NUTRITIONAL AND TOXICOLOGICAL ASPECTS OF SOME FOOD COLORS: EFFECTS ON *IN VITRO* PROTEIN AND STARCH DIGESTIBILITIES AND THE MUTAGENIC POTENTIAL USING AMES TEST

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ชื่อวิทยานิพนธ์ ปัญหาทางโภชนาการ และพิษวิทยา ของสีผสมอาหาร: ผลต่อการย่อยโปรตีน และแป้งในหลอดทดลอง และการประเมินฤทธิ์ก่อกลายพันธุ์ โดยใช้เอมส์เทสต์

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บทคัดย่อ

สารสกัดของสีธรรมชาติ 6 ชนิด คือ กระเจี๊ยบ คำฝอย อัญชัน เตย น้ำตาลไหม้ ถ่านกาบมะพร้าว และ สีผสมอาหารสังเคราะห์ 7 ชนิด คือ ปองโซ 4 อาร์, ตาร์ตราซีน, เออริโธรซิน, ซันเซ็ต เย็ลโลว์ เอ็ฟ ซี เอ็ฟ, อินดิโกคาร์มีน, บริลเลียนท์บลู เอ็ฟ ซี เอ็ฟ, ตาร์ตราซีน ผสม อินดิโกคาร์มีน อัตราส่วน 1:1 ถูกนำมาศึกษาถึงฤทธิ์ก่อกลายพันธุ์ของผลิตภัณฑ์ที่เกิดจากปฏิกริยาของสีผสมอาหาร กับไนไตรท์, ผลต่อความสามารถ ในการย่อย ของโบไวนซีรั่มอัลบูมินในหลอดทดลอง โดยการย่อยสลายด้วยเอ็นไซม์ pepsin ตามด้วย trypsin และ chymotrypsin วิเคราะห์หา กลุ่มอะมิโนที่ปลดปล่อยออกมาด้วย 2,4,6 trinitrobenzenesulfonic acid, และผลต่อความสามารถในการย่อยแป้งในหลอดทดลองโดยการย่อยสลายด้วยเอ็นไซม์ α-amylase วิเคราะห์หากลุ่มมอลโทสที่ปลดปล่อยออกมาด้วย 3,5-dinitrosalycyclic acid

ผลการทดลองแสดงถึง สีธรรมชาติ และสีสังเคราะห์ ที่ใช้ในการทดสอบทั้งหมดสามารถลดการย่อยของโบไวนซีรั่มอัลบูมินและแป้งอย่างมีนัยสำคัญในการทดสอบที่มีการเพิ่มเกลือไนไตรท์พบว่า การย่อย ของโบไวนซีรั่ม อัลบูมิน

และแป้งลดลงกว่าเดิม อย่างมีนัยสำคัญ การยับยั้งการย่อย โปรตีน และแป้งอาจขึ้นอยู่กับ องค์ประกอบของสารเคมี ในสีผสมอาหารธรรมชาติ และสีผสมอาหารสังเคราะห์ ได้แก่ ตัวยับยั้งเอ็นไซม์ย่อยโปรตีนและแป้ง แทนนิน สารฟีนอลอื่น ๆ และ สารโมเลกุลเล็ก หรือ ไอออน ที่สามารถยับยั้ง การทำงาน ของเอ็นไซม์ ผลิตภัณฑ์ที่ได้จาก สีผสมอาหาร ทำปฏิกิริยา กับเกลือไนไตรท์ อาจแสดงฤทธิ์คล้ายตัวยับยั้ง ที่จะลดการย่อย ของโบไวนซีรั่มอัลบูมิน และแป้ง อย่างไรก็ตาม กลไกการยับยั้ง ยังไม่ทราบแน่นอน

ผลิตภัณฑ์ที่เกิดจากปฏิกริยาของสีธรรมชาติกับไนไตรท์ทั้งหมด มีฤทธิ์ก่อกลายพันธุ์แบบ direct mutagen ต่อเชื้อแบคทีเรีย *Salmonella typhimurium* ทั้งสายพันธุ์ TA98 และ TA 100 ซึ่งแสดงว่าเกิดการกลายพันธุ์ทั้งแบบ frameshift และ base-pair substitution ผลิตภัณฑ์ที่เกิด จากปฏิกริยาของสีผสมอาหารสังเคราะห์กับไนไตรท์ ที่ใช้ในการทดสอบบางตัว คือ ปองโซร์ 4 อาร์ เออริโธซิน และ ซันเซ็ต เย็ลโลว์ เอฟ ซี เอฟ แสดงฤทธิ์ก่อกลายพันธุ์ต่อเชื้อแบคทีเรีย *Salmonella typhimurium* สายพันธุ์ TA 98 เฉพาะ ปองโซร์ 4 อาร์ ที่ แสดงฤทธิ์ก่อกลาย พันธุ์ต่อสายพันธุ์ TA100 ด้วย

Thesis Title Nutritional and Toxicological Aspects of Some Food Colors: Effects on *In Vitro* Protein and Starch Digestibilities and The Mutagenic Potential Using Ames Test

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Abstract

The effects of six natural food colors namely, *Clitorea ternatea* Linn. (อัญชัน), *Hibiscus sabdariffs* Linn. (กระเจี๊ยบ), *Pandanus amaryllifolius* Roxb. (เตย), *Carthamus tinctorius* Linn. (คำฝอย), Caramelized coconut sugar (น้ำตาลไหม้), Carbon black from coconut skin (ถ่านกาบมะพร้าว) and seven synthetic food colors namely, Ponceau 4 R, Erythrosine, Brilliant Blue FCF, Indigo Carmine, Sunset Yellow FCF, Tartrazine, Tartrazine with Indigo Carmine 1:1 were studied after being interacted with nitrite the apparent *in vitro* digestibilities of bovine serum albumin (BSA) and starch as well as on their mutagenic potential. The determination of amino group with 2,4,6 trinitrobenzenesulfonic acid (TNBS) after an *in vitro* hydrolysis of pepsin followed with trypsin and chymotrypsin, the determination of maltose with 3,5-dinitrosalycylic acid after an *in vitro* hydrolysis of α-amylase were performed.

Most of natural food colors and synthetic food colors significantly decreased the digestion of BSA and starch. Being added with nitrite, BSA and

starch were poorly digested. The inhibitory actions may depend on the properties of food color components e.g. protease and amylase inhibitors, tannins, other phenolic substances and the inhibition of enzymatic activity by specific small molecules and ion. Nitrosated products of food colors also decreased BSA and starch digestibilities. Nevertheless, the mode of action of food colors and nitrite on BSA and starch digestion were required further investigation.

The nitrosated products of natural food colors contained direct mutagens indicated by the revertants of *Salmonella typhimurium* TA 98 and TA 100. It was interpreted that some direct mutagens of frameshift and base-pair substitution were formed during nitrosation. Some synthetic colors such as ponceau 4 R, erythrosine and sunset yellow FCF were mutagenic to TA 98. Only ponceau 4 R was mutagenic to TA 100 after nitrite treatment.

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LIST OF ABBREVIATION

ALA	alanine
ARG	arginine
BSA	bovine serum albumin
g	gram
GLY	glycine
hr	hour
ILE	isoleucine
LEU	leucine
LYS	lysine
M	molar
mg	milligram
ml	millilitre
min	minute
mM	millimolar
$Na_2B_4O_7$ $10H_2O$	di-sodium tetraborate decahydrate
NaH_2PO_4	sodium dihydrogen phosphate
nm	nanometre
NO_2	nitrite
NaOH	sodium hydroxide
Na_2SO_3	sodium sulphite
OD	optical density
TNBS	trinitrobenzenesulfonic acid
TRP	tryptophan

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CHAPTER I

INTRODUCTION

The use of food colors have a long history. Colors are very important in foods and food products since they impart eye appeal. Unfortunately perhaps, many people have a tendency to "eat with their eyes," and it is possible to satisfy many people more because of appearance than flavor or nutritive value.

The colors of foods are the result of natural pigments or of added colorants. The natural pigments are a group of substances present in animal and vegetable products. The added colorants are regulated as food additives, or call synthetic food colors.

The most important natural pigments from higher plant sources are the chlorophylls and the anthocyanins. The former are responsible for the green color of plants, but more importantly act as the catalytic agent in the photosynthetic process which is so valuable in the production of food. The latter, the anthocyanins are phenolic glycosides and are soluble in water. *Clitorea ternatea* Linn. (อัญชัน), *Hibiscus sabdariffs* Linn. (กระเจี๊ยบ), *Pandanus amaryllifolius* Roxb. (เตย), *Carthamus tinctorius* Linn. (คำฝอย), carmelized coconut sugar (น้ำตาลไหม้), carbon black from coconut skin (ถ่านกาบมะพร้าว) are among edible plants or plant products used as food color in Thailand. Since these plants were used for a long time without any signs of acute toxicity, the users assume that the colors were generally recognized as safe.

Ponceau 4 R (Newcoccine), Red No. 3 (erythrosine), Blue No.1 (brillant blue FCF), Blue No.2 (indigo carmine), Yellow No.6 (sunset yellow FCF), Yellow No.5 (tartrazine) and tartrazine with indigo carmine are synthetic colors that permitted for use in food under the provision of Ministry of Public Health in

Thailand. All of these synthetic colors showed no significant mutagenic potential on *Salmonella typhimurium* in the Ames test (1).

In Thailand the use of food colors (natural food colors and synthetic food colors) is extensively promoted in food industries. These food colors may be consumed in dairy products or other protein rich food. Therefore, considerable effort has to be put forth in elucidating those factors in food that may constitute a hazard to man or his domesticated animals when consumed.

One group of compounds that has received much attention is the digestive enzyme inhibitors. Examples of protease inhibitors and amylase inhibitors are few, but potentially important. Since such antinutritional substances may possibly exist in edible portion of natural food colors and exert their inhibitory effects on proteolytic enzymes to reduce protein hydrolysis in human stomach as well as stach hydrolysis in the small intestine. It was, therefore, of interest to investigate the possible effects of food colors both natural and synthetic as inhibitor of protein and starch digestibility.

The other food additive, nitrite, is a precursor of nitroso compounds. It presents in many foods as an intentionally additive as well as a naturally occurring substance (2). This additive is mainly used in fermented meat products to prevent the occurrence of *Clostridium botulinum* toxin. It is very common to find that most consumer eat this type of food with coloring soft drink. Since it is noted that the environment of pepsin hydrolysis, pH between 1-3.5, is similar to that of nitroso compound formation (3-5) and nitrite of the fermented meat can interact with other component of meal including coloring agents in the gastric condition to form nitrosated products. It is an unavoidable situation that the two phenomena, namely protein digestion and nitrosation, occur simultaneously. Unfortunately, there is no information on the effect of such products on nutrient utilization such as protein hydrolysis in the stomach. Therefore, the investigation on the effects of food

colors as well as of food colors with nitrite on enzymatic digestions of protein and starch has been done and reported.

In addition to the effect on nutrient digestion, interaction between two compounds in the gastric simulated condition sometimes produces toxic products. Nitroso compounds are among the most known potent carcinogens formed *in vivo* by nitrosating some chemicals with nitrite (6,7). A number of nitrosatable precursors are present in food. Possible human risk arising from the nitrosated products of these precursors has been discussed elsewhere (8,9). Sufficient evidences have been shown that N-nitroso compounds do occur in the gut.

It has been demonstrated that interaction of nitrosable compounds especially secondary and tertiary amines with nitrite under the acidic condition could give rise to mutagenic N-nitroso compounds either in chemical systems (10) or in human stomach (11). In view of correlation between positive results in mutagenicity assay, the evidences available today indicates that most carcinogenic nitroso compounds are mutagenic (12).

Food colors are among the nitrosatable precursors to which human are unnecessarily exposed. The formation of hazardous substance by food color-nitrite interaction is an important problem in safety evaluation of food colors. Kangsadalampai and Rojanapo (Unpublished data,1988) reported that the ethanol extract of a natural food color *Carthamus tinctorius* Linn. was proved mutagenic with *S. typhimurium* both strain TA 98 and TA 100 in the presence of metabolic activation in the Ames test. In addition, the nitrosated products of crude ethanol extract was mutagenic towards both strains in the absence of metabolic activation. These results did not, however, agree with Morimoto *et al.* (13) who found that water and methanol extract of *C. tinctorius* did not exhibite mutagenic towards *S. typhimurium* strains TA 98 and TA 100 in the Ames test.

Since such a controversy information on risk of nitrosated food colors have never distributed to the consumer; therefore, the reaction products of the nitrosation on food colors had been presently evaluated for their mutagenicity as well as their digesting inhibitory effects.

CHAPTER II

REVIEW OF LITERATURE

Food Colors

Natural food colors They have a long history of safe use for human consumption, although it is difficult to verify by modern toxicological procedures. The colors of foods are the result of natural pigments. The natural pigments are a group of substance present in vegetables, animals and mineral products. These pigments in this experiment can be divided into the following:

Chlorophylls Chlorophylls are green pigments responsible for the color of leafy vegetables and some fruits e.g. *Pandanus amaryllifolius* Roxb. (เตย). In green leaves, the chlorophylls are broken down during senescence and the green color tends to disappear. Chlorophylls in many fruits are present in the unripe state and gradually disappears as the yellow and red carotenoids take over during ripening. In plants, chlorophyll is isolated in the chloroplastids (14).

The chlorophylls are tetrapyrrole pigments in which the porphyrin ring is in the dihydro form and the central metal atom is magnesium. In the book of Principles of Enzymology for the Food Sciences by Whitaker (15) said that there are two chlorophylls, *a* and *b*, which occur together in a ratio of about 1:25. Chlorophyll *b* differs from chlorophyll *a* in that the methyl group on carbon 3 is replaced with an aldehyde group. The structural formula of chlorophyll *a* is given in Figure 1. Chlorophyll is a diester of a dicarboxylic acid (chlorophyllin); one group is esterified with methanol, the other with phytyl alcohol. The magnesium is removed very easily by acids giving pheophytins *a* and *b*. The action of acid is

especially important for fruits that are naturally high in acid. However, it appears that the chlorophyll in plant tissues is bound to lipoproteins and is protected from the effect of acid. Heating coagulates the protein and lowers the protective effect. The color of the pheophytins is olive-brown. Chlorophyll is stable in alkaline medium. The phytol chain confers insolubility in water on the chlorophyll molecule. Upon hydrolysis of the phytol group, the water-soluble methyl chlorophyllides are formed. This reaction can be catalyzed by the enzyme chlorophyllase. In the presence of copper or zinc ions, it is possible to replace the magnesium, and the resulting zinc or copper complexes are very stable.

In addition to those reactions described above, it appears that chlorophyll can be degraded by yet another pathway. Chichester and Mc Feeters (16) reported on chlorophyll degradation in frozen beans, which were related to fat peroxidation. In this reaction, lipoxidase may play a role, and no pheophytins, chlorophyllides, or pheophorbides were detected. The reaction required oxygen and was inhibited by antioxidants.

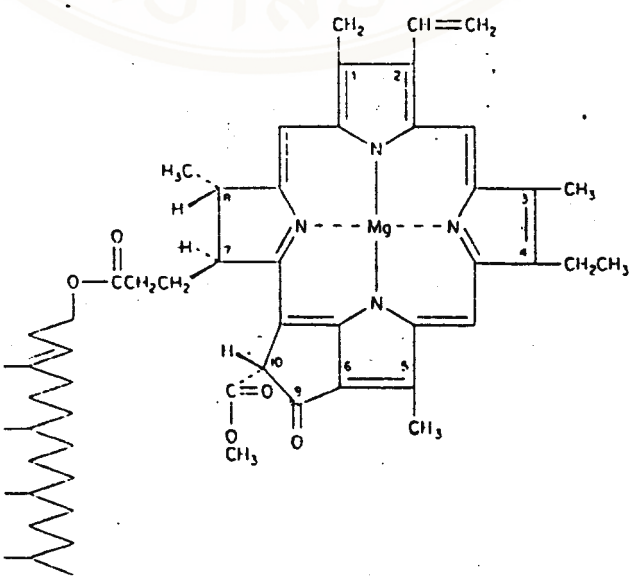


Figure 1 Structure of chlorophyll *a*.

Anthocyanins The anthocyanin pigments are present in the sap of plant cells; they take the form of glycosides and are responsible for the red, blue, and violet colors of many fruits and vegetables e.g. *Clitoria ternatea* Linn. (อัญชัน), *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) (17). When the sugar moiety is removed by hydrolysis, the aglucone remains and is called anthocyanidin. The sugar part usually cosists of one or two molecules of glucose, galactose and rhamnose. The basic structure consist of 2-phenyl-benzopyrylium or flavylium with a number of hydroxy and methoxy substituents. Most of the anthocyanins are derived from 3,5,7,-trihydroxyflavylium chloride (Figure 2) and the sugar moiety is usually attached to the hydroxyl group on carbon 3 (14). Anthocyanin pigments can easily be destroyed when fruits and vegetables are processed. High temperature, increased sugar level, pH, and ascorbic acid can affect the rate of destruction (18).

Relatively little is known about the metabolic fate of anthocyanins despite their widespread occurrence and sizeable daily intake, estimated at 185-215 mg/day (19). The metabolism of anthocyanins and anthocyanidins has been studied in vivo in rats and in vitro with intestinal microorganisms. Scheline (20) incubated cyanidin chloride anaerobically with rat cecal bacteria for 22 h and found no detectable metabolites.

Griffiths and Smith (21) and Scheline (22) observed that pelargonidin was degraded by similar in vitro incubation to *p*-hydroxyphenyl lactic acid. The other fission product was presumably phloroglucinol (1,3,5-trihydroxy benzene). Delphinidin was administered to a rat intragastrically and an unidentified metabolite was found in the urine. Two metabolites were found *in vitro* by incubation with cecal microflora. It is unknown to what extent anthocyanins or their aglycones are absorbed following ingestion, although the studies mentioned above indicate that ring fission and glycoside hydrolysis occur to a limited extent with certain anthocyanins.

Early studies with experimental animals indicated urinary excretion of less than 2% of oral anthocyanins from grapes (22,23). Which cyanidin, the most widespread anthocyanidin, is resistant to catabolism by intestinal bacteria, an unusual finding in view of its hydroxylation pattern, when present in other flavonoid compounds, e.g. flavonols, predisposes those compounds to bacterial ring fission. Cyanidin and delphinidin have been tested for mutagenicity in the *Salmonella*/mammalian microsome assay and were found to be non mutagenic (24). These findings are in sharp distinction to certain other flavonoids, namely, the flavonols (25).

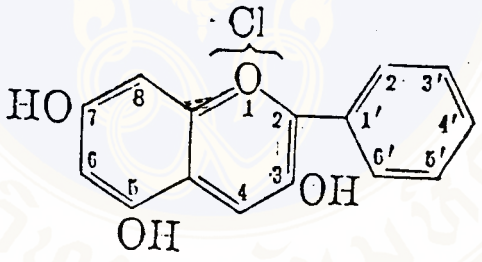


Figure 2 3,5,7-trihydroxyflamylium chloride, the basic structure of the anthocyanins.

Carotenoids The name of this group, carotenoids, is derived from carotene, the main coloring matter in the root of the carrot. Carotenoids are found throughout the plant world, in leaves, fruits if red or yellow colored, flowers, and roots e.g. *Carthamus tinctorius* Linn. (คำฝอย) (17). The naturally occurring carotenoids, with the exception of crocetin and bixin, are tetraterpenoids. They have a basic structure of eight isoprenoid residues arranged as if two 20-carbon units, formed by head-to-tail condensation of four isoprenoid units, had joined tail to tail. There are two possible ways of classifying the carotenoids. The first system

recognizes two main classes, the carotenes, which are hydrocarbons, and the xanthophylls, which contain oxygen in the form of hydroxyl, methoxyl, carboxyl, keto, or epoxy groups. The second system divides the carotenoids into three types, a cyclic, monocyclic, and bicyclic.

Natural carotenoid food colors are annatto, oleoresin of paprika, and unrefined palm oil. A pigment named crocin has been isolated from saffron. Crocin is a glycoside containing two molecules of gentiobiose. When these are removed, the dicarboxylic acid crocetin is formed (Figure. 3). It has the same general structure as the aliphatic chain of the carotenes. Also obtained from saffron is the bitter compound picrocrocin. It is possible to imagine a combination of two molecules of picrocrocin and one of crocin; this would yield protocrocin. Protocrocin is directly related to zeaxanthin and this has in fact been found in saffron (26).

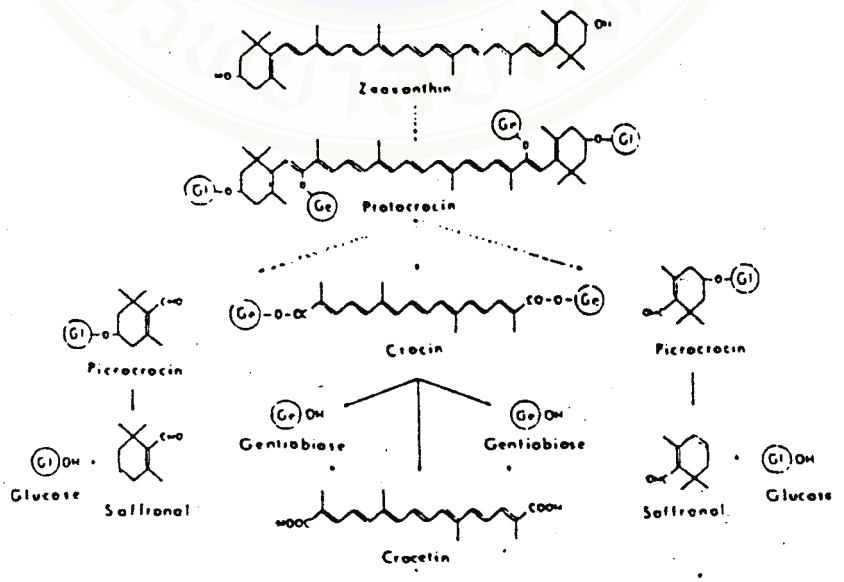


Figure 3 Relationship between crocin and picrocrocin and the carotenoids.

Caramels Caramel is a complex polysaccharide of unidentified chemical structure prepared by heating a food-grade carbohydrate such as glucose, sucrese, or starch in the presence of a catalyst acetic, sulfurous, or citric acids or bases such as ammonium, calcium, or sodium hydroxides. Chemical composition varies with method of preparation. About 50% of the colorant is digestible carbohydrate, 25% is nondigestible carbohydrate, and 25% is melanodins (27,28). Caramel can be considered a natural constituent of the diet since it is found when certain foods are cooked or when sucrose is heated.

Toxicologically, there is no distinction between such naturally produced caramels and those produced commercially from food-grade carbohydrates, with the exception of caramels produced by the ammonia-ammonium sulfate process (29). Mutagenic potential of caramel was evaluated in *Saccharomyces cerevisiae* D4 and *Salmonella typhimurium* (strains TA 1535, TA 1537 and TA 1538) both with and without activation by mammalian liver homogenates. No mutagenicity was observed in these tests, but caramel exhibited high toxicity for the *Salmonella* strains and thus was tested only up to 350 ppm. (30).

In rats fed 30 ml of 10% and 20% caramel solutions, from starch hydrolysates with ammonium hydroxide and sulfurous acid catalysts, daily for 100 days, the average absorption was about 30% of the does. Remaining caramel was extracted from feces. Measured absorption would include degradation by intestinal microflora (31).

Sources of natural food colors used in Thailand

Roselle (กระเจี๊ยบแดง), *Hibicus sabdariffa* Linn. (32) (Family Malvaceae) An annual erect shrub with red or green stem, practically unbranched or with branches near the base; stem glabrous or slightly hairy with minute tubercles; leaves serrate, lower leaves ovate and undivided, upper ones palmately 3-5 lobed; flowers large, yellow with dark crimson eye: epicalyx united at the base and adnate to calyx: calyx connate below, free above, dark purple, fleshy; capsules ovoid, pointed, villous; seeds numerous, large, reniform, blackbrown, covered, with minute, stout, stellate hairs. The flowers yield a yellow dye of little commercial value. The chief pigment is a flavonol glycoside, hibiscitrin, hibiscetin, gossypitrin and sabdaritrin are also present. The waxy matter separated from the flowers contains a phytosterolin, hydrocarbons and minor quantities of sitosterol. The phytosterolin gives on hydrolysis a sitosterol and glucose.

Safflower (คำฝอย), *Carthamus tinctorius* Linn. (33) (Family Compositae) An erect annual herb, 1-3 ft. high, with spinosely serrate leaves and globular flower heads, with yellowish or orange-red florets and smooth four-angled achenes without any pappus. Numerous races of safflower are known under cultivation varying markedly in botanical features and in oil and dye contents. Safflower florets contain principally two colouring matters, carthamin which is scarlet-red and insoluble in water, and safflower yellow which is soluble in water. A third compound, isocarthamin, which gradually reverts to carthamin on standing, has been recently isolated.

Butterfly Pea (อัญชัน), *Clitoria ternatea* Linn. (33) (Family Leguminosae) A pretty perennial climber with conspicuous blue or white flower, found commonly throughout Thailand. The blue dye from the corollas of the flowers can be used as a substitute for litmus. The seeds also yield a blue dye. The seeds contain a fixed oil and a bitter resinous principle. Both seeds and root-bark contain tannin. The chemical in this flower is anthocyanins.

Pandan (เตย), *Pandanus amaryllifolius* Roxb. (34) (Family Pandanaceae) Pandan is a cluster of annual plants, densely branched shrub, rarely erect. Stem up to 1 m. high, supported by aerial roots; single leaves and green, 50-75 cm. long, ensiform, caudate acuminate, coriaceous, with spines on the margins andon the midrib.

We can make color food additives by put this plant to water and filter with gauze. This color is green and fragrance. Color substance in this plant is chlorophills.

Synthetic food colors Dairy products, beverages, baked goods, spices, jellies, candy, maraschino cherries, meat product casings, and Easter egg dyes are a few uses of synthetic colors. Synthetic colorants, used commercially, are also known as certified color additives in many country. There are two types, FD&C dyes and FD&C lakes. FD&C indicates substances approved for use in food, drug, and cosmetic use by U.S. federal regulations. Dyes are water-soluble compounds that produce color in solution. They are manufactured in the form of powders, granules, pastes, and dispersions. They are used in foods at concentrations of less than 300 ppm (FAD, unpublished data, 1986). Lakes are made by combining dyes with alumina to form insoluble colorants, which have dye contents in the range of 20 to 25 percent. The lakes produce color in dispersion and can be used in oil-

based foods when insufficient water is present for the solubilization of the dye. Synthetic colors currently used in this experiment are as follow.

Table 1 Test Agents (35).

Trivial name	FD&C or C.I.name	C.I. number	Chemical class
Ponceau 4 R	New Coccine	16255	azo dye
Erythrosine	FD&C red No.3	45430	xanthene
Tartrazine	FD&C Yellow No.5	19140	azo dye
Sunset Yellow FCF	FD&C Yellow No.6	15985	azo dye
Brilliant Blue FCF	FCFFD&C Blue No.1	42090	Triphenylmethane
Indigo carmine	FD&C Blue No.2	73015	Indigoid

Ponceau 4 R (New Coccine) The color is regularly used in candy, preserves, beverages, and canned and frozen vegetable. The chemical structure of this color is shown in Figure 4. Molecular weight of Ponceau 4R is about 604.48. They have light red powder or granules, water soluble, sparingly soluble in ethanol and color not less than 82% total coloring matter (35). The chemical structure of this color is shown in figure 4.

Metabolism studies indicate that Ponceau 4R is rapidly absorbed from the gastrointestinal tract and undergoes extensive metabolism with the formation of naphthionic acid and 7-hydroxy-8-aminomaphthalalene-1, 3-disulfonic acid. Following a single dose of ^{14}C-labelled coloring to previously untreated rats, mice and guinea-pigs or rats given repeated doses of unlabeled coloring for 28 days, no marked accumulation of radioactivity in any tissue was found (36).

The acute and short-term studies showed no significant toxic effects. In the Ames test, Ponceau 4R had no mutagenic effect on *Salmonella typhimurium* (37). No adverse effects on reproductive function were noted and no teratogenic potential was detected (38). The recent long-term studies in rats confirmed the absence of carcinogenic potential, thus offering further assurance. Nepthrotoxicity was seen in the high doses. The Committee of the European Communities (ECC), reports of the Scientific Committee for Food, Fourteenth Series, (1983) established an ADI of 0-4 mg/kg body weight based on the no-adverse level in the long-term mouse study of 500 mg/kg body weight .

Tartrazine (FD&C Yellow No.5) The color is regularly used in desserts (e.g. dessert powders, puddings, custards, gelatin, ice cream), candy, preserves, beverages (e.g. carbonated soda beverages and flavored drink powders), prepared meats, and canned and frozen vegetables. It is also used in pharmaceuticals and cosmetics. Molecular weight of this color is about 534.37. They have light orange powder or granules, water soluble, sparingly soluble in ethanol and color not less than 85.0% total coloring matters (35). The chemical structure of this color is shown in Figure 4.

Commission of the European Communities comments about this color. The following data were available: metabolic studies, an early *in vitro* mutagenicity study, acute and short-term toxicity studies, multigeneration reproduction studies, and teratology studies, several long-term studies in the mouse and rat and summaries of recent U.S. long-term studies in the mouse and rat. The color is metabolized by azo reduction and absorption of the breakdown products with subsequent excretion in feces and urine. The acute and short-term tests revealed no obvious toxic effects. Using the Ames test, tartrazine had no mutagenic effect on *Salmonella typhimurium* (1). But in tests with *Salmonella typhimurium* TA 100, TA 1535, TA 98, and TA 1538 tartrazine showed mutagenic activity with TA 100

strain without metabolic activation (39). Reproductive function was not affected and no teratogenic potential was noted in rats and rabbits. The available long-term studies reveal no carcinogenic potential and the apparent absence of any adverse effects in the recent U.S. studies offers additional reassurance. The Committee maintained the ADI of 0-7.5 mg/kg body weight established previously on the basis of the no adverse-effect level of 750 mg/kg body weight in the earlier long-term rat study.

Erythrosine (FD&C Red No. 3) The color is regularly used in desserts (e.g. dessert powders, puddings, custards, gelatin, ice cream), candy, preserves, beverages (e.g. carbonated soda beverages and flavored drink powders), prepared meats, and canned and frozen vegetables. It is also used in pharmaceuticals and cosmetics. Molecular weight of this color is about 879.87. They have light red powder or granules, water and ethanol soluble, and color not less than 85.0% total coloring matters (35). The chemical of this color is shown in Ffigure 4. Metabolic studies confirmed that erythrosine is absorbed only to a small extent from the gastrointestinal tract in rats and man (40). Biochemical studies of thyroid function and of the metabolism of thyroid hormones indicated that erythrosine inhibits the deiodination of thyroxine to triiodothyronine, and, at high dose levels, activates secretory mechanisms for thyrotropin in the pituitary. There is some information on metabolism including the contribution to human iodine intake from the ingested color. The significance to man of the neurophysiological effects related to neuronal transmission observed *in vitro* and to the activity and behavior of treated animals is not clear (41,42).

The short-term studies showed only specific effects due to the iodine released on ingestion, but no other significant toxic effects were seen (43). Erythrosine had no mutagenic effect on *Salmonella typhimurium* (1). The earlier multigeneration study revealed no effects reproductive function, and the earlier

long-term studies in rats and mice showed no carcinogenic potential. The more recent Japanese studies apparently confirmed this. The previous ADI was reduced to 0-1.25 mg/kg of body weight and made temporary. The previously established temporary ADI was withdrawn and a new temporary ADI of 0-0.6 mg/kg of body weight was allocated based on the results of studies of the biochemical effects of erythrosine on thyroid hormone metabolism and regulation, in which a no-effect level of 2.5 g/kg of the diet, equivalent to 125 mg/kg of body weight, was established.

Sunset Yellow FCF (FD&C Yellow No.6) The color is regularly used in desserts (e.g. dessert powders, puddings, custards, gelatin, ice cream), candy, preserves, beverages (e.g. carbonated soda beverages and flavored drink powders), prepared meats, and canned and frozen vegetables. It is also used in pharmaceuticals and cosmetics. Molecular weight of this color is about 452.37. They have light orange powder or granules, water soluble, and color not less than 85.0% total coloring matters (35). The chemical structure of this color is shown in Figure 4.

The color is metabolized by the azo-reduction and some of the breakdown products are absorbed and excreted in bile and urine. The color was tested for mutagenic effect in a concentration of 0.5 g/100 ml in cultures of *Escherichia coli*. No mutagenic effect was found (44). Sunset Yellow was not found to be mutagenic in the "rec-assay" (45).

Several mutagenicity tests using *Salmonella* strains have been employed to assess the mutagenic activity of Sunset Yellow. Sunset Yellow was not found to be mutagenic in any of these tests (1). The mutagenic tests showed no genotoxic activity. Acute and short-term tests showed no obvious toxic effects. Reproductive function was not affected and no teratological potential noted. The long-term studies in rats, mice and dogs revealed no carcinogenic potential. The committee

established an ADI of 0-2.5 mg/kg body weight based on the long-term dog study, being the most sensitive species, with a no-adverse-effect level of 250 mg/kg body weight (Commission of the European Communities, 1983).

Brillant Blue FCF (FD&C Blue No.1) The color is regularly used in desserts (e.g. dessert powders, puddings, custards, gelatin, ice cream), candy, preserves, beverages (e.g. carbonated soda beverages and flavored drink powders), prepared meats, and canned and frozen vegetables. It is also used in pharmaceuticals and cosmetics. Molecular weight of this color is about 792.86. They have light blue powder or granules, water soluble, and color not less than 85.0% total coloring matters (35). The chemical structrre of this color is shown in Figure 4.

Metabolism studies show that Brilliant Blue FCF is poorly absorbed from the gastro-intestinal tract, and undergoes subsequent rapid and complete biliary excretion (46). The acute and short-term studies showed no significant toxic effects. In the Ames test, brilliant blue FCF had no mutagenic effect on *Salmonella typhimurium* (1,47).

Indigo carmine (FD&C Blue No.2) The color is regularly used in desserts (e.g. dessert powders, puddings, custards, gelatin, ice cream), candy, preserves, beverages (e.g. carbonated soda beverages and flavored drink powders), prepared meats, and canned and frozen vegetables. It is also used in pharmaceuticals and cosmetics. Molecular weight of this color is about 466.36. They have light blue powder or granules, water soluble, and color not less than 85.0% total coloring matters (35). The chemical structure of this color is shown in Figure 4.

Indigo carmine does not appear to be readily absorbed from the gastro-intestinal tract or to be extensively decomposed within the body. Using ^{35}S-labeled

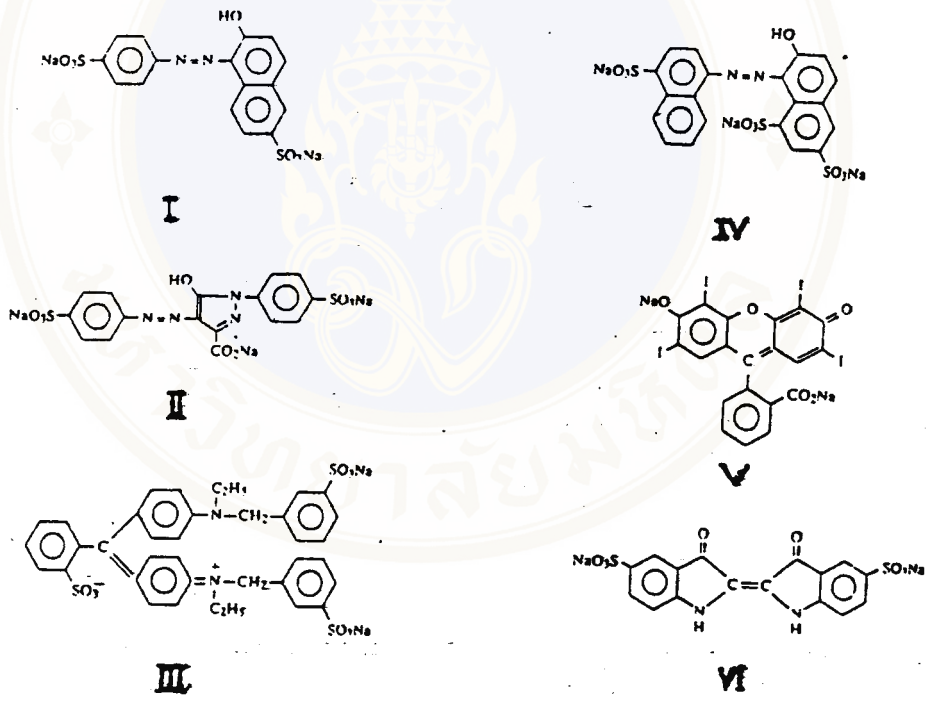
material, Lenthco & Webb (48), showed that 10% of an iv dose in rats was present in the bile and 63% in the urine within 6 hr, the majority as the unchanged coloring. On the other hand, 60-80% of an oral dose was present in the feces and this was considered to indicate a lack of absorption rather than biliary excretion.

A similar lack of absorption and decomposition was indicated in studies in the pig reported by Gaunt, Kiss, Grasso & Ganglolli (49), who found blue-colored intestinal contents, feces and urine. Nevertheless some metabolism dose take place, since Lethco & Webb (48) found isatin-5-sulphonic acid and 5-sulphoanthranilic acid in the bile and urine of rats following oral or iv doses of the coloring. These same products are known to be produced by oxidation in solutions of the coloring (50), but the process is much accelerated in vivo since both products were found within 2 hr in the urine of iv-treated rats whereas the 5-sulphoanthranilic acid occurred only after 12 wk in solution. These latter authors also established that the coloring decomposed *in vitro* in contact with rat intestinal contents and that *in vivo* 5-sulphoanthranilic acid was more rapidly absorbed than the intact coloring. Therefore, it seems likely that some of the indigo carmine may be oxidized in the intestine and that the products may be preferentially absorbed

Indigo carmine was not acutely toxic when administered orally, the LD_{50} values being in excess of 2.5 and 2.0 g/kg in mice and rats, respectively (FDA unpublished data cited by the Joint FAO/WHO Expert Committee on Food Additives,1970). In man, iv injection of indigo carmine (10 ml of 0.8% aqueous solution) was shown to have a vasopressor effect probably mediated by the sympathetic nervous system. This finding was confirmed by Wu & Johnson (51) using only 5 ml of 0.8% solution and they suggested that indigo carmine should be used with caution for renal function studies and urological studies in hypertensive or cardiac patients.

The acute and short-term studies showed no significant toxic effects.

In the Ames test, Indigo carmine had no mutagenic effect on *Salmonella typhimurium* (1). No adverse effects on reproductive function were noted and no teratogenic potential was detected. In long-term studies no evidence of carcinogenicity was found in 20 male and 20 female rats given diet containing 1% indigo carmine for 2 yr. or in groups of 24 rats given diets containing 0, 0.5, 1.0, 2.0, or 5.0% indigo carmine for 2 yr (52).



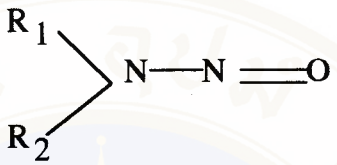
I Sunset Yellow FCF II Tartrazine III Brilliant Blue FCF

IV Ponceau 4 R V Erythrosine VI Indigo carmine

Figure 4 Chemical structures of certified color dyes.

N-nitroso Compounds

The compound classified as the N-nitroso group will have the following general structure:



where R_1 and R_2 can be virtually any organic groups. N-nitroso compounds may be categorized into the N-nitrosamines and the N-nitrosamides. For N-nitrosamines, R_1 and R_2 can be either alkyl, aryl, alkaryl groups, or in some cases, salicylic. They can be derived from dialkyl-, alkaryl-, diaryl-, or cyclic secondary amines, A closely related class of compounds, N-nitrosamides, where R_1 is an alkyl or aryl group and R_2 is an acyl group, can be derived from N-akylurea, N-alkylcarbamates and simple N-alkylamides. Moreover, N-nitroso derivatives have been shown to be derived from amidines, guanidines, cyanamides, hydroxylamine, hydrazine, hydrazone and hydrazide (6).

Basically, three things are needed to form a N-nitrosamine: an amine or other nitrosatable compounds, a nitrosating agent, and the proper chemical environment. N-nitrosamines can be formed by the chemical reaction of various precursor entities. The amine fragment, R_1R_2N, may be derived from primary, secondary or tertiary amines, mixed polyamines, amino acids and quaternary ammonium compounds. The nitrosyl groups, NO, may come from nitrogen oxides (NO, NO_2, N_2O_3, and N_2O_4), nitrite (nitrite salt or nitrous acid) or NOX where X is anion: NOCl, $NOBF_4$, NO-NCS or NO-3-nitrocarbazole (6).

Toxicological of nitroso compounds have been implicated as mutagens,

carcinogens, and teratogens in the human environment. Nitrosamines are suspected etiologic agents in some forms of human cancer, e.g., esophageal cancer, especially in instances where the disease occurs in localized patterns (53,54). It is possible that exposure to nitrosamines can occur through the ingestion of nitroso compounds or through simultaneous ingestion of nitrosatable substances and nitrite.

N-nitroso compounds are mutagenic and have been studied both from the viewpoint of fundamental genetics and as mutagenic hazards in the environment. It is now established that many nitrosamines and nitrosamides are potent mutagens in a variety of unicellular and multicellular organisms (55-57).

Acute tissue injury induced by nitrosamines, relatively stable nitroso compounds, expressed as a single oral LD_{50} dose in adult rats, range from 5 mg/kg for dicyclohexylnitrosamine to 7.5 g/kg for N-nitrosoethyl-2-hydroxyethylamine (58). The chemical and pharmacological properties of dimethylnitrosamine, the simplest and most extensively studied dialkylnitrosamine, are believed to be representative of this class of compounds. The dialkylnitrosamines, with some exceptions, are selectively hepatotoxic, causing hemorrhagic centrilobular necrosis in a wide range of animal species.

A variety of such agents are now known and have been classified according to mode of action, e.g., alkylators, intercalators, etc. As alkylating agents, N-nitroso compounds are extremely potent point mutagens in addition to producing chromosome breaks and aberrations. Both N-nitrosamines and nitrosamides have been widely used as test agents in mutagenesis studies in animal. While the nitrosamides have been shown to be mutagenically active in microorganisms and in *Drosophila*, the nitrosamines are mutagenic only in the latter. The lack of mutagenic effect by the nitrosamines in microorganism is attributed to the inability of microbes to metabolize these compounds to active mutagenic forms (59).

Mammalian activation system is necessary in mutagenesis in microorganisms which can significantly enhance the mutagenic activity of a chemical is a prerequisite in the study of the mutagenicity of nitrosamine. The ability of NADPH-dependent microsomal enzymes to form mutagenic compounds from dialkyl nitroso compounds has been demonstrated by numerous investigators. Gabridge and Legator (55) have shown that DMN induced mutation in (his) G46 strain of *S. typhimurium* after activation in the host-mediated assay. Dimethylnitrosamine was also shown to be mutagenic to *S. typhimurium* following *in vitro* activation in the liquid culture assay (57).

Many epidemiological studies demonstrated positive correlations between the ingestion of nitrate and the development of gastric cancer (59,60). However, the relationship was insignificant (61). The endogenous formation of N-nitroso compounds was hypothesized to be a causative factor (60). N-nitroso compounds can be formed from nitrite and secondary amines and amides under acidic conditions. Hence the stomach is found to be an important place for endogenous formation (6). Groenen et al. (62) demonstrated that the endogenous formation of volatile N-nitrosamines in the gastric juice of healthy persons was occurred a few hours after the consumption of a nitrate-rich meal.

The main source of nitrite in the stomach is ingested nitrate. It is reduced to nitrite by bacteria present in the oral cavity (57). In the human diet, about 75% of the daily nitrate intake is derived from vegetables (63).

Formation of N-nitroso compounds in simulated gastric juice have been demonstrated. Alam *et al.* (3) reported *in vivo* synthesis of nitrosopiperidine from nitrite and piperidine in the stomach and small intestine of rat and also with rat gastric contents *in vitro*. Sen et al. (4) showed that nitrosamines were synthesized *in vitro* with human gastric juice and *in vivo* laboratory animals. Klein et al. (5) detected nitrosodiphenylamine by incubated nitrite and diphenylamine (pH 2) at

37°C for 2 hrs. Knowles *et al.* (64) investigated the nitrosation of bovine serum albumin under the conditions of pH and temperature normally recorded in the human stomach. This series of reactions showed that beside nitrosamine formation there was other nitrosation reactions which could take place under stomach conditions and yields modified amino acids.

Several substances have been shown to catalyze the formation of nitroso compounds. Lijnsky *et al.* (10) examined the interaction between nitrite and six tertiary nitrogen compounds *in vitro*, usually at 37°C 4 hrs and at a pH between 3.5-5.5, representative the different types of chemical structure: oxytetracy cline (antibiotic), aminopyrine (analgesic), disulphiram (antialcoholic), nikethamide (respiratory stimulant), tolazamide (oral hypoglycaemic), and piperine (found in peper), all of which gave measurable yields of nitrosamine.

The Salmonella/mamaliam Microsomal Assay (Ames test)

The simplicity, sensitivity, and accuracy of this method for screening large numbers 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. McCann *et al.* (65) discussed several aspects of the experimental basis for their current assessment of the value of the test as a useful predictive tool:

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weaknesses 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 tested the current status of carcinogenicity tests of these chemicals in animals.

4. The evidence that the correlation between carcinogenicity and mutagenicity in the *Samonella*/microsome 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.

The *salmonella* test was first validated in a study of 300 chemicals, most of which were known carcinogens. It was subsequently validated in studies by the Imperial Chemical Industries, the National Cancer Center Research Institute in Tokyo, and the International Agency for Research on Cancer. Nearly 90% of the carcinogens tested were mutagenic in these studies. However, McCann *et al.* (65) estimated the correlation to be about 83%. All the validations show that the test fails to detect a few classed of carcinogens such as polychlorinated pesticides. Prior to the initial development of the *Salmonella*/microsome assay there were several studies that employed bacterial systems to detect mutagenic agents. However, one of the problems with these earlier approaches was the use of screening techniques that did not employ bacterial strains designed to detect broad range of mutagenic mechanism. Therefore Maron and Ames (66) have developed a set of *S. typhimurium* strains that are permeable to a wide range of chemicals and also are partially deficient in DNA repair.

The *Salmonella* tester strains The reverse mutation system of *S. typhimurium* uses the genetically well-defined histidine requiring mutants developed by Ames and his colleagues. The *Salmonella* histidine reverse mutation is based on the use of several selected *S. typhimurium* strains that revert from histidine dependence (auxotroph) to histidine independence (prototroph). Maron and Ames

(66) had collected and characterized a large number of *S. typhimurium* strains containing mutations in different gene of the histidine operon (Table 2). Maron and Ames (66) had been developed the newly *Samonella* tester strains to make them more effective in detecting mutagens that were not previously detected with the original strains. The newly standard tester strains contain other mutations that greatly increase their ability to detect mutagens such as

- rfa mutation which causes partial loss of the lipopolysaccharide barrier that coat the surface of the bacteria and increases permeability to large molecules that do not penetrate the normal cell wall .

- uvr B mutation is a deletion of a gene coding for the DNA excision repair system, resulting increase sensitivity in detecting many mutagens .

- R-factor plasmid (pKM 101). The strains containing the plasmid show greatly enhanced response to chemical shown to be mutagenic and also give clear positive response to chemical describe as weak, borderline or non mutagens with the original set of tester strains. Furthermore, MacPhee reported that pKM 101 contains gene products associated with error-prone repair which may be responsible for the enhance sensitivity seen in these strains.

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

Genotypes of the *Samonella typhimurium* strains used for mutagenesis testing are shown in Table 2. The standard tester strains, TA 97, TA98, TA 100 and TA 102 contain the R-factor plasmid, pKM 101. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. These standard tester strains are recommended for general mutagenesis testing. TA 98 is derived from TA 1538 by introduction of

plasmid pKM 101. It can detect mutagens that causes frameshift mutation with a DNA sequence (-CGCGCGCG-), which can be reverted to histidine independence by a variety of mutagens that act by adding or deleting base pairs. While TA 100, the R-factor plasmid derivative of TA 1535, can detect mutagens that cause base pair substitutions. The others *Samonella* 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.

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

Histidine mutation				LPS	Repair	R-factor
hisD6610 his 01242 = TA 98	his D3052	his G46	hisG428 (pAQ)			
TA 90	TA 1538	TA 1535	-	rfa	uvr B	-R
(TA 97)	(TA 98)	(TA 100)	-	rfa	uvr B	+R
-	TA 1978	TA 1975	-	rfa	+	-R
TA 110	TA 94	TA 92	-	+	+	+R
-	TA 1534	TA 1950	-	+	uvr B	-R
-	-	TA 2410	-	+	uvr B	+R
TA 98	TA 1964	TA 1530	-	gal	uvr B	-R
-	TA 2641	TA 2631	-	gal	uvr B	+R
-	-	-	(TA 102)	rfa	+	+R

However, some mutagens affect only one strain of frameshift mutation strains (TA 1538 or TA 98) or only base-pair substitution strains (TA 1535 or TA 100), 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 for the other.

The Mutagenicity Test Using *Samonella typhimurium*

Plate incorporation test The test is the standard method that has been used for test the mutagenicity of chemicals. This test consists of combining the test compound, the bacterial tester strain and s9 mix in soft agar which is poured onto a minimal agar plate. Positive and negative controls are also include in each assay. After incubation at 37° C for 48 hr revertant colonies are counted. For initial screening, chemicals were tested in concentrations over a three-log dose range in the presence and absence of s9 mix. And positive or questionable result should be confirmed by demonstrating a dose-response relationship using a narrower range of concentrations. For most mutagens, there is a concentration range that produces a linear dose-response curve and the number of revertants per plate reported for a mutagen should be taken from the region of the curve. However, a few mutagens such as 9-aminoacridine, MNNG, diethylsalfate and ethymethanesulfonate produce non linear dose-response curve. The compounds that are negative can be retested using the preincubation method.

Preincubation method Some mutagens, such as dimethyl and diethyl-nitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahagi *et al.* (67) in

which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen, s9 mix, and bacteria for 20-30 min at 37° C and then added the top agar. The assay has also been used to detect the mutagenicity of 10 carcinogenic nitrosamines and several carcinogenic alkaloids . The mutagenic activity of aflatoxin B_1, benzidine, benzo (a)-pyrene, and methyl methanesulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay. The increased activity is attributed to the fact that the test compound, s9, and bacteria are incubated at higher concentrations in the preincubation assay than in the standard plate incorporation test. The procedure described below is based on recommendations of Matsushima (unpublished data, 1983).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay requires an extra step and therefore involves more work than the standard test but many laboratories use it routinely because of the increased sensitivity for some compounds. Its use in screening assays has been recommended by De Serres and Shelby.

Positive control (diagnostic mutagens) In each experiment positive mutagenesis controls using diagnostic mutagens to confirm the reversion properties and specificity of each strain. The characteristic reversion patterns of the standard strains to some diagnostic mutagens are described by Maron and Ames (66).

Evaluation criteria for Ames assay Because the procedures to be used to evaluate the mutagenicity of the test article are semiquantitative, the criteria used to determine positive effects are inherently subjective and based primarily on an historical data base. Most data sets should be evaluated using the following criteria:

1. Strains TA-1535, TA-1537, and TA-1538. If the solvent control value is within the typical range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to three times the solvent control value, is considered mutagenic.

2. Strains TA-98 and TA-100. If the solvent control value is within the normal range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to twice the solvent control value is considered mutagenic. Occasionally a doubling is not necessary for TA 100 if a clear dose-related pattern is observed over several concentrations.

3. Pattern. Because TA 1535 and TA 100 are derived from the same parental strain (G46), and because TA 1538 and TA 98 are derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen, and such a pattern is sought. Generally, if a strain responds to a mutagen in inactivation tests, it should do so in activation tests.

4. Reproducibility. If a test article produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute and other extenuating factors may enter into a final evaluation decision. However, these criteria can be applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established. It must be emphasized that modifications of the procedure involving preincubation conditions or source of s9 mix are necessary for evaluation of specific chemicals or classes of chemicals.

Protein Digestibility

Protein digestibility is an important parameter in protein quality evaluation. It indicates the efficiency of protein to be used by the body. Protein digestion is begun in the stomach. Parietal cells release hydrochloric acid to denature dietary protein. This loss of structure makes protein more susceptible to subsequent action by digestion enzymes. Hydrochloric acid acts also as a primer to convert pepsinogen, the inactive precursor enzyme which is produced by chief cells of the stomach under the influence of the gastro-intestinal hormone gadtrin, to pepsin (68).

Pepsin hydrolyzed only those bonds in which the carboxyl group is provided by either phenylalanine or tyrosine. However, only a minimal amount of protein hydrolysis is accomplished in the stomach (69).

As soon as stomach contents enter the duodenum, the intestinal hormone pancreozymin stimulates the cell of the pancrease to release their complement of proteolytic enzymes these inactive precursor enzymes, trypsinogen, chymotrypsinogen, proelastase and procarboxypeptidase are secreted into the duodenum where they are transformed into active participants in the digestive process. Trypsinogen is converted to trypsin by the action of enterokinase, an intestinal enzyme produced by epithelial cells lining the duodenum. Trypsin activates the precursor enzymes chymotrypsinogen, proelastase and procarboxypeptidase to active enzymes chymotrypsin, elastase and carboxypeptidase.

Each of these enzymes acts only on specific peptide bond. Trypsin selects only those bonds whose carboxyl groups are provided by arginine and lysine residue. Chymotrypsin similarly attacks only those peptide bonds where the carboxyl group is contributed by tyrosine, phenylalanine and tryptophan. Elastase is the only enzyme able to hydrolyze the protein elastin, found in muscle meats. Because they attack interior peptide bonds only, these three enzymes are known as

endopeptidase. Through their action, the polypeptide chains of large protein molecules become progressively more fragmented into shorter peptide chains (70).

The carboxypeptidases, A and B, initiate peptide bond splitting from the carboxyl end of a peptide chain. Carboxypeptidase A requires Zn^{2+} and can attack carboxyl terminal amino acid whose carbon skeleton is either aromatic or aliphatic. Carboxypeptidase B attacks peptide linkages in which the carboxy-terminal amino acid is arginine or lysine. The completion of the digestive process is achieved by two other groups of proteolytic enzymes, the aminopeptidase and dipeptidase found in both the mucosa and lumen of the small intestine (71).

Proteolytic enzymes as a determinant of protein digestibility

1. **Pepsin** Pepsin is a gastric acid protease. It is formed from a single polypeptide chain of 321 amino acid residues and has a molecular weight of 33,000. It is active even at pH 1, where most enzymes or proteins undergo rapid denaturation. Pepsin is formed in the stomach mucosa from pepsinogen. Its specificity has a broad range of substrates including as esterase activity.

Activity of enzyme is inhibited by substrate-like epoxide (72), diazoketone (73), diazo-acetyl norleucine methyl ester (74).

2. **Trypsin** Trypsin, the serine protease, is one of the major proteases of the pancrease which is formed in the intestinal tract from an inactive precursor, trypsinogen. It is composed of 233 amino acid residues and molecular weight is 23,800. Its optimum pH is in the range from 7 to 9 for proteins and most synthetic substrates.

Trypsin is highly specific for certain peptide bonds. These are the bonds linking the carboxyl group of the two basic amino acids, lysine and arginine, to the amino group of any other amino acids.

Inhibitors of trypsin were reported. Benzamidine-2HCl, p-amino

benzamidine, phenylguanidine sulfate, cyclohexylcarboxamidine-HCl, cyclohexylguanidine-HCl, 2- phenylacetamidine-HCl and acetamidine-HCl were found to be competitive inhibitors of trypsin. Benzamidine-2HCl and p-amino benzamidine-2HCl were the most potent small molecule competitive inhibitors of trypsin (75). w-Amidinophenylalkyl amidinophenyl ether and modification of the compound included lengthening of the alkane chain, variation in the position of the amidino groups and substitution of halogen on the benzene ring, acted as competitive reversible trypsin inhibitor (76). Some naturally occurring substances in various plants e.g. legumes, cereals and potatoes were also demonstrated to act as antitrypsin properties (77).

3. **Chymotrypsin** Chymotrypsin is one of the serine proteases. The number of amino acid residues is 241, and the molecular weight is 25,300. The enzyme shows optimum activity in the pH range of 7 to 9, which is conformed to its natural action in the small intestine from chymotrypsinogen .

Chymotrypsin has specific activity for peptides of amino acids with aromatic rings such as tyrosine, phenylalanine and tryptophan or a relatively bulky side chain of hydrophobic nature, such as isoleucine and leucine.

Factors affect protein digestibility: Naturally occurring antinutritional proteins in plants Antinutritional proteins in plants are of great importance since they can limit bioavailability of proteins in both human and animals. The two main types of antinutritional proteins presents are the protease inhibitors and the lectins (78). Furthermore, there are some compounds which act as antinutritional protein e.g. phytate, tannins.

1. **Protease inhibitors** By definition, protease inhibitors are substances that inhibit proteolytic enzyme activity and are specific in their interactions with proteases. In plants, the specific protease inhibitors are polypeptides and proteins.

Plant protease inhibitor can essentially be divided into four catagories, namely those that specifically inhibit the serine proteases (e.g. trypsin, chymotrypsin, thrombin, plasmin and elastin), those that inhibit the sulphydryl proteases (e.g. papain, bromelain and ficin), those that inhibit metalloproteases (e.g. carboxypeptidase A and B and the aminopeptidase) and finally those that inhibit the acidic proteases (e.g. pepsin and renin) (78,79).

The protease inhibitors have since been demonstrated to be extremely widespread in their distribution thoughout the plant kingdom. The location of protease inhibitors are not necessarily restricted but distributed in varies parts of plant. Several processes are commonly used to destroy protease inhibitors, these are heat treatment, germination and fermentation (77-81).

Physiology of the protease inhibitors in plants are poorly understood despit the fact that they often form an important parts of the protein found in the tissue. They may merely act as storage of reserve protein, or they may, because of their peculiar properties actually regulate the endogenous protein enzymes and hence control protein turnover and metabolism. Alternatively, they may be part of the plants defence mechanisms against the attacks of insects and micro-organisms (77-79).

The protease inhibitors in plants were clearly caused in growth depression in animal experiment (82) and decreased protein digestibility (81,83-85). This effects have been extensively stride in biochemical properties. It has been noticed that the primary structure contains repetitive sequences or regions of internal homology. They frequently contain the reactive (inhibitory) sites of the inhibitor which interact with the active site of the relevant protease.

Studies on the properties of these inhibitors have enable the reactive sites of many naturally occurring protease inhibitors have enable to draw up certain tentative rules for these reactive sites (77-79). For example, the ARG^{63}-ILE^{64}

reactive peptide bond in the soybean (kunitz) trypsin inhibitor, consists of 181 amino acids and two disulfide bonds, can be modified in various ways by chemical and enzymatic means without the inhibitor losing it activity. The ARG^{63} can be replaced by LYS^{63}, in which case the modified inhibitor behaves like the native form, or by TRP^{63} where the protein becomes a good inhibitor of chymotrypsin. Alternatively the inhibitor will tolerate the replacement of ILE^{64} by either ALA^{64}, LEU^{64}, or GLY^{64} whilst still retaining activity.

The reactive site of inhibitors is identified to single reactive site, double reactive sites and multiple reactive sites. Reactive site may be not only specifies certain enzyme but also other enzymes which have acted to reactive site of inhibitor (78).

2. **Lectins** Lectins or (phyto) hemagglutinins are proteins that possess a specific affinity for certain sugar molecules and able to agglutinate erythrocyte. Most hemagglutinins of higher plants are found in seeds. They also presence in tubers, plant saps, leaves, stems and bark (81).

Physiological roles of the lectins include as antibodies to counteract soil bacteria, involvement in plant protection against pathogenic bacteria and against fungal attack during seed inhibition and early seeding growth, their role in the development and differentiation of embryonic cells, and that of transport of stored sugar (78). It has been suggested that in dry seed the lectins are enzymatically inactive proteins which acquire catalytic properties during germination (86).

The toxic effects of some raw legume seeds caused growth retardation in experimental animals (83,87-88) and some times even death when incorporated into the diet (87,89-90). Jaffe (81) summarized that toxic symptoms produced by lcetins also depended on the animal species and even on the special animal strains. Honavar *et al.* (87) showed that the level of antitryptic activity related to the level of hemagglutinins of crude extracts of raw legumes. The property of lectins could

also decrease activity of pepsin (83).

3. **Phytate** Phytic acid, which is the hexaphosphate of myoinositol, is a common constituent of plant tissue. Its able to chelate di and trivalent metal ions such as calcium, magnesium, zinc, copper and iron to form insoluble complex, phytate which are not readily absorbed from the intestinal tract.

Kratzer (91) noted that phytate may also caused protein to be more resistant to proteolytic digestion. Phytate significantly decreased on pepsin digestion of both casein and BSA (92). This effect may be the result of the formation of phytate-protein complexes into indigestible form. Morr (93) reported that protein-calcium-phytate complexes may also tended to inhibit the digestibility of soy protein at acid pH value.

Possible protease inhibitors of polyphenolic compound The important of protease inhibitors are widely recognized. Polyphenolic compounds that occur naturally in plant have been studied on this effects. A broad classification of the major groups of plant polyphenolic compounds is shown in Table 3.

Tannins Tannins are any polyphenolic substances that have molecular weight between 500-3000. Tannins may be classified as hydrolyzable, that is, degradable by enzyme or acid to yield a sugar residue and a phenolcarboxylic acid and condensed tannins, which are polymeric flavonoids.

Condensed tannins were likewise believed to be responsible for the growth depression that had been observed when sorghum was feed to experimental animals (94). There appeared to be little doubt that the growth depression caused by sorghum tannins were due to an adverse effect on protein and dry matter digestibility (95-100).

Beans had been shown to contain polyphenolic compounds (tannins), which could partially contribute to their low protein digestibility (101). Nitrogen in

grain of high-tannins varieties was poorly digested in chick and rat (100). This effect related to the fact that tannins interfered the binding of the dietary protein into an indigestible form (102,103). Hagerman and Butler (104) showed that tannins significantly decreased the susceptibility to tryptic hydrolysis of bovine serum albumin. Heat treatment (105) and dehulling (106) reduced tannins and increased *in vitro* protein digestibility.

Table 3 The classification of major groups of plant polyphenolic compounds (94)

Carbon skeletons	Plant polyphenols
C_6	simple phenols e.g. phenol C_6H_5OH
C_6-C_1	phenolic acids (hydrolysable tannins) e.g. gallic acid , ellagic acid
C_6-C_2	hydroxyphenyl acetic acids
C_6-C_3	coumarins, isocoumarins,chromones
$(C_6$-$C_3)_2$	lignans
$(C_6$-$C_3)_n$	lignin
C_6-C_3-C_6	flavanones flavones flavonols anthocyanidins flavanols isoflavones chalcones aurones e.g. flavan-3-ol
$(C_{15})_2$	procyanidines biflavonyls
$(C_{15})_n$	polymeric procyanidins (condensed tannins)
C_6-C_1-C_6	benzophenones xanthones
C_6-C_2-C_6	stibenes
C_6, C_{10} and C_{14}	quinones

Starch Digestibility

The average daily consumption of carbohydrate is about 350 gm per day, comprising about 50 to 60% of caloric intake. About 55% of carbohydrate is eaten as starch. Starch is a polymer of glucose with very long straight chains of 1-4 bonds and side branches with 1-6 bonds at the branching points (107).

Preliminary luminal digestion of carbohydrates occurs with the action of salivary and pancreatic amylase on starch but release of monosaccharides occurs only with final digestion by the oligosaccharidases embedded in the glycocalyx of the brush border of the small intestinal mucosa. Digestion of carbohydrate begins in the mouth with the action of salivary amylase, which has the same specificity as a pancreatic amylase. These amylases split only 1-4 bonds, which have at least two glucoses on either side of the action of amylase on starch are maltose, maltotriose, and α-limit dextrins. The latter consist of an 1-6 branching point plus two to six additional glucose in 1-4 linkage. In the initial stages of digestion by amylase, starch is broken into particles of several thousand daltons. Digestion of starch by salivary amylase continues in the stomach until the pH falls below 4.0, which for the interior portions of the meal may take 1 to 2 hours. After a large mixed meal, about 30% of starch is digested in stomach (68).

Digestion of starch by pancreatic amylase continues in the lumen of the small intestine. The end-products of digestion of starch by amylase and the disaccharides, lactose, and sucrose are hydrolyzed to monosaccharides by enzymes embedded in the glycocalyx of the microvilli of the brush border of the small intestinal mucosa. These enzymes act as immobilized surface catalysts and are secreted into the luminal fluid only in trace amounts. The specific enzymes split the disaccharides into their constituent monosaccharides. In addition to acting on maltose, maltase also splits both 1-4 bonds of maltotriose and all of the 1-4 bonds

of α-limit dextrins. Thus, the α-limit dextrins are completely broken down to individual glucose molecules by maltase acting at the 1-4 bonds and isomaltase at the 1-6 bond (107).

Amylases The amylases are glycosidases that catalyze the hydrolysis of α-1,4-glucose polymers by the transfer of a glucosyl residue (donor) to H_2O (acceptor). Alpha-amylase (1,4--D-glucan glucanohydrolase, EC 3.2.1.1) occurs widely in all living organisms, while β-amylase (1,4--D-glucan maltohydrolase, EC 3.2.1.2) is found in seeds of higher plants and in sweet potatoes, oats, corn, rice, and sorghum. The commercially used α-amylase is mostly obtained from *Bacillus licheniformis* or *A. spergillus oryzae* (108).

Both amylases have molecular weights in the range of 50,000, except sweet potato β-amylase, which is a tetramer of 197,000 daltons. Alpha-amylase contains one Ca^{2+} per mole of protein, which is essential for stabilization of the enzyme. In contrast, β-amylase requires no metals. The optimum pH of α-amylase varies depending on the source (6.0-7.0 for mammalian, 4.8-5.8 for *A. oryzae*, 5.85-6.0 for *B. subtilis*). For β-amylase, the pH optimum for activity ranges from 5.0 for wheat, malt, and sweet potato to 6.0 for soybean.

Alpha-amylase cleaves α-1,4-glucose polymers at internal positions (endo-attack) to yield oligosaccharide fragments with the C-1 hydroxyl group in the α-configuration. Beta-amylase is an exoglycosidase that successively cleaves maltosyl units from the non reducing end of the polymer to yield maltose in the β-configuration.

Inherent in its sequential attack, the action of β-amylase stops at the α-1,6 branch point in the starch molecule. Alpha-amylase, with its random attack, can bypass the branch points in the polymer. However, the presence of an α-1,6 branching point is known to render the neighboring α-1,4 linkage resistant to

attack by amylases.

The α-amylase from *Bacillus amyloliquefaciens* has been partially sequenced and the three-dimensional structure of α-amylase from porcine pancreas established by x-ray crystallography. The enzyme molecule has a dimension of 50 A°, with the active site situated in a deep cleft that runs 30 A°, separating the protein into two different-sized domains. Two specific binding sites are identified, one deeply in the active-site region and the other located on the surface of the molecule (108).

Factor affact starch digestibility

1. **Amylase inhibitors** There are four types of amylases. These are: (a) α-amylase, (b) β-amylases, (c) glucoamylases and the (d) pullulanase-type (debranching) amylases. The only well-described type of naturally-occurring protein amylase inhibitors are those against the α-amylases from higher animals and insects. These inhibitors, with the possible exception of the one from corn, are not effective against higher plant and microbial α-amylases or against the other three types of amylases. Quite recently, there have been discussions of the possibility of a glycoamylase-type inhibitor.

There are also small peptide inhibitors of α-amylase found in certain *Streptomyces*. Two carbohydrate α-amylase inhibitors, Acarbose and Amylostatin, have been described. Their structures are shown in Figure 5. The inhibitors are very similar in structure (109).

Naturally occurring inhibitors of pancreatic amylase were first discovered in aqueous extracts of wheat, rye, and kidney beans. They were found to have the properties of a protein and to be quite thermostable. Inhibition occurred in a noncompetitive manner. The wheat-derived inhibitor is active against the amylase from a number of mammalian species, including rat, mouse and dog, whereas the

kidney bean-derived inhibitor was found to have substantial activity only against dog amylase. More recently a new class of amylase inhibitors have been isolated from culture broths of *Actinomycetes*. These inhibitors are complex oligosaccharides with a molecular weight of 500-5000. Some are fully competitive inhibitors, while others are noncompetitive .

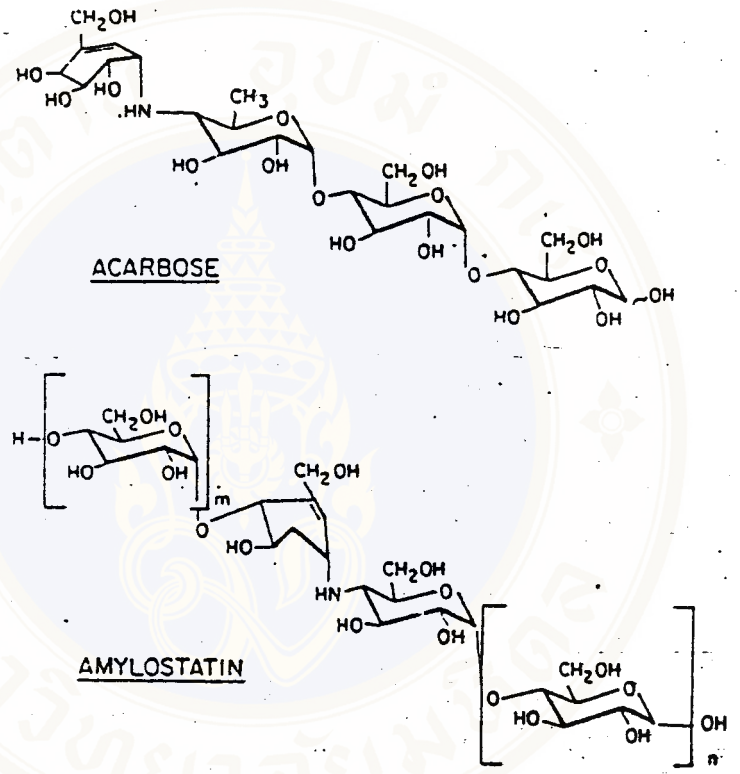


Figure 5 Structures of Acarbose and Amylostatin.

The protein α-amylase inhibitors form a very tight complex with salivary and pancreatic α-amylases. For example, the complex between the red kidney bean protein inhibitor and porcine pancreatic α-amylase at 30°C and pH 6.9 was calculated to be 3.5×10^{-11} M. The inactive complex forms slowly, requiring 60-120 minutes to reach complete reaction depending on pH and inhibitor and enzyme concentrations. The red kidney bean protein inhibitor does not inhibit plant and microbial α-amylases; only those from higher animals and insects are inhibited.

It appears that there is an initial rapid complex formed between the enzyme and inhibitor which is still active. Then, a much slower conformational change occurs leading to loss of activity.

Unlike the protein protease inhibitors, complexation between the red kidney bean protein α-amylase inhibitor and α-amylase does not appear to involve binding at the active site of the α-amylase. Evidence for this includes ability of the complex to bind maltose (a competitive inhibitor of α-amylase), starch, Sephadex and to still hydrolyze small substrates.

The red kidney bean α-amylase inhibitor contains 9-10% covalently bound carbohydrate. Removal of up to 70% of the carbohydrate does not affect the activity of the inhibitor. The glyco groups, removed from the protein, do not inhibit -amylase at 3.5 X 10^4 times the concentration of the inhibitor. Chemical modification studies indicate that histidine and tyrosine residues in the inhibitor may be important for its activity. In summary, the knowledge of the mechanism of action of the red kidney bean α-amylase inhibitor indicates that an initial complex is formed between inhibitor and enzyme which does not involve the active site of the enzyme (complex still fully active). Subsequently, there is a conformational change in the complex which destroys the ability of α-amylase to hydrolyze large substrates but does not prevent their binding to the enzyme (109).

Proteins inhibitory of α-amylase are found in many biological fluids. However, only the protein inhibitors found in legumes and in wheat have been extensively investigated. Recently, it has been shown that all insect α-amylases tested, except one, are inhibited by the red kidney bean α-amylase inhibitor. Bjouck *et al.* (110) have suggested that the wheat α-amylase inhibitors may be active against attack of the wheat by insects during storage. With one exception the plant α-amylase inhibitors do not have any activity against higher plant or

microbial amylases tested. The three α-amylase inhibitors of maize have reported to inhibit maize α-amylase, indicating a possible physiological role of these inhibitors in maize.

The nutritional significance of the α-amylase inhibitors is largely unknown. It is known that low levels of inhibitory activity can be detected in regularly cooked food products. Whitaker (109) reported that α-amylase inhibitor, fed to humans as an impure preparation, has no effect on the caloric value of the starchy meal.

2. **Phytate** Phytic acid, which is the hexaphosphate of myoinsitol, is a common constituent of plant tissue. Its able to chelate di and trivalent metal ions such as calcium, magnesium, zinc, copper and iron to form insoluble complex, phytate which are not readily absorbed from the intestinal tract. Knuckles and Betschart (92) noted that phytate and other myo-inositol phosphate esters inhibited the *in vitro* digestion of starch.

Possible amylase inhibitors of polyphenolic compound The important of amylase inhibitors are widely recognized. Polyphenolic compounds that occur naturally in plant have been studied on this effects. A broad classification of the major groups of plant polyphenolic compounds is shown in Table 3.

Tannins Tannins are any polyphenolic substances that have molecular weight between 500-3000. Tannins may be classified as hydrolyzable, that is, degradable by enzyme or acid to yield a sugar residue and a phenolcarboxylic acid and condensed tannins, which are polymeric flavonoids.

Condensed tannins were likewise believed to be responsible for the growth depression that had been observed when sorghum was feed to experimental animals (94). Featherston and Rogler (111) observed a decrease protein and

carbohydrate digestibility for rats and chicks fed sorghum with an above average tannin content. Tannins also form complexes with starch. The formation of such tannin-starch complexes are shown to decrease the *in vitro* amylolysis of sorghum starch (112) and several legume starches (113).

Statement of Problem

Food colors certified by the Thai food and Drug Administration (FDA) seem to be safe. However, information on interaction between color and other food additives such as nitrite in terms of producing nitrosated compounds was never incorporated in to safety evaluation. Interestingly, while protein and starch digestion occurs in the stomach, the situation is also suitable for nitrosating reaction. Generally, food colors were contained in several foods. Therefore, if nitrite is available from processed food, the possibility of nitrosated products formation seems to be unavoidable.

The purpose of this study was to assess the effects of seven synthetic organic colors and six natural food colors on apparent protein digestibility of bovine serum albumin and starch digestibility. Also, mutagen formation was determined after interaction with nitrite. This assessment would be helpful in considering the use of synthetic organic colors and natural food colors in order to avoid the unfavorable effects of color on nutritional status of consumers.

Experimental Objectives

1. To evaluate the effects of synthetic organic colors and natural food colors with and without nitrite on *in vitro* protein and starch digestibility.

2. To evaluate the mutagenic potential of nitrosated products of both synthetic organic colors and natural food colors.

CHAPTER III

MATERIALS AND METHODS

Sample Preparations

Natural food colors Six natural food colors used in this experiment were obtained from drugstores in Bangkok, homemade products and also from wild trees grown in Salaya Campus, Mahidol University. All of them were stored refrigerated. Detail on names and usable parts are shown in Table 4. Each sample was pulverized and kept frozen in a bottle fitted with a plastic stopper until study.

Table 4 Name, part and source of natural food colors used in this experiment.

Botanical name	Thai name	Part	Source
Clitorea ternatea Linn.	อัญชัน	Flower	S
Hibiscus sabdariffs Linn.	กระเจี๊ยบ	Flower	M
Pandanus amaryllifolius	เตยหอม	Leaf	H
Carthamus tinctorius Linn.	คำฝอย	Flower	M
Caramelized coconut sugar	น้ำตาลไหม้	-	H
Carbon black from Coconut skin	ถ่านกาบมะพร้าว	-	H

Note: S = grown in Salaya Campus M = Bangkok markets H = homemade

Synthetic organic colors Seven synthetic organic colors namely, Ponceau 4 R (newcoccine), Red No. 3 (erythrosine), Blue No.1 (brillant blue FCF), Blue No.2 (indigo carmine), Yellow No.6 (sunset yellow FCF), Yellow No.5 (tartrazine) and the combination of tartrazine and indigo carnine (1:1) were obtained from the Royal Thai Food and Drug Administration, Ministry of Public Health. These sample were packed in aluminium foil. They were dissolved in distilled water and made to 1 mg/ml before each study.

Effect of Colors on Protein Digestibility

Reagent Bovine serum albumin (fraction V powder), trypsin (type II), chymotrypsin (type II), porcine pepsin, sodium dihydrogen phosphate were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). TNBS (2,4,6-trinitrobenzenesulfonic acid) trihydrate form was obtained from Fluka AG, (Buchs, Switzerland) Calcium chloride, barbitone, sodium hydroxide, sodium nitrite, sodium sulfite were obtained from BDH Chemical Ltd. (Poole, England). Sodium chloride, hydrochloric acid, di-sodium tetraborate decahydrate were obtained from E. Merck (Dermstadt, Germany). Other chemicals were of reagent grade.

The study on the effects of food colors with and without nitrite was shown in Figure 6.

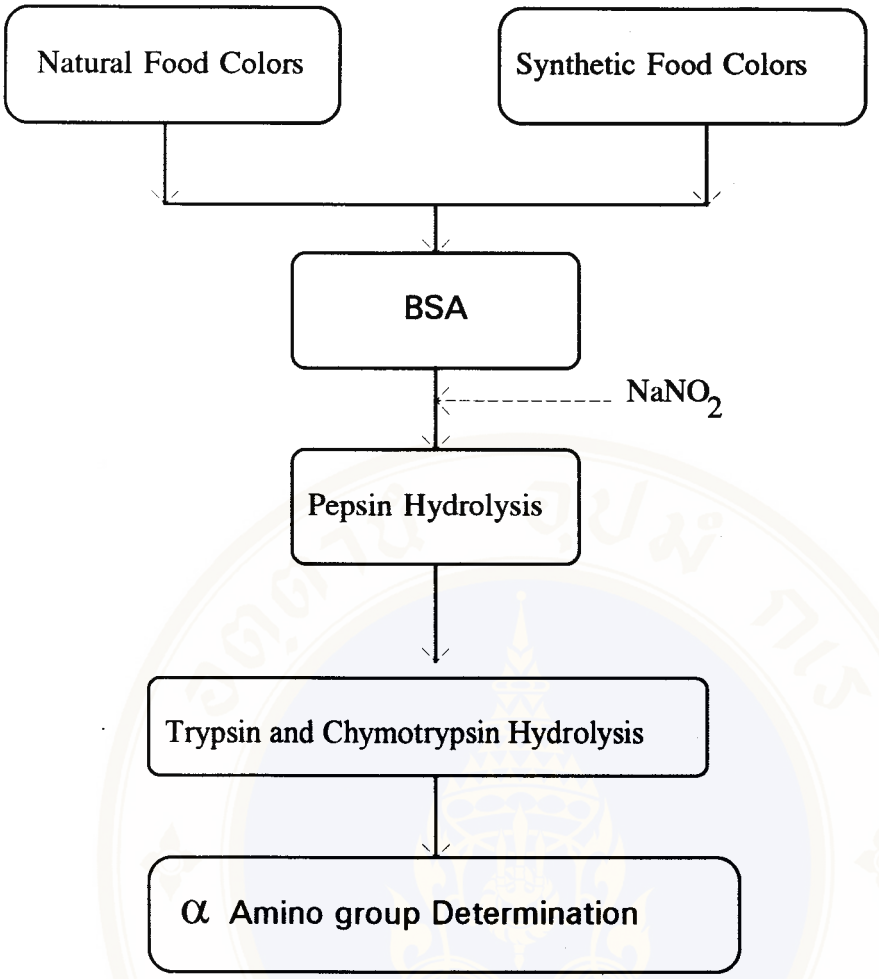


Figure 6 Experimental design on protein digestibility

In vitro protein digestibility with a multienzyme system (pepsin, trypsin, chymotrypsin) of Romero and Ryan was followed as described below.

Reagents for amino acid determination Solution A was weekly prepared by dissolving 1.26 g Na_2SO_3 in deionized distilled water and adjusted to 100 ml. Solution B was prepared by dissolving 11.99 g NaH_2PO_4 in deionized distilled water and adjusted to 1000 ml. Solution C was prepared by dissolving 3.99 g NaOH in approximately 800 ml deionized distilled water; then, 38.14 g $Na_2B_4O_7$ was added into the sodium hydroxide solution and the final solution was adjusted to be 1000 ml. Solution D was freshly prepared by mixing 1.5 ml solution A with 98.5 ml of solution B.

Color forming reagent was prepared by dissolving TNBS (trihydrate) 1 g in deionized distilled water (2 ml). The solution was kept frozen in a bottle fitted with a plastic stopper during storage.

Procedure Prepared sample (0.5, 1.0, 1.5 ml) was added into 12 mg BSA in 2.5 ml of 0.5 M sodium chloride. The pH of the solution was adjusted to 1.5 (the optimal pH for pepsin) with 0.08 M hydrochloric acid in 0.5 M sodium chloride. The reaction mixture was maintained at 37° C in shaking water bath. Then 1.2 ml of pepsin (1 mg/ml, containing 100 mM calcium chloride) was added to the reaction mixture, keeping the enzyme-BSA ratio 1:10. The reaction mixture was digested enzymatically at 37° C for 4 hrs. To each digest, 0.08 M sodium barbital (pH 10.3) buffer was added and the pH was brought to 8 for subsequent digestion with trypsin and chymotrypsin. A mixture of equal volume (0.6 ml) of enzyme trypsin and chymotrypsin (1 mg/ml containing 100 mM calcium chloride) was added to the reaction mixture maintained at 37° C and the digestion was continued for 4 hrs. At the end of the digestion, the reaction mixture was heated in a boiling water bath for 5 min to arrest the enzyme reaction. The volume of reaction mixture was made to 25 ml with deionied distilled water. An aliquot of this digest was filtrated and the supernatant was diluted 10 foles with deionized distilled water. Substrate with enzyme (control), substrate without enzyme and enzyme only (blanks) were prepared in each digestion.

The determination of α-amino group with TNBS method of Field was used with slight modification. Diluted supernatant (0.5 ml) was added to 0.5 ml of borate buffer (solution C) . Then 0.02 ml of 1.1 M TNBS solution was added and the solution was rapidly mixed. After 5 minutes, the reaction was stopped by adding 2.0 ml of solution D (1.5 ml 0.1 M sodium sulphite containing 98.5 ml 0.1 mM sodium dihydrogen phosphate) and absorbance at 420 nm was determined. Optical density (OD) of sample was calculated to percentage relatively to that of control.

Effects of nitrite on protein digestibility In order to determine the nitrite effect on the digestion of BSA, sodium nitrite (containing nitrite 1.2 mg/tube) was added to the reaction mixture before starting the protein digestion described earlier. Absorbance (OD value) of sample, deminused appropriate blank, was calculated and was shown as percentage of control (without nitrite).

Statistical Analysis Analysis of variances was based on calculated requirement at defined levels of main effect of sample levels or nitrite and interaction of sample levels and nitrite at p-value <.05 (114).

Effect of Colors on Starch Digestibility

Reagents Porcine pancreatic α-amylase (type IV A), sodium phosphate, 3,5-dinitrosalicylic acid were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Calcium chloride, sodium hydroxide, sodium nitrite were obtained from BDH Chemical Ltd. (Poole, England). Sodium chloride, hydrochloric acid, sodium potassium tartrate tetrahydrate, starch were obtained from E. Merck (Dermstadt, Germany). Other chemicals were of reagent grade.

Dinitrosalicylic acid color reagent was prepared by dissolved 1.0 gm of 3,5-dinitrosalcylic acid in 20 ml 2 N NaOH. Then 30.0 g sodium potassium tartrate tetrahydrate was added slowly and diluted to a final volume of 100 ml with carbondioxide free distilled water.

Procedure Starch solution (1%) was prepared by dissolving 1.0 g soluble starch in 100 ml of 0.02 M sodium phosphate buffer pH 6.9 with 0.006 M sodium chloride. It was heated in a boiling water bath for 20 min to dissolve the starch.

Sample (0.005, 0.05, 0.5 ml) was added into 5 ml 1% starch. The pH of the solution was adjusted to 6.9 (the optimal pH for alpha amylase) with sodium hydroxide in 0.5 M sodium chloride or hydrochloric acid in 0.5 M sodium chloride and volume was made up to 10 ml with deionized water.

Adjusted sample (0.5 ml) was added to the reaction tube containg enzyme solution consisted of 1.5 mg of porcine pancreatic α-amylase in 8 ml of 0.02 M sodium phosphate buffer pH 6.9 with 2 ml of 1 mM calcium chloride. Sample with enzyme, sample blank and enzyme blank were prepared in each digestion. Tubes were incubated at 25° C for exactly 3 minutes. Released maltose was determined according to the method of Bernfeld (115) using dinitrosalicylic acid. Dinitrosalicylic acid color reagent (1 ml) was added to the reaction tube and left in a boiling water bath for 5 minutes. The mixture was cooled to room temperature and 5 ml deionized water was added. Absorbance of sample at 540 nm was determined and calculated to percentage with respected to control.

Effects of nitrite on starch digestibility In order to determine the nitrite effect on starch digestibility, nitrite (1.2 mg/tube) was added to the reaction mixture before starting the digestion as described earlier. Absorbance of sample, deminused appropriate blank, was calculated.

Statistical analysis Analysis of variances was based on calculated requirement at defined levels of main effect of sample levels or nitrite and interaction of sample levels and nitrite at p-value <.05 (114).

Mutagenicity Study on Food Colors

Chemicals, reagent and nutrient broth D-biotin, sodium nitrite and ammonium sulfamate were purchased from Sigma Chemical Co. (St.Louis, Missouri, U.S.A.). L-Histidine monohydrochloride, sodium-chloride (NaCl) were obtained from BDH Chemicals Ltd.(Poole, England), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), citric acid monohydrate GR ($C_6H_8O_7.H_2O$), anhydrous di-potassium hydrogen phosphate (K_2HPO_4), sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4.4H_2O$), anhydrous D(+)-glucose, potassium chloride (KCl) GR, sodium dihydrogen phosphate

($NaH_2PO_4.2H_2O$), di-sodiumhydrogen phosphate (Na_2HPO_4), were obtained through E. Merck, (Darmstadt, Germany). Bacto agar was purchased from Gibco BRL (Life Technologies Ltd., Paisley, England). Nutrient broth was purchased from Oxoid Ltd., (Basingstoke, Hants., England). Aminopyrene (Aldrich, St. Louis U.S.A.) was used as standard nitrosatable compound in this assay.

The bacterial tester strains *Salmonella typhimurium* strains TA98 and TA100 were originally provided by Prof. B.N. Ames, University of California at Berkely, California, U.S.A. Overnight culture of strains TA 98 or TA 100 were prepared in Oxoid nutrient broth No.2 at 37° C with shaking. Manipulation of the cultures was done as suggested by Maron and Ames (66) (Appendix 1).

Agar

Minimal agar plate It contained 30 ml of minimal glucose agar medium consisting of 1.5 % Bacto-Difco agar and 2% glucose in Vogel-Boner medium E. (Appendix 1). Sterile petri dishes were used.

Top agar It contained 0.6% agar and 0.5% NaCl. It was autoclaved. Ten ml of a sterile solution of 0.5 mM L-histidine. HCl and 0.5 mM biotin was added to each 100 ml of the top agar and mixed throughly by swirling (Appendix 1).

Sample preparation Before mutagenicity determination, each sample was dissolved (100 mg/ml) in distilled water. To obtain a bacterial free sample, it was either passed through a sterile membrane filter or autoclaved at 121° C for 15 min.

Nitrosation Each sample was interacted with nitrite using the method described by Takeda and Kanaya (116). To 0.1 ml of the sample in a tube fitted with a plastic stopper, 0.650 ml of 0.2 N hydrochloric acid which contained sufficient acid to acidify the reaction mixture to pH 3.0-3.5, and 0.250 ml of 2 M sodium nitrite were added. The final concentration of nitrite was 500 mM. The reaction tube was shaken at 37° C for 4 hr. The reaction was stopped by placing the tube in an ice bath. An aliquote (0.250 ml) of 2 M ammonium sulfamate was

added to the reaction mixture in order to decompose the residual nitrite, and the whole was allowed to stand for 10 min in an ice bath.

Direct mutagenesis assay Among nitroso compounds occured during the gastric digestion of food, only N-nitrosamide possed direct mutagenic activity. Therefore, in this study we use the non-activation procedure of Ames test.

Sample was mixed with 0.5 ml of NaH_2PO_4-KCl buffer pH 7.4 and 0.1 ml of fresh overnight culture of tester strain. The contents were incubated at 37° C for 20 min. After incubation, two ml of molten top agar (45° C) was added, mixed well and poured onto a minimal glucose agar plate. His^+ revertant colonies were counted after incubation at 37° C for 48 hours in the dark. The procedure is shown in Figure 7. Duplicate plates were carried out for each concentration of the extract. Negative control containing the bacteria and solvent (without test chemical) was required to establish the spontaneous mutation of each tester strain. Positive controls contained nitrosated aminopyrine were used as positive control for strains TA 98 and TA 100. The mutagenicity of each sample was presented as number of histidine revertants per plate. The sample expressed its mutagenicity higher than 2 times of spontaneous revertants with a dose-response relationship was evaluated mutagenic (117).

Tester strain : *Salmonella typhimurium* TA 98 and TA 100

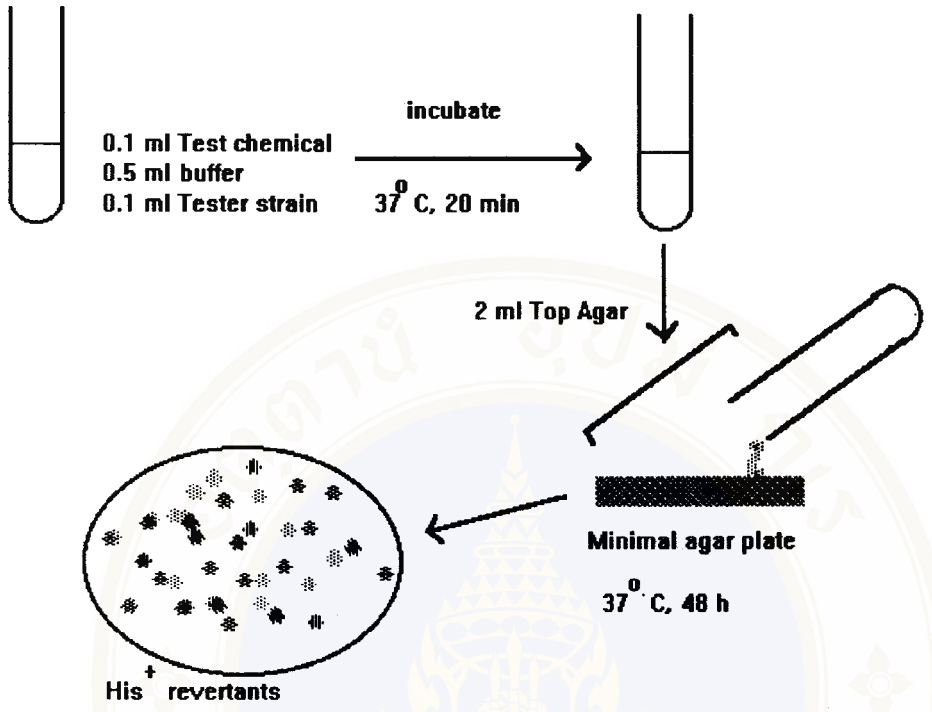


Figure 7 Steps in mutagenicity evaluation using the Ames test (pre-incubation modification)

CHAPTER IV

RESULTS AND DISCUSSION

The effects of six natural food colors and seven synthetic food colors themselves and interacted with nitrite were studied on the *in vitro* digestion of bovine serum albumin (BSA), the *in vitro* digestion of starch and mutagenicity. Nitrite at 1.2 mg/tube (the amount selected for this study) were not shown to affect on BSA digestion (118). However, the amount higher than 1.2 mg/tube could deviate the digestion of protein and starch.

Effects of Food colors on *In Vitro* BSA Digestibility

The effects of natural food colors and synthetic organic colors with and without nitrite were classified by analysis of variances. The results show that all natural food colors (Figure 8-13) and three synthetic food colors, namely tartrazine (Figure 15), erythrosine (Figure 16), and ponceau 4 R (Figure 17) have affected on BSA digestibility. The alteration of BSA digestibility did not depend only on the main effects of food color or nitrite treatment but also on the effects of color-nitrite interaction. The effects of food colors and nitrite treatment at 1.2 mg/tube were presented to inhibit digestion of BSA. Those were shown by the effects of synthetic food colors such as sunset yellow FCF (Figure 14), brilliant blue FCF (Figure 19) and mixture of indigo carmine and tartrazine (1:1) (Figure 20). The decrease of BSA digestibility was illustrated by nitrite treatment effect on indigo carmine (Figure 18).

The effects of the extracts of natural food colors on the digestion of BSA

were explained on the property of the naturally occurring antinutritional protein in plant. The most important natural pigments from higher plant sources were the chlorophylls and the anthocyanins. The anthocyanins were phenolic glycosides and were presented for the red, blue colors of many plants such as *Clitoria ternatea* Linn. (อัญชัน), *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) (113). Citric acid, waxes, resin and pectin were other chemicals have found (119). The color of *Carthamus tintorius* Linn. (คำฝอย) was from flower. The chemicals of this flower were arachidic acid, safflor yellow A, safflor yellow B, safrole yellow and phytol. It was indicated that the decrease of BSA digestibility was directly related to the concentration of food colors. Natural constituent normally present in many plant such as phytate and polyphenolic compound should take responsibility in decreasing of such phenomenon.

The effects of synthetic food colors on digestibility of BSA were enhanced or decreased the activity. Unfortunately, no machanism was known. The decrease in activity of enzyme might be occured via the binding of enzyme, metal and substrate. Enomoto, *et al.* (120,121) studied about the influence of food-color upon protein digestive action of trypsin. They indicataed that when the amount of food-color was increased, the digestive action of trypsin was suppressed. The suppressing effect was observed at less concentration than that ordinarily used for food. The addition of calcium ion or magnesium ion showed good results for the promotion of the digestive action.

Effect of Colors Treated with Nitrite on *In Vitro* BSA Digestility

Nitrosomorpholine prepared as suggested by Takeda and Kanaya (116) in gastric liked condition was tested by Hualmukda (118). It was shown that nitrosomorpholine reduced the digestion of BSA. Limitation of the available of

substrate via nitroso compounds formation was the possible explanation. It was shown by Knowles et al. (64) that nitrosation of BSA under gastric condition presented the decreasing of tyrosine to form nitroso compounds and its derivatives i.e. nitrosotyrosine, 3-nitrosotyrosine, 3-4-dihydroxyphenylalanine.

The results of natural food colors and synthetic food colors treated with nitrite on the BSA digestion explained another possible harmful effects of nitroso compounds. Previous study showed that nitroso compounds were formed by Thai medicinal plants interacted with nitrite (Kangsadalampai, unpublished communication) and in gastric condition (3,4). Experiment to prove the hypothesis that nitroso compounds may affect on BSA digestibility was then designed.

The amount of nitrite 1.2 mg/tube did not significantly influence on BSA digestibility (116). On the other hand, nitrite at the no effect level has potentiated the inhibitory effect on BSA digestibility of many food colors in this study. It was possible that not only the components of food color can caused this effect but also their nitrosated products may involved. Challis (122) reported that phenolic compounds could react with nitrite to form p-nitrosophenol which may be faster than the nitrosation of secondary amines. Hence, nitrosation of anthocyanins which are phenolic glycosides in natural food colors might be possible and, therefore, it would occur more rapidly than nitrosation of BSA digestion.

p-Nitrosophenolic compounds may indirectly relate to the decrease of BSA digestibility. Davis et al. (123) suggested that phenolic compounds which was capable of tautomerism could produce nitrosophenolic compounds, which showed catalytic activity of nitrosation reaction, higher than those without effect of nitrosophenolic. The overall effects were likely depended upon whether the steady state concentration of the nitrosophenol, as determined by the relative rates of its formation and of its destruction by oxidation by oxidation to the nitrosophenol. However, this phenoemenon depended upon the relative degrees of inhibition and

catalysis of nitroso formation exerted by the concentration and type of phenolic compound and nitrosophenolic, respectively. Nitroso compounds in this reaction directly decreased amount of substrate, subsequently the decreasing of BSA digestibility was occurred.

Interestingly, it was shown that the additive of nitrite to the digesting mixture resalted in the higher protein digestibilities (Figure 8, 19). Butyee (unpublished data, 1993) tested the formation of nitroso compounds of some food colors. It was found that the extracts of *Hibiscus sabdaiffa* Linn. (กระเจี๊ยบ), *Carthamus tinctorius* Linn. (คำฝอย), and brilliant blue FCF were converted to nitroso compounds. It was suggested that nitrite prefered to involve in nitrosation rather in inhibition the BSA digestion.

Effects of Colors on *In Vitro* Starch Digestibility

The effects of six natural food colors and seven synthetic food colors on the *in vitro* digestion of starch were examined. It was concerned that nitrite may posed as an inhibitory factor on starch digestibility. They could interact with nitrite to form nitroso compounds. Therefore, it was possible that nitroso compounds may be occured and influenced on the starch digestibility. A study on the effect of nitroso compound on starch digestibility was designed.

Nitrosomorpholine produced by the interaction between morpholine and nitrite was tested on starch digestibility. The data showed that nitrosomorpholine was an inhibitor of the starch digestion (Butyee, unplublished data).

The effects of natural food colors and synthetic organic colors with and without nitrite were classified by analysis of variances. The results showed that six nutural food colors, namely *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ), *Carthamus tinctorius* Linn. (คำฝอย), *Clitorea ternatea* Linn. (อัญชัน), *Pandanus amaryllifolius*

Roxb. (เตย), caramelized coconut sugar (น้ำตาลไหม้), carbon black from coconut skin (ถ่านกาบมะพร้าว) (Figure 21-26) and seven synthetic food colors, namely sunset yellow FCF, tartrazine, erythrosine, Ponceau 4 R, indigo carmine, brilliant blue FCF, the mixture of tartrazine and indigo carmine (1:1) (Figure 27-33) affected on starch digestibility. The decrease of starch digestibility not only by the main effects of food color or nitrite treatment but also the effects of food color-nitrite interaction was shown.

The effects of natural food colors on the digestion of starch were explained in detail of the property of naturally occurring antinutritional carbohydrate in plant. It was indicated that the decrease or increase of starch digestibility was directly related to the concentration of natural food colors.

The extract of natural food colors in this study could solubilize substances e.g. chlorophylls, flavonoid and anthocyanin. Meanwongyat (17) reported that the colors of *Clitorea ternatea* Linn. (อัญชัน) and *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) are flavonoid and anthocyanin. Chlorophyll is the color of *Pandanus amaryllifolius* Linn. (เตย). Most flavonoids occur as glycosides in which the C_6-C_3-C_6 aglycone part of the molecule is esterified with a number of different sugars. The flavonoids are unique in that they do not occur as glycosides but show reactivity through polymerization into "condensed tannins" (113). They can acted as tannins to from complexes with starch. The formation of such tannin-starch complexes are shown to decrease the in vitro amylolysis of sorghum starch (112) and several legume starches (113).

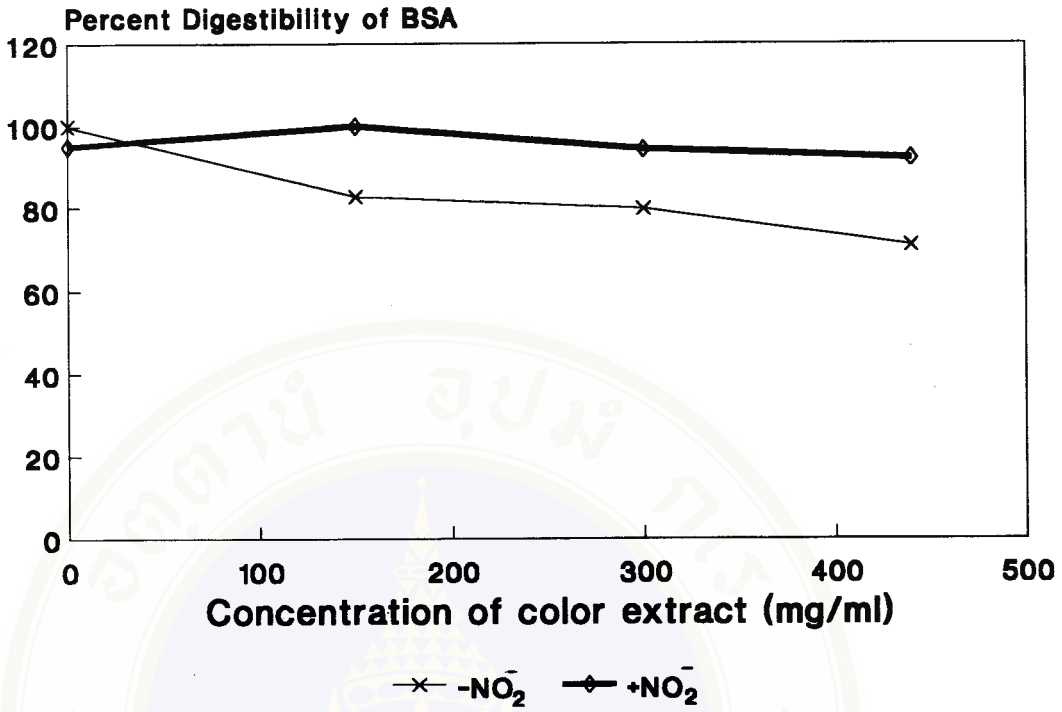
Synthetic food colors compounds represent four distinct chemical classes: azo dyes, triphenylmethane dyes, xanthene dyes and indigoid dyes. The effects of food colors on digestibility of starch were decreased the activity. Unfortunately, no machanism was known. Adachi and Yamaha (124) studied about effect of food dyes on pancreatic amylase. They indicated that food dyes could inhibited

digestion of amylase. It may decrease starch digestibility.

Effect of Colors Treated with Nitrite on *In Vitro* Digestibility of Starch

The results of natural food colors and synthetic food colors treated with nitrite on the starch digestibility explained another possible harmful effects of nitroso compound. It was possible that their nitrosated products may inhibitory effect on starch digestibility. Davis et al. (123) suggested that phenolic compounds which was capable of tautomerism could produce nitrosophenol which showed catalytic activity of nitrosation reaction higher than nitroso compounds formation without effect of nitrosophenol. The overall effects were likely to depend upon whether the steady state concentration of the nitrosophenol, as determined by the relative rates of its formation and of its destruction by oxidation to the nitrosophenol. However, this depended upon the relative degree of inhibition and catalysis of n-nitroso formation exerted by the concentration and type of phenol and the nitrosophenol respectively. Nitroso compounds in this reaction directly decreased amount of substrate, subsequently the decreasing of starch digestibility was occurred.

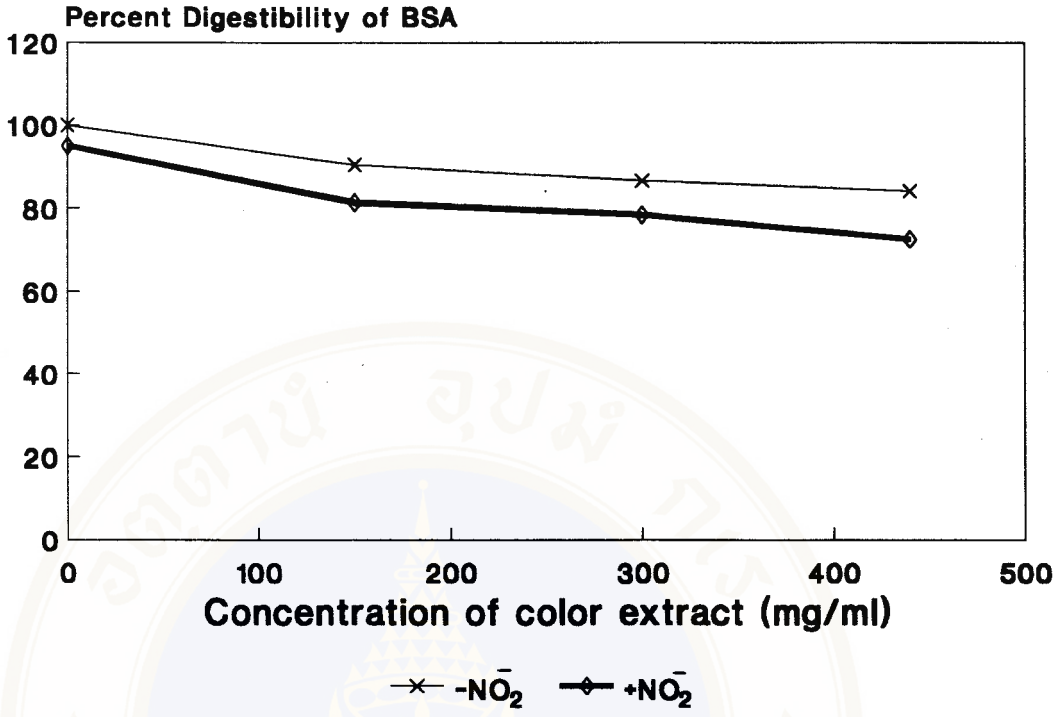
Interestingly, it was shown that the additive of nitrite to the digesting mixture resalted in the higher starch digestibilities (Figure 21, 22). Butyee (unpublished data, 1993) tested the formation of nitroso compounds of some food colors. It found that the extracts of *Hibiscus sabdaiffa* Linn. (กระเจี๊ยบ), *Carthamus tinctorius* Linn. (คำฝอย), and brilliant blue FCF were converted to nitroso compounds. Therefore, it was suggested that nitrite prefered to involve in nitrosation rather in inhibition the starch digestion.



Concentration of	Concentration of color extract (mg of dry weight/ml)			
nitrite (mg/ml)	0.00	150	300	440
0.0 ($-NO_2^-$)	100.00	82.77	79.71	70.94
	(±0)	(±0.06)	(±4.47)	(±2.13)
0.48 ($+NO_2^-$)	95.07	99.99	94.33	92.16
	(±1.66)	(±1.46)	(±3.49)	(±0.58)

Statistical explanation (Appendix 2)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

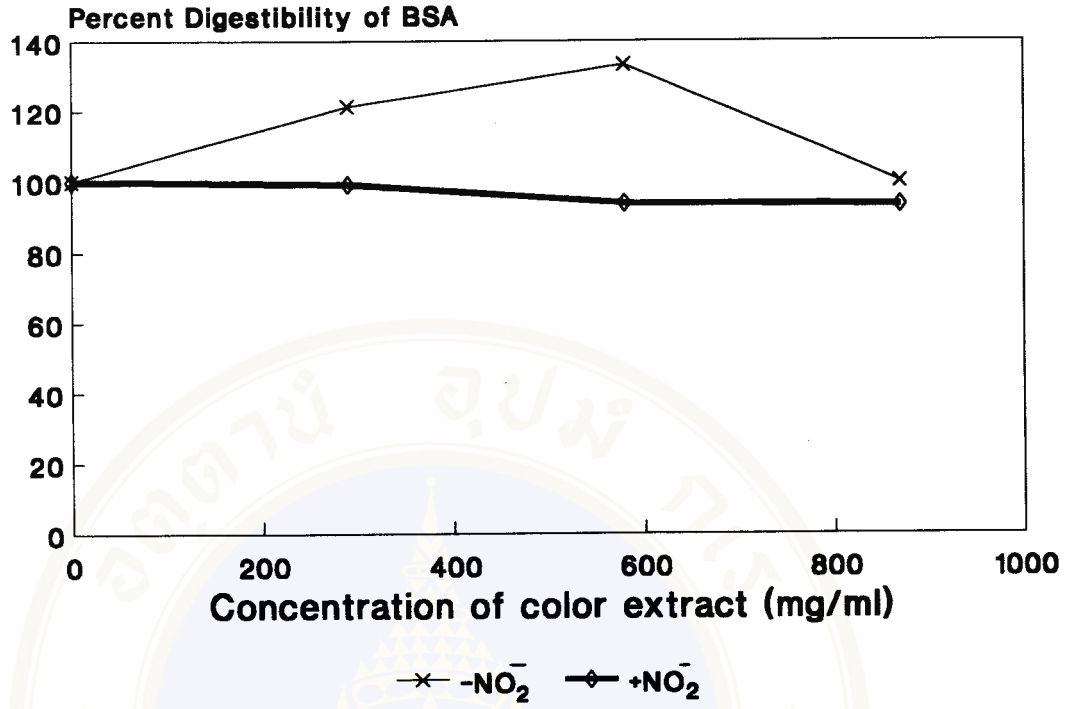
Figure 8 Effects of *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg of dry weight/ml)			
	0.00	150	300	440
0.0 ($-NO_2^-$)	100.00 (0.00)	90.54 (±0.71)	86.66 (±0.22)	84.12 (±1.07)
0.48 ($+NO_2^-$)	95.07 (±1.66)	81.39 (±0.07)	78.47 (±0.08)	72.63 (±0.47)

Statistical explanation (Appendix 3)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

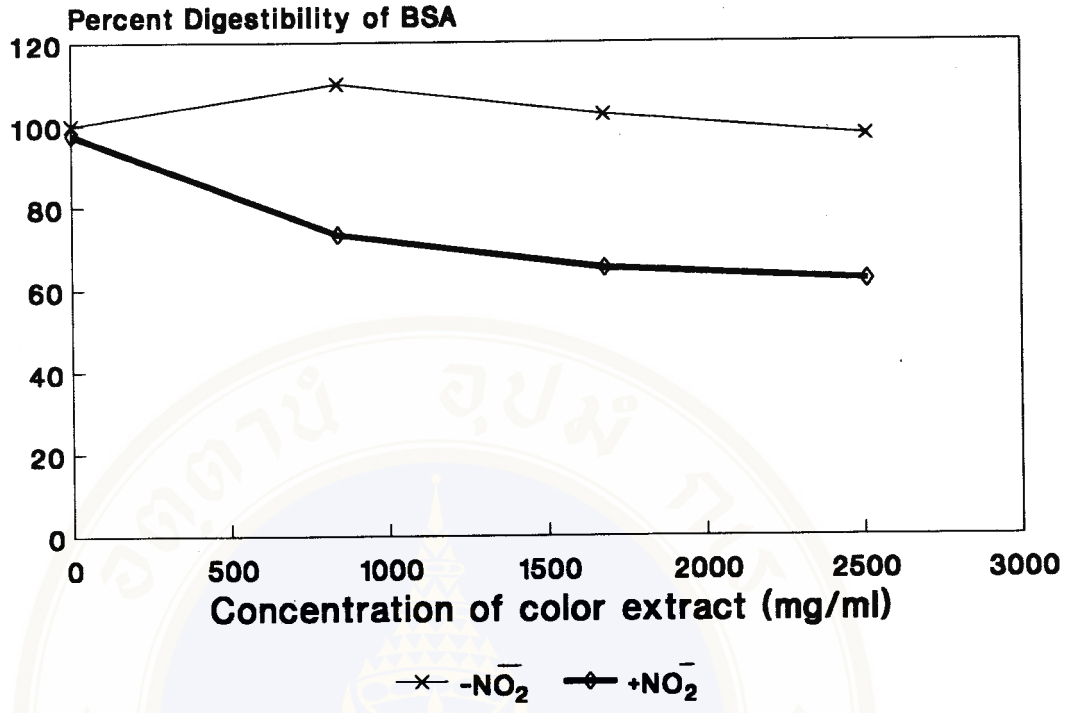
Figure 9 Effects of *Carthamus tinctorius* Linn. (คำฝอย) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg of wet weight/ml)			
nitrite (mg/ml)	0.00	290	580	870
0.0 ($-NO_2^-$)	100.00	121.33	133.33	99.98
	(±0.00)	(±0.37)	(±0.08)	(±1.53)
0.48 ($+NO_2^-$)	95.07	99.09	93.80	93.43
	(±1.66)	(±0.19)	(±0.35)	(±0.34)

Statistical explanation (Appendix 4)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

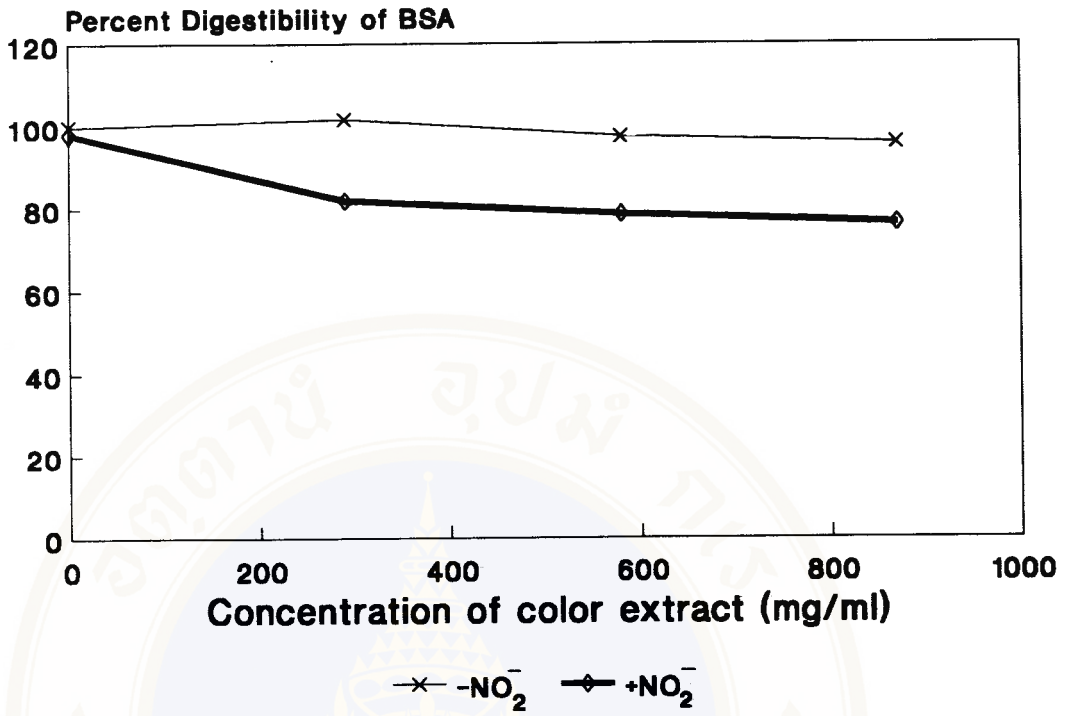
Figure 10 Effects of *Clitorea ternatea* Linn. (อัญชัน) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg of wet weight/ml)			
	0.00	840	1,680	2,510
0.0 ($-NO_2^-$)	100.00 (±0.00)	110.06 (±1.33)	102.54 (±4.35)	97.44 (±1.36)
0.48 ($+NO_2^-$)	97.72 (±1.95)	73.32 (±.01)	65.15 (±1.63)	62.15 (±1.56)

Statistical explanation (Appendix 5)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

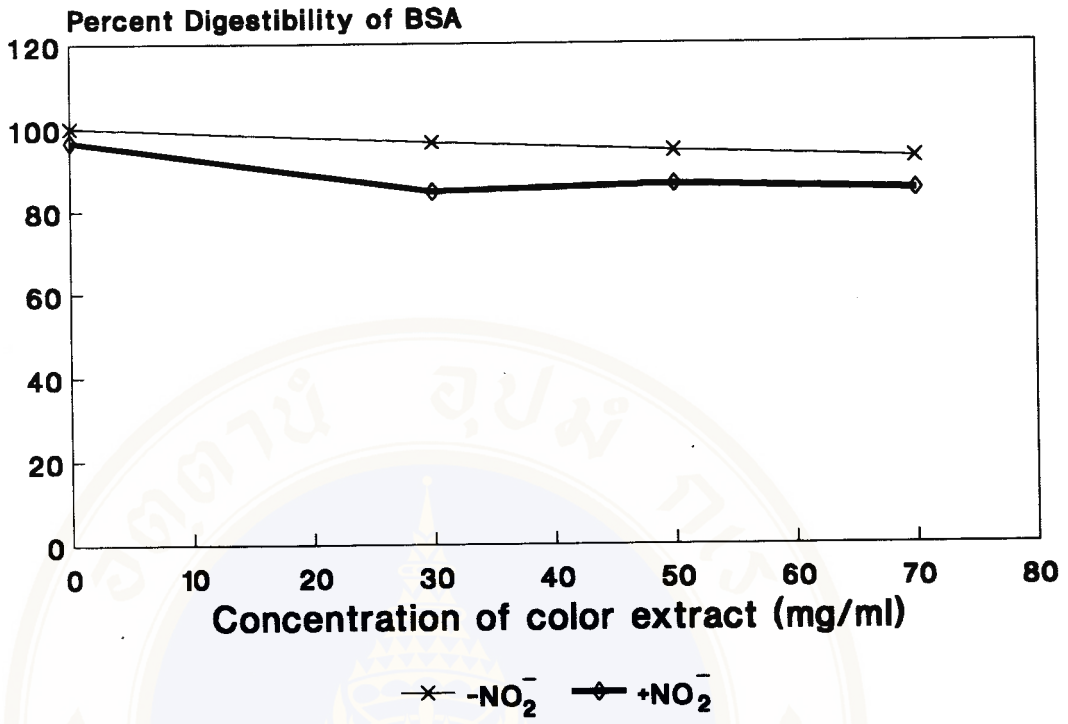
Figure 11 Effects of *Pandanus amaryllifolius* Roxb. (เตย) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	290	580	870
0.0 ($-NO_2^-$)	100.00 (±0.00)	101.83 (±1.83)	97.58 (±2.43)	95.94 (±0.79)
0.48 ($+NO_2^-$)	98.17 (±0.72)	81.93 (±0.54)	78.61 (±0.87)	76.40 (±0.85)

Statistical explanation (Appendix 6)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

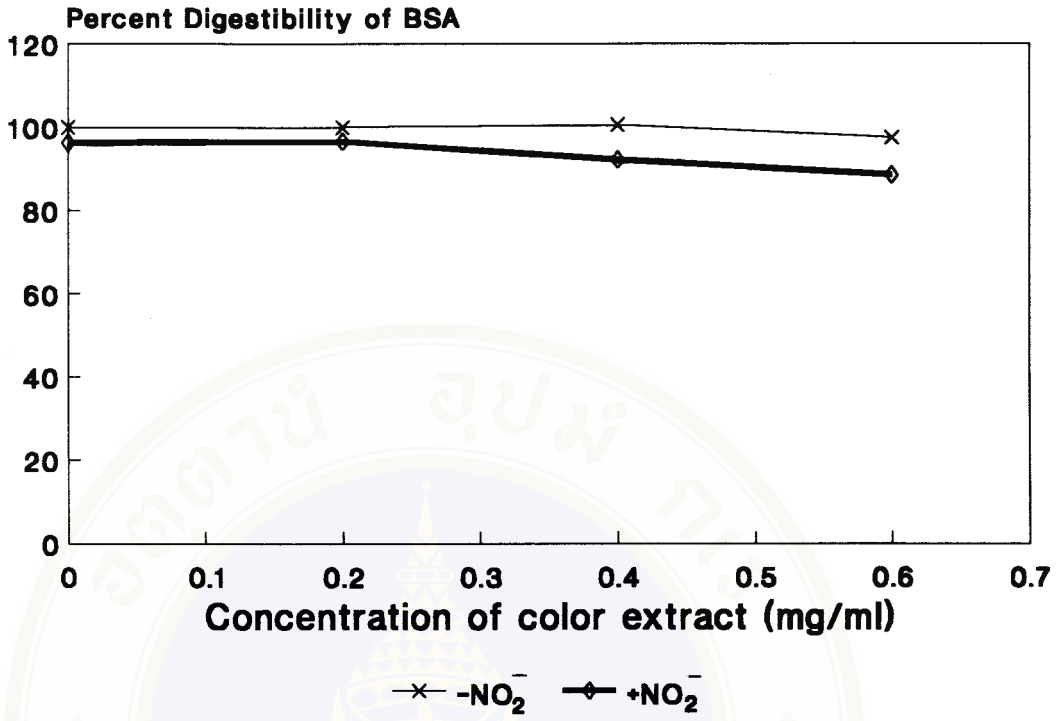
Figure 12 Effects of caramelized coconut sugar (น้ำตาลไหม้) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	30	50	70
0.0 ($-NO_2^-$)	100.00	96.53	94.44	92.73
	(±0.00)	(±0.38)	(±2.13)	(±0.63)
0.48 ($+NO_2^-$)	96.71	84.68	86.32	84.93
	(±0.15)	(±2.39)	(±0.75)	(±0.39)

Statistical explanation (Appendix 7)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

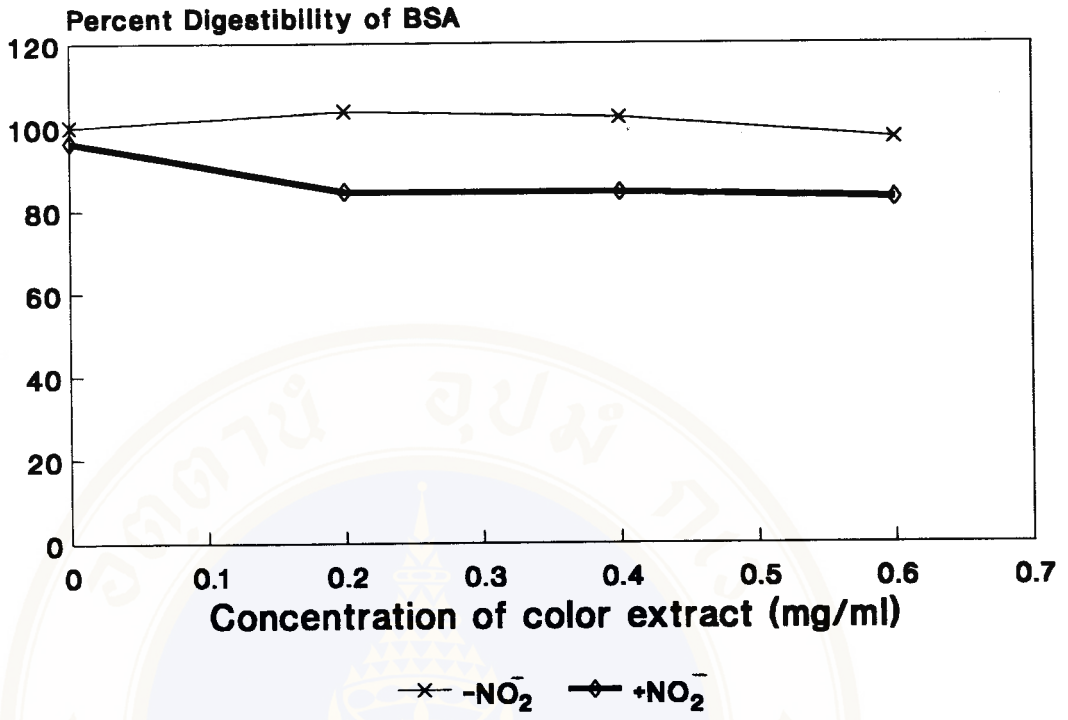
Figure 13 Effects of carbon black from coconut skin (ถ่านกาบมะพร้าว) with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00 (±0.00)	100.03 (±1.15)	100.66 (±0.66)	97.56 (±1.12)
0.48 ($+NO_2^-$)	96.31 (±2.23)	96.65 (±1.88)	92.21 (±2.31)	88.63 (±2.22)

Statistical explanation (Appendix 8)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite		+

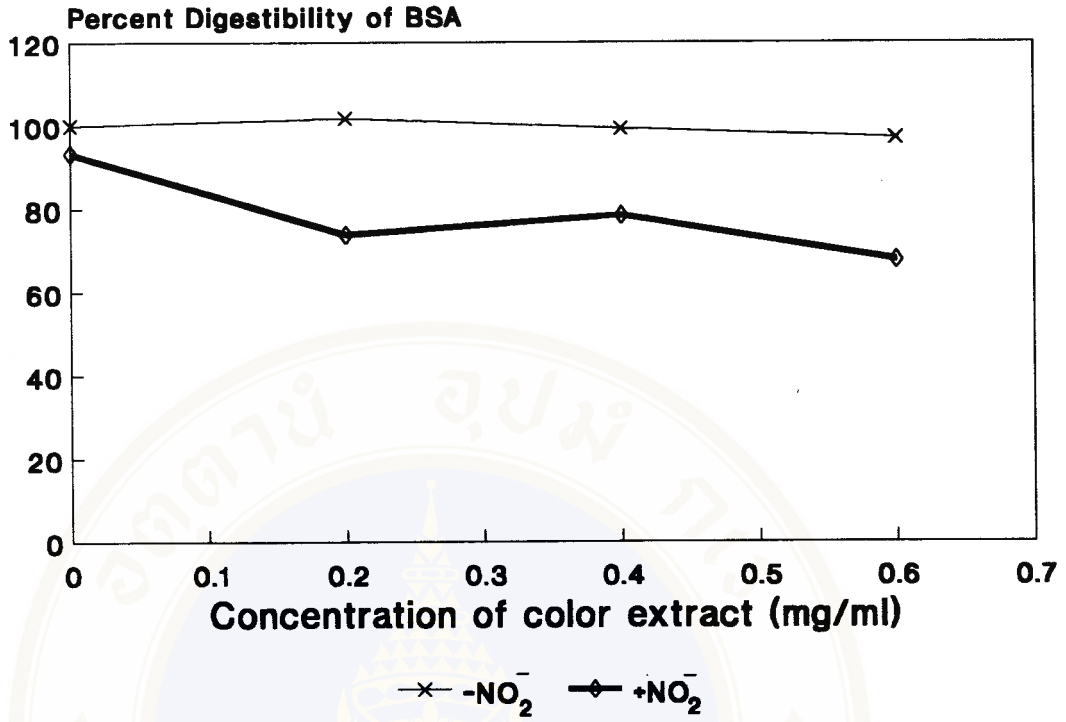
Figure 14 Effects of Sunset Yellow FCF with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00 (±0.00)	103.76 (±0.20)	102.28 (±0.489)	97.39 (±0.63)
0.48 ($+NO_2^-$)	96.31 (±2.23)	84.52 (±1.94)	84.43 (±0.488)	83.07 (±1.19)

Statistical explanation (Appendix 9)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

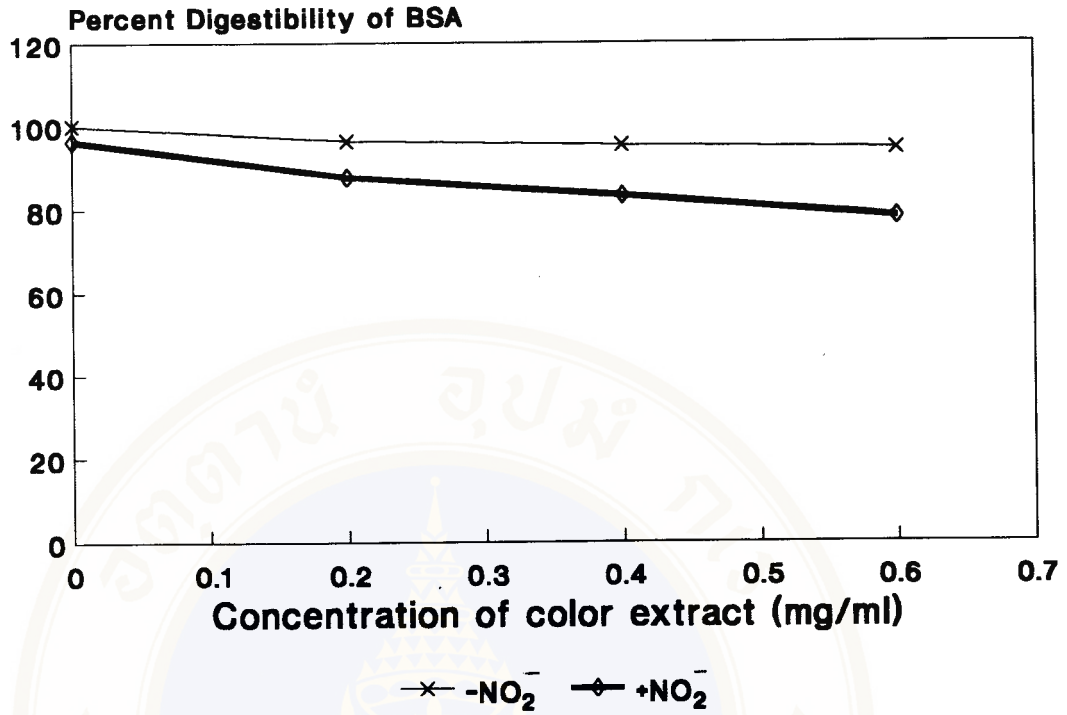
Figure 15 Effects of Tartrazine with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00 (±0.00)	100.86 (±2.03)	99.23 (±1.18)	97.11 (±2.58)
0.48 ($+NO_2^-$)	96.31 (±2.23)	73.95 (±0.78)	78.61 (±1.61)	68.01 (±0.24)

Statistical explanation (Appendix 10)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

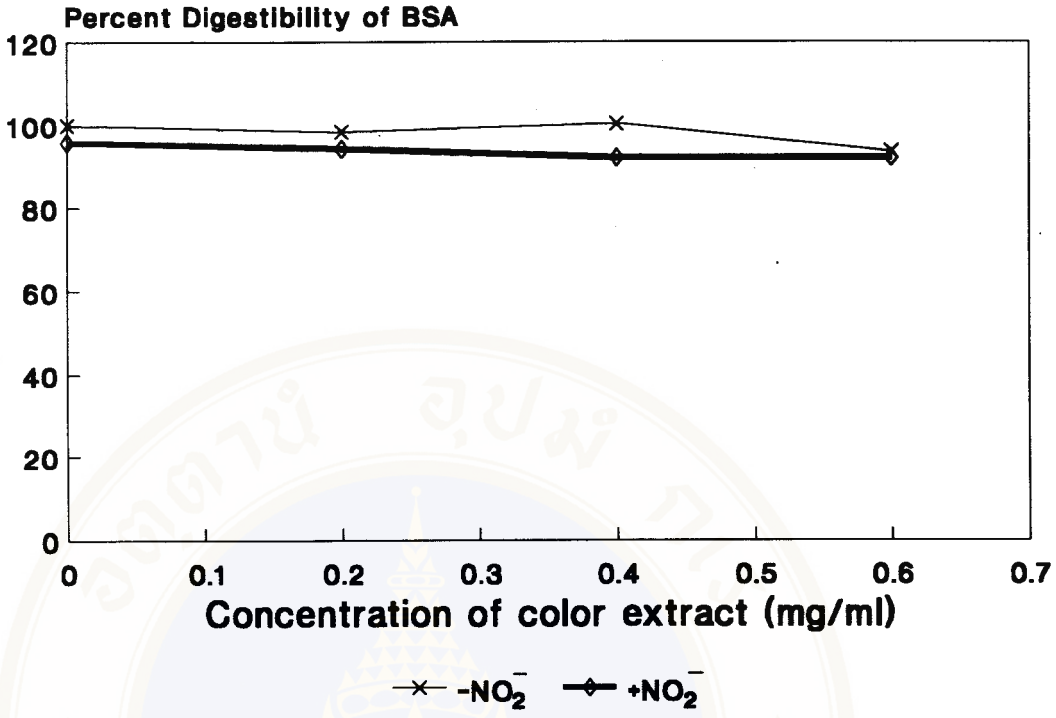
Figure 16 Effects of Erythrosine with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00	96.25	95.25	94.46
	(±0.00)	(±2.11)	(±2.28)	(±2.66)
0.48 ($+NO_2^-$)	96.31	87.70	83.24	78.23
	(±2.23)	(±0.94)	(±1.01)	(±0.53)

Statistical explanation (Appendix11)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

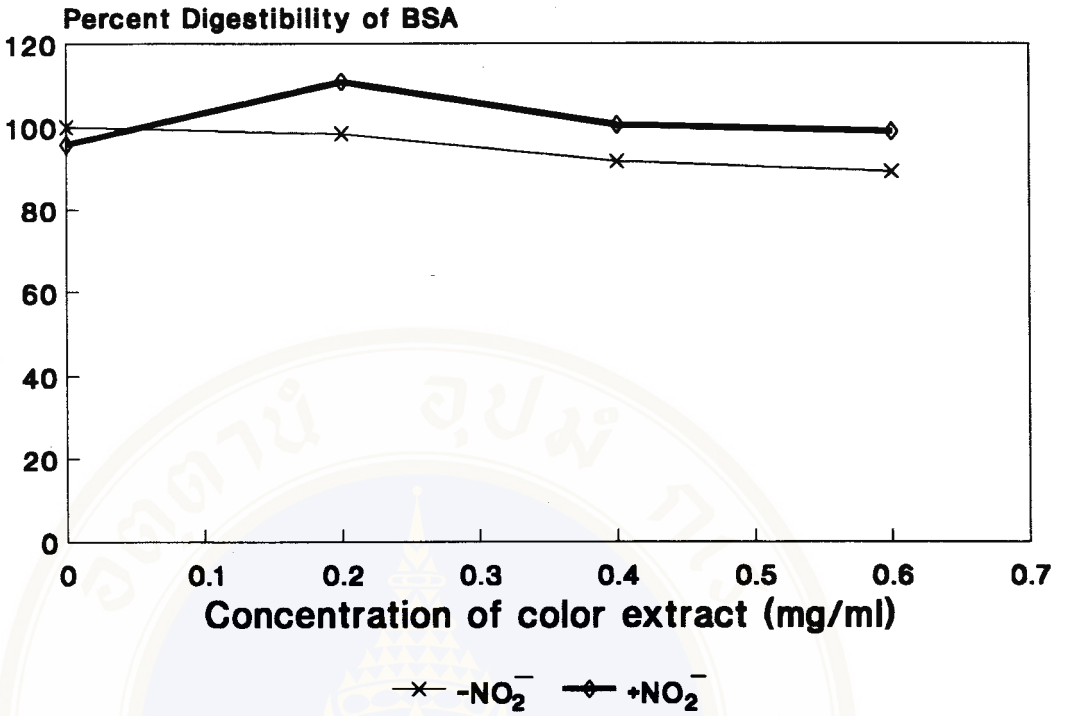
Figure 17 Effects of Ponceau 4 R with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00 (±0.00)	98.45 (±0.480)	100.36 (±1.37)	93.69 (±0.61)
0.48 ($+NO_2^-$)	95.85 (±2.52)	94.34 (±3.23)	92.18 (±2.55)	92.23 (±3.71)

Statistical explanation (Appendix 12)	Effect	No effect
Effect of color on BSA digestibility		+
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite		+

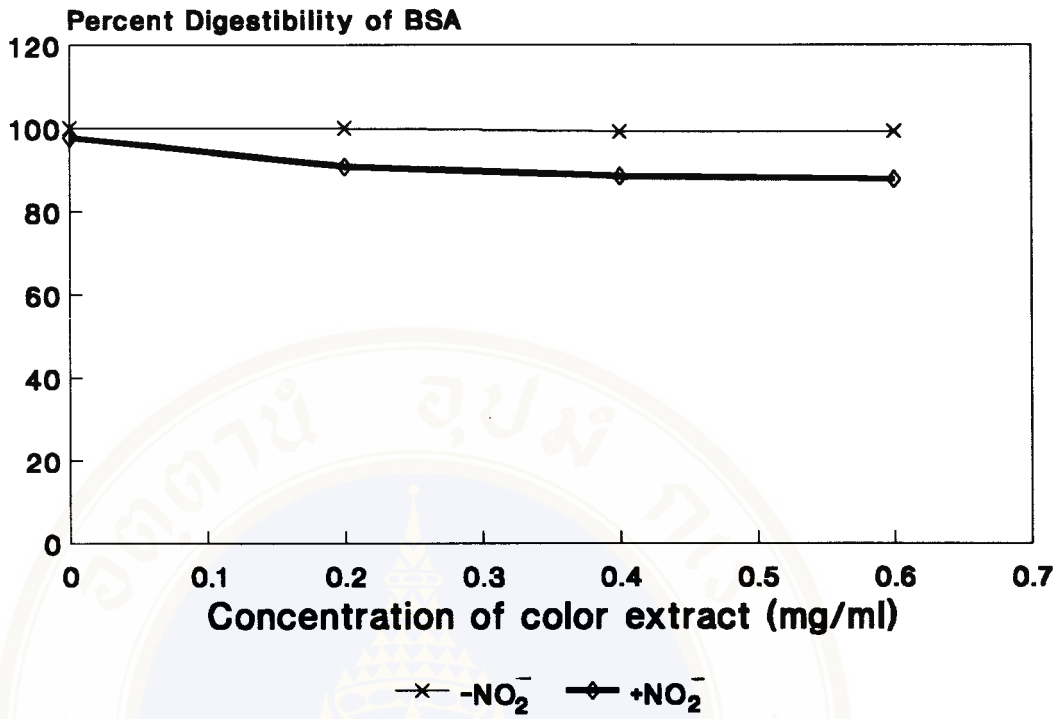
Figure 18 Effects of Indigo carmine with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml)			
	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00 (±0.00)	98.44 (±0.481)	91.76 (±1.45)	89.31 (±1.41)
0.48 ($+NO_2^-$)	95.85 (±2.52)	110.86 (±4.19)	100.52 (±4.35)	99.02 (±3.83)

Statistical explanation (Appendix 13)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite		+

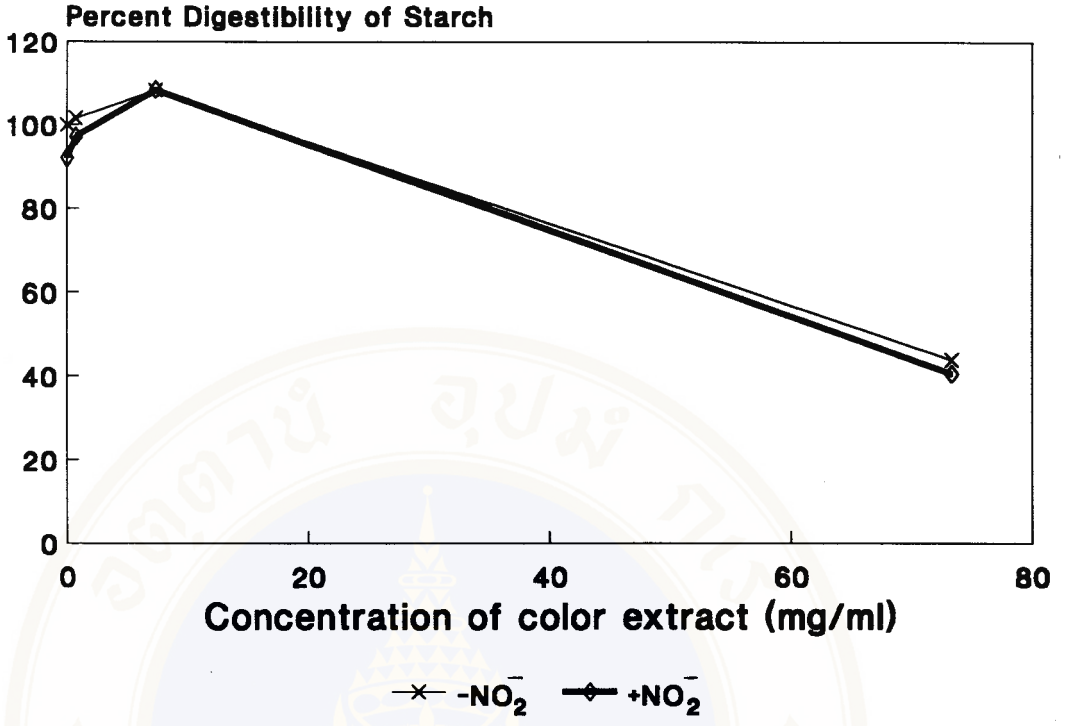
Figure 19 Effects of Brilliant Blue FCF with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg/ml)			
nitrite (mg/ml)	0.00	0.20	0.40	0.60
0.0 ($-NO_2^-$)	100.00	100.01	99.14	99.28
	(±0.00)	(±0.36)	(±0.482)	(±1.16)
0.48 ($+NO_2^-$)	97.72	90.88	88.54	87.83
	(±1.95)	(±2.28)	(±2.22)	(±0.53)

Statistical explanation (Appendix 14)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite		+

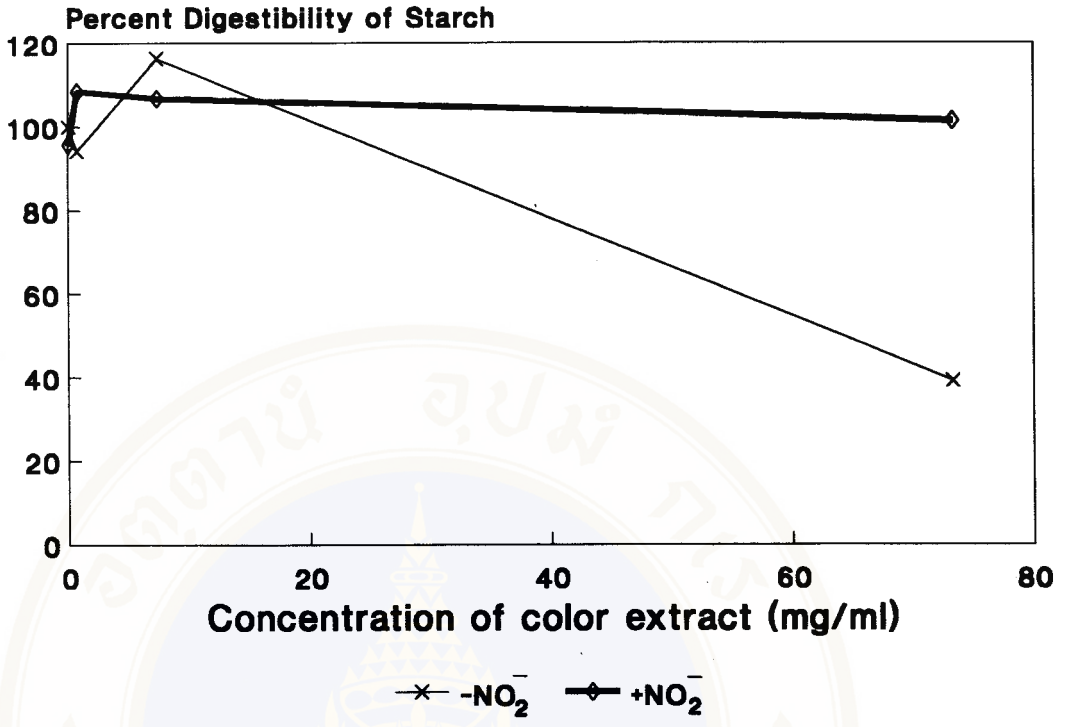
Figure 20 Effects of Tartrazine with Indigo Carmine 1:1 with and without nitrite on *in vitro* digestibility of BSA (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg of dry weight/ml)			
	0.00	0.73	7.33	73.34
0.0 ($-NO_2^-$)	100.00 (±0)	101.64 (±0.22)	108.32 (±0.64)	43.89 (±0.50)
0.48 ($+NO_2^-$)	92.11 (±0.50)	97.23 (±0.22)	108.40 (±0.86)	40.54 (±0.43)

Statistical explanation (Appendix 15)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

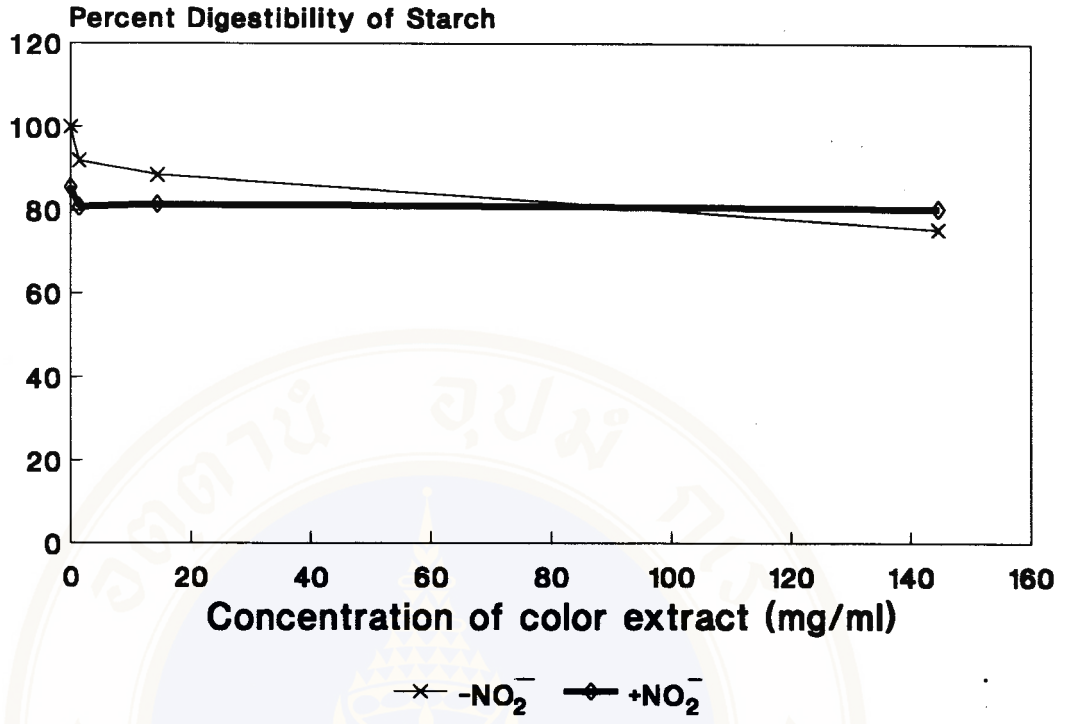
Figure 21 Effects of *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg of dry weight/ml)			
nitrite (mg/ml)	0.00	0.73	7.33	73.34
0.0 ($-NO_2^-$)	100.00	94.24	116.36	39.12
	(±0)	(±0.21)	(±0.14)	(±0.14)
0.48 ($+NO_2^-$)	95.74	108.47	106.76	101.35
	(±0.29)	(±2.29)	(±0.22)	(±0.21)

Statistical explanation (Appendix 16)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

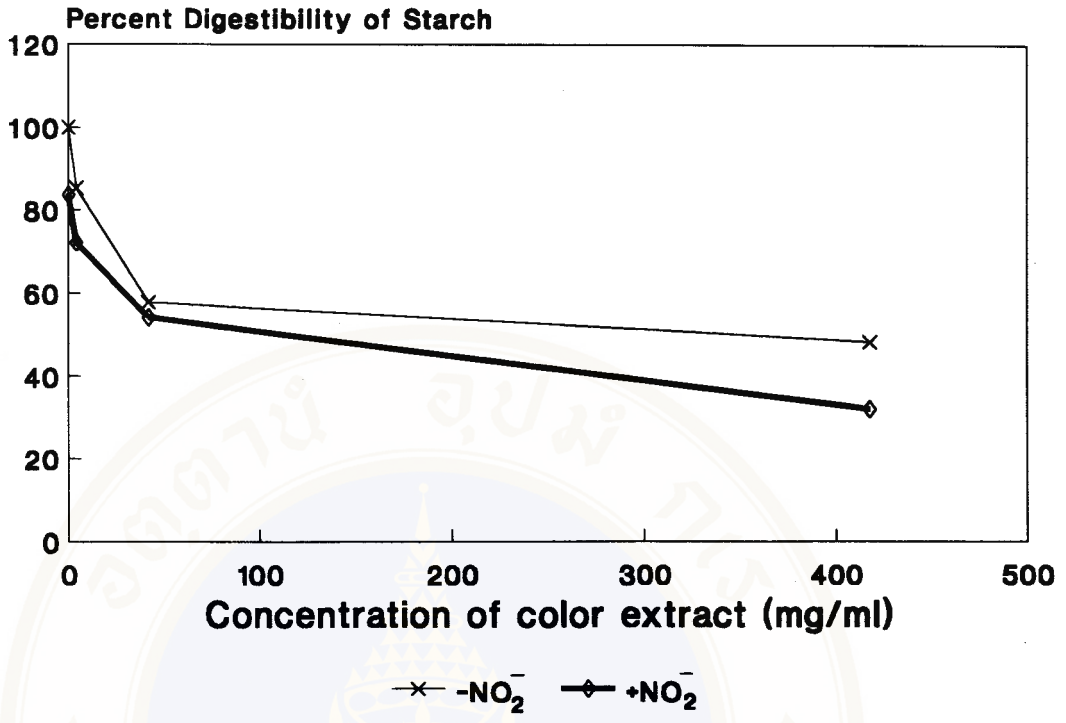
Figure 22 Effects of *Carthamus tinctorius* Linn. (คำฝอย) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg of wet weight/ml)			
	0.00	1.44	14.46	144.60
0.0 ($-NO_2^-$)	100.00 (±0)	91.12 (±0.15)	88.52 (±0.43)	75.61 (±0.29)
0.48 ($+NO_2^-$)	85.61 (±0.22)	80.78 (±0.21)	81.42 (±0.29)	80.51 (±3.18)

Statistical explanation (Appendix 17)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

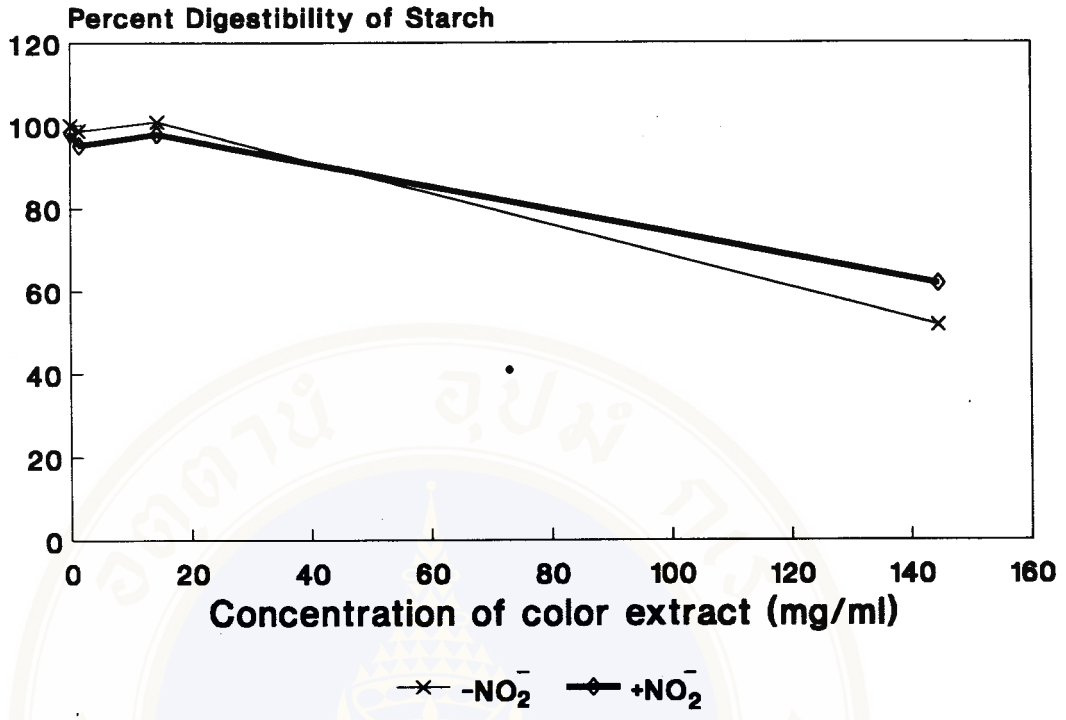
Figure 23 Effects of *Clitorea ternatea* Linn. (อัญชัน) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg of wet weight/ml)			
nitrite (mg/ml)	0.00	1.44	14.46	144.60
0.0 ($-NO_2^-$)	100.00	85.37	57.84	48.34
	(±0)	(±0.07)	(±0.14)	(±0.07)
0.48 ($+NO_2^-$)	83.72	72.12	54.21	32.11
	(±0.06)	(±0.14)	(±0.17)	(±0.21)

Statistical explanation (Appendix 18)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

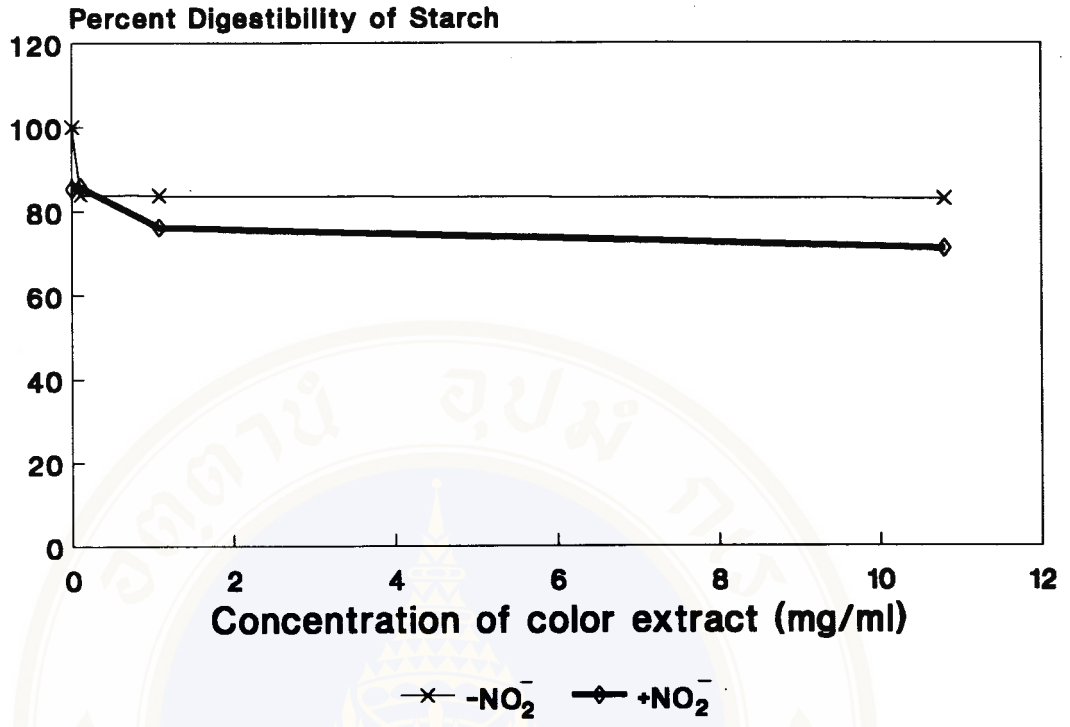
Figure 24 Effects of *Pandanus amaryllifolius* Roxb. (เตย) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg/ml)			
nitrite (mg/ml)	0.00	1.45	14.45	144.50
0.0 ($-NO_2^-$)	100.00	98.69	100.79	51.77
	(±0)	(±0.26)	(±0.13)	(±0.66)
0.48 ($+NO_2^-$)	86.37	95.15	97.84	61.86
	(±0.26)	(±0.52)	(±0.20)	(±0.26)

Statistical explanation (Appendix 19)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

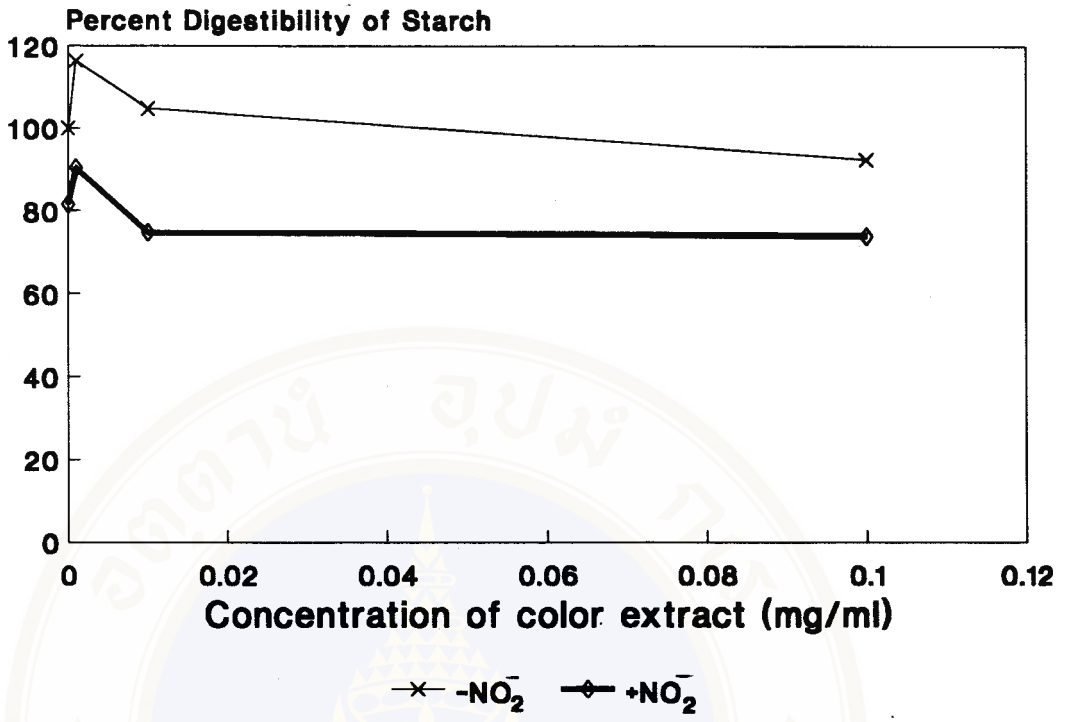
Figure 25 Effects of Caramelized coconut sugar (น้ำตาลไหม้) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg/ml)			
nitrite (mg/ml)	0.00	0.11	1.08	10.80
0.0 ($-NO_2^-$)	100.00	84.03	83.82	83.06
	($\pm$0)	($\pm$0.28)	($\pm$0.21)	($\pm$0.14)
0.48 ($+NO_2^-$)	85.49	85.90	76.25	76.25
	($\pm$0.07)	($\pm$0.21)	($\pm$0.14)	($\pm$0.14)

Statistical explanation (Appendix 20)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

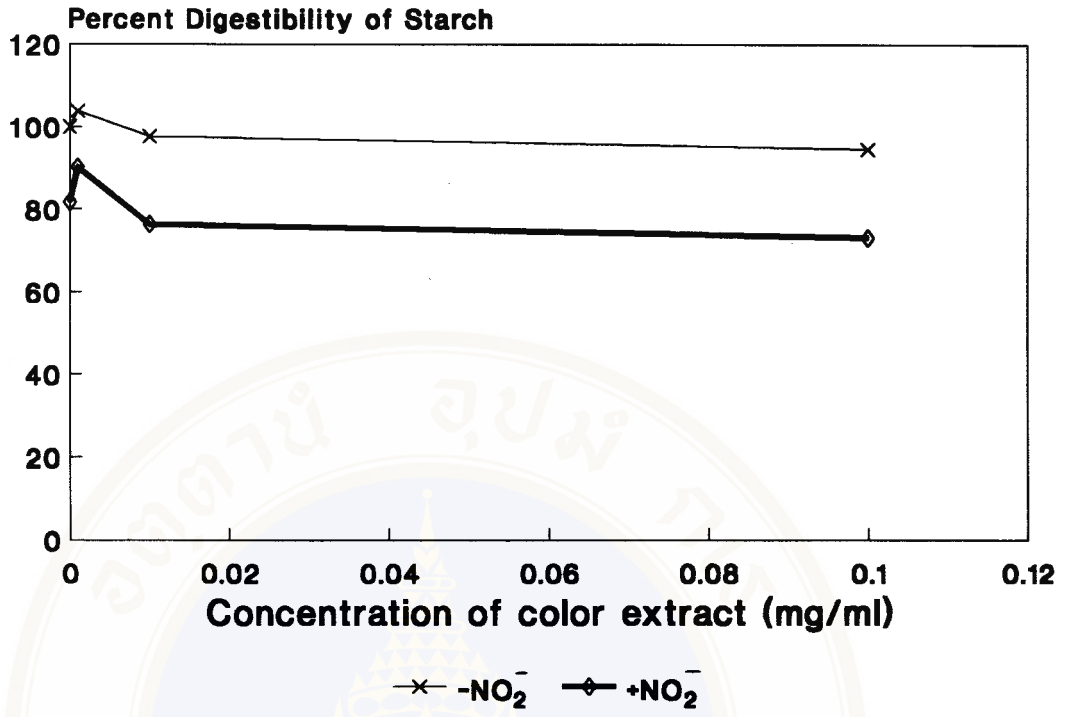
Figure 26 Effects of Carbon black from coconut skin (ถ่าน) with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg/ml)			
nitrite (mg/ml)	0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00	116.33	104.76	92.52
	(±0)	(±0.68)	(±2.72)	(±1.36)
0.48 ($+NO_2^-$)	81.63	90.48	74.83	74.15
	(±1.36)	(±2.04)	(±1.36)	(±3.40)

Statistical explanation (Appendix 21)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

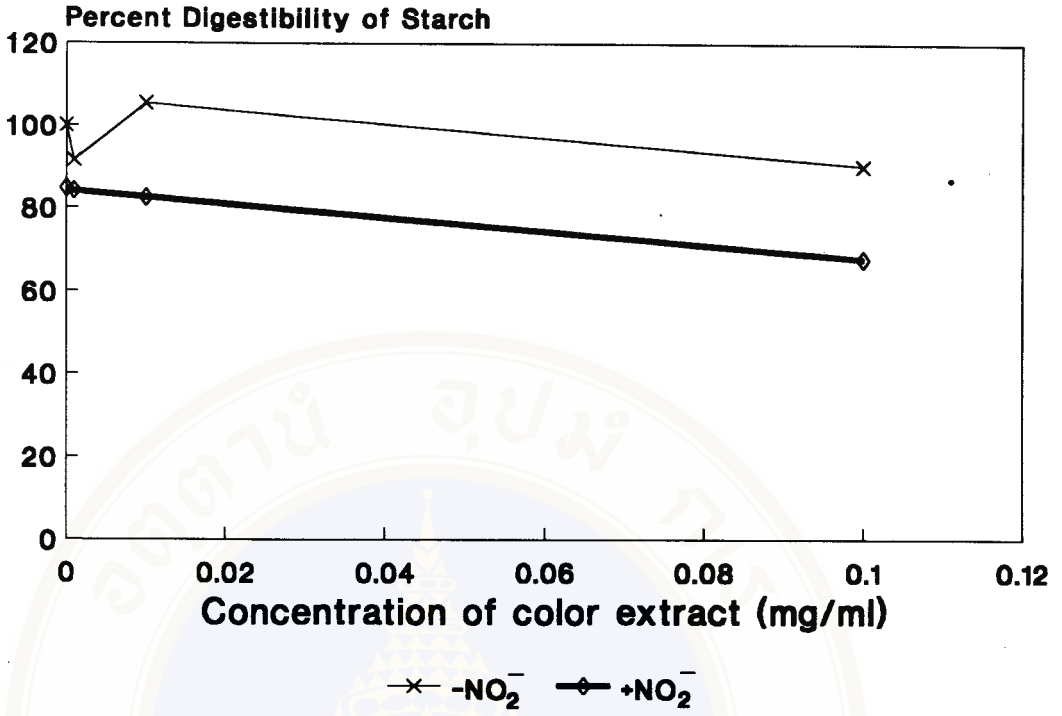
Figure 27 Effects of Sunset Yellow FCF with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00 (±0)	103.89 (±1.04)	97.73 (±0.32)	94.70 (±0.26)
0.48 ($+NO_2^-$)	81.83 (±0.33)	90.30 (±0.13)	76.46 (±0.13)	73.29 (±0.07)

Statistical explanation (Appendix 22)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

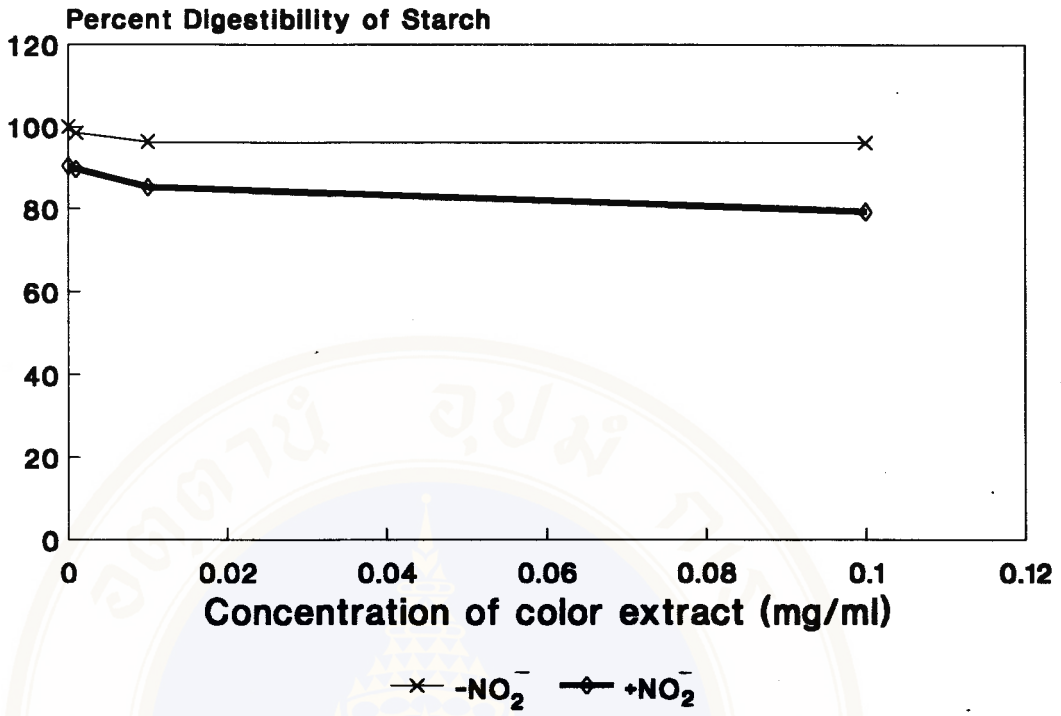
Figure 28 Effects of Tartrazine with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00 (±0)	91.60 (±0.22)	105.37 (±0.14)	90.26 (±0.488)
0.48 ($+NO_2^-$)	84.75 (±0.15)	84.11 (±0.07)	82.49 (±0.43)	67.94 (±2.83)

Statistical explanation (Appendix 23)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

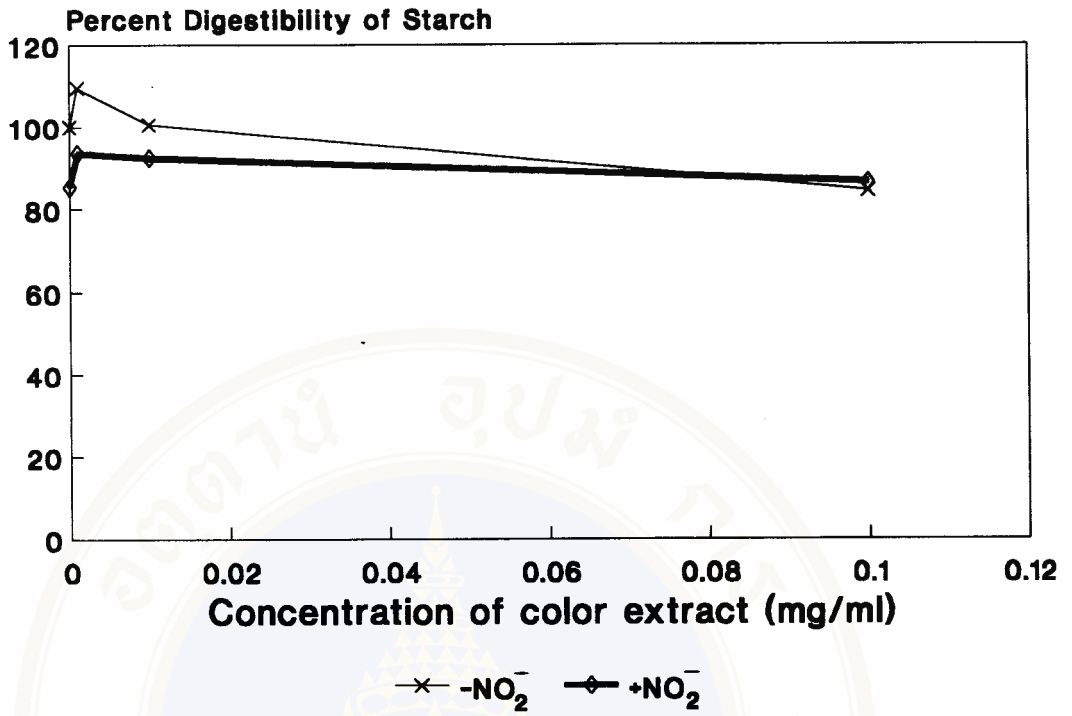
Figure 29 Effects of Erythrosine with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.001	0.01	0.10
0.0 (-NO_2^-)	100.00 (±0)	98.53 (±0.27)	96.38 (±0.13)	96.12 (±0.14)
0.48 (+NO_2^-)	90.42 (±0.34)	89.75 (±0.07)	85.32 (±0.20)	79.36 (±0.14)

Statistical explanation (Appendix 24)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

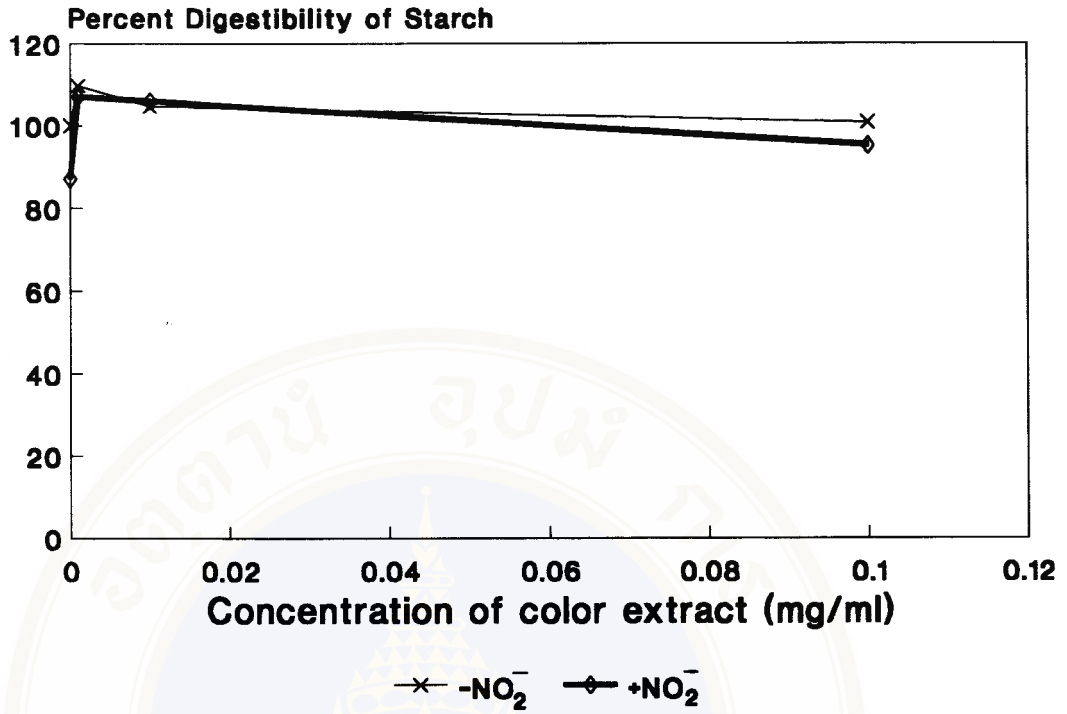
Figure 30 Effects of Ponceau 4 R with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00 (±0)	109.54 (±0.07)	100.54 (±0.14)	84.80 (±0.93)
0.48 ($+NO_2^-$)	85.47 (±0.14)	93.60 (±0.13)	92.47 (±0.20)	86.00 (±0.27)

Statistical explanation (Appendix 25)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

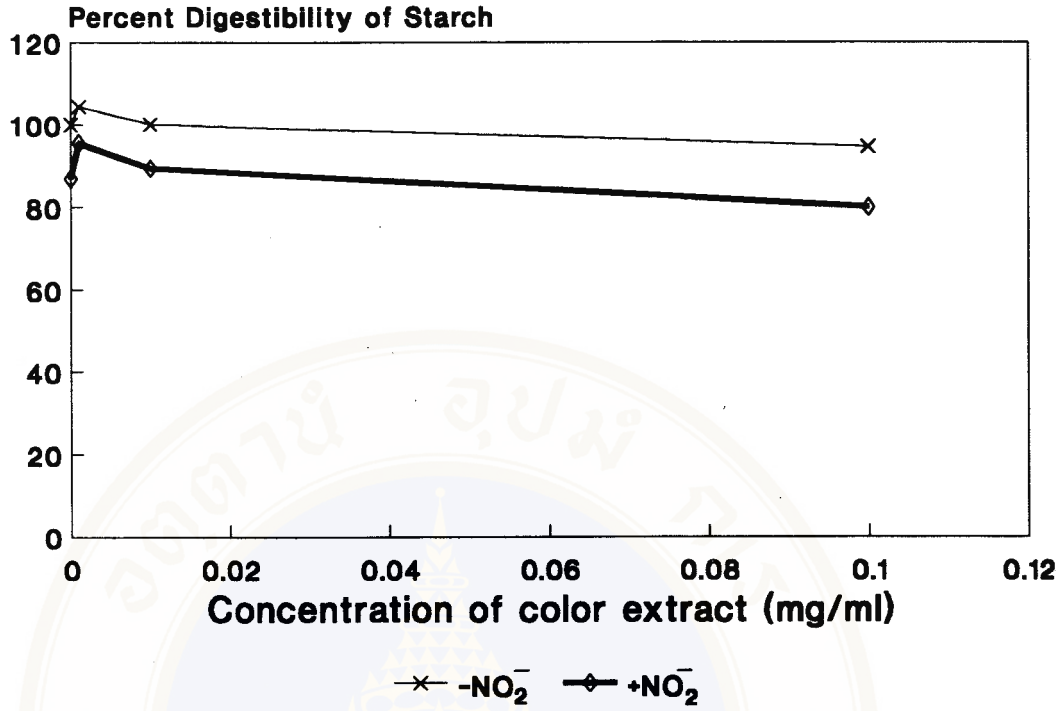
Figure 31 Effects of Indigo carmine with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of nitrite (mg/ml)	Concentration of color extract (mg/ml) 0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00 (±0)	109.71 (±0.07)	104.75 (±0.20)	100.81 (±0.14)
0.48 ($+NO_2^-$)	87.15 (±0.14)	107.50 (±0.40)	105.96 (±0.20)	95.25 (±0.20)

Statistical explanation (Appendix 26)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

Figure 32 Effects of Brilliant Blue FCF with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)



Concentration of	Concentration of color extract (mg/ml)			
nitrite (mg/ml)	0.00	0.001	0.01	0.10
0.0 ($-NO_2^-$)	100.00	91.92	88.52	75.61
	(±0)	(±0.15)	(±0.43)	(±0.29)
0.48 ($+NO_2^-$)	85.61	80.78	81.42	80.51
	(±0.22)	(±0.21)	(±0.29)	(±3.18)

Statistical explanation (Appendix 27)	Effect	No effect
Effect of color on BSA digestibility	+	
Effect of nitrite on BSA digestibility	+	
Interaction of color and nitrite	+	

Figure 33 Effects of Indigo carmine with tartrazine 1:1 with and without nitrite on *in vitro* digestibility of starch (Results expressed as percent of control with neither color nor nitrite.)

Mutagenic Potential of Food Colors and Their Products after Nitrite Treatment

The mutagenicity of each sample was presented as number of histidine (His^+) revertants per plate. Table 5 presents the mutagenic activity of water extracts of six natural food colors after reacted with nitrite (500 mM) at pH 3.0-3.4. All nitrite treated natural food colors contained direct mutagens represent as revertants of *Salmonella typhimurium* TA 98 and TA100. It was suggested that the nitrosated products were mixture of direct mutagens classified as frameshift mutation and base-pair substitution inducers.

Mutagenicity of the extract of any plant after nitrite treatment seems not to be surprised. Water extractions of Thai medicinal plants, namely *Andrographis paniculata* nees, *Carthamus tinctorius* Linn., *Cassia alata* Linn., *Cassia angustifolia* Vahl, *Cassia fistula* Linn., *Centella asiatica* (Linn.) Urban, *Curcuma domestica* Val., *Curcuma zedoaria* Rosc., *Cyperus rotumdus* Linn., *Oroxylum indicum* Vent. and *Zingiber officinale* Roscoe. were mutagenic after interaction with nitrite (Kangsadalampai and Ieamworapong, unpublished observation). Namiki *et al.* (125) tested the mutagenicity of pepper, chili perper and laurel after treatment with nitrite. The extract of Chinese cabbage was also found mutagenic on TA 100 after interacted with nitrite (126).

Mutagens occurred from the interaction of natural compounds and nitrite may not belong to only mutagenic nitrosamides. Tiedink *et al.* (127) and Butyee (unpublished data, 1993) found that there was no correlation between the number of induced revertants on *S. typhimurium* TA 100 and the amount of nitroso compounds formed. Nitro compounds which is the other class of nitrogen containing mutagenic compounds may be involved and will be discussed later.

Table 6 shows the mutagenic potential of seven synthetic organic colors after reacted with nitrite (500 mM) pH 3.0-3.4. The nitrite treated erythrosine and sunset yellow FCF demonstrated their mutagenicity towards *S.typhimurium* strain TA 98. It was implied that the products of these food colors after treatment with nitrite were mainly direct-acting mutagens which could induce frame shift mutation.

Only ponceau 4R demonstrated the stronger mutagenic than other to both strain TA 98 and TA 100 after nitrite treatment. It was determined that the direct mutagenic compounds that could induce both frameshift mutation and base-pair substitution were formed during nitrite treatment under acid condition.

The products of synthetic food colors after nitrite treatment, namely brillant blue FCF, tartrazine, indigo carmine and tartrazine with indigo carmine were not mutagenic towards both *S. typhimurium* strains TA 98 and TA 100. The nitrite treatment products of tartrazine with indigo carmine showed its partial killing effect towards both strains and the nitrosated products of indigo carmine exhibited partial killing effect on tester strain TA 98.

It is perhaps significant that the two azo compouds (Ponceau 4 R and sunset Yellow FCF) of this study exhibited direct mutagenicity for *Salmonella typhimurium* only after nitrite treatment. It may explain by two hypothesis : (1) the colors could be nitrosated to be mutagenic nitrosamides or (2) some nitro aromatic compounds was occurred.

The first hypothesis seems to be unwarrant since the double bond of the azo bond of the compounds is rather stable which should not be cleavaged by normal gastric condition. Therefore, the second hypothesis might help to explain the mutagenicity of the products after incubation the colors with nitrite

In general, nitro aromatics exhibit potent mutagenicity for microbial test systems and many examples have been reported. Perhaps most notable are the hair

dye components studied by Ames *et al.*, (128) including nitrophenylene diamines and nitrophenols. Other mutagenic nitro aromatics include trinitrotoluene (129), nitroimidazoles (130), p-nitrobenzoic acid, 4-chloro-3-nitrobenzophenone (1), nitrofurans (131), p-nitrobiphenyl and 2-nitronaphthalene (65) and nitroanthraquinones (132). It seems fairly certain that the potency of these aromatic nitro compounds is due to their activation by nitro reductase and probably other enzymes (133) of the bacterial tester strains.

Erythrosine, the only xanthene dye tested in this study, was also found to be weak mutagenic in *Salmonella* after nitrosation. It is suggested that the addition of nitro portion during the treatment eased the dyes to be suitable substrate for nitroreductase of the tester strains. The products of the nitrite treatment erythrosine may have similar in structure to the dye phloxin which was found mutagenic in *Escherichia coli* (45). However, erythrosine (2', 4', 5', 7' tetraiodo-9-(2-carboxyphenyl)-6-hydroxy-3-isoxanthenone) itself lacked mutagenicity in the *Salmonella* system (19).

It was not too surprising that some xanthene dyes exhibited mutagenicity (1). As might be expected of such planar molecules, they primarily affect frameshift mutations, although there was considerable variation in both the specificity of tester strains for these agents and the potency of reversion elicited.

Brilliant Blue which is the triphenylmethane dye of this study did not converted to be mutagenic after nitrite treatment. Thus, it confirmed the safeness of the dye as such. Brown *et al.* (1) tested twelve other triphenylmethane dyes without nitrite treatment and these are also nonmutagenic for the *Salmonella*/microsome test.

The results of this study indicated that some currently approved food color additives which are proved to be nonmutagenic in the Salmonella/microsome test could turn to be mutagenic after nitrite treatment. Variety of treatment conditions other than nitrite treatment may yield specific mutagenic responses with other dyes.

The evaluation of other test conditions such as treatment with sulfite or its related compounds with the *Salmonella*/microsome test as well as the use of other mutagenicity tests based on other microorganisms such as *Saccharomyces cerevisiae* and *Escherichia coli* or more complicate organism such as *Drosophilla melanogaster* should be conducted.

Table 5. Mutagenicity of crude water extract of natural food colors after nitrosation in dilute HCl solution on *S.typhimurium* TA 98 and TA 100

Name	Amount (μg)	No.of His^+-Revertants/plates	
		TA 98	TA100
Clitorea ternatea Linn. (อัญชัน)	0	27±3	113±2
	2,892	41±3	209±12
	5,785	43±4	**285±11**
	11,570	**66±15**	**481±26**
Hibiscus sabdariffs Linn. (กระเจี๊ยบ)	0	26±4	120±9
	1,467	37±4	164±19
	2,934	47±9	**290±28**
	5,869	**84±13**	**470±4**
Pandanus amaryllifolius Roxb. (เตย)	0	26±4	102±2
	8,357	38±2	162±2
	16,714	**71±1**	**241±8**
	33,428	**82±10**	**318±45**
Caramelized coconut sugar (น้ำตาลไหม้)	0	26±4	102±2
	1,136	27±5	108±5
	2,934	39±6	110±9
	4,544	44±5	166±10
	9,088	**60±7**	**210±8**

Table 5. Mutagenicity of crude water extract of natural food colors after nitrosation in dilute HCl solution on *S.typhimurium* TA 98 and TA 100 (cont.)

Name	Amount (µg)	No.of His^+-Revertants/plates TA 98	TA 100
Carthamus tinctorius Linn. (คำฝอย)	0	26±4	102±2
	1,467	**93±4**	**226±15**
	2,934	**136±25**	**548±8**
	4,401	**183±16**	ND
	5,868	PK	**745±16**
Carbon black from coconut skin	0	16±6	121±1
	200	23±6	128±2
	400	27±3	132±5
	800	**40±4**	165±11
Positive Control: Aminopyrene	0	20±5	110±4
	0.038	299±152	ND
	0.617	534±248	205±40
	0.4834	ND	374±114

Results are means of 2 separate experiments

PK = Partial killing effect

ND = Not determined

Table 6. Mutagenicity of crude water extract of synthetic food colors after nitrosation in dilute HCl solution on *S.typhimurium* TA 98 and TA 100

Name	Amount (µg)	No.of His^+-Revertants/plates	
		TA 98	TA 100
Ponceau 4 R	0	17±2	109±2
	200	**154±12**	**295±25**
	400	**349±11**	**645±9**
	600	**525±56**	ND
	800	PK	**1638+315**
Erythrosine	0	17±2	107±2
	200	32±6	118±21
	400	**52±3**	144±5
	600	PK	154±45
	800	PK	PK
Brilliant Blue FCF	0	17±2	109±17
	200	20±5	110±4
	400	23±2	116±8
	800	19±4	124±18
Indigo Carmine	0	17±2	109±2
	200	29±5	91±11
	400	23±2	112±1
	800	PK	99±11

Table 6. Mutagenicity of crude water extract of synthetic food colors after nitrosation in dilute HCl solution on *S.typhimurium* TA 98 and TA 100 (cont.)

Name	Amount (µg)	No.of His^+-Revertants/plates	
		TA 98	TA 100
Sunset Yellow FCF	0	17±2	109±2
	200	14±5	94±1
	400	17±1	100±4
	800	25±1	108±5
	1,600	**41±1**	181±12
Tartrazine	0	17±2	109±2
	200	21±4	107±9
	400	16±5	104±11
	800	25±5	104±5
Tartrazine with Indigo Carmine 1:1	0	19±2	107±2
	80	33±2	ND
	200	28±7	109±9
	400	PK	133±3
	600	ND	126±5
	800	PK	PK

Table 6. Mutagenicity of crude water extract of synthetic food colors after nitrosation in dilute HCl solution on *S.typhimurium* TA 98 and TA 100 (cont.)

Name	Amount (μg)	No.of His^+-Revertants/plates TA 98	TA 100
Positive Control: Aminopyrene	0	20±5	110±4
	0.038	299±152	ND
	0.617	534±248	205±40
	0.4834	ND	374±114

Results are means of 2 separate experiments

PK = Partial killing effect

ND = Not determined

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

General discussion

The effects of natural food colors and synthetic food colors on the digestibility of BSA and starch with and without nitrite were classified by analysis of variance. The result showed that almost all of them were presented to inhibit digestion of BSA and starch. Only indigo carmine have not affected on BSA digestibility.

Natural food colors were prepared from plant. Tannins-like compounds and other phenolic compounds may be presented as in part of the extracts of plants. They might be form complex with BSA and starch (113). It was possible that the effects of food colors on digestibility of BSA and starch were either enhanced or decreased the activity. It depended on types and amount of tannins-like compounds or phenolic compounds.

Synthetic food colors were organic compounds. They may formed complexes between an enzyme, metal and substrate. It may be decrease BSA and starch digestibilities. Unfortunately, no mechanism was known.

The results of natural food colors and synthetic food colors treated with nitrite on the BSA and starch digestibility explained another possible harmful effects of nitroso compound. Limitation of the available of substrate via nitroso compounds formaton was the possible explanation. It was shown by Knowles *et al.* (64) that nitrosation of BSA under gastric condition presentd the decreasing of tyrosine to form nitroso compounds and its derivatives i.e. 3-nitrosotyrosine, 3-4

dihydroxyphenylalanine. Hualmukda (118) reported that the amount nitrite 1.2 mg/tube did not significantly influence on digestibility. Thus, nitrite at the no effect level has potentiated the inhibitory effect on digestibility in this study. It was possible that not only the properties of food colors components can caused this effect but also their nitrosated products may involved. Nitrosamine in this reaction directly decreased amount of substrate, subsequently the decreasing of digestibility was occurred. It was difficult to predict the mechanism. The follow up study in detail was still necessary to elucidate the phenomenon.

The study on mutagenic activity after nitrosation of natural food colors and synthetic food colors showed that almost all of them were mutagenic to *Salmonella typhimurium* strains TA 98 and TA 100. Only brillant blue FCF, tartrazine, indigo carmine and tartrazine with indigo carmine were not mutagenic. The nitrosated products were direct mutagenic to *Salmonella typhimurium* strains TA 98 and TA 100. It was suggested only N-nitrosamides possed direct mutagenic activity while N-nitrosamine require metabolic activation to become mutagenic (124). Hence, the mutagenic compounds may likely to be N-nitrosamide rather than N-nitrosamine.

In vivo nitrosation seems to be common since nitrite is foundable in food either as intentional food additive or natural component of food. Nitrite in the stomach is also derived from ingested nitrate, which is reduced to nitrite by bacteria present in the oral cavity (60). Many food colors were taken before meal, food colors and nitrite interaction which in turn to the formation of some mutagen. It must be kept in mind that the result obtained from *in vitro* model cannot be solely extrapolated to human. Biological factors such as gastric emptying and adsorbtion of reactants cannot be accounted for simulated gastric model. Under gastric conditions, transiently higher localized gastric nitrite concentrations than used in the present model system may occur prior to dilution and decay of nitrite through

disproportionation and reaction with other gastric constituents (134). The actual risk of nitrosation of these food colors *in vivo* remains to be further elucidated.

Conclusion

The effects of natural food colors and synthetic food colors on the digestibility of BSA and starch with and without nitrite were classified by analysis of variance. The result showed that almost all of them were presented to inhibit digestion of BSA and starch. Only indigo carmine have not affected on BSA digestibility.

The inhibitory effects on BSA and starch digestibility were depend on the amount and types of inhibitors existed in each food colors as well as their nitrosated products. Hence, precaution on using food colors should be concerned to avoid the limit of protein and carbohydrate availability during digestion. Protein-rich meal will be helpful in counteracting the effects. In addition to, food color interacted with nitrite enhanced the inhibitory effect on protein and starch digestibility. Thus, consumption of nitrite containing processed food should be avoid in order to maintain the ability of protease enzyme as it would be.

The mutagenic activity of six natural food colors and seven synthetic food colors plus nitrite under acidic conditions in the present study implied that certain nitrosable compounds were present in the sample. The concentrations of food colors and nitrite used in the experiments might be the upper end at those expected to be present in the stomach during ingestion of food colors and diet. Extrapolation of *in vitro* studies to human should be done carefully. Whenever possible, extensive kinetic and metabolic studies in the intact mammalian organism should follow an *in vitro* observation. Such comparative data at hand can the results finally be interpreted and put into perspective.

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APPENDIX 1 MANIPULATION OF THE TESTING STRAINS

1. PREPARATION OF STOCK SOLUTION AND MEDIA

1.1 Vogel-Bonner medium E stock salt solution (VB Salt)

Ingredient	1 litter	2 litter
Distilled H_2O	670 ml.	1340 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	10 g	20 g
Citric acid monohydrate	100 g	200 g
Potassium phosphate dibasic (anhydrous)(K_2HPO_4)	500 g	1000 g
Sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$)	175 g	350g

Add salts in the order indicated to water and allowed each salt to dissolve completely before adding the next. Filter the solutions and then autoclave at 121° C for 20 min.

1.2 Minimal glucose agar plate

Ingredient	300 ml	350 ml
Basto agar	4.5 g	5.25 g
Distilled H_2O	280 ml	330 ml
VB salts	6 ml	7 ml
40% glucose	15 ml	17.5 ml

Add agar to distilled water in a glass bottle. Autoclave at 121° C for 20 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

1.3 Oxoid nutrient broth No. 2. Dissolve 2.5 g of nutrient broth No.2 in 100 ml distilled H_2O. Transfer 12 ml of nutrient broth for each L tube. Autoclaved at 121° C for 20 min.

1.4 Top agar

Ingredient	200 ml	300 ml
Bacto agar	1.2 g	1.8 g
Sodium chloride (NaCl)	1.0 g	1.5 g
Distilled H_2O	200 ml	300 ml

Dissolve ingredients in water. Store in a glass bottle. Autoclave for 20 min at 121° C and then add 20 ml and 30 ml of 0.5 mM histidine-0.5 mM biotin for 200 ml and 300 ml of Top agar respectively.

1.5 0.1 M L-histidine HCl stock

Ingredient	100 ml
L-histidine HCl	2.096 g
Distilled H_2O	100 ml

Dissolve 2.096 g of L-histidine HCl (MW 209.6) in 100 ml distilled water. Autoclave at 121° C for 20 min.

1.6 1 mM L-histidene HCl stock

Ingredient	100 ml
0.1 M L-histidine HCl	1 ml
Distilled H_2O	99 ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water. Autoclave at 121° C for 20 min.

1.7 1 mM biotin stock

Ingredient	100 ml
Biotin	24.43 mg
Distilled H_2O	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121° C for 20 min.

1.8 0.5 mM L-histidine HCl-0.5 mM biotin

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

Mix and autoclave at 121° C for 20 min.

1.9 NaH_2PO_4 -KCl buffer

Ingredient	330 ml
0.5 M $NaPO_4$ pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled H_2O	213.5 ml

Mix and autoclave at 121° C for 20 min.

1.10 0.15 M KCl

Ingredient	1,000 ml
Potassium chloride	11.5 g
Distilled H_2O	1,000 ml

Mix and autoclave at 121° C for 20 min.

1.11 0.16 M $MgCl_2$

Ingredient	100 ml
$MgCl_2$.$6H_2O$ (MW 203.31)	3.253 g
Distilled H_2O	100 ml

Mix and autoclave at 121° C for 20 min.

2. PROCEDURE FOR REISOLATION AND GROWING CULTURE

Tester strains, TA 98 and TA 100 are grown in Oxoid nutrient broth No.2 and incubated overnight in a 37° C shaking water bath. The growth period should not exceed 16 hr. These cultures are resolution by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 1 mM biotin. These plates are incubated at 37° C for 48 hr. After incubation, the 5 single colonies per strain TA 98 and TA 100 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 0.99 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 400 ul of the culture aseptically into sterile cryotubes (Nunc). The tubes should be filled nearly full and then transfer to 80° C freezer.

3. CONFIRMING GENOTYPE OF TESTER STRAINS

The broth cultures of TA 98 and TA 100 are used to confirm genotypes in the following ways.

3.1 HISTIDINE REQUIREMENT

The histidine 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 is required for each tester strains. Each of the plates is applied on the surface with 0.1 ml of 1 mM biotin,0.3 ml of 0.1 M His-HCl,0.3 ml of 0.1 M His-HCl plus 0.1 ml of 1 mM biotin and no application (plate b,c,d,a respectively). Made a single streak of each strains across these plates. Five strains could be tested on the same plate. Incubated at 37° C for 48 hr. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

3.2 R FACTOR

The R-factor strains (TA 97, TA 98, TA 100 and TA 102) should be tested routinely for the presence of the ampicillin 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 2.0 mole molten top agar containing 0.5 mM histidine and 0.5 mM biotin. 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 mix 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 hours. The absence of the clear zones of inhibition around the discs indicate resistance to ampicillin.

3.3 rfa MUTATION

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

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

4. 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 : 0.1 ml of DMSO (solvent in the experiment) is added to capped culture tube. Add 0.5 ml of NaH_2PO_4-KCl buffer pH 7.4, 0.1 ml of fresh overnight culture of TA 98 or TA 100, followed by 2.0 ml of molten top agar. Mixed and then poured on minimal glucose agar plate. Rotated plates and left it to become harden. Incubated at 37° C for 48 hours and the histidine revertant colonies were counted.

5. 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 are nitrosoaminopyrene. Tester strain which highly response to positive mutagens must be collected.

Procedure : The procedure is as described in spontaneous reversion. Nitrosoaminopyrene are used instead of water in the experiment. The characteristic of the stock culture for TA 98 and TA 100 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 carcinegen.

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

Reference

Maron D.M.,Ames B.N. <1983>. Revised methods for the Salmonella mutagenicity test. Mut Res 113:173-215.

Appendix 2 Analysis of variance of *Hibicus sabdariffa* Linn. (กระเจี๊ยบ) on BSA digestiblity

Source of Variation	Sum of Squares	DF	MeanSquare	F	Signif of F
Main Effects	1128.522	4	282.130	26.950	.000
COLOR	549.638	3	183.213	17.501	.001
NITRITE	578.884	1	578.884	55.297	.000
2-way Interactions	405.624	3	135.208	12.916	.002
COLOR NITRITE	405.624	3	135.208	12.916	.002
Explained	1534.146	7	219.164	20.935	.000
Residual	83.749	8	10.469		
Total	1617.895	15	107.860		

Appendix 3 Analysis of variance of *Carthamus tinctorius* Linn. (คำฝอย) on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	096.804	4	274.201	234.242	.000
COLOR	811.869	3	270.623	231.186	.000
NITRITE	284.934	1	284.934	243.411	.000
2-way Interactions	22.199	3	7.400	6.321	.017
COLOR NITRITE	22.199	3	7.400	6.321	.017
Explained	1119.003	7	159.858	136.562	.000
Residual	9.365	8	1.171		
Total	1128.367	15	75.224		

Appendix 4 Analysis of variance of *Clitorea ternatea* Linn. (อัญชัน) on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	2237.370	4	559.343	364.477	.000
COLOR	895.797	3	298.599	194.572	.000
NITRITE	1341.574	1	1341.574	874.192	.000
2-way Interactions	783.267	3	261.089	170.130	.000
COLOR NITRITE	783.267	3	261.089	170.130	.000
Explained	3020.638	7	431.520	281.186	.000
Residual	12.277	8	1.535		
Total	3032.915	15	202.194		

Appendix 5 Analysis of variance of *Pandanus amaryllifolius* Roxb. (เตย) on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	3979.395	4	994.849	122.837	.000
COLOR	859.614	3	286.538	35.380	.000
NITRITE	3119.781	1	3119.781	385.209	.000
2-way Interactions	879.017	3	293.006	36.178	.000
COLOR NITRITE	879.017	3	293.006	36.178	.000
Explained	4858.412	7	694.059	85.698	.000
Residual	64.791	8	8.099		
Total	4923.203	15	328.214		

Appendix 6 Analysis of variance of Caramelized coconut sugar (น้ำตาลไหม้) on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	1297.431	4	324.358	107.262	.000
COLOR	390.217	3	130.072	43.014	.000
NITRITE	907.214	1	907.214	300.008	.000
2-way Interactions	233.646	3	77.882	25.755	.000
COLOR NITRITE	233.646	3	77.882	25.755	.000
Explained	1531.077	7	218.725	72.331	.000
Residual	24.192	8	3.024		
Total	1555.268	15	103.685		

Appendix 7 Analysis of variance of Carbon black from coconut skin (ถ่านกาบมะพร้าว) on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	461.440	4	115.360	40.232	.000
COLOR	220.026	3	73.342	25.578	.000
NITRITE	241.414	1	241.414	84.194	.000
2-way Interactions	36.837	3	12.279	4.282	.044
COLOR NITRITE	36.837	3	12.279	4.282	.044
Explained	498.276	7	71.182	24.825	.000
Residual	22.939	8	2.867		
Total	521.215	15	34.748		

Appendix 8 Analysis of variance of Sunset Yellow FCF on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	236.862	4	59.215	10.907	.003
COLOR	77.282	3	25.761	4.745	.035
NITRITE	159.580	1	159.580	29.393	.001
2-way Interactions	22.867	3	7.622	1.404	.311
COLOR NITRITE	22.867	3	7.622	1.404	.311
Explained	259.728	7	37.104	6.834	.007
Residual	43.434	8	5.429		
Total	303.162	15	20.211		

Appendix 9 Analysis of variance of Tartrazine on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	886.914	4	221.728	64.150	.000
COLOR	127.636	3	42.545	12.309	.002
NITRITE	759.278	1	759.278	219.673	.000
2-way Interactions	148.395	3	49.465	14.311	.001
COLOR NITRITE	148.395	3	49.465	14.311	.001
Explained	1035.308	7	147.901	42.791	.000
Residual	27.651	8	3.456		
Total	1062.960	15	70.864		

Appendix 10 Analysis of variance of Erythrosin on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	2123.387	4	530.847	104.342	.000
COLOR	510.361	3	170.120	33.438	.000
NITRITE	1613.026	1	1613.026	317.053	.000
2-way Interactions	396.769	3	132.256	25.996	.000
COLOR NITRITE	396.769	3	132.256	25.996	.000
Explained	2520.156	7	360.022	70.765	.000
Residual	40.701	8	5.088		
Total	2560.857	15	170.724		

Appendix 11 Analysis of variance of Ponceau 4 R on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	714.275	4	178.569	30.030	.000
COLOR	304.718	3	101.573	17.081	.001
NITRITE	409.556	1	409.556	68.875	.000
2-way Interactions	84.647	3	28.216	4.745	.035
COLOR NITRITE	84.647	3	28.216	4.745	.035
Explained	798.921	7	114.132	19.193	.000
Residual	47.571	8	5.946		
Total	846.492	15	56.433		

Appendix 12 Analysis of variance of Indigo Carmine on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	132.739	4	33.185	3.327	.069
COLOR	52.591	3	17.530	1.758	.233
NITRITE	80.147	1	80.147	8.036	.022
2-way Interactions	23.093	3	7.698	.772	.542
COLOR NITRITE	23.093	3	7.698	.772	.542
Explained	155.832	7	22.262	2.232	.142
Residual	79.788	8	9.974		
Total	235.620	15	15.708		

Appendix 13 Analysis of variance of Brilliant Blue FCF on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	427.453	4	106.863	6.787	.011
COLOR	248.629	3	82.876	5.263	.027
NITRITE	178.824	1	178.824	11.357	.010
2-way Interactions	163.774	3	54.591	3.467	.071
COLOR NITRITE	163.774	3	54.591	3.467	.071
Explained	591.227	7	84.461	5.364	.015
Residual	125.969	8	15.746		
Total	717.196	15	47.813		

Appendix 14 Analysis of variance of Indigo Carmine with Tartrazine 1:1 on BSA digestibility

Source of Variation	Sum of Squares	DF	Mean Square	F	Signif of F
Main Effects	330.191	4	82.548	19.316	.000
COLOR	113.145	3	37.715	8.825	.006
NITRITE	217.047	1	217.047	50.788	.000
2-way Interactions	39.426	3	13.142	3.075	.091
COLOR NITRITE	39.426	3	13.142	3.075	.091
Explained	369.617	7	52.802	12.356	.001
Residual	34.188	8	4.274		
Total	403.805	15	26.920		

Appendix 15 Analysis of variance of *Hibiscus sabdariffa* Linn. (กระเจี๊ยบ) on starch digestibility

Sourcd of Variation	Sum of Squares	DF	Mean Squre	F	Signif of F
Main Effects	10850.915	4	2712.729	5686.988	.000
COLOR	10790.270	3	3596.757	7540.271	.000
NITRITE	60.645	1	60.645	127.137	.000
2-way Interactions	32.329	3	10.776	22.591	.000
COLOR NITRITE	32.329	3	10.776	22.591	.000
Explained	10883.243	7	1554.749	3259.389	.000
Residual	3.816	8	.477		
Total	10887.060	15	725.804		

Appendix 16 Analysis of variance of *Carthamus tinctorius* Linn. (คำฝอย) on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	4722.327	4	1180.582	539.567	.000
COLOR	3743.106	3	1247.702	570.243	.000
NITRITE	979.221	1	979.221	447.538	.000
2-way Interactions	3206.149	3	1068.716	488.440	.000
COLOR NITRITE	3206.149	3	1068.716	488.440	.000
Explained	7928.476	7	1132.639	517.655	.000
Residual	17.504	8	2.188		
Total	7945.980	15	529.732		

Appendix 17 Analysis of variance of *Clitorea ternatea* Linn. (อัญชัน) on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	631.026	4	157.757	59.718	.000
COLOR	438.857	3	146.286	55.376	.000
NITRITE	192.169	1	192.169	72.744	.000
2-way Interactions	213.504	3	71.168	26.940	.000
COLOR NITRITE	214.504	3	71.168	26.940	.000
Explained	844.531	7	120.647	45.670	.000
Residual	21.134	8	2.642		
Total	865.664	15	57.711		

Appendix 18 Analysis of variance of *Pandanus amaryllifolius* Roxb. (เตย) on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	6982.250	4	1745.562	39072.468	.000
COLOR	6372.407	3	2124.136	47546.405	.000
NITRITE	609.843	1	609.843	13650.655	.000
2-way Interactions	107.348	3	35.783	800.957	.000
COLOR NITRITE	107.348	3	35.783	800.957	.000
Explained	7089.598	7	1012.800	22670.391	.000
Residual	.357	8	.045		
Total	7089.956	15	472.664		

Appendix 19 Analysis of variance of Caramelized coconut sugar (น้ำตาลไหม้) on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	4820.191	4	1205.048	5035.983	.000
COLOR	4795.041	3	1598.347	6679.609	.000
NITRITE	25.150	1	25.150	105.105	.000
2-way Interactions	283.799	3	94.600	395.339	.000
COLOR NITRITE	283.799	3	94.600	395.339	.000
Explained	5103.990	7	729.141	3047.136	.000
Residual	1.914	8	.239		
Total	5105.905	15	340.394		

Appendix 20 Analysis of variance of Carbon black from coconut skin (ถ่าน) on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	815.087	4	203.772	3560.500	.000
COLOR	558.847	3	186.282	3254.908	.000
NITRITE	256.240	1	256.240	4477.275	.000
2-way Interactions	154.460	3	51.487	899.624	.000
COLOR NITRITE	154.460	3	51.487	899.624	.000
Explained	969.547	7	138.507	2420.125	.000
Residual	.458	8	.057		
Total	970.005	15	64.667		

Appendix 21 Analysis of variance of Sunset Yellow FCF on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	2985.255	4	746.314	102.476	.000
COLOR	845.268	3	281.756	38.688	.000
NITRITE	2139.988	1	2139.988	293.841	.000
2-way Interactions	98.954	3	32.985	4.529	.039
COLOR NITRITE	98.954	3	32.985	4.529	.039
Explained	3084.209	7	440.601	60.499	.000
Residual	58.262	8	7.283		
Total	3142.471	15	209.498		

Appendix 22 Analysis of variance of Tartrazine on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	1767.521	4	441.880	1279.862	.000
COLOR	382.007	3	127.336	368.815	.000
NITRITE	1385.515	1	1385.515	4013.003	.000
2-way Interactions	40.382	3	13.461	38.988	.000
COLOR NITRITE	40.382	3	13.461	38.998	.000
Explained	1807.903	7	258.272	748.059	.000
Residual	2.762	8	.345		
Total	1810.665	15	120.711		

Appendix 23 Analysis of variance of Erythrosine on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	1686.874	4	421.718	170.760	.000
COLOR	532.743	3	177.581	71.905	.000
NITRITE	1154.131	1	1154.131	467.324	.000
2-way Interactions	156.515	3	52.172	21.125	.000
COLOR NITRITE	156.515	3	52.172	21.125	.000
Explained	1843.389	7	263.341	106.631	.000
Residual	19.757	8	2.470		
Total	1863.146	15	124.210		

Appendix 24 Analysis of variance of Ponceau 4 R on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	670.694	4	167.674	2395.123	.000
COLOR	137.431	3	45.810	654.374	.000
NITRITE	533.264	1	533.264	7617.371	.000
2-way Interactions	38.818	3	12.973	185.309	.000
COLOR NITRITE	38.918	3	12.973	185.309	.000
Explained	709.613	7	101.373	1488.060	.000
Residual	.560	8	.070		
Total	710.173	15	47.345		

Appendix 25 Analysis of variance of Indigo carmine on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	904.819	4	226.205	873.905	.000
COLOR	556.343	3	185.448	716.447	.000
NITRITE	348.476	1	348.476	1346.278	.000
2-way Interactions	183.199	3	61.066	235.920	.000
COLOR NITRITE	183.199	3	61.066	235.920	.000
Explained	1088.018	7	155.431	600.483	.000
Residual	2.071	8	.259		
Total	1090.089	15	72.673		

Appendix 26 Analysis of variance of Brilliant Blue FCF on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	654.783	4	163.696	2041.889	.000
COLOR	560.644	3	186.881	2331.101	.000
NITRITE	94.139	1	94.139	1174.254	.000
2-way Interactions	108.297	3	36.099	450.286	.000
COLOR NITRITE	108.297	3	36.099	450.286	.000
Explained	763.080	7	109.011	1359.774	.000
Residual	.641	8	.080		
Total	763.721	15	50.915		

Appendix 27 Analysis of variance of Indigo carmine with Tartrazine 1:1 on starch digestibility

Source of Variation	Sum of Squre	DF	Mean Squre	F	Signif of F
Main Effects	885.209	4	221.302	825.254	.000
COLOR	327.777	3	109.259	407.436	.000
NITRITE	557.432	1	557.432	2078.710	.000
2-way Interactions	18.701	3	6.234	23.246	.000
COLOR NITRITE	18.701	3	6.234	23.246	.000
Explained	903.910	7	129.130	481.537	.000
Residual	2.145	8	.268		
Total	906.056	15	60.404		