand. Bangkok: Asia Books; 1998. P.47.
- Isono K, Yourno J. Chemical carcinogens as frameshift mutagens : *Salmonella* DNA sequence sensitive to mutagenesis by polycyclic carcinogens. *Proc Natl Acad Sci (U.S.A.)* 1974;71: 1612-7.
- Ito N , Hasegawa R, Sano M, Tamano S, Esumi H, Takayama S, Sugimura T. A new colon and mammary carcinogen in cooked food 2-amino-1- methyl-6- phenylimidazo [4,5-b]- pyridine (PhIP). *Carcinogenesis* 1991; 12: 1503-6.
- Jackson LS, Hargraves WA. Effect of time and temperature on the formation of MeIQx and DiMeIQx in a model system containing threonine, glucose and creatinine. *J Agri Food Chem* 1995; 43: 1678-84.

- Jackson LS, Hargraves WA, Stoup WH, Diachenko GW. Heterocyclic aromatic amines content of selected beef flavors. *Mutat. Res* 1994 ; 320:113-24.
- Jagerstad M, Laser Reutersward A, Olsson R, Grivas S, Nyhammar T, Olsson R, Dahlqvist A. Creatinin (in)e and Maillard reaction products as precursors of mutagenic compounds : effect of various amnio acid. *Food Chem* 1983b ; 12 : 255-64.
- Jagerstad M, Laser Reutersward A, Oste A, Dahlqvist A, Grivas S, Olsson K, Nyhammar T. Creatine and Maillard reaction products as precursors of mutagenic compounds formed in fried beef. In: Waller GR, Feather MS. editors, *The Maillard Reaction in Foods and Nutrition ACS Symposium. Serie 215.* Washington DC: American Chemical Society: 1983a. p. 507-19.
- Jain AK, Shimoi K, Nakamura Y, Kada T, Hara Y Tamita I. Crude tea extract decrease the mutagenic activity of N-methyl-N'-nitro-N- nitroso guanidine in vitro and in intragastric tract of rats. *Mutat Res.* 1989 ; 210: 1-8.
- Ji.H , Yu MC, Stillwell WG, Skipper PL, Roos RK Henderson BE, Tannenbaum SR. Urinary excretion of 2-amino-3,8-dimethylimidazo [4,5 - f] quinoxaline on white, black and Asian men in Los Angeles Country. *Cancer Epidemiol Biomarker & Prev* 1994;3:407-11.
- Johansson M, Jagerstad M. Occurrence of mutagenic/ carcinogenic heterocyclic amines in meat and fish products, including pan residues, prepared under domestic conditions. *Carcinogenesis* 1994 ; 15: 1511-8.
- Johansson M, Jagerstad M. Influence of pro and antioxidant on the formation of

- mutagenic carcinogenic heterocyclic amines in a model system. *Food Chem* 1996; 56: 69-75.
- Johansson M, Skog K, Jagerstad M. Effect of edible oils and fatty acids on the formation of mutagenic heterocyclic amines in a model system. *Carcinogenesis*, 1993 ; 14: 89-94.
- Jones C. Weisburger J. L-Tryptophan inhibits formation of mutagens during cooking of meat and in laboratory models. *Mutat Res* 1988 ; 206 : 343-9.
- Josephy PD, Greez P, Nohmi T. Recent advances in the construction of bacterial genotoxicity. *Mutat Res* 1997;386(1): 1-23.
- Kangsadalampai K, Butryee C, Manconphol K. Direct mutagenicity of the polycyclic aromatic hydrocarbon containing fraction of smoked and charcoal broiled food treated with nitrite in acid solution. *Food Chem Toxicol* 1996; 35:213-8.
- Kapadia GJ, Rao S, Morton JF. Herbal tea consumption and esophageal cancer. In : Stich HF, editor. *Carcinogens and mutagens in environment*. Boca Raton, Florida: CRC Press; 1983. p. 3-12.
- Kasai H, Yamaizumi Z, Shiomi T, Yokoyama S, Miyazawa T, Wakabayashi K, *et al.* Structure of a potent mutagen isolated from fried beef. *Chem Lett* 1981 ; 4: 485-8.
- Kasai H, Yamaizumi Z, Wakabayashi K, Nagao M, Sugimura T, Yokoyama S, *et al.* Potent novel mutagens produced by broiling fish under normal conditions. *Proc Jpn Acad* 1980 a ; 56 B : 278-83.

- Kasai H, Yamaizumi Z, Wakabayashi K, Nagao M, Sugimura T, Yokoyama S, *et al.*
Structure and chemical synthesis of Me-IQ , a potent mutagen isolated from
broiled fish. Chem Lett 1980 b; 1391-4.
- Katiya SK, Agarwal R , Wood GS , Mukhtar H. Inhibition of 12-*O*-tetradecanoyl
phorbol -13-acetate-caused tumor promotion in 7,12-dimethyl benz[a]
anthracene-initiated SENCAR mouse skin by a polyphenolic fraction
isolated from green tea. Cancer Res 1992; 52: 6890-7.
- Kato T, Kikugawa K, Hayatsu H. Occurrence of the mutagens 2-amino-3,4,8-
trimethyl - imidazo [4,5- *f*] quinoxaline (4,8-Me, IQx) in some Japanese
smoked, dried fish products. J of Agric and Food Chem 1986; 34: 810-4.
- Kato T, Tadokoro N, Tsutsui M, Kikugawa K. Transformation of arylamines
into direct-acting mutagens by reaction with nitrite. Mutat Res. 1991; 249:
243-54.
- Kaufman BD, Liberman JS, Tyshetsky VI. Some data concerning the incidence
of esophageal cancer in the Gurjev region of the Kazakh SSR
(Russ). Vopr Onkol 1965; 11: 78-85.
- Keating GA, Layton DW, Felton JS. Factor determining dietary of heterocyclic
amines in cooked foods. Mutat Res 1999 ; 443 : 149-56.
- Khan SG, Katiyar SK, Agarwal R. Enhancement of antioxidant and phase II
enzymes by oral feeding of green tea polyphenols in drinking water to
SKH-1 hairless mice: Possible Role in cancer chemoprevention.
Cancer Res 1992; 52: 4050-2.
- Khan WA, Wang ZY, Athar M. Inhibition of the skin tumorigenicity of ($\pm$) 7 β ,
8 α -dihydroxy-9 α ,10 α -epoxy-7, 8, 9, 10-tetrahydrobenzo (a) pyrene by

- tannic acid, green tea polyphenols and quercetin in Sencar mice. *Cancer Lett* 1988; 42: 7-12.
- Kigashimoto M, Akada Y, Sata M, Kinouchi T, Kuwahara T, Ohnishi Y. Inhibitory effect of tea extracts on the mutagenicity of 1- Methyl-1,2,3,4-tetrahydro β -carboline-3-carboxylic acid on treatment with nitrite in the presence of ethanol. *Food Chem Toxicol* 2000 ; 38: 7-13.
- Kikugawa K, Nagao M. Nitrosatable precursors of mutagen in Food. In: Hayatsu H, editor. *Mutagen in food : Detection and prevention*. Boca Raton : CRC ; 1991. p. 68-85.
- Kinlen IJ, McPherson K. Pancreas cancer and coffee and tea consumption : a case – control study. *Br J Cancer* 1984; 49: 93-6.
- Kinouchi T, Manabe Y, Yakisaka K, Ohinishi Y. Biotransformation of 1-aminopyrene, 1-nitropyrene in intestinal anaerobic bacteria. *Microbial Immunol* 1982; 26: 993-1005.
- Knize M.G, Dolbear FA, Carroll KL, Moore DH, Felton JS. Effect of cooking time and temperature on the heterocyclic amines content of fried beef patties. *Food Chem Toxicol* 1994 ; 32: 595-603.
- Knize M.G., Roper M., Shen N. H. Felton J.S. Proposed structure for an amino-dimethylimidazofuropyridine mutagen in cooked meats. *Carcinogenesis* 1990 ; 11: 2259-62.
- Kohlmeier L, Weterings KGC, Steck S, Kok FJ. Tea and cancer prevention : An evaluation of the epidemiologic literature. *Nutr. and Cancer*. 1997; 27 (1): 1-13.
- Kono S. Green tea and colon cancer. *Jpn J Cancer Res* 1992; 83(6): 669.



- Kuroda Y, Hara Y. Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutat Res* 1999; 436: 69-97.
- La Vecchia C, Negri E, Franceschi SD, Avanzo B, Boyle P. Tea consumption and cancer risk. *Nutr Cancer* 1992;17: 27-31.
- Laser-Reutersward A, Skog K, Jagerstad M. Mutagenicity of pan-fried bovine tissue in relation to their content of creatine, creatinine, monosaccharides and free amino-acids. *Food Chem Toxicol* 1987 ; 25: 755-62.
- Laskin JD, Heck D, Laskin DL. Inhibitory effects of a green tea polyphenol fraction on 12-0-tetradecanoylphorbol-13-acetate-induced hydrogen peroxide formation in mouse epidermis. In : Huang MT, Ho CT, Lee CY, editors. *Phenolic compounds in foods and health II: Antioxidant & cancer preventing*. Washigton DC: Amer Chem Soc; 1992. p. 308-14.
- Lee HH, Wu HY, Chuang YC, Chang AS, Chao HH, Chen KY, *et al*. Epidemiologic characteristics and multiple risk factors of stomach in Taiwan. *Anticancer Res* 1990; 10(4): 875-81.
- Levin DE, Hollstein MC, Christman MF, Schwiers EA, Ames BN. A new *Salmonella* tester strain (TA102) with A : T base pairs at the site of mutation detects oxidative mutagens. *Proc Natl Acad Sci (U.S.A.)* 1982;79: 7445-9.
- Lijinsky W, Shubik P. Benzo (a) pyrene and other polynuclear hydrocarbons in charcoal-broiled meat. *Science* 1964 ; 145 : 53-5.
- Liu L, Castonguay A. Inhibition of the metabolism and genotoxicity of 4-(methylnitrosamino)-1-(3-pyridyl) -1-butanone (NNK) in rat hepatocytes by (+)-catechin. *Carcinogenesis* 1991; 12:1203-8.

- Lou FQ. A study of prevention of atherosclerosis with tea-pigment. Presented at the Int Tea - Quality - Human Health Sympo (China) 1987; p. 141-3.
- Lynch AM, Knize MG, Boobis AR, Gooderham NJ, Davies DS, Murray S. Intra and interindividual variability in systemic exposure in human to 2-amino-3,8-dimethylimidazo-[4-5, f]quinoxaline and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]-pyridine carcinogens present in cooked beef. *Cancer Res.* 1992; 52: 6216-23.
- Maron DM, Ames BN, Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 1983 ; 113 : 173-215.
- Marquardt H, Rufino F, Weisburger JH. Mutagen activity of nitrite-treated food; human stomach cancer may be related to dietary factors. *Science* 1977; 196: 1000-1.
- McCann J, Ames BN. Detection of carcinogens as mutagens in the *Salmonella*/microsome test : Assay of 300 Chemicals: Discussion. *Proc Natl Acad Sci (U.S.A.)* 1976 ; 73: 950-54.
- McCann J, Ames BN. The *Salmonella*/microsome mutagenicity test : Predictive value for animal carcinogenicity. In : Hiatt HH, Watson JD, Winsten JA, editors. *Origins of Human Cancer*, Cold Spring Harbor laboratory. New York : Cold Spring Harbor; 1977.p.1431-50.
- McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens as mutagens in the *Salmonella*/microsome test : Assay of 300 chemicals. *Proc Natl Acad Sci. (U.S.A.)* 1975a; 72: 5135-9.

- McCann J, Spingarn NE, Kobori J, Ames BN. Detection of carcinogens as mutagens : bacterial tester strains with R factor plasmids. Proc Natl Acad Sci (U.S.A.) 1975b;72: 979-83.
- Memik F, Nak SG, Gulten M, Ozturk M. Gastric carcinoma in northwestern Turkey epidemiologic characteristics, J Environ. Pathol Toxicol Oncol 1992; 11:335-8.
- Mirvish SS. Formation of N-nitroso compounds : Chemistry, kinetic and in vivo occurrence. Toxicol Appl Pharmacol. 1975; 31: 325-51.
- Mirvish SS. The etiology of gastric cancer : intragastric nitrosamide formation and other theories. J Natl Cancer Inst 1983; 7: 629-47.
- Muramatsu M, Matsushima T. Formation of MeIQx and 4,8-DiMeIQx by heating mixtures of creatinine, amino acids and monosaccharides. Mutat Res 1985 ; 147: 266-7.
- Nader CJ , Spencer LK, Weller RA. Mutagen production during pan-broiling compared with microwave irradiation of beef. Cancer Lett 1981;13: 147-51.
- Nagahara A, Ohshita K, Nasuno S. Relation of nitrite concentration to mutagen formation in soy sauce. Food Chem toxicol 1986;24:13-5.
- Nagao M , Honda M , Seino Y , Yahagi T, Sugimura T. Mutagenicities of smoke condensates and the charred surface of fish and meat. Cancer lett 1977 ; 2:221-6.
- Nagao M, Takahashi Y, Yamanaka H. Mutagen in coffee and tea. Mutat Res 1979; 68: 101-6.
- Nakamura M, Kawabata T. Effect of Japanese green tea on nitrosamine formation in vitro. J Food Sci 1981 ; 46: 306-7.

- Namiki K, Yamanaka M, Osawa T, Namiki M. Mutagen formation by nitrite-spice reaction. *J Agric Food Chem* 1984;32 : 948-52.
- Negishi C, Wakabayashi K, Tsuda M, Sato S, Sugimura T, Saito H, *et al.* Formation of 2-amino-3,7,8-trimethylimidazo[4,5-*f*] quinoxaline, a new mutagen by heating mixture of creatinine , glucose and glycine. *Mutat Res* 1984 ; 140: 55-9.
- Negishi C, Wakabayashi K, Yamaizumi J, Saito H, Sato S, Jagersted M. Identification of 4,8-DiMeIQx, a new mutagen.(Abstract). *Mutat Res* 1985 ; 147 : 267-68.
- Newmark HL. Plant phenolics as potential cancer prevention agents. In: Back N, Cohen IR, Kritchewsky D, Lajtha A, Paoletti R, editors. *Dietary phytochemicals in cancer prevention and treatment*. New York: Plenum Press; 1996. p.25-34.
- Nilson L, Overvick E, Fredholm L, Levin O, Nard CE, Gustafsson JA. Influence of frying fat on mutagenic activity in lean pork meat. *Mutat Res* 1986; 171: 115-21.
- Norell SE, Ahlbom A, Erwald R, Jacobson G, Lindberg-Navier I, Olin R, *et al.* Diet and pancreatic cancer : A case-control study. *Am. J Epidemiol.* 1986; 124: 894-902.
- Oguni I, Chen SJ, Lin PZ, Hara Y. Protection against cancer risk by Japanese green tea. *Prev. Med* 1992; 21: 332.
- Ohgaki H, Takayama S, Sugimura T. Carcinogenicities of heterocyclic amines in cooked food. *Mutat Res* 1995; 259:399-410.

- Ohshima H, Bartsch H. Quantitative estimation of endogenous nitrosation in human by monitoring N-Nitrosoproline excreted in the urine. *Cancer Res* 1981; 41: 3658-62.
- Osawa T, Ishibashi H, Namiki M, Yamanaka M, Namiki K. Formation of mutagens by pepper-nitrite reaction. *Mutat Res* 1984;91: 291-3.
- Overvik E, Kleman M, Berg I, Gustafsson JA. Influence of creatine, amino acids and water on the formation of the mutagenic heterocyclic amines found in cooked meat. *Carcinogenesis* 1989 ; 10:2293-301.
- Overvik E, Nilsson L, Fredholm L, Levin O, Nord CE , Gustafsson JA. Mutagenicity of pan residues and gravy from fried meat. *Mutat Res* 1987 ; 187 : 47-55.
- Peerawong K, Kangsadalampai K. Mutagenic potential of various chickens extracts after nitrosation. *Mahidol J* 1998 ; 5 (1) : 5-8.
- Piacek-Llanes BG and Tannenbaum SR. Formation of an activated N-nitroso compound in nitrite treated fava beans (*Vicia faba*). *Carcinogenesis* 1982; 3:1379-84.
- Pignatelli B, Bereziat JC, Descotes G, Bartsch H. Catalysis of nitrosation in vitro and in vivo in rats by catechin and resorcinol an inhibition by chlorogenic acid. *Carcinogenesis* 1982; 3(9) : 1045-9.
- Purchase IFH, Longstaff E, Ashby J, Styes JA, Anderson D, Lefevre PA, Westwood FR. Evaluation of six short term tests for detecting organic chemical carcinogens and recommendations of their use. *Nature (London)* 1976;264: 624-27.

- Robbana-Barnet S, Rabache M, Rialland E, Fradin J. Heterocyclic amines : Occurrence and prevention in Cooked food. *Environ Health Perspect* 1996 ; 104 (3) : 280-8.
- Ruch RJ, Cheng SJ, Klauning JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 1989; 10: 1003-8
- Sasagawa C, Muramatsu M, Matsushima T. Formation of direct mutagen from amino-imidazoazaarenes by nitrite treatment. (Selected Abstracts of the 16th Annu. Meet. Environmental Mutagen Society of Japan. 27-28 October 1987, Kyoto , Japan) . *Mutat Res* 1988 ; 203 : 386.
- Schiffman M.H, Felton J.S. Fried foods and the risk of colon cancer. *Am J Epidemiol* 1990;131: 376- 8.
- Shephard SE, Schlatter CH, Lutz WK. Assessment of risk of formation of carcinogenic N-nitroso compounds from dietary precursors in the stomach. *Food Chem. Toxicol* 1987 ; 25 (1) : 91-108.
- Shi ST, Wang ZY, Smith TJ, Hong JY, Chen WF, Ho CT, *et al.* Effect of green tea and black tea on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone bioactivation , DNA methylation, and lung tumorigenesis in AJ mice. *Cancer Res* 1994;5:4641-7.
- Shibamoto T. Possible mutagenic constituents in nitrite-treated soy sauce. *Food Chem Toxicol* 1983; 21:745-7.
- Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, *et al.* The prostate: A target for carcinogenicity of 2-amino-1-methyl-6- phenylimidazo

[4,5-*b*] pyridine (PhIP) derived from cooked food. *Cancer Res* 1997; 57: 195-8.

Sinha R, Knize MG, Salmon CP, Brown ED, Rhodes D, Felton JS, *et al.*
Heterocyclic amine content of pork products cooked by different methods and to varying degrees of doneness. *Food Chem Toxicol* 1998 b; 36 : 289-97.

Sinha R, Rothman N, Brown EO, Salmon CP, Knize MG, Swanson CA, *et al.*
High concentrations of the carcinogen 2-amino-1-methyl-6- phenylimidazo-[4,5-*b*] pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res* 1995 ; 54: 6154-9.

Sinha R, Rothman N, Salmon CP, Knize MG, Brown ED, Swanson CA, *et al.*
Heterocyclic amine content of beef cooked by different methods to varying degrees of doneness and gravy made from meat drippings. *Food Chem Toxicol* 1998 a ; 36 : 279-87.

Skog K. Cooking procedures and food mutagens : A Literature Review. *Food Chem Toxicol* 1993 ; 31 (9) : 655-75.

Skog K, Augustsson K, Steineck G, Stenberg MM, Jagerstad M. Polar and non-polar heterocyclic amines in cooked fish and meat products and their corresponding pan residues. *Food Chem Toxicol* 1997; 35: 555-65.

Skog K, Jagerstad M. Effect of monosaccharides and disaccharides on the formation of food mutagens in model system. *Mutat Res* 1990; 230: 263-72.

Skog K, Steineck G, Augustsson K, Jagerstad M. Effect of cooking temperature on the formation of heterocyclic amines in fried meat products and pan residues. *Carcinogenesis* 1995 ; 16 : 861-67.

- Spingarn N, Garvie - Gould C, Vuolo L, Weisburger JH. Formation of mutagens in fried beef patties. *Cancer Lett* 1981 ; 13:93-7.
- Stavric B. Biological significance of trace level of mutagenic heterocyclic amines in human diet : a critical review. *Food Chem Toxicol* 1994 ; 32: 977-94.
- Steineck G, Hagman U, Gerhardsson M, Norell SE. Vitamin A supplements, fried foods, fat and urothelial cancer, A case- referent study in Stockholm in 1985-1987. *Int J Cancer* 1990; 45: 1006-11.
- Stellman JM, McCann M, Warshaw L, Brabant C, Finklea J, Coppee GH, *et al.* In: *Encyclopedia of occupational health and safety*. 4 edition. Geneva International labor office. 1998. p. 43-50.
- Stich HF. Teas and tea components as inhibitors of carcinogen formation in model systems and man. *Prev Med* 1992; 21: 377-84.
- Stich HF, Rosin MP, Bryson L. Inhibition of mutagenicity of a model nitrosation reaction by naturally occurring phenolics, coffee and tea. *Mutat Res* 1982; 95: 119-28.
- Stocks P. Cancer mortality in relation to national consumption of cigarettes, solid fuel, tea and coffee. *Br J Cancer* 1970; 24: 215-25.
- Sugimura T. Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat Res* 1985;150: 33-41.
- Sugimura T. Studies on environmental chemical carcinogenesis in Japan. *Science* 1986; 233: 312-8.
- Sugimura T. Multistep carcinogenesis : A 1992 perspective. *Science* 1992; 258: 603-7.

- Sugimura T, Fujimura S. Tumour production in glandular stomach of rat by N-methyl-N-nitrosoguanidine. *Nature (London)* 1967; 216: 943.
- Sugimura T, Kawachi T, Nagao M, Yahagi T, Seino Y, Okamoto, T, *et al.* Mutagen principle (s) in tryptophan and phenylalanine pyrolysis products. *Proc Jpn Acad* 1977b ; 53: 58 - 61.
- Sugimura T, Nagao M , Kawachi T, Honda M, Yahagi T, Seino Y, *et al.* Mutagens-carcinogens in food ; with special reference to highly mutagenic pyrolytic products in broiled foods. In : Hiatt HH, Watson JD, Winston JA, editors. *Origin of Human Cancer*. New York: Cold Spring Harbor; 1977a.p.1561-77.
- Sugimura T, Nagao M, Wakabayashi K. Heterocyclic amines in cooked food: Candidates for causation of common cancer. *J Natl. Cancer Inst* 1994 ; 86: 2-4.
- Sugimura T, Sato S. Mutagens - carcinogens in Food. *Cancer Res (Suppl.)* 1983 ; 43 : 2415s-21s.
- Sugimura TS, Sato S, Nagao M, Yahagi T, Matsushima T, Seino Y, *et al.* Overlapping of carcinogens and mutagens. In : Magee PN *et al.*, editors. *Fundamentals in cancer prevention*. Tokyo: University of Tokyo Press; 1976. p. 191-215.
- Sugimura T, Wakabayashi K. Mutagens and carcinogens in the diet. In : Pariza MW, Felton JS, Aeschbacher HU, Sato S, editors, *Mutagens and carcinogens in the diet*. New York : Wiley Liss, 1990 ; p 1-18.
- Sugimura T, Wakabayashi K, Nagao M, Esumi H. A new class of carcinogens : heterocyclic amines in cooked food. In: Parke DV, Loannides C, Walker R,

editors. Food nutrition and chemical toxicity. Great Britain: Smith Gordon; 1993. p. 259-76.

Synderwine EG, Yamashita K, Adamson RH, Sato S, Nagao M, Sugimura T, Thorgeirsson SS. Use of the ^{32} P. postlabeling method to detect DNA adducts of α -amino-3-methylimidazo [4,5-*f*]-quinoline (IQ) in monkey fed IQ: identification of the N-(deoxyquarasin-8-yl) IQ adduct. Carcinogenesis (London) 1988; 9: 1739-43.

Tajima K, Tominaga S. Dietary habits and gastrointestinal cancer : a comparative case control study of stomach and large intestinal cancers in Nagoya. Jpn J Cancer Res (Gann) 1985; 76: 705-16.

Takahashi M, Toyoda K, Aze Y, Furuta K, Mitsumori K, Hayashi Y. The rat urinary bladder as a new target of heterocyclic amines carcinogenicity; Tumor induction by 3-amino-1-methyl-5H- pyrido [4,3-*b*] indole acetate. Jpn J Cancer Res. 1993; 84: 852-8.

Takayama S, Nakotsuru Y, Ohagaki H, Sata S, Sugimura T. Atrophy of salivary glands and pancreas of rats fed on diet with amino-methyl- α - carboline. Proc Jpn Acad 1985; 61(Ser B): 277-80.

Tanaka K, Hayatsu T, Negishi T, Hayatsu H. Inhibition of N-nitrosation of secondary amines in vitro by tea extracts and catechin. Mut Res 1998; 412: 91- 8.

Tanizawa H, Toda S, Sazuka Y, Taniyama T, Hayashi T, Arichi S, Takino Y. Natural antioxidants.I. Antioxidative components of tea leaf (*Theasinensis*). Chem Pharm Bull 1984; 32: 2011-4.

- Taylor RT, Fultz E, Knize MG. Mutagens formation in a model beef boiling system. III. Purification and identification of three heterocyclic amine mutagens - carcinogens. J of Environ Sci and Health 1985; A20: 135- 48.
- Taylor RT, Fultz E, Knize MG. Mutagen formation in a model beef supernatant fraction IV. Properties of the system. Environ Health Prospect 1986 ; 67: 59-74.
- Trichopoulos D, Ouranos G, Day NE, Tzonou A, Manousos O, Papadimitriou C, Trichopoulos A. Diet and cancer of the stomach : a case-control study in Greece. Int J Cancer 1985; 36: 291-7.
- Tsuda M, Negishi C, Makino R, Sato S, Yamaizumi Z, Hirayama T, Sugimura T. Use of nitrite and hypochlorite treatments in the determination of the contributions of IQ-type and non-IQ-type heterocyclic amines to mutagenicities in crude pyrolyzed materials. Mutal Res 1985; 147 : 335- 341.
- Turesky RJ, Bur H, Huynh-Ba T, Aeschbacher HU, Milon H. Analysis of mutagenic heterocyclic amines in cooked beef products by high-performance liquid chromatography in combination with mass spectrometry. Food Chem. Toxicol 1988 ; 26:501-9.
- Uchida S, Edamatsu R, Hiramatsu M, Mori A, Nonak G, Nishioka I, *et al.* Condensed tannins scavenge oxygen free radical. Med Sci Res 1987; 15: 831-2.
- Ushiyama H, Wakabayashi K, Hirose M, Itoh H, Sugimura T., Nagao M. Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation. Carcinogenesis 1991;12:1417-22.

- Uyeta M, Taue S, Mazaki M. Mutagenicity of hydrolysate of tea infusions. *Mutat Res* 1981; 88: 233-40.
- Vahl M, Gry J, Nielson PA. Mutagens in fried pork and the influence of the frying temperature. *Mutat Res* 1988 ; 203:239.
- Victora CG, Munoz N, Day NE, Barcelos JB, Peccin DA, Braga NM. Hot beverages and esophageal cancer in southern Brazil : a case control study. *Int J Cancer* 1987; 39: 710-6.
- Wakabayashi K, Nagao M, Chung TH, Yin MQ, Karai L, Ochial M, *et al.* Appearance of direct acting mutagenicity of various food stuff produced in Japan and South East Asia on nitrite treatment. *Mutat Res* 1984;158:119-24.
- Wakabayashi K, Nagao M, Esumi H, Sugimura T. Food derived mutagens and carcinogens. *Cancer Res (Suppl.)* 1992 ; 52 : 2092s - 8s.
- Wakabayashi K, Nagao M, Ochial M, Fujita Y, Tahira T, Nakayasu M, *et al.* Recently identified nitrite-reactive compounds in food : occurrence and biological properties of the nitrosated products. In : Bartsch H, O'Neill IK, Schulte-Herman R, editors. The relevance of N-nitroso compounds to human cancer: Exposures and mechanisms. Lyon France : IARC Scientific Publ. No. 84 International Agency for Research on Cancer; 1987.p. 287-91.
- Wakabayashi K, Ochial M, Saito H, Tsuda M, Suwa Y, Nagao M, Sugimara T. Presence of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid, a precursor of a mutagenic nitroso compound in soy sauce. *Proc Natl Acad USA* 1983;80:2912-6.
- Wakabayashi K, Sugimura T. Heterocyclic amines formed in the diet: Carcinogenicity and its modulation by dietary factors. *J Nutr Biochem* 1998 ; 9 : 604-12.

- Wakabayashi K, Ushiyama H, Takahashi M, Nukaya H, Kim, S.B, Hirose M, *et al.*
Exposure to heterocyclic amines. *Environ Health Perspect* 1993; 99: 129-33.
- Wang H, Wu Y: The blocking effect of Chinese tea on N-nitrosation in vitro and in vivo. In: Prasad KN, Meyskens FL Jr, editors. *Nutrients and cancer prevention*. New Jersey: Human Press; 1990. p. 546- 49.
- Wang YY, Vuolo LL, Spingarn NE, Weisburger JH. Formation of mutagens in cooked food. V. The mutagen reducing effect of soy protein concentrates and antioxidants during frying of beef. *Cancer Lett* 1982; 16: 179-89.
- Wang ZY, Das M, Bickers DR. Interaction of epicatechins derived from green tea with rat hepatic cytochrome P-450. *Drug Metab Dispos Biol Fate Chem* 1988; 16: 98-103.
- Wang ZY, Zhou ZC, Bickers DR. Inhibition of chemical and photocarcinogenesis in murine skin by green tea polyphenols. *Proc Am Assoc Cancer Res* 1990; 31:159.
- Ward MH, Sinha R, Heineman EF, Rothman N, Markin R, Weisenburger DD, *et al.* Risk adenocarcinoma of the stomach and esophagus with meat cooking method and doneness preference. *Int. J. Cancer* 1997; 71 : 14- 9.
- Wattenberg LW. Inhibition of neoplasia by minor dietary constituents. *Cancer Res* 1983;43:2448s-53s.
- Wattenberg LW. Chemoprevention of cancer. *Cancer Res* 1985;45:1-8.
- Wei CI , Kitamura K , Shibamoto T. Mutagenicity of Maillard browning products obtained from a starch glycine model system. *Food Chem. Toxicol* 1981 ; 19 : 749-51.

- Weisburger JH. Nutritional approach to cancer prevention with emphasis of vitamins, antioxidants, and carotenoids. *Am J Clin Nutr* 1991;53:226s-37s.
- Weisburger JH, Horn CL. Human and laboratory studies on the causes and prevention of gastrointestinal cancer. *Scand J of Gastroenterology* 1984; 104 suppl: 15-26.
- Weisburger JH, Nagao M, Wakabayashi K, Oguri A. Prevention of heterocyclic amines formation by tea and tea polyphenols. *Cancer Lett* 1994; 83: 1-2.
- Widmark EMP. Presence of cancer - producing substances in roasted food. *Nature* 1939 ; 143: 984.
- Williams GM, Wynder EL. Diet and cancer: Asynopsis of causes and preventions strategies. In: Watson RR, Siraj IM, editors. *Nutrition and cancer prevention*. New York: CRC Press; 1996. p. 1-12.
- World Health Organization, International Agency for Research on Cancer, Tea, Mate, Methylxanthine and Methylglyoxal. *International Agency for research on Cancer Monograph on the Evaluation of Carcinogenic Risks to Human*, Vol 51, Lyon LARC Press:1991. p 201-71.
- Wu Y, Wang H, Li J, Han C. The inhibitory effect of Chinese tea and its polyphenol on in vitro and in vivo N-nitrosation. *Biomed and Environ Sci*. 1993 ; 6: 237-58.
- Wynder EL, Gori GB. Contribution of the environment to cancer incidence : An epidemiologic exercise. *J Natl Cancer Inst* 1977 ; 58: 825-32.
- Xu Y, Han C: The effect of Chinese tea on the occurrence of esophageal tumors induced by N-nitrosomethylbenzylamine formed in vivo. *Biomed Environ Sci* 1990; 3: 406-12.

- Xu Y, Ho CT, Amin SG, Han C, Chung FL. Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis in A/J mice by green tea and its major polyphenol as antioxidants. *Cancer Res* 1992;52:3875-9.
- Yahagi T, Degawa M, Seino Y, Matsushima T, Nagao M, Sugimura T, Hashimoto Y. Mutagenicity of carcinogenic azo dyes and their derivatives. *Cancer Lett* 1975; 1: 91-6.
- Yamaizumi Z, Kasai H, Nishimura S, Edmons CG, Mc Closkey JA. Stable isotope dilution quantification of mutagen in cooked food by combined liquid chromatography - thermospray mass spectrometry. *Mutat. Res* 1986 ; 173 : 1-7.
- Yamamoto T, Tsuji K, Kosuge T, Okamoto T, Shudo K, Takeda K, *et al.* Isolation and structure determination of mutagen substances in L-glutamic acid pyrolysate. *Proc Jpn Acad* 1978 ; 54B : 248-50.
- Yamane T, Takahashi T, Kuwata K, Oya K, Inagake M, Kitao Y, *et al.* Inhibition of *N*-methyl-*N'*-nitrosoguanidine-induced carcinogenesis by (-)-epigallocatechin gallate in the rat glandular stomach. *Cancer Res* 1995;55:2081-4.
- Yang CS, Chen L, Lu MJ, Landau JM. Effect of tea on carcinogenesis in animal models and humans. In: the American Institute for Cancer Research. *Dietary Phytochemicals in Cancer Prevention and Treatment*. New York: Plenum Press; 1996. p. 51-61.
- Yang CS, Lu SH. Effect of nutrition on carcinogenesis : Mechanisms involving nitrosation. In: Prasad KN, Meyskens FL Jr, editors. *Nutrition and cancer prevention*. New Jersey: Human Press; 1990. p. 53-70.

- Yang CS, Wang ZY. Tea and cancer. *J Natl Cancer Inst* 1993;85(13):1038-49.
- Yang D, Tannenbaum S, Buchi C, Lee G. 4-chloro-6-methoxyindole is the precursor of a potent mutagen (4-chloro-6-methoxy-2-hydroxyl-1-nitroso-indolin-3-oneoxine) that form during the nitrosation of the fava bean (*vicia faba*). *Carcinogenesis* 1984; 5(10): 1212-24.
- Yano M, Wakabayashi K, Tahira T, Arakawa N, Nagao M, Sugimura T. Presence of nitrosatable mutagen precursors in cooked meat and fish. *Mutat Res* 1988; 202:119-123.
- Yen GC, Hsieh PP. Possible mechanism of antimutagenic effect of Maillard reaction products prepared from xylose and lysine. *Agric Food Chem* 1994; 42:133-7.
- Yokota M, Narita K, Kosuge T, Wakabayashi K, Nagao M, Sugimura T, *et. al.* A potent mutagen isolated from a pyrolysate of L-ornithine. *Chem Pharm Bull* 1981 ; 29 : 1473-5.
- Yoshida D, Fukuhara Y. Formation of mutagen by heating creatine and amino acids. *Agric Biol Chem* 1982 ; 46: 1069-70.
- Yoshida D, Matsumoto T, Yoshimura R, Matsuzaki T. Mutagenicity of amino- α -carbolines in pyrolysis products of soybean globulin. *Biochem Biophys Res Commun* 1978 ; 83:915-20.
- Yoshida D, Saito Y, Mizusaki S. Isolation of 2-amino-3-methyl-imidazo-[4-5-f]quinoline as mutagen from the heated product of a mixture of creatine and proline. *Agric Biol Chem* 1984 : 48 : 241-3.
- Yoshiawa S, Horiuchi T, Sugimura M. Penta-O-galloyl- β -D-glucose and (-) epigallocatechin gallate : Cancer prevention agent. In: Huang MT, Ho CT,

Lee CY, editors. Phenolic compounds in Food and health II Antioxidant & cancer prevention. Washington DC: Am Chem Soc; 1992. p. 316-25.

Yu GP, Hsie CC. Risk factors for stomach cancer : a population – based case-control study in Shanghai. Cancer Causes Control 1991;2:169-74.

Zhang XM. Wakabayashi K, Liu ZC, Sugimura T, Nagao M. Mutagenic and carcinogenic heterocyclic amines in Chinese cooked foods. Mutat Res 1988 ; 201 : 181-8.

Zhao B, Li X, He R, Cheng S, Wenjuan X. Scavenging effect of extract of green tea and natural antioxidants on active oxygen radical. Cell Biophys 1989; 14: 175-85.

APPENDIX

MANIPULATION OF THE TESTING STRAINS

1. Preparation of Stock Solution and Media

1.1 Vogel Bonner media E stock salt solution (VB salt)

Use : Minimal agar

Ingredient	Per liter
Warm distilled H ₂ O (45°C)	670 ml
Magnesium sulfate (MgSO ₄ .7H ₂ O)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K ₂ HPO ₄)	500 g
Sodium ammonium phosphate (NaH ₂ NH ₄ PO ₄ . 4H ₂ O)	175 g

Add salts in the order indicated above to warm water and allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Filter the solutions and then autoclave at 121°C for 15 min.

1.2 Minimal glucose agar plate

Use : Mutagenicity assay

Ingredient	Per liter
Bacto agar	15 g
Distilled H ₂ O	930 ml
VB salts	20 ml
40% glucose	50 ml

Add agar to distilled water in a glass bottle. Autoclave at 121°C for 15 min.

When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose.

Mix and pour 30 ml into each sterile petri plate. Minimal glucose agar plates were kept in incubator at 37°C before using. (The VB salts and 40% glucose should be autoclaved separately).

1.3 Oxoid nutrient broth No. 2

Use : Growing culture

Dissolve 2.5 g of nutrient broth No. 2 in 100 ml distilled H₂O. Transfer 12 ml Nutrient broth for each 50 ml erlenmeyer flask (covered with sterile guaze). Autoclave at 121°C for 15 min.

1.4 Top agar

Use : Mutagenic assay

Ingredient	100	ml
Bacto agar	0.6	g
Sodium chloride (NaCl)	0.5	g
Distilled H ₂ O	100	ml

Dissolve ingredients in distilled water. Store in a glass bottle. Autoclave for 15 min. at 121°C and then add 10 ml of a sterile solution of 0.5 mM L - histidine / biotin and mixed thoroughly by swirling.

1.5 0.1 M L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	10	ml
L-histidine HCl	0.2096	g
Distilled H ₂ O	10	ml

Dissolve 0.2096 g of L-histidine HCl (MW 209.63) in 10 ml distilled water.

Autoclave at 121°C for 15 min. Store in a glass bottle at 4°C.

1.6 1 mM L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	100	ml
0.1 M L-histidine HCl	1	ml
Distilled H ₂ O	99	ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water. Autoclave at 121°C for 15 min.

1.7 1 mM biotin stock

Use : Fortification of minimal agar plate.

Ingredient	100	ml
Biotin	24.43	mg
Distilled H ₂ O	100	ml

Dissolve biotin (MW 244.3) in distilled H₂O. Warm it until dissolve completely.

Autoclave at 121°C for 15 min.

1.8 0.5 mM L-histidine / biotin solution.

Use : Mutagenicity assay (add 10 ml to 100 ml of Top agar)

Ingredient	200	ml
1 mM L-histidine HCl	100	ml
1 mM biotin	100	ml

Mix and autoclave at 121°C for 15 min.

1.9 1 M potassium chloride

Use : Na_3PO_4 - KCl buffer

Ingredient	100	ml
Potassium chloride	7.456	g
Distilled water	100	ml

Mix and autoclave at 121°C for 15 min.

1.10 0.5 M sodium phosphate pH 7.4

Use : Na_3PO_4 - KCl buffer for mutagenicity assay

Ingredient

0.5 M	Sodium dihydrogen phosphate (NaH_2PO_4) (MW 120) (30 g / 500 ml)
0.5 M	Disodium hydrogen phosphate dihydrate. ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) (MW 177.99) (44.5 g / 500 ml)

Dissolve 44.5 g disodium hydrogen phosphate dihydrate in 300 ml of distilled water. Add 0.5 M sodium dihydrogen phosphate until to pH 7.4, then adjust volume to 500 ml. Sterilize by autoclaving for 15 min at 121°C.

1.11 Na_3PO_4 - KCl buffer

Use : mutagenicity assay

Ingredient	330	ml
0.5 M Na_3PO_4 pH 7.4	100	ml
1 M KCl	16.5	ml
Distilled H_2O	213.5	ml

Autoclave for 15 min at 121°C .

2. Recipes for Some Reagents and Test Chemicals

2.1 2M sodium nitrite

Use : Nitrosation

Ingredient	10	ml
Sodium nitrite	1.38	g
Distilled water to	10	ml

Autoclave for 15 min at 121°C .

2.2 2M ammonium sulfamate

Use : reaction mixture

Ingredient	10	ml
Ammonium sulfamate	2.28	g
Distilled water to	10	ml

Dissolve ammonium sulfamate in distilled water and adjust volume. Autoclave for 15 min at 121°C .

2.3 0.2 N hydrochloric acid

Use : reaction mixture

Ingredient	100	ml
Conc. Hydrochloric acid	1.66	ml
Sterile distilled water	98.34	ml

Dissolve conc hydrochloric acid in sterile water. Store in sterile glass tubes or bottles with screw caps.

Note : Preparation of 0.2 N HCl must be used sterile technique because hydrochloric acid cannot be autoclaved.

2.4 0.3 mg/ml aminopyrene

Use : standard solution for mutagenicity assay

Ingredient	1	ml
Aminopyrene	3	mg
Acetonitrile	1	ml

Dissolve aminopyrene in acetonitrile. Store in sterile vial with screw caps in the freezer. Preparation of this solution must be used sterile technique.

Ingredient	1	ml
3 mg/ml aminopyrene	0.1	ml
Acetonitrile	0.9	ml

Dissolve 3 mg/ml aminopyrene in acetonitrile. Store in sterile vial with screw cap in the freezer. This preparation must be used sterile technique.

2.5 8 mg/ml ampicillin solution

Ingredient	10	ml
Ampicillin sodium	80	mg
Distilled water to	10	ml

2.6 0.1% crystal violet

Ingredient	10	ml
Crystal violet	10	mg
Distilled water to	10	ml

2.5 and 2.6 : Store at 4°C. in glass bottle with screw cap.

3. Procedure for Reisolation and Growing Culture

Tester strains, TA98 and TA100 are grown in Oxoid nutrient broth No. 2 and incubated overnight in a 37°C shaking water bath. The growth period should not exceeded 16 h. These cultures are re-isolated by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1mM biotin. These plates are incubated at 37°C for 48 h. After incubation, the 4 single colonies per strain TA98 and TA100 are picked up and grown in Oxoid nutrient broth No. 2 overnight at 37°C in shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 400 µl of the culture aseptically into sterile cryotubes (Nunc). The tubes then transfer to a -80 °C freezer.

4. Confirming Genotype of Tester Strains

The broth cultures of TA98 and TA100 are used to confirm genotypes in the following ways.

4.1 Histidine requirement

The his⁻ character of the strains is confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin

Procedure :

plate a	no histidine and biotin
plate b	0.1 ml of 1 mM biotin
plate c	0.3 ml of 0.1 M His-HCl
plate d	0.3 ml of 0.1 M His-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strains. Each plate is applied on the surface with 0.1 ml of 1 mM biotin or 0.3 ml of 0.1 M His-HCl, or 0.3 ml of 0.1 M His-HCl plus 0.1 ml of 1 mM Biotin or no application (plate b, c, d, a, respectively). Made a single streak of each strains across these plates. Four strains could be tested on the same plate. Incubated at 37°C for 24 h. The growing of bacteria on histidine plus biotin pate is the result of histidine requirement.

4.2 R-Factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure : For each tester strain, add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.1 M histidine - HCl followed by adding 20 ml of molten top

agar containing 0.5 mM Mixed and poured on a minimal glucose agar plate. Rotated the plate to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and rfa mutation (see the next section) are performed in the same plate by dividing the plate into 2 areas, one for R-factor and the other for rfa mutation. For R-factor, commercial ampicillin disc or filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 h. The absence of the clear zones of inhibition around the disc indicate resistance to ampicillin.

4.3 Rfa Mutation

Strains having the deep rough (rfa) character should be tested for crystal violet sensitivity.

Procedure : Pipetted 0.1 % solution of crystal violet to the sterile filter paper disc (1/4 inch) and transferred the disc to plates, seeded with bacteria (the procedure is similar to R-factor). Incubated at 37°C for 48 h. The clear zone appeared around the disc indicated the presence of the rfa mutation that permitted crystal violet to enter and kill bacteria.

5. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a

frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure : 0.2 ml of sterile distilled water (solvent in the experiment) is added to capped tube. Add 0.5 ml of NaPO₄-KCl buffer pH 7.4 in the absence of metabolic activation, 0.1 ml of fresh overnight culture of TA98 or TA100, followed by 2.0 ml of molten top agar. Mixed and then poured on minimal agar plates, rotate plates and left it to become harden. Incubated at 37°C for 48 h. and the his⁺ revertant colonies were counted.

6. The Response to Standard Carcinogen

Standard carcinogens or positive carcinogens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen which used in these experiments is aminopyrene in the absence of metabolic activation. Tester strain which highly response to positive mutagens must be collected.

Procedure : The procedure is as described in spontaneous reversion except aminopyrene (0.06, and 0.12 µl per plate for TA98 and TA100, respectively) are used instead of sterile distilled water in absence of S-9 mix, respectively. The characteristic of stock culture for TA98 and TA100 as the source of bacteria for mutagenicity is

- a) contained R-factor (pKM 101) and rfa mutation
- b) His⁺ requirement
- c) low spontaneous reversion

d) highly response to standard carcinogen

After the characteristic of the culture was tested, the mutagenicity test was started.



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