STUDY ON THE MAILLARD REACTION IN THAI SOY SAUCE

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It is well known that the Maillard reaction, or nonenzymatic browning reaction in foods and biological systems is a very complex reaction which stems from the interaction between amino compounds and reducing sugars. In soy sauce, the Maillard reaction is known as the main cause of the dark brown color and the characteristic flavor formation. Despite many studies, which have tried to explain the mechanism of the Maillard reaction in Japanese soy sauce, the results did not represent soy sauce manufactured in other regions due to the variation in ingredients and processing conditions.

The Maillard reaction occurring during processing and storage of two conventional Thai soy sauces were investigated for the chemical changes of browning, reducing sugar (RS), reactive amino group (RAG), 5% TCA-precipitated protein (PP), Amadori products (ARP), and 5-hydroxymethyl furaldehyde (HMF). The development of the Maillard reaction during moromi fermentation was a 2-stage consecutive mechanism. During the first stage (i.e., the first 3 days of fermentation), browning, RS, RAG, and ARP increased rapidly while pH decreased. In the second stage, these changes occurred slowly. During fermentation, the increase of HMF was the index of the progress of the Maillard reaction. After the moromi fermentation, raw soy sauce, pasteurized soy sauce (without seasoning), and cooked soy sauce (seasoned and pasteurized) were studied for the duration of the Maillard reaction during storage at 37°C for 3 months. The Maillard reaction in raw soy sauce continued progress in a similar pattern as in moromi fermentation, while HMF and PP highly accumulated in cooked soy sauce during storage. This suggested that seasoning and heat treatment of the soy sauce product enhanced the progress of the Maillard reaction.

The effects of brine concentration and aeration on the progress of the Maillard reaction were investigated during moromi fermentation (20 days). Koji was fermented in brine at 18, 20, 22, and 24% (w/v) salt. The Maillard reaction occurred in the same pattern as previously reported. Brine concentration did not affect on the progress of the Maillard reaction during fermentation, except the 24% brine gave higher amounts of PP. To investigate the involvement of oxidative browning during moromi fermentation, the moromi in 20% brine was aerated at 20 ml/min for 20 days and then compared with the non-aerated system. Although the aeration did not affect brown color development, it influenced the low RS, RAG, PP, total soluble protein (SP), and pH and caused the turbidity of the system. Thus, the oxidative browning was not apparently involved with the Maillard reaction to develop the brown characteristic of Thai soy sauce.

When compared with Japanese soy sauce during storage test, both of the Thai soy sauces had an excess of RS, while the Japanese soy sauce had an excess of RAG and the accumulation of HMF was the important index for the progress of the Maillard reaction. The increase of RS in Thai soy sauce during the storage test was confirmed by the sugar analysis by HPLC. Arabinose, xylose, glucose, and sucrose were present in all samples of soy sauce. The arabinose and xylose types of 5C sugar were involved in the progress of the Maillard reaction in Thai soy sauce and Japanese soy sauce, respectively.

The presence of metal ions (Fe^{2+}, Fe^{3+}, and Cu^{2+}) and potassium iodide (KI) in soy sauce at various concentrations did not directly affect the Maillard reaction during storage. However, Fe^{2+} caused a high accumulation of PP and a high loss of ARP while KI caused less PP. Hence, KI could be added as an iodine supplement in soy sauce without enhancing the Maillard reaction.

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ปฏิกิริยาแบบ Maillard หรือปฏิกิริยาการเกิดสีน้ำตาลแบบไม่ใช้เอนไซม์ในอาหารและสิ่งมีชีวิตเป็นปฏิกิริยาที่ซับซ้อน ซึ่งเกิดขึ้นจากปฏิกิริยาระหว่างสารประกอบอะมิโนและน้ำตาลรีดิวซ์ ปฏิกิริยา Maillard นี้เป็นสาเหตุสำคัญในการเกิดสีน้ำตาลเข้มและกลิ่นหอมของซีอิ๊ว แม้ว่าจะมีงานวิจัยหลายชิ้นพยายามอธิบายถึงกลไกการเกิดปฏิกิริยานี้ในซีอิ๊วของญี่ปุ่น ทว่าผลที่ได้อาจไม่สามารถนำมาใช้อธิบายถึงการเปลี่ยนแปลงของซีอิ๊วที่ผลิตในพื้นที่อื่นๆได้ เนื่องจากความแตกต่างของส่วนประกอบและสภาวะในการผลิต

ในการศึกษาถึงกลไกการเกิดปฏิกิริยา Maillard ในซีอิ๊วของไทยในระหว่างการผลิตและการเก็บรักษานั้นได้ศึกษาการเปลี่ยนแปลงของสีน้ำตาล, น้ำตาลรีดิวซ์, หมู่อะมิโน, โปรตีนที่ตกตะกอนด้วย 5% TCA, สารประกอบ Amadori และ 5-hydroxymethylfuraldehyde (HMF) จากการทดลองพบว่าปฏิกิริยาการเกิดสีน้ำตาลนั้นเริ่มต้นตั้งแต่กระบวนการหมักโมโรมิ ซึ่งการเกิดขึ้นนี้เป็นกลไกสองขั้นที่ต่อเนื่องกัน ขั้นแรกเกิดในช่วงสามวันแรกของการหมักโดยสีน้ำตาล น้ำตาลรีดิวซ์ หมู่อะมิโนและสารประกอบ Amadori เพิ่มขึ้นอย่างรวดเร็ว ในขณะที่ค่าพีเอชลดลง ส่วนขั้นที่สองมีการเปลี่ยนแปลงเกิดขึ้นอย่างช้าๆ โดยการเพิ่มขึ้นของ HMF ตลอดระยะเวลาของการหมัก เป็นตัวบ่งชี้ถึงการเกิดขึ้นของปฏิกิริยา Maillard หลังจากการหมักโมโรมิแล้วนำซีอิ๊วดิบ ซีอิ๊วพาสเจอร์ไรซ์ (ไม่มีการปรุงรส) และ ซีอิ๊ว (ผ่านการปรุงรสและพาสเจอร์ไรซ์) มาศึกษาถึงการดำเนินไปของปฏิกิริยาในระหว่างการเก็บรักษาที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลาสามเดือน ปฏิกิริยา Maillard ในซีอิ๊วดิบเกิดขึ้นต่อเนื่องมาจากขั้นการหมักโมโรมิในลักษณะเดียวกัน แต่ในซีอิ๊วที่ผ่านการปรุงรสและพาสเจอร์ไรซ์นั้น HMF และโปรตีนที่ตกตะกอนด้วย 5% TCA เพิ่มขึ้นอย่างรวดเร็วในซีอิ๊วตลอดระยะของการเก็บรักษา แสดงให้เห็นว่าการปรุงรสและการให้ความร้อนกับซีอิ๊วนั้น เป็นการเร่งปฏิกิริยา Maillard

ในการศึกษาผลของความเข้มข้นของน้ำเกลือและอากาศต่อการเกิดปฏิกิริยา Maillard ในระหว่างการหมักโมโรมิ (20 วัน) นั้น พบว่าปฏิกิริยาการเกิดสีน้ำตาลแบบ Maillard ในโคจิที่หมักด้วยน้ำเกลือเข้มข้น 18, 20, 22 และ 24% เกิดขึ้นลักษณะเดียวกับการศึกษาที่ผ่านมา โดยที่ความเข้มข้นของน้ำเกลือไม่มีผลต่อการเกิดขึ้นของปฏิกิริยาในระหว่างการหมัก ยกเว้นที่ความเข้มข้นของน้ำเกลือ 24% ซึ่งทำให้เกิดโปรตีนที่ตกตะกอนด้วย 5% TCA มากขึ้น ในระหว่างการหมักโมโรมิ พบว่าเมื่อเติมอากาศให้โมโรมิในน้ำเกลือเข้มข้น 20% ที่อัตราเร็ว 20 มิลลิลิตรต่อนาที เป็นเวลา 20 วันเปรียบเทียบกับโมโรมิที่ไม่เติมอากาศแล้วการเกิดสีน้ำตาลไม่แตกต่างกัน แต่มีผลให้น้ำตาลรีดิวซ์ หมู่อะมิโน โปรตีนที่ตกตะกอนด้วย 5% TCA และโปรตีนที่ละลายอยู่ในซีอิ๊วเกิดน้อยลง รวมทั้งมีค่าพีเอชลดลงและทำให้ระบบขุ่น ดังนั้นออกซิเจนไม่มีส่วนร่วมอย่างเด่นชัดกับปฏิกิริยา Maillard ในซีอิ๊ว

เมื่อเปรียบเทียบปฏิกิริยา Maillard ในซีอิ๊วไทยและซีอิ๊วญี่ปุ่นในระหว่างการเก็บรักษาแล้ว พบว่าซีอิ๊วไทยทั้งสองมีน้ำตาลรีดิวซ์ที่มากเกินพอ ส่วนซีอิ๊วญี่ปุ่นมีหมู่อะมิโนมาก การเพิ่มขึ้นของ HMF เป็นตัวบ่งชี้ที่สำคัญในการเกิดปฏิกิริยา Maillard ซึ่งการเพิ่มขึ้นของน้ำตาลรีดิวซ์ในซีอิ๊วไทยนี้สามารถยืนยันได้จากผลการวิเคราะห์ด้วย HPLC โดยน้ำตาลในซีอิ๊วประกอบไปด้วย อาราบิโนส ไซโลส กลูโคสและซูโครส สำหรับน้ำตาล 5C ที่มีส่วนร่วมในการเกิดปฏิกิริยา Maillard ในซีอิ๊วไทยคืออาราบิโนส ส่วนในซีอิ๊วญี่ปุ่นคือไซโลส

อิออนของโลหะ (Fe^{2+}, Fe^{3+}, และ Cu^{2+}) และโปแตสเซียมไอโอไดด์ที่ความเข้มข้นต่างๆ ซึ่งมีอยู่ในซีอิ๊วในระหว่างการเก็บรักษาไม่มีผลโดยตรงต่อการเกิดปฏิกิริยา Maillard อย่างไรก็ตาม Fe^{2+} ทำให้โปรตีนที่ตกตะกอนด้วย 5% TCA เพิ่มขึ้นและปริมาณสารประกอบ Amadori ลดลง ในขณะที่โปแตสเซียมไอโอไดด์มีผลทำให้โปรตีนที่ตกตะกอนด้วย 5% TCA ลดลง ดังนั้นโปแตสเซียมไอโอไดด์สามารถเติมในซีอิ๊วเพื่อเพิ่มปริมาณไอโอดีนได้ โดยไม่เร่งให้เกิดปฏิกิริยา Maillard

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
et al.	Et alli (Latin), and others
i.e.	Id. Est (Latin), that is
e.g.	Example gratia (Latin), for example
Fig	Figure
Da	Dalton
UV	Ultraviolet
MW	Molecular weight
µl	Microliter
ml	Milliliter
nm	Nanometer
µm	Micrometer
mm	Millimeter
cm	Centimeter
cm^2	Square centimeter
mg	Milligram
g	Gram
kg	Kilogram

LIST OF ABBREVIATIONS

(continued)

sec	Second
min	Minute
hr	Hour
w/w	Weight by weight
w/v	Weight by volume
ppm	Part per million
μM	Micromolar
mM	Millimolar
M	Molar
N	Normality

CHAPTER I

INTRODUCTION

The most important nonenzymatic browning reaction occurring in foods is known as the Maillard reaction. The overall reaction is very complex series of reactions stemming from the initial interaction between aldehydes, ketones, or reducing sugars with amino compounds such as amines, amino acids, peptides, and proteins. This reaction is concerned as causative factor for the production of flavor and brown color in many foods during processing and storage. Furthermore, this reaction is known to widely occur in biological system and contribute to pathogenesis and lesion in diabetes and aging.

Despite much effort during five decades has been spent on the elucidation of the reaction mechanism, a complex network of steps comprised in the Maillard reaction is far from being clearly understood. To understand its roles in complex food systems, many studies on the Maillard reaction have been conducted in model systems, that are, simple amine/sugar mixtures. Besides monitoring the browning, many works have explained the reaction mechanism by determining intermediates such as 1-amino-1-deoxy-2-ketoses (Amadori compounds) and 5-Hydroxymethyl-2-furaldehyde (HMF). In addition, the influencing factors of the reaction such as reaction conditions (pH, temperature, time, water activity (A_w), light, metal ions, and oxygen) and concentration of reactants (reducing sugars and amino compounds, amino-carbonyl group ratio) are investigated.

Soy sauce is one of the complex systems, which has been known that the Maillard reaction involved in its browning. This reaction causes the development of flavor and brown color characteristics of the products. Even if there are several valuable researches published to date covering different aspects of the Maillard reaction mechanisms in Japanese soy sauce, those mechanisms are not completely matched with the mechanisms of Thai soy sauce due to the variation in ingredients and processing. The aim of this thesis was to study the mechanism and roles of the Maillard reaction in Thai soy sauce.

In this thesis, two conventional brands of Thai soy sauce were studied. The Maillard reaction mechanism was investigated during processing and storage, together with the influences of aeration, metal ions, KI, and sugar supplement. Browning, reducing sugar, reactive amino group, 5-Hydroxymethyl-2-furaldehydes (HMF), 5% trichloroacetic acid-precipitated protein (TCA-precipitated protein: PP), and Amadori products were measured as the Maillard reaction indices.

It is expected that the results would lead to the more understanding about the mechanism of the Maillard reaction in Thai soy sauce products and this knowledge can be applied in browning and flavor control in industrial production.

CHAPTER II

LITERATURE REVIEW

1. Chemistry of the Maillard reaction

The Maillard reaction is the most common non-enzymatic browning reaction that occurs in food (1, 2, 3, 4) and biological system (5, 6). It can be defined as an interaction between aldehydes, ketones, and reducing sugars with amines, amino acids, peptides, and proteins, initiating a sequence of consecutive and parallel reactions (4, 7, 8).

Accordingly, after heating reducing sugar to react with amines for several days an extraordinarily complex mixture of compounds is obtained (9). These compounds are present in widely ranging from gas at room temperature to water-insoluble melanoidins (1, 9). Volatile and soluble products contribute to aroma and taste while less soluble products associate with color and texture formation (1).

Despite the reaction was first theorized by Ling Who in 1908 that the color produced in the brewing process comes from a reaction between sugars and protein (3), the man who gave the name and made this reaction well-known was Louis Camille Maillard in 1912 (1, 3, 10). The progress of the Maillard reaction research since 1950s has been provoked by developments in analytical chemistry (1).

Hodge (1953) has given the classical scheme illustrating the pathway of the reaction and categorizing the reaction to seven types of pathway (lettered A through G) as follows (4):

I. Initial stage (colorless; no absorption in near-UV)

A. Sugar–amine condensation

B. Amadori rearrangement

II. Intermediate stage (colorless or yellow, with strong absorption in near-UV)

C. Sugar dehydration

D. Sugar fragmentation

E. Amino acid degradation

III. Final stage (highly colored)

F. Aldol condensation

G. Aldehyde-amine polymerization; formation of heterocyclic nitrogen compounds

Following from A, pathways of B, C, D, E, F, and G can spontaneously occur (4). The interrelationships of these reactions are shown in Fig 1.

The partial mechanisms for browning can be considered as part of the total sugar-amine condensation theory. All the reactions are known to occur in browning model systems, but the browning processes in foods is not well understood (4).

1.1 Initial state of Sugar-amine reaction

1.1.1 A. Sugar-amine condensation

The first stage in the overall reactions is combining of the amino acid with the sugar in an equi-molar ratio (4) and is reversible (2, 4, 10). The condensation starts from opening of cyclic form of the sugar (4), following with addition of uncharged amine to the acyclic aldehydic sugar or the cation generated from the sugar by mutarotation (2, 3, 7), and subsequent elimination of one molecule of water to form *N*–substituted glycosylamine (3, 7).

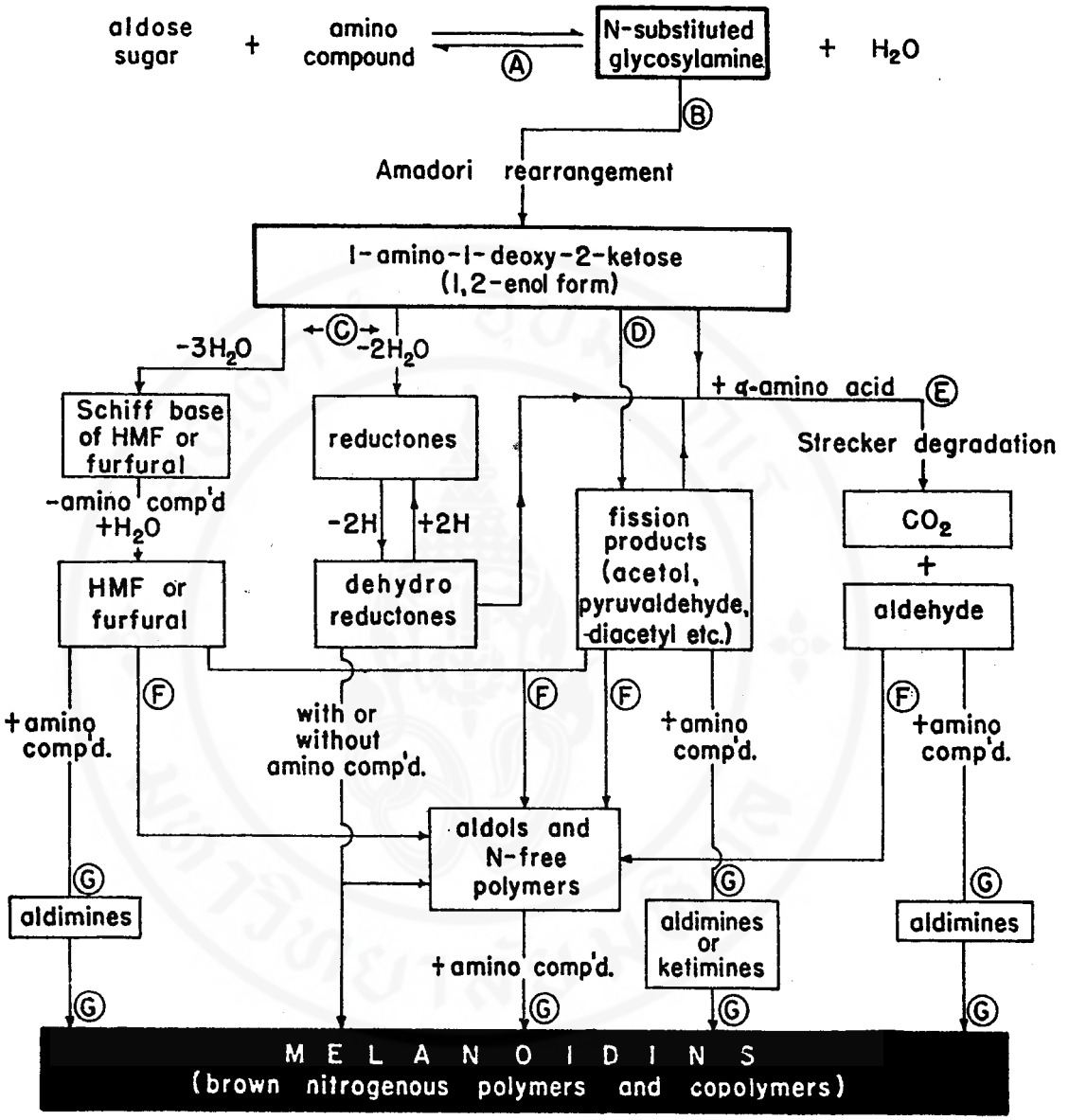
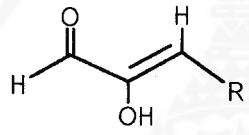


Figure 1: Schematic of the Maillard reaction derived by Hodge (1953) (4). A. Sugar–amine condensation, B. Amadori rearrangement, C. Sugar dehydration, D. Sugar fragmentation, E. Amino acid degradation, F. Aldol condensation, and G. Aldehyde-amine polymerization; formation of heterocyclic nitrogen compounds

Most ketoses and hexoses exist less than 0.05% as the open aldehydic form in water solution because the stability of which depends on the conformational orientation of the hydroxyl group. The formation of acyclic cation $[R–CH=OH]^+$ by general acid catalytic enhances the reactivity of the carbonyl function (2). Among carbonyl compounds, α,β-unsaturated aldehydes (furaldehyde) and α-dicarbonyl compounds (diacetal and pyruvaldehyde) are highly reactive (10).



α,β - Unsaturated aldehyde

α -Dicarbonyl compound

The trivalent nitrogen atom of the amine acts as a nucleophile attacking towards the carbonyl group at C1 (2, 3), followed by the elimination of the hydroxyl ion (2). Bases can catalyze this reaction by removing a proton from the nucleophile and converting nitrogen atom from a weak nucleophile to a strong nucleophile (3). The initial product of the reaction is *N*-substituted glycosylamine (2, 3, 4, 10). The mechanism for the development of *N*-substituted glycosylamine is shown in Fig 2.

The yield of glycosylamine is affected by the amount of water present (10). A substantial amount of this compound is formed when the amount of water present is low (10). This implies its importance in concentrated or dried food systems (4, 10). Moreover, in concentrated and dehydrated food, the reversible sugar-amine condensation goes toward completion (4).

1.1.2 Amadori rearrangement

The *N*-substituted glycosylamine quickly rearranges into the more stable 1-amino-1-deoxy-2-ketose (2, 3, 4, 9, 10, 11). In case the reducing sugar is an

aldose, this process is known as Amadori rearrangement (2, 3, 4, 11). A similar rearrangement in ketoses is known as Heyns rearrangement (11), which changes a ketosylamine to 2-amino-2-deoxyaldose (3, 4). Both of these rearrangements bring about the same transformation (10). Amadori compounds are present, alongside other products, in heated foods, stored foods, dehydrated fruit, dried vegetable, and milk products, as well as in soy sauce (9).

The Amadori and Heyns rearrangements are a key reaction for browning (4).

Aldoses The rearrangement of glycosylamine to Amadori rearrangement product (ARP) is acidic catalyzed (Fig 3) (2, 4, 11). The initial step in the Amadori rearrangement is suggested as *N*-protonation of glycosylamine. The mechanism is interpreted to involve the addition of the proton to the ring oxygen rather than the nitrogen atom, because the iminium ion is stable and unrèactive (2). The amine salt is in equilibrium with the cation of the Schiff base (iminium ion) (2, 4).

Hodge (1953) suggested that the C2 hydroxyl of an aldose is essential for the occurrence of a significant degree of browning (4). The elimination of the hydrogen atom at C2 yields the enol form of 1,2-emaminol (2). Tautomerization of the enolic glycosylamine leads to the same end product of 1-amino-1-deoxy-2-ketose (2, 4). The mechanism of the Amadori rearrangement depends upon the *N*-substitution of the amino component. The formation of the iminium ion is favored by strongly basic amine (2).

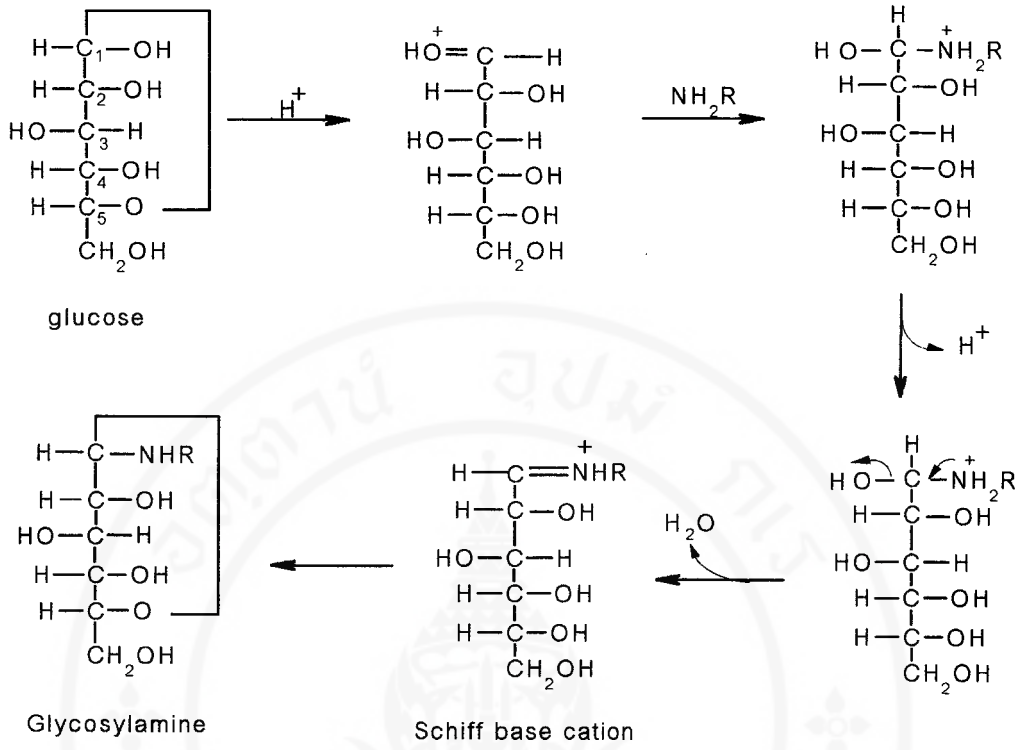


Figure 2: The mechanisms for the formation of *N*-substituted glycosylamine (2).

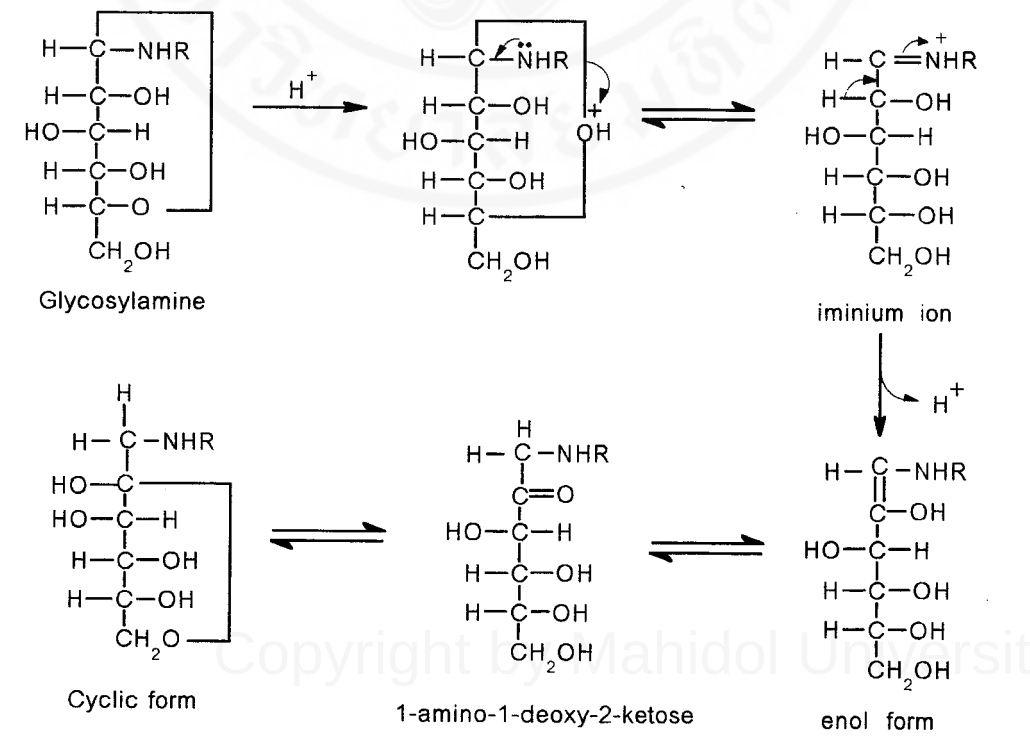


Figure 3: Acid-catalyzing mechanisms for the Amadori rearrangement (2).

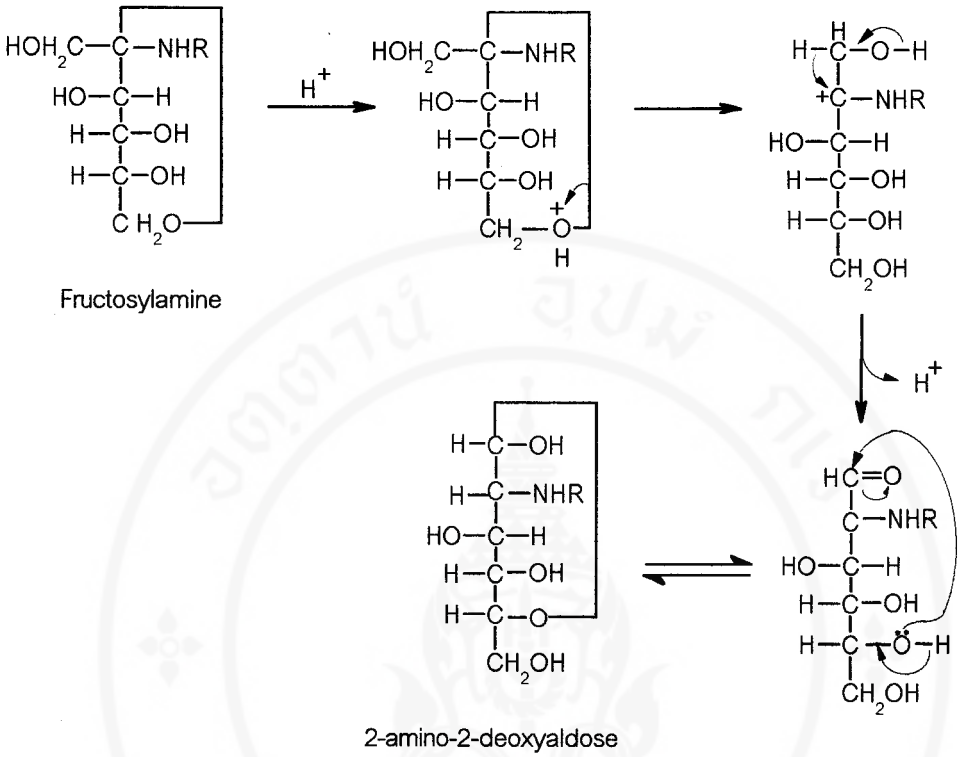


Figure 4: Acid-catalyzing mechanisms for the Heyns rearrangement (10).

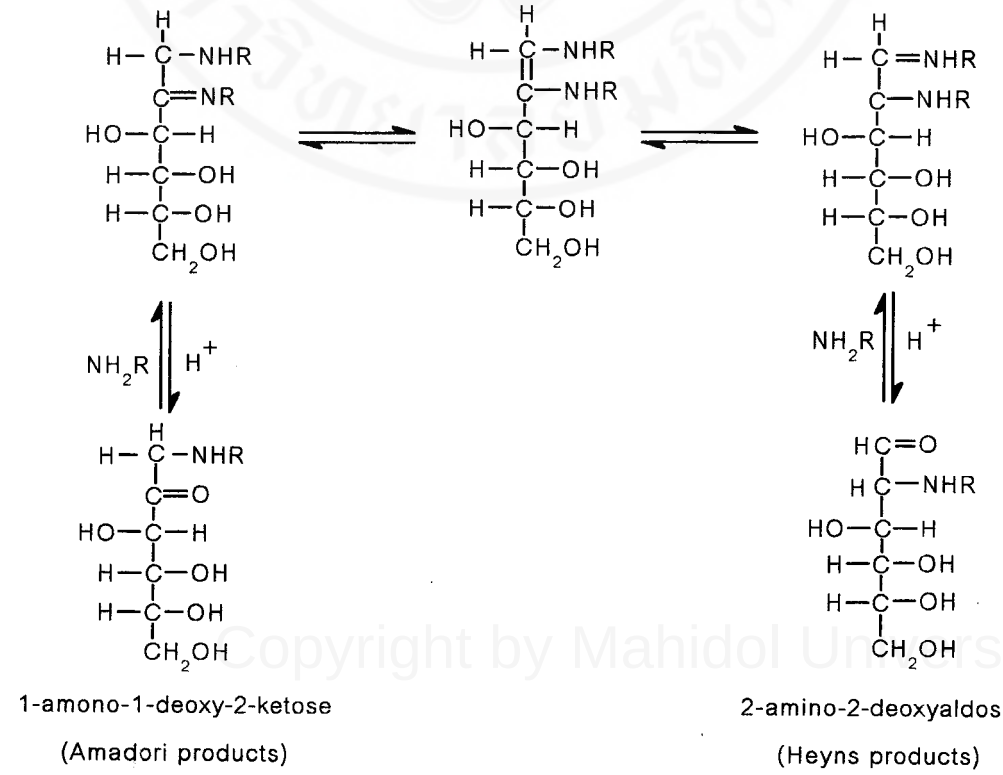


Figure 5: The conversion of Amadori products to a Heyns products (11).

Once the Amadori rearrangement products are important for browning. It has been shown that blocking the Amadori rearrangement step could block the formation of brown pigment entirely (3, 4). In the presence of oxygen and trace amounts of transition metals, Amadori products can undergo autoxidation and produce α-dicarbonyl compound (11).

Ketoses Although the Amadori rearrangement occurs after aldose-amine condensation, very little is known about the first reaction by which ketoses undergo browning. Hodge (1953) reported that ketose gets browning with amino acid at a rate somewhat faster than aldoses (4). The Heyns rearrangement shows the same rearrangement with Amadori rearrangement starting with fructose instead of glucose (10). The product from the rearrangement of ketosylamine is 2-amino-2-deoxyaldose (Fig 4) (3, 4).

The reaction of *keto* sugars such as fructose and Amadori products with amine nucleophiles is known to proceed through imine intermediate and leads to the formation of Heyns products (11). It is shown that the structure of Heyns products is unstable in the presence of amino acids and can react to produce Amadori products (9, 11). In the absence of free amino acids, the browning rates of Heyns products were found to be slower than those of Amadori (Fig 5) (11).

1.2 Intermediate stage

The initial stage of browning (reaction A and B) cannot be detected by UV spectrophotometric measurements; however, before visible browning begins, a strong UV absorption appears to announce the beginning of the intermediate stage (4).

The intermediate stage involves the removal of the amino group from the amino-sugar moiety followed by formation of more reactive compounds. Some of these fluorescent compounds and brown pigments may occur but at very low concentration (3).

Hodge (1953) concluded the three degradation pathways of the 1-amino-1-deoxy-2-ketose (Amadori rearrangement product; ARP); dehydration of the sugar moiety, fragmentation of the sugar moiety, and the Strecker's degradation of α-amino acid (4).

1.2.1 C. Sugar dehydration

There are two types of sugar dehydration reactions (3, 4); both of them depend on the pH of the system (3, 4, 9, 11).

Furfural formation Under acidic condition, the Amadori products exists in its salt form (2). The Amadori products degrade via 1,2-enolization into 3-deoxy-2-hexoluloses (3-deoxyosone) (2, 3, 4, 9, 11) with the loss of 3 molecules of water (4, 16, 17) to yield 5-hydroxymethyl-2-furaldehyde (HMF) as shown in Fig 6 (2, 4, 11, 12).

The mechanism involves the protonation to nitrogen atom. The 1,2-enolization is assisted by the withdrawal of electrons from C1 by the positive charged nitrogen atom (2, 11). 1,2-Enolization undergoes elimination of the C3 hydroxyl groups to yield the 2,3-enol (2), which is readily hydrolyzed at C1 of Schiff base to the 3-deoxyglycosulose (2, 11) or 3-deoxyosone (9).

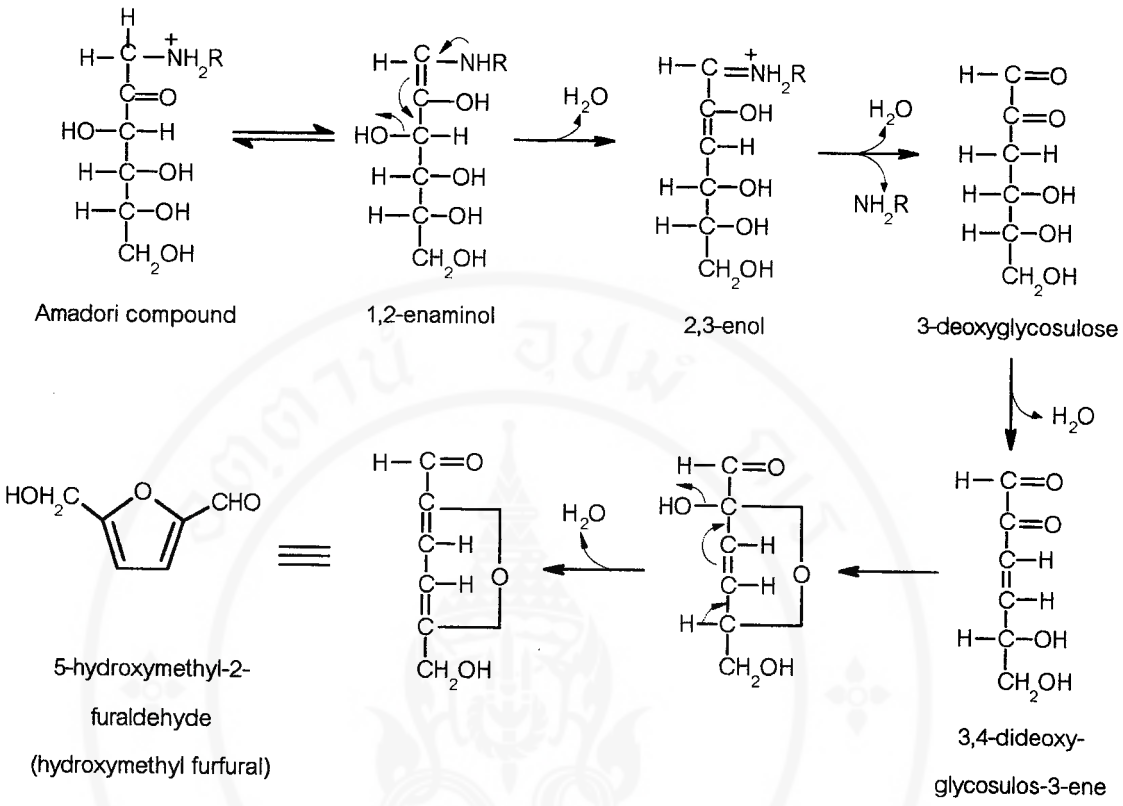


Figure 6: The mechanism of Hydroxymethyl furfural under acidic condition (2).

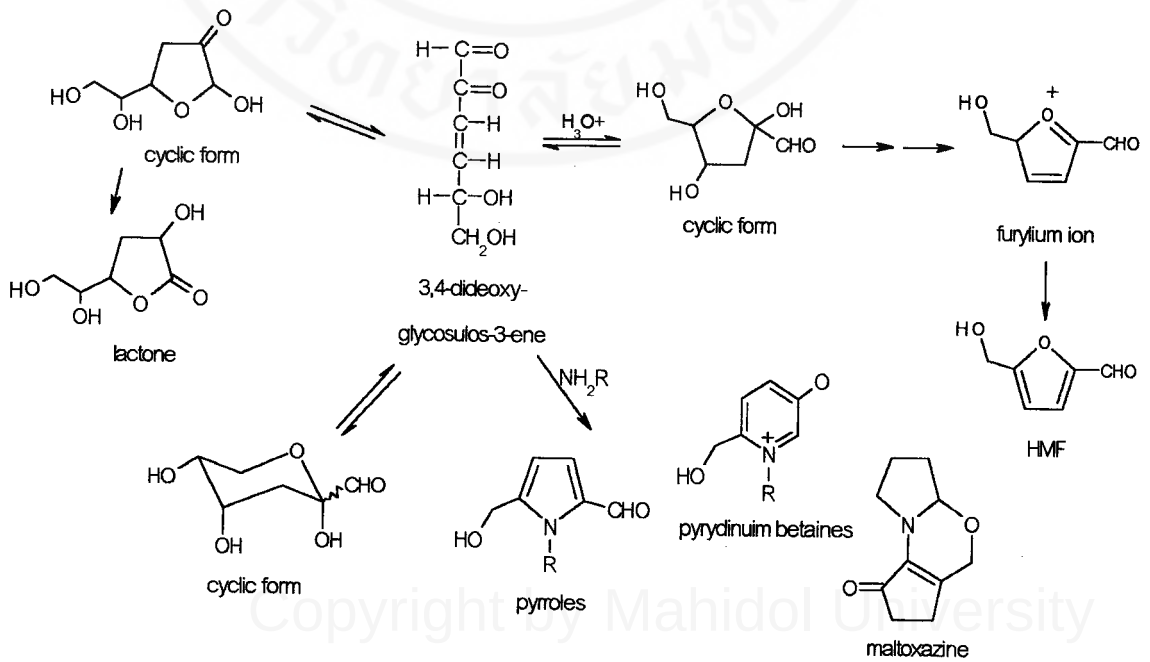


Figure 7: Reaction of 3-deoxyosone under acidic condition (11).

Under acidic condition, the stability of 3-deoxyosone, which is reactive α-dicarbonyl intermediate, is dependent on the temperature due to the fact that it exists mainly in cyclic forms (11). Further elimination of hydroxyl group at C4 yields an unsaturated glucosulose-3-ene (2). Cyclization and dehydration of the latter yields the hydroxymethyl furfuraldehyde (HMF) from hexose or furfuraldehyde from pentoses (3, 11, 13).

The reactivity of 2-ketohexose is higher because the 1,2-enolization step occurs more easily than with an aldose (13). Although HMF is the main product isolated at acidic pH, lactone and pyranones also occurred. Amadori products are converted into HMF though direct dehydration of cyclic form and formation of furylium ion (11). In the presence of primary amino compounds, the formation of HMF is suppressed on the expense of substituted pyrroles (11), and pyrazine derivative (2). The HMF is known as a precursor of browning (14). Hence, it is often used as an indicator of browning reaction (12, 15). The reaction of 3-deoxyosone to form many products under acidic condition is shown in Fig 7.

Yaylayan and Huyghues-Despointes (1994) suggested that the formation of pyrroles is particularly important. This is because of the reactivity of the hydroxymethyl carbon toward nucleophilic substitution reaction that may lead to protein crosslinking (11).

Morales and Jiménez-Pérez (1998) suggested that the formation of bound-HMF (b-HMF) occurs via the acidic degradation of protein-bound Amadori products (Lactulosyllysine), which is different from total and free HMF. They concluded that HMF in milk and milk-like product has two formation routes: by degradation of lactose and by the Maillard reaction (12).

Maltoxazine or 8-Oxo-1,2,3,3a,5,6,7,8-octahydrocyclopenta[d]pyrrolo-[2,1-b][1,3]oxazine is the major product, when proline (a secondary amino acid) is reacted with glucose (9, 11, 16). Yaylayan and Mandeville (1994) proposed the mechanism of maltoxazine formation initiated by base catalyzed 2,3-enolization of the proline Amadori product without formation of deoxyosones as shown in Fig 8 (16).

Reductone formation Parallel to the HMF formation, but to a lesser extent, enolization involving the C3 atom favors the formation of a 2,3-enidiol (3), and 1-deoxy-2,3-hexodiulose (1-deoxyosones) are formed under acidic condition (11, 13). The degradation pathway of 2,3-enolization, with the loss of two molecules of water, generates reductone (2, 3, 4, 9).

Hodge (1953) suggested that in dry state, or in non-aqueous solvents with the presence of amines, reductones are formed. It is possible that the reductones possess the structure of furfurals without furan ring closure (4).

The reactions of 1-deoxyosones derived from disaccharides differ in some respects from those of monosaccharide. The main product in reaction mixtures of disaccharide with primary amines is the pyridone (9, 13), that is known to bind metals such as iron (9) and aluminium (9, 11) tightly. Further typical products of disaccharide in the presence of primary and secondary amines are the pyrrole, the cyclopentenone, and the furanes (9, 11).

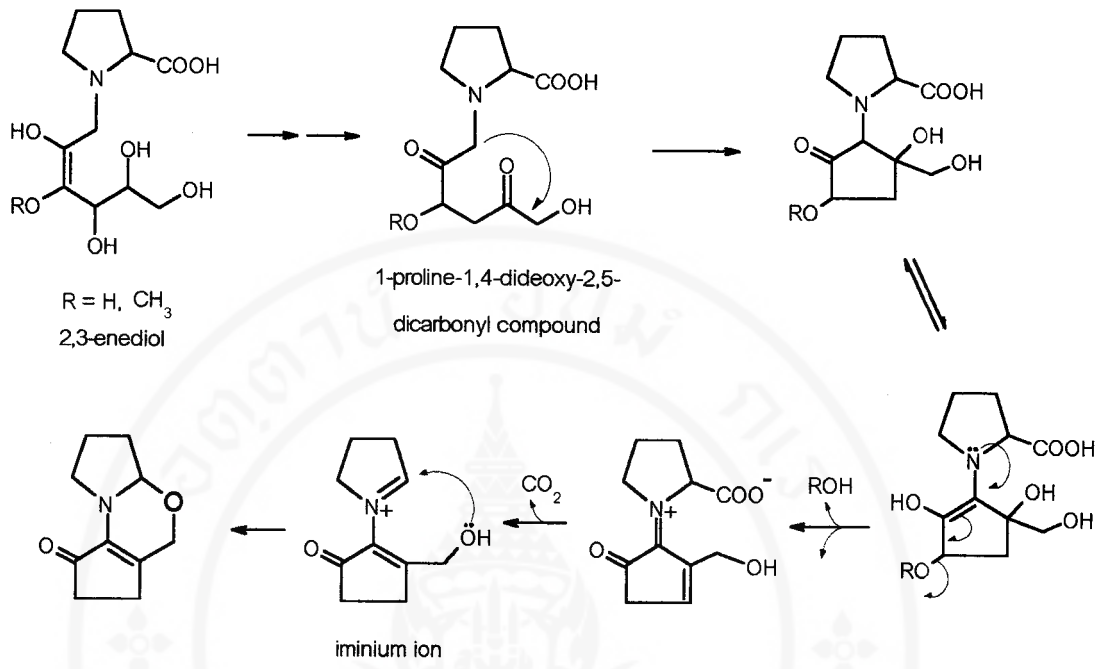


Figure 8: The mechanism of formation of maltoxazine from intact proline Amadori compound (11).

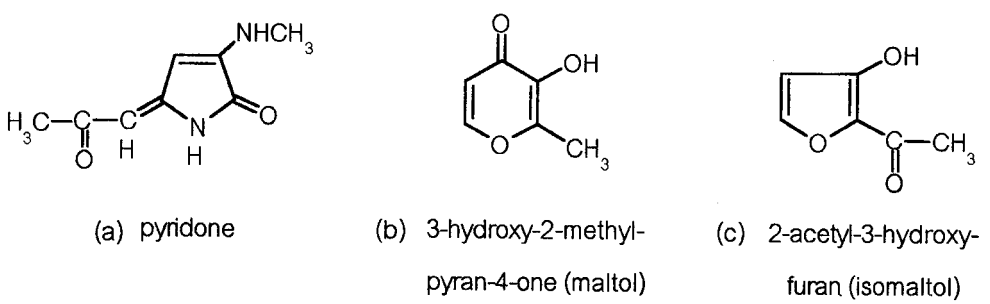


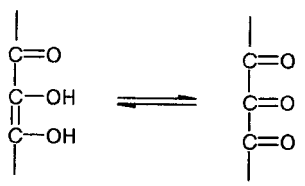
Figure 9: The structure of pyridone (a), maltol (b), and isomaltol (c) (13).

Fructose, in addition to being the predominant of 1,2-enediol, can form the 2,3-enediol; hence the spectrum of its degradation products is wider than with glucose (13). Elimination of an OH-group on C1, and corresponding intermediary steps, from 2-acetyl-3-hydrofuran (isomaltol) or 3-hydroxy-2-methyl-pyran-4-one (maltol) (4, 9, 11, 13), whereas OH-group elimination from C4 forms hydroxyacetylfuran (13). However, maltol is formed under normal conditions of the Maillard reaction from disaccharide only (13).

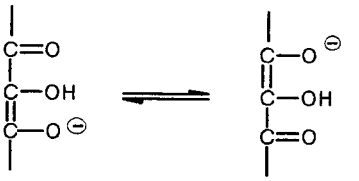
Diacetylformosine or 2,4-dihydroxy-2,5-di-methyl-3-furanone can be formed from 1-deoxyosones via 1,6-di-deoxy-2,4,5-hexotriulose (actually the end product is in cyclic form) (13). Diacetylformosine and others intermediate products, which retain a carbonyl group in the vicinity of an enediol group, are called reductones (9, 13). Ascorbic acid is the best known reductones (4, 13), a class characterized by strong reducing power in acidic media, even at low temperature (13).

Hodge (1953) suggested that all reductones would be found to undergo similar browning reactions, as they all have the some type of conjugated unsaturation (4). Conjugated unsaturation is necessary for stability of the reductone (3, 4).

In acidic media, reductone is oxidized into its dehydro compound in these reaction:



Reductones are stable at pH<6, as resonance effect stabilized monoanions, while at high pH the dianion is unstable in the presence of oxygen:



The reaction outlined above takes place even under mild condition in the presence of amino compounds. Reductones formed in this way, in food act as natural antioxidant (13).

Huyghues-Despointes and Yaylayan (1996) proposed that three distinct mechanisms require reduction steps for the formation of the reductones: through hydride transfer from formic acid; through cyclic dimerization of α-hydroxy carbonyl compounds followed by electrocyclic ring opening to produce redox products; and by disproportionation of enediols with α-dicarbonyl compounds via double proton transfer (17).

1.2.2 D. Sugar fragmentation

The second reaction that occurs with Amadori products is fragmentation of sugar moiety (3, 4). Amadori products, deoxyosones and other sugar derivatives formed under Maillard reaction conditions can undergo retro-aldol reactions (9, 11, 17) especially under basic conditions (17). Retro-aldol reactions produce more reactive 2, 3, 4, and 5 Carbon-containing sugar fragments (2-C, 3-C, 4-C, and 5-C) (11, 17) containing α-hydroxy carbonyl (4) and α-dicarbonyl moiety those are more reactive to amines than 6-C sugars (17).

Retro-aldol reactions become important at higher pH and can contribute to the formation of brown polymer (11). In the presence of amino compounds, the

browning is greatly accelerated via the participation of the primary or secondary amino group (4).

Oxidative fragmentation reaction of deoxyosones are known between the α-diketo function, leading to 1-C and 5-C sugar fragments from the 3-deoxyosone, and to 2-C and 4-C sugar fragments from the 1-deoxyosone (9).

Huyghues-Despointes and Yaylayan (1996) indicated that 3-C sugar fragments are produced by a retro-aldol reaction at C3-C4 of Amadori compound. The 2-C and 4-C sugar fragments are produced simultaneously by retro-aldol cleavage at C4-C5 of 1-deoxyosone, although the major retro-aldol cleavage is initiated at C2-C3 from the Amadori compound, after a carbonyl migration (17).

Recently, the free radical formation has been suggested involving sugar fragmentation (2, 11). Several studies on the free radicals formation in the Maillard reaction have been published (18, 19, 20). Electron spin resonance (ESR) spectral data have provided evidence that the pyrazinium radical cation (11) or pyridinium radical (9) is the source of the free radical activity (11). The pyrazine nucleus is assumed to be formed by the condensation of two enaminols produced by a retro-aldol-type cleavage of the imine precursor of Amadori product (11).

Namiki and Hayashi (1973, 1975) used ESR spectroscopy to investigate free radicals formation in the amino-carbonyl reaction, and identified the free radical products as *N,N'*-dialkylparazinium radical cations (20, 21). Their results showed that the alkyl side chains on the pyrazinium nitrogen atoms are derived from the amine, while the carbon atoms come from the sugar (20). They concluded that the radical does not locate in a highly conjugated structure such as melanoidins but it exist at a particular position in some products formed during early stage of the Maillard

reaction (20, 21). Moreover, they studied the effects of oxygen on the free radicals reaction, and found that oxygen is not required or inhibit the radical formation (20).

The free radicals develop rapidly in an early stage of the Maillard reaction (19, 20, 21, 22) and decreases while the formation of the Amadori compound and 3-deoxosone remains increasing (2). The accepted pathway of free radical formation involves a reverse-aldol reaction of the glycosylamine to give the 2-carbon enaminol, glycoaldehyde alkylimene, and condensation of the latter to form the unstable dialkyldihydropyrazine, which is readily oxidized to the dialkylparazinium product via the cation radical intermediate (Fig 10). It has been shown that the dialkylparazinium product is likely the active intermediate for the polymerization in browning (2).

These free radicals occur at neutral pH (2), increases under basic condition and high temperature (18). Cämmerer and Kroh (1996) suggested that the free radicals increase with increasing pH especially at pH above 8 and decrease at pH above 10 due to the liability of the free radical products before disappears above 11 (22).

Djilas and Milic' (1994) studied the influence of four naturally occurring phenolic compounds, ellagic acid, gallic acid, ferulic acid, and syringic acid, as inhibitors of the formation of 1,4-pyrazine cation free radical and pyrazine derivatives in the Maillard reaction. The results confirmed that naturally occurring phenolic compounds interact in the Maillard reaction responsible for the formation of free pyrazine cation radical (18).

Robert and Lloyd (1997) found the two series of the cyclic cation radical formation during the reaction between glycolaldehyde and glyceraldehyde with *N,N'*-dialkylethylenediamines (19). The first is 1:1 sugar: amine product, the species I

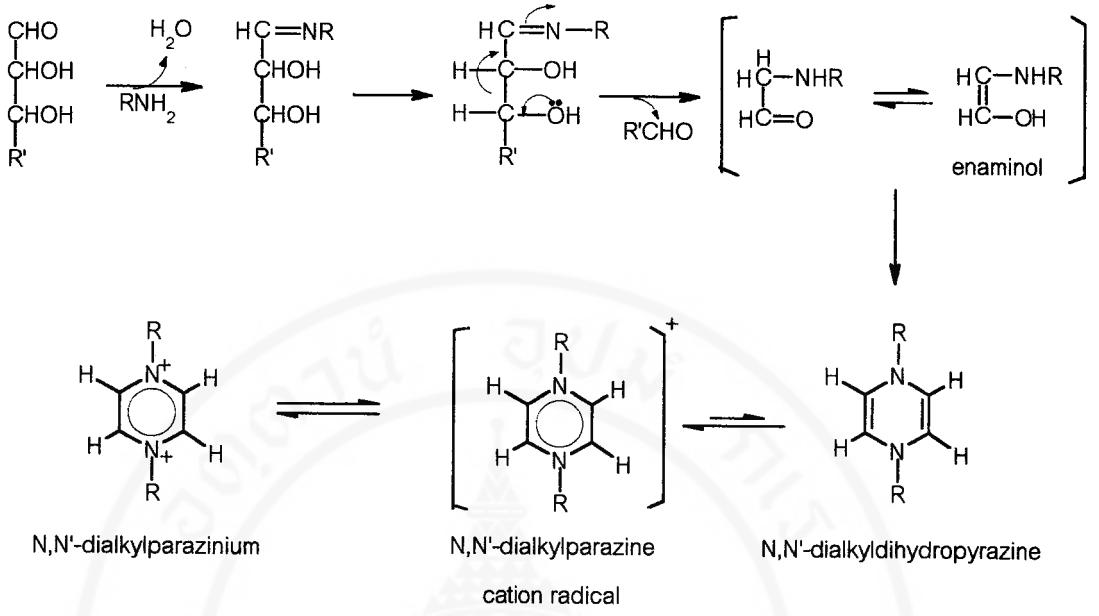


Figure 10: The formation of *N,N'*-disubstituted pyrazine cation radical via the fragmentation of the glycosylamine (2).

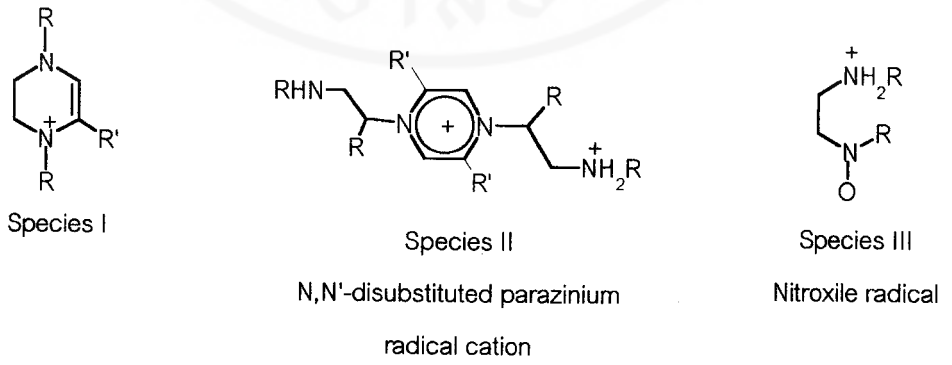


Figure 11: The structure of species I (a), species II (b), and species III (c) radical (19).

radical, in which the nitrogen atom in 1-ethylenediamine molecule is bridged by a sugar molecule.

The second, and more stable, is 2:2 products, the species II radical. In addition, nitroxide radicals, the species III radical, are found when methanol is used as the medium (23). The structure of these cyclic radicals is shown in Fig 11.

1.2.3 E Strecker degradation

Further reaction sequence as shown in Fig 12, the Strecker degradation of α-amino acids brings about the reduction of the α-dicarbonyl compounds (2, 4, 13), mainly of the deoxyosones (9) and reductone (3, 4). This interaction, involving transamination, provides an aminoketone (13), an aldehyde containing one carbon less than the original amino acid (3, 4, 10), and carbon dioxide (3, 4, 10, 13).

The α-dicarbonyl compounds are osones (10) and are active agent for undergoing the nucleophilic addition reaction with amino acid via Strecker degradation (11). The enol form of α-amino acid decarboxylates is ready to yield the enaminol, and then undergoes self-condensation to brown polymer or hydrolysis to the amine and the aldehyde (2). The aldehydes formed, so-called Strecker aldehydes (11, 13), constitute many of important flavor compounds in food systems (2, 9).

Hodge (1953) indicated that the Strecker aldehydes are a source of browning since they can condense with themselves, with sugar fragments, with furfurals, and other dehydration products, or with aldimines and ketimine to form brown pigments (4). The aminoketone formed can yield pyrazine derivatives, the powerful aroma constituents (13). Pyrazine is one of the most important volatiles distributed widely in food systems, especially food processed at high temperatures and low water environment (24). 3,6-Dihydroxyparazine formed by the condensation of

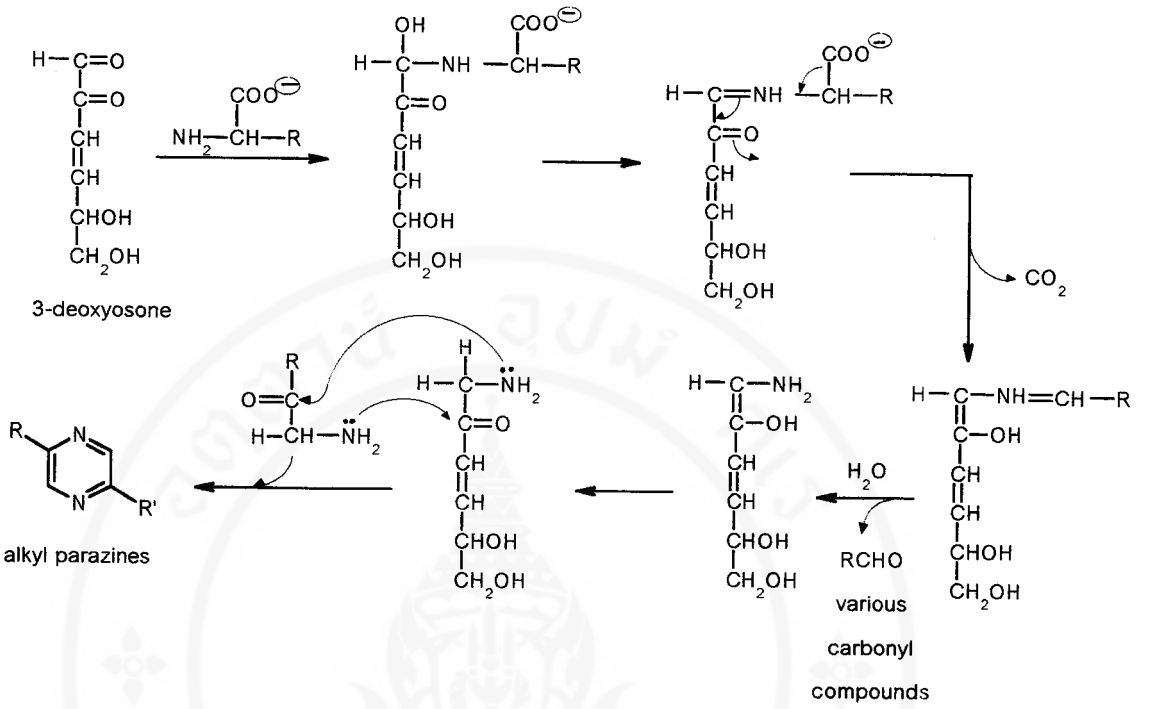


Figure 12: The mechanism of alkyl pyrazine formation via Strecker degradation (2).

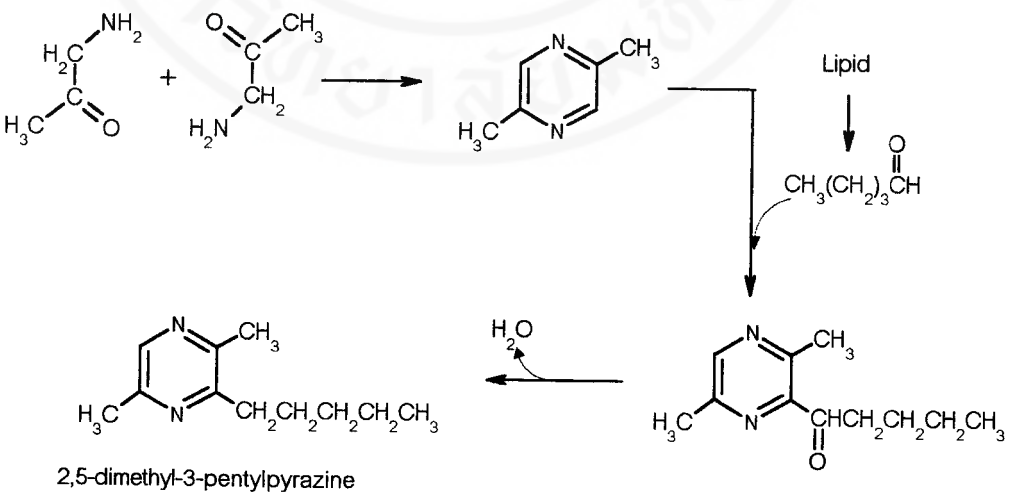


Figure 13: Mechanism for the formation of 2,5-dimetyl-3-pentylpyrazine (25).

aminoketones reacts with pentanal, a lipid oxidation product, and results in the formation of 2,5-dimethyl-3-pentylpyrazine (Fig 13) (25).

Shu (1998) suggested that Strecker degradation convert the α-dicarbonyls into α-aminocarbonyls, which in turn are condensed (26) followed by oxidation (2), to form pyrazines (26). Because sugar degradation also provides α-dicarbonyls, pyrazines can be formed directly from the Strecker degradation alone (26). The previous studies found that other type of pyrazines in addition to alkyl pyrazine is predominant at high temperature above 150°C (27).

During the Maillard reaction and thermal degradation of sugars, some active intermediates such as acetol (1-hydroxy-2-propanone) (24, 25) and acetoin (3-hydroxy-2-butanone) (26) can be produced (24). These intermediates react with ammonia, released from amino acids and the sugar degradation product (26), to generate α-aminoketone and then form pyrazines (24).

Shu (1998) also suggested that the ammonia released after decarbonylation of an α-amino acid reacts with acetoin (a simple acyloins, the product from sugar degradation) to generate pyrazine. As shown in Fig 14, it is proposed that the α-amino acid is decarboxylated to generate the reactive intermediate, 1-hydroxyamine, deamination of which leads to the formation of the Strecker aldehydes (26).

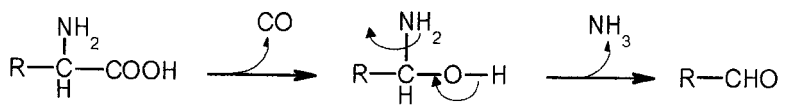


Figure 14: Proposed mechanism of deamination from α-amino acid (26).

Under acidic condition, ammonia and α-hydroxy carbonyl reactive intermediates are released. The ammonia can react further with dicarbonyl compounds to produce pyrrole (11, 28).

Chen and Ho (1999) indicated two general pathways of pyrrole formation in the Maillard reaction. One is the reaction of furans or 3-deoxyosone with amino acids or amines, and the other pathway may also exist for pyrrole compound generation, such as pyrrolysis of amino acids (24).

Transformation into pyrrole compounds (as shown in Fig 15) is also possible by reaction of a α-dicarbonyl compound with a 1-amino-1-deoxyketone (13) or cycliclization of the amino carbonyl product of Strecker degradation (2)

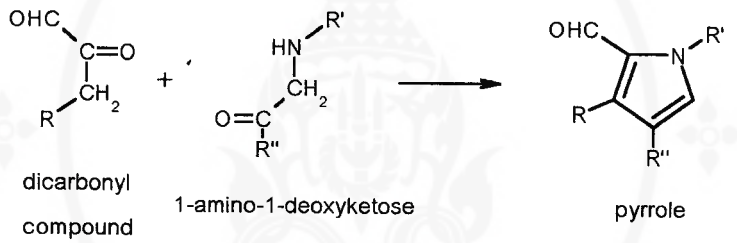


Figure 15: The transformation into pyrrole (13).

The production of carbon dioxide parallels aldehyde production and color formation (3). Hodge (1953) suggested that the liberation of carbon dioxide come from the carbonyl group of the amino acid rather than from the sugar moiety (4). This fact can be demonstrated by using isotope tracing technique (10).

The Strecker degradation pathway, however, is not the major color-producing reaction (3, 4) but possibly is important concomitant reaction leading to melanoidins formation (4).

1.3 Advance stage

In the final stage of browning, the intermediates polymerize and unsaturated, fluorescent, colored polymers are formed (4). The chief reactions involved are thought to be aldol condensation, aldehyde-amine polymerization, and the formation of heterocyclic nitrogen compounds such as pyrroles, imidazoles, pyridines, and parazine (3, 4).

1.3.1 F. Aldol condensation

Since amine catalysts are present, and aldehydes can be generated by sugar dehydration (C), by sugar fission (D), and by Strecker degradation (E), it is evident that aldol condensation (F) is a highly probable reaction for melanoidins formation (4).

Amino compounds (partially amine salts) are effective catalysts for the aldol condensation of acetaldehyde (4) and crotonaldehyde [$CH_3CH=CHCOH$] or 2-butanal, which is stabilized by having conjugated double bonds (5).

Hodge (1953) suggested that nitrogen-free aldols in general would react with amino compounds, aldimines, and ketimines to produce nitrogenous melanoidins, since aldol [$CH_3CHOHCH_2CHO$] is known to undergo browning with glycine. He also indicated that the aldol condensation of diacetyl proceeds by first forming the ordinary aldol between two molecules, then intramolecular condensation takes place to yield 2,5-dimethyl-*p*-quinone (4).

1.3.2 G. Aldehyde-amine polymerization and formation of heterocyclic nitrogen compounds

The development of color is an extremely important feature of the Maillard reaction (29, 30), but relatively little is known about the chemical nature of the compounds responsible for visual color (9, 29, 30, 31).

Colored Maillard reaction products (CMRP) may be divided in to two general classes: the low molecular weight color compounds which typically comprise two to four linked rings (29, 30) and the melanoidins which are brown polymers and possess molecular weights of several thousand Dalton (9, 29, 30, 31).

The melanoidins are widely in molecular weight and contain several chromophores depending on the amino acid-sugar ratio and conditions of their condensation (3). Melanoidins are the result of polymerization of the unsaturated carbonyl compounds formed in the Maillard reaction with amines (2).

Rizzi (1997) indicated that CMRP could be considered a mixture of dyes and pigments because of their inhomogeneous chemical nature and wide molecular weight distribution. He also suggested that CMRP can be dyes, that is, soluble individual molecules with molecular weight less than 500 Da or soluble polymers; or they can be pigments, that is, colored, insoluble polymers (31).

Formation of CMRP of molecular weight less than 500 Dalton (MW < 500 Da) in amines or amino acids with aldose.

Many low-molecular weight (<500 Da) substances were usually separated by organic extraction of complex reaction mixture in low yield (31). Although several of the information available on the low-molecular weight colored Maillard products have been carries out in methanol (32, 33, 34) or ethanol (31), few

studies have reported structural data for colored compounds formed in aqueous solution (30, 35, 36, 37).

Labuza and Baisier (1992) found that neutral or acidic amino acids and sugars form melanoidins with lower molecular weights than those formed by basic amino acids (3). The structure of low molecular weight colored products identified from different model systems is shown in Table 1.

Ames and coworkers (1993) isolated and purified novel colored Maillard product by repeated semi-preparative Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC), prior to analysis by electronic absorption and Nuclear Magnetic Resonance (NMR) spectroscopy and by low and high resolution Fast Atom Bombardment Mass Spectrometry (FAB-MS) (30).

Rizzi (1997) indicated that furfurals, structurally related pyrroles, and 3-furanones, reaction products of deoxyosones, react with each other to form CMRP (Fig 16). In this case, the formation of CMRP suggested a high degree of reactivity for the C2 methylene group in furanone. A derivative of acethylformoin, a well-known Maillard intermediate similarly originated from 1-deoxyosones, is identified as yellow-colored furfurylidene derivatives in the reaction of γ-pyranones with furfural (31).

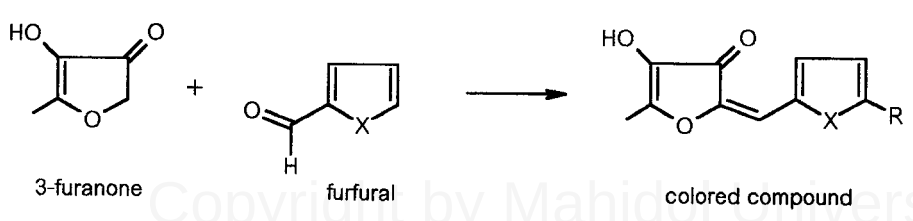
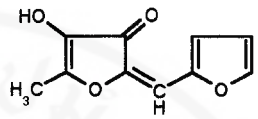
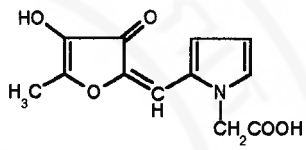
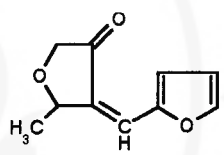
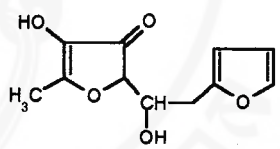
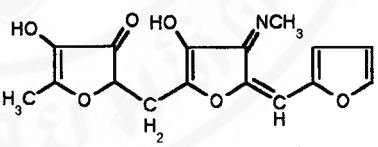
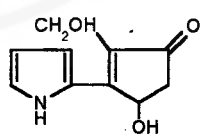
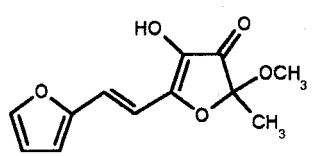
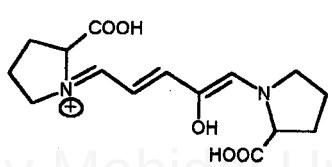
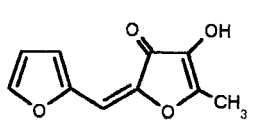


Figure 16: Formation of CMRP from 3-furanone and heterocyclic aldehydes (31).

Table 1. Colored, low molecular weight reaction products identified in different model systems.

Reactants	Product	Ref.
Xylose or arabinose + glycine or lysine (Aqueous system)		30
Xylose + glycine (Aqueous system)		30
Xylose + glycine (Aqueous system)		30
Xylose + glycine (Aqueous system)		30
Xylose + lysine (Aqueous system)		30
Xylose + lysine (Aqueous system)		30
Xylose + alanine (Aqueous system)		34
Furan-2-carboxaldehyde + N-(1-deoxy-D-fructos-1-yl)-L-proline (Methanolic system)		36
Furan-2-carboxxaldehyde+ L-proline (Aqueous system)		37

Moreover, Hofman (1998) suggested that acetyllformoin acts as a chemical switch in the Maillard reaction determining different reaction pathways in the presence of primary and secondary amino acids (34).

It is questionable that whether some low molecular weight colored condensation products isolated from Maillard model systems represent some melanoidins substructures or not. Tressel et al. (1998a) reported that low molecular weight Maillard products might play an important role in the formation of melanoidins (33).

Characterization of visible chromophores in complex polymeric Maillard reaction products

Very little is known about the chemical structures responsible for visible colors of Maillard polymers (31), the high molecular weight substances or melanoidins (9). The empirical formula for melanoidins was thought to be $C_{67}H_{76}N_5O_{32}$ (10), derived from the elementary analysis such as ^{1}H, ^{13}C, ^{15}N NMR (9, 31, 32, 33, 36, 37), ESR (31), and HPLC (36, 37).

The light absorption of melanoidins in the ultraviolet and visible regions shows that condensation reaction participates only to a limited extent in the linking of the monomer (9). A possible structure of melanoidins may be composed of repeating units of Schiff base formed between 3-deoxyosone and its enamine as shown in Fig17. Although polymers of furan and pyrrole structures have also been suggested, the proposed structure consists of ether bonds and reductone systems (2).

The carboxyl acidity in the melanoidins structure can be connected with the relative contribution of the sugar; the higher the sugar-amino acid ratio, the higher the carbonyl group concentration and the higher the concentration of total melanoidins

pigments (3). The backbone of melanoidins is probably a sugar moiety on which various containing groups are grafted. This suggested the fragmentation and recombination of the original sugar chain occur (3, 31).

Rizzi (1994) divided the systems of colored structure to three types, namely; (a) simple models consisting of reducing sugars plus amines or amino acids; (b) sugars plus proteins; and (c) sugars themselves (i.e., caramelization) (31).

(a) The simplest model systems between reducing sugar and amino acids reveal the formation of dehydrated sugar intermediates which further react in an unexplained manner to produce the Maillard polymer. The CMRP development is dependent to incorporation of the amino acid. Besides conjugated unsaturation, N-containing free radicals have occasionally been mentioned as a possible source of visual color (31).

(b) For the development of CMRP in sugar-protein reactions, sugar reacts at ε-amino group of protein to form chromopheric, reactive side chains that eventually undergo crosslinking with other protein moiety to produce insoluble brown pigments. In addition, the numerous fluorescence is identified as precursors of polymeric CMRP (31).

(c) Brown-colored formation in sugar caramelization is similar to the Maillard reaction in that dehydration and condensation of carbohydrate moieties are intimately involved (13, 31). Furan derivatives such as furfural, 5-hydroxymethyl furfural (13, 31), and 2-hydroxyacetylfuran have recently been proposed as precursor of regular, polymeric structure responsible for brown color (31).

Hofman (1998a) studied the Maillard reaction leading to nitrogen-containing colorants by using the mixture of furan-2-carboxaldehyde, a major

dehydration product from pentoses, and L-proline in aqueous system. He identified the unknown intense yellow 5-(*S*)-(2-carboxy-1-pyrrolidinyl)-2-hydroxy-(*E,E*)-2,4-pentadienal-(*S*)-(2-carboxypyrrolidine)imine as the colored main product comprised four rings with an amino acid moiety incorporated (36).

Additional studies on the reaction of pentoses and aniline revealed that either the completed amino acid moiety can be incorporated into colored structures or only the nitrogen atom can be transferred into colorants via the Strecker degradation with deoxyosones (37).

Tressel et al. (1998a) reported the formation and characterization of linear (type I) melanoidins-like polymers from N-methyl-2-(hydroxymethyl)pyrrole (33). Further experiment also demonstrated a novel route to form melanoidins-like polymers from the predominant low molecular weight Maillard products yielded from hexoses and pentoses (32). Figure 18 summarizes the fragmentation pathways of different sugars leading to polymers of types II and I.

In conclusion, the mechanism for the Maillard reaction contains several distinct routes to melanoidins formation. The routes, which stem from the Amadori rearrangement of sugar-amine condensation products leading to the 1,2-enol and, from it, the accelerated production of brown pigments occurs through several different routes (4).

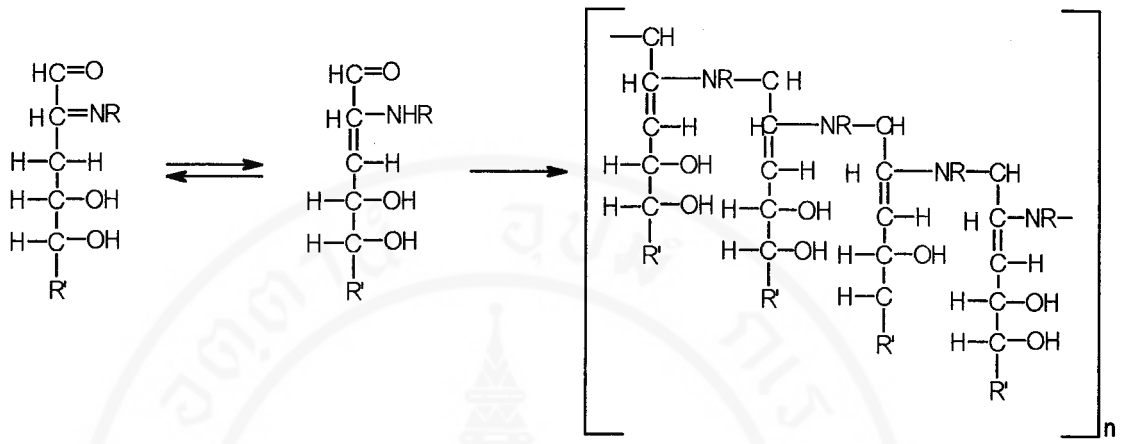


Figure 17: The proposed structure of repeating units of melanoidins (2).

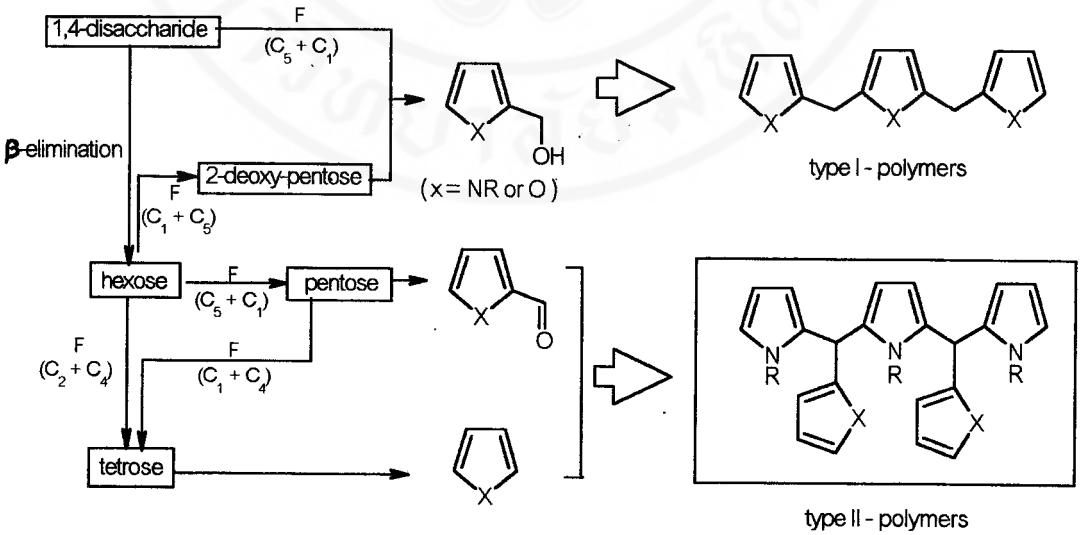


Figure 18: Maillard transformation of hexoses, pentoses, and disaccharides into melanoidins-like polymers (R= alkyl or amino acid residue) (33).

2. Factor Influencing the Maillard reaction

Several factors influence the progression of the Maillard reaction. Among these are nature and concentration of the reactants (3, 9, 10, 38, 39), sugar-amine ratio (3, 38), pH (3, 10, 40, 41), temperature (3, 10, 38, 40, 41), water activity or a_w (10, 29, 42), metal ions (1, 43, 44), and oxygen (44).

2.1 Types of sugar

When crystalline sugar is dissolved in a solvent, it undergoes mutarotation (3, 45), the equilibrium reaction between the α-pyranosyl, α-furanosyl, β-pyranosyl, β-furanosyl, and acyclic forms as shown in Fig 19. Only the acyclic or aldehydic chains from with aldose or ketose residue can participate in the Maillard reaction. The bond between oxygen and hydrogen of the hydroxyl group attached to the C1 carbon is slightly acidic in aldose. Hence, the bond weakens as the pH increases, and the rate of ring-to-aldehyde transformation increases rapidly with the pH (3).

Although disaccharide such as sucrose, maltose, and lactose has no active carbonyl groups, they participate in the Maillard reaction only after cleavage of the glycosidic bond (9, 45).

Pischetsrieder and Severin (1994) suggested that the Maillard reaction of disaccharide leads to the formation of compounds that differ in structure form those obtained from monosaccharide such as glucose or fructose. They also proposed the different pathway of the decomposition of mono- and disaccharide as shown in Fig 20 (46). However, the influence of sugar structure is noted that pentose has a higher reactivity for the Maillard reaction more than hexoses and disaccharide (9, 10). The susceptibility of sugars for browning from fastest to slowest order as follow: ribose > D-xylose > L-arabinose > mannose > glucose > disaccharide (3, 6, 45).

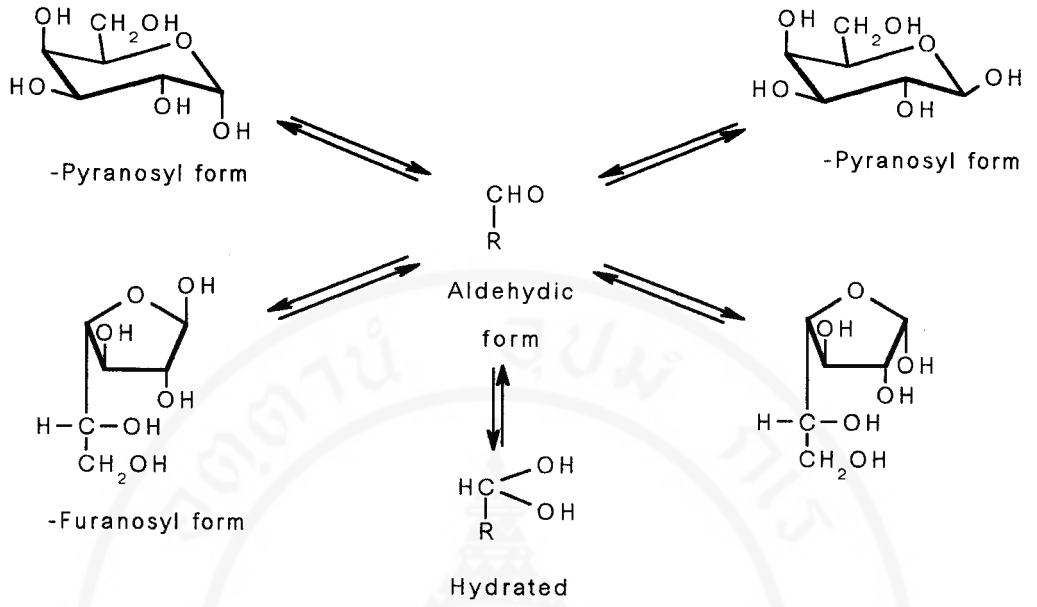


Figure 19: Mutarotation of sugars in solution (4).

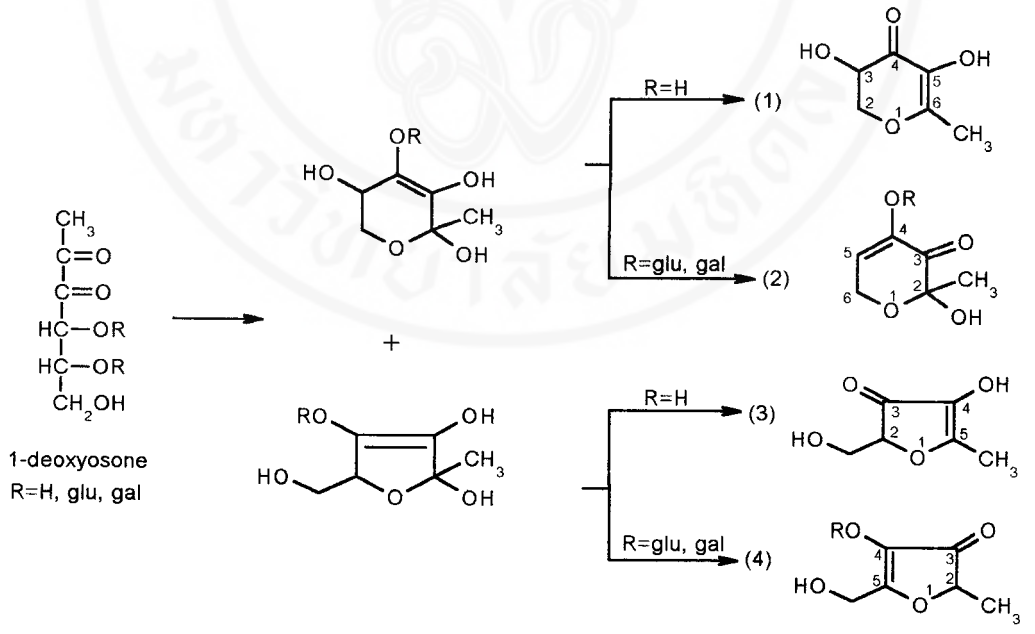


Figure 20: Different pathways of the further decomposition of mono- and disaccharide; (1) 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one,

(2) 4-(glycosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-ones,

(3) 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone, and

(4) 4-(glycosyloxy)-5-(hydroxymethyl)-2-methyl-3(*2H*)-furanones (46).

2.2 Types of amino acid

The type of amino acids plays an important role on browning contribution (14). Inspite, lysine is often hypothesized as being the most reactive amino acid due to its two reactive amino groups, other amino acids classified as the most reactive are glycine (35), tryptophan, and tyrosine (3). Proline and amino acids with hydrophobic side chains react more slowly than the other amino acids (3, 14).

Gögus et al. (1997) studied the different browning formation different amino acid models. They concluded that proline is the least reactive amino acid compared to arginine and glutamine (14).

It is shown that sugars and amines undergo browning in aqueous solution in proportion to the basic strength of the amines employed. Hodge (1953) suggested that the rate of primary reaction, sugar-amine condensation, increased with the basicity of the amino component (4).

Generally, in the Maillard reaction, primary amines are more important than secondary amines. Thus, in protein, it is the primary amino group of the lysine residue that reacts predominantly, and free amino acid occurred in foods. Primary amino acids are more important than secondary amino acids (9, 34).

Browning of protein is related to the amount of ε-amino group in which relating to browning are lysine residue (34). The other potential nitrogen groups are histidine and tryptophan, and the number of end-terminal amino groups (3, 34).

2.3 Sugar-amine ratio

The ratio of reducing sugar to amino acid has been suggested as an important factor in determining the rate of Maillard browning (47).

Hodge (1953) stated that in the initial stage of the reaction, the sugar condenses with amino acid at the ratio of 1 to 1. This ratio increases and approaches 1.5 to 1 during the latter stages of the reaction (4). The different ratios effect on the velocity of the Maillard reaction (3, 47). Of critical concern is that when one compare browning data between labs or within any experiment, the ratio between reducing sugar and amine must be evaluated (3).

Labuza and Baisier (1992) found that the browning rate increases with increasing of initial glucose: lysine ratio (3). Mistry et al. (1995) used high ratio of sugar to amino acid as model systems and explained that excess sugar would increase the rate of the Maillard reaction (47). In contrast, Renn and Sathe (1997) studied the effect of L-leucine and D-glucose on the Maillard reaction and found that samples with excess leucine has higher browning rate than samples with glucose/leucine ratio to 1:1 or 2:1 (48).

2.4 pH

The pH has a very significant effect on the Maillard reaction (3). The rate of Maillard reaction is low at low pH and increases progressively as the pH increased (49). Keeping pH of the system at lower than the pKa value can minimize the carbonyl formation (45). This occurs typically between pH 9.0 and 10.5 (3, 45).

The pH of the system significantly influent the type of products formed (39). Under alkaline condition (>pH 7), imine (Schiff base) and Amadori compound mainly undergo chain fragmentation to form 2- and 3-carbon fragments (1), which quickly

react further to form melanoidins (4). At strongly acidic pH (<pH 5), the Amadori product eliminate the original amine ($R\text{-}NH_2$) (45) to generate a 3-deoxyosone (1, 2, 3, 4). At intermediate pH (pH 5-7), a similar fragmentation with loss of amine leads to a 1-deoxyosone (1, 4).

The pH dependence of the initial stage of the Maillard reaction is related to the amount of unprotonated form of the amino acid (3), which is the form that can ready react with reducing sugar (2, 3, 4). The higher pH, the higher the percent of amino acids that is in the unprotonated form (10). The pH also influences amount of sugar acyclic form (2, 9, 10). Labuza and Baisier (1992) showed that the amount of acyclic form increased with increasing pH for most sugars (3, 10). This leads to enhancing of browning rate as Renn and Sathe (1997) reported. The browning rates increase with the increasing of reaction mixture pH up to 10, while less browning occurs below pH 6 (38).

Tai and Ho (1998) studied the influence of pH on the volatile formation. They concluded that thiophene derivatives, polysulfides, N-containing heterocyclic compounds, and carbonyls are favorable products in the reactions at higher pH, while furan derivatives are dominated in quantity at pH 3 to 6 (40).

2.5 Temperature

Temperature effects the rate of browning, that is, the browning rate increases with raising temperature (10, 14, 38, 45, 50, 51, 52, 53). In model systems, the development of browning increases 2 to 3 times for each 10°C raises in temperature (10). High temperature (> 100°C) is often used to reduce the time required for browning, but could change the significance of various steps and thus would not be representative of typical food storage conditions (3).

Labuza and Saltmarch (1981) observed the effect of temperature on browning and protein quality losses in whey powder. They noted that the rate of browning and the loss of protein quality increase with storage temperature. The major finding in their studies is that the reaction rate with fluctuating condition of 25 to 45°C is greater than constant condition (25, 35, and 45°C) at the calculated mean temperature (50).

Shue and Lawrence (1994) studied the effect of temperature on the volatile compounds formed in the reaction of glucose and ammonium hydroxide. They noted that the number of volatile compounds increase as the reaction temperature increases from 75 to 150°C and the optimal temperature to generate the highest yield of pyrazine is found to be 120°C (27).

Eichner and Schuirmann (1994) investigated the influence of temperature on the formation of imidazoquinolines and imidazoquinoxalines (IQ compounds). They found that IQ compounds are formed at moderate temperature and increasing temperature causes a large increase in the rate of formation (42). Bates et al. (1998) indicated that the color formation in a starch-glucose-lysine system is temperature dependent due to a linear relationship between 120 and 155°C at all pH values in Arrhenius plots (54). Hidalgo et al. (1999) found that the color and fluorescence increased as the temperature increased during incubation of Bovine Serum Albumin (BSA) with carbohydrate or an oxidized lipids (35).

Further experiment result in agreement with the previous paper. The Maillard and oxidized lipid/protein reactions are not favored under the same reaction conditions. At low or moderate temperature oxidized lipid/protein reactions is more important for causing antioxidant activity. On the other hand, carbohydrate/protein reaction causes more antioxidant activity at high temperature (41).

2.6 Water activity (A_w)

Moisture content and water activity (A_w) have important effects on the rate of browning (10). As A_w is increased, browning increases to maximum at A_w of 0.5-0.8 and then decreases at greater A_w (3, 10, 45). When A_w is increased, the solution become less viscous as water molecules are less bound (3, 45). At low A_w, the solution viscosity is limited (45) because the water is tightly bound to surface polar sites by hydrogen bonds and is generally unavailable for reaction (3). Reduction of browning rate during storage can be achieved by using a high A_w, but is not practical for commercial because of the unmanageably high viscosity (45).

Labuza and Saltmarch (1981) reviewed that the influence of the state of water, as characterized by its thermodynamic availability or A_w, on the rate of browning and substrate loss in reduced moisture systems (50). Eichner and Schuirmann (1994) also studied the influence of A_w on the formation of IQ compounds and concluded that A_w of the food plays an important role in the IQ compounds formation (52).

Lu et al. (1995) and Bates et al (1998) suggested that the browning rate depends on A_w, i.e., the color development increase as A_w increases, while the activation energy was inversely related to A_w (54, 55). Thus the effect of water on the activation step of the browning reaction was less likely (55). Dayies et al. (1998) hypothesized that only soy protein underwent browning in the absence of glucose; otherwise the rate of browning and fluorescence increased with increasing temperature and A_w (56).

In case of moisture migration, water and solute diffusion rates increase with increasing temperature. These increase by many orders of magnitude as the temperature passes trough the glass transition temperature (T_g) (49). There are two

papers reported the influence of A_w and T_g, the temperature at which an amorphous system changes from a relatively immobile and stable glass to a system where molecular mobility is increased and changes (57), on the chemical stability of foods (57, 58).

Karmas and Karel (1995) pointed out that A_w, glass transition, and crystallization of the matrix influence the brown pigment formation (57). Another paper of Bell et al. (1998) studied that the effects of A_w and T_g on the kinetics of glycine loss via the Maillard reaction. They found that rate constants are higher in systems with lower glass transition temperature at constant water activity (58).

2.7 Metal ions and oxygen

Metal ions and oxygen have been well known to catalyze the Maillard reaction; however, some metal ions like iron (II) can also affect color formation in latter stages of the reaction (31). It is still unclear about the effects of oxygen and transition metals on the formation of advance glycation end products (AGE) under physiological condition (44).

Hayase et al. (1996) investigated the effects of oxygen and transition metal ions on the Maillard reaction of lysozyme and glucose under physiological conditions. They noted that transition metals accelerated the formation of Amadori compounds via Schiff base without oxygen interferes. In addition, they proposed the reaction pathway for AGE in a lysozyme-glucose reaction system as summarizes in Fig 21.

However, the participation of oxygen and transition metals in the formation of AGE via 3-deoxyosone has not been sufficiently investigated (44)

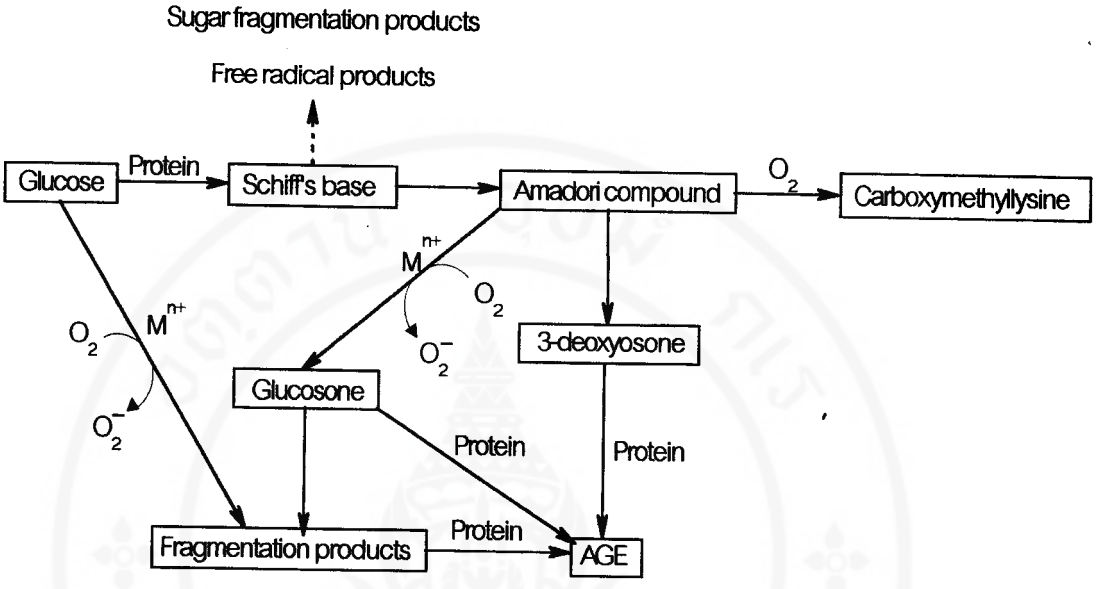


Figure 21: Proposed reaction pathway for advanced glycation end products (AGE) formed by the protein-glucose reaction system (44).

Note: Solid line, experimentally proven pathway; dotted line, speculated pathway

Rizzi (1997) reported that iron (II)-catalyzed glucose-glycine reaction produces a red colored, nondializable melanoidins, with retained 60% of the iron from the original reaction mixture and Amadori compound also generates a red-brown color when it is allowed to react with iron (II) plus molecular oxygen (31).

O'Brien and Morrissey (1997), using potentiometric proton-liberation experiments, demonstrated binding of Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ca^{2+} by the glucose-glutamate Maillard reaction product (MRP). The strength of binding occurred in the order $Mg^{2+} > Cu^{2+} = Ca^{2+} > Zn^{2+}$ (43).

Narayan (1998) indicated that copper, iron, and cobalt salts may enhance amino-carbonyl interaction, while manganese inhibits the rate of browning in air (49).

3. Significance of the Maillard reaction for foods

The great interest shown by the food industry in the Maillard reaction largely stems from a desire to produce and control the characteristic aromas and colors (9). Whilst contributing to an improvement of the organoleptic and appearance properties of foods, through aroma and color development, browning is often an undesirable side-effect of obligatory heat treatments applied for microbiological (sterilization and drying) or nutritional (cooking) reasons and for convenience (storage) (59).

Due to the multiplicity of the food systems, the complexity of the chemical reactions and the large variety of heat treatments involved, the biological out come of the Maillard reaction for foods have been classified as nutritional, and toxicology effects (1, 57).

3.1 Nutritional effects

The nutritional effects of the Maillard reaction in foods are due both to the chemical modification of essential nutrients, which thereby become unavailable and to the presence of Maillard products, which reduce the bioavailability, and disturbs the metabolism of other nutrients (57).

The physiochemical changes (color and texture) taking place in proteins during long-term storage of foods often decrease digestibility and reactive amino acid (23) such as lysine (23, 50, 57) arginine, histidine, possibly tryptophan, and cysteine (50). From a nutritional standpoint, the compromise to protein quality occurs at the first stage of the Maillard reaction. This is because mammalian proteolytic enzymes do not digest the Amadori compounds and the biological availability of lysine in food is limited (49).

Protein bioavailability is also affected by cross-linking phenomena of 2-carbon fragments, and lead to indigestible forms of protein (1, 23). Rizzi (1994) suggested that multiple amino acid residues i.e. lysine plus arginine can be involved in cross-linked protein (1). Maillard products exhibit antinutritive effects by involving complexation with metal ions or micronutrients causing the urinary excretion of zinc, copper, and iron (1, 43).

3.2 Toxicity

The interest of the toxicity of Maillard reaction products comes from the observation that the Maillard reaction involving creatinine (a typical molecule of animal tissue) or pyrolyzed proteins represent a carcinogenic potential due to the formation of heterocyclic amines (1, 59).

Cooked muscle foods seem to contain more mutagens than other food (1). A particularly potent class of mutagens, IQ compounds, may be formed by the Maillard reaction of reducing sugars, amino acids, and creatine/creatinine (1, 42). The other heterocyclic amines detected in foods are pyrolysis products from tryptophan, glutamic acid, phenylalanine, and soybean globulin (1, 59).

Generally, the mutagenic activity observed *in vitro* correlates quite well with the formation of brown pigments (4, 59). Not only that, Amadori compounds have also been shown mutagenic activity via *N*-nitoso derivatives, nitrosamine (4). Examples of heated foods with such a weak mutagenic activity are some commercial caramel preparations, hydrolyzed plant proteins, and roasted coffee. However, it has been confirmed in animal feeding studies that melanoidins containing foods such as roasted coffee and roasted coaco are neither mutagenic nor carcinogenic (59).

4. Soy sauce

Soy sauce is a soybean based liquid product with a dark reddish-brown color (60), salty taste, and pleasant odor suggestive of its meaty flavor (61). The recorded used of soy sauce dates back 2700 years in China (60) and introduced to Japan over more than 1300 years ago (60, 62).

Soy sauce is produced and consumed mostly in Asian countries. Owing to progress in world trade, soy sauce is now consumed almost worldwide (60). The soy sauce manufacturing in the countries of South East Asia such as Indonesia, Malaysia, the Philippines, Singapore, and Thailand is usually a very old process with the manufacturing technique similar to those used in China (61, 63). Most of the soy

sauce is produced in small-scale factories following traditional Chinese techniques handed down from generation to generation (63).

In general, soy sauce carried different names, that are, Chiang-yu in Chinese, Shoyu in Japanese, Kanjang in Korean (61), Ketjap or Kecap in Indonesian, Tayo in Philippinoes (60), and See-ieu in Thai (60, 61).

The soy sauce factories in Thailand are very small when compared with the modern and sophisticated fermentation factories in Japan (61). The production in Thailand is relatively small in comparison to Japan, and is consumed mostly in the local market, while small quantity is exported (64). There are approximately 57 soy sauce factories, which are located in Bangkok area and neighboring provinces such as Chonburi and Samutprakarn (61).

Commonly, soy sauce is divided into two categories. One is fermented soy sauce and the other is chemical soy sauce (63). In fermented soy sauce, the proteins and carbohydrates contained in the raw materials are hydrolyzed very slowly at about 30°C for over 3 months (63), whereas in chemical soy sauce they are hydrolyzed very quickly by hydrochloric acid at over 80°C for 3 days (65).

According to Thai soy sauce, fermented soy sauce is classified into four types: white soy sauce, salty dark soy sauce, dark soy sauce, and sweet soy sauce (61, 66, 67, 68). The first two types have a salty taste and nature high nitrogen contents whereas the latter two have a sweet taste which results from the mixing of fermented soy sauce with sugar, molasses dextrose, or liquid glucose (61).

Table 2: Amino acids in soybean (63)

Amino acids	%
Valine	5.17 – 5.48
Leucine	7.59 – 8.45
Isoleucine	5.15 – 5.53
Methionine	1.28 – 1.53
Glutamic acid	17.90 – 19.20
Arginine	7.22 – 8.30
Histidine	2.16 – 2.52
Lysine	5.97 – 7.07
Tryptophan	1.42 – 1.64
Phenylalanine	4.80 – 5.31
Threonine	3.58 – 4.06

4.1 Raw materials used in the production of soy sauce

The major raw materials used in soy sauce production are soybeans, wheat, and brine.

4.1.1 Soybeans

Soybeans are a well-known source of vegetable protein for Asian people (61). Soybeans contained high valuable amino acids as shown in Table 2 (65).

The chemical composition of the beans of varies among growing areas and species. In general, soybeans contain 34.3% protein, 26.7% carbohydrate, 17.5% fat, 4.5% fiber, 5% ash, and 12% water (61). In addition, it contains high levels of minerals and vitamins, e.g. B_6 (pyridoxine), biotin, choline, inosital, calcium, potassium, magnesium, and iron (65).

Although either whole soybeans or defatted soybean flaked can be used for production of soy sauce (60), fermented soy sauce in Thailand usually uses whole soybeans. This is become the solid residual remained after draining out the sauce, is able to make a lower grade soybean paste product (61). The advantage of whole soybeans over defatted soybeans is approximately 10% higher the protein contents than defatted soybeans (62). Furthermore, soy sauce made from whole soybeans has been reported to have lighter color, better color stability, higher alcohol and glycerol contents, smaller amount of lactic acid and reducing sugar, and better organolaptic evaluation than soy sauce made from defatted soybeans (61, 62).

4.1.2 Wheat

Wheat is the major source of carbohydrate among the raw materials used to make soy sauce (62). Although either whole-wheat grains or wheat flour is used for production of high quality soy sauce (61), wheat flour is particularly used

more than whole-wheat grain (69, 70, 71). The roles of wheat in soy sauce manufacture are as follow:

a) To reduce the moisture content of cooked soybeans from 60% to about 45%, which is unfavorable for the growth of undesirable bacteria and yeast but adequate for mold growth (61, 66).

b) To assist in obtaining the highest growth of the mycelium of mold when the starting materials, soybeans and wheat, are mixed together (65).

c) To serve as the major source of carbohydrates those are the precursor of sugar, alcohol, and organic acid (61).

d) To serve as the source of lignin and glycosides, the precursor of the vanillic flavor, and glutamic acid, which contributes to an important taste of soy sauce (61, 65).

Although the use of wheat decreases the total nitrogen content of soy sauce, it contributes to aroma (61), flavor, and color of the product (65). Yokotsuka (1986) noted that wheat bran is able to use instead of wheat flour (62). The result is the higher nitrogen content, the lower alcohol content, a darker color (62), and inferior color stability due to the increased amount of pentose in soy sauce (61).

4.1.3 Salt

Salt is an essential mineral for human. Sea salt is generally used in soy sauce production (65, 66). The role of salt has been demonstrated as follow:

a) To select the type of microorganisms that grow in the moromi fermentation stage and during the aging process (61, 66)

b) To permit the development of the flavor and aroma forming lactic acid bacteria and yeast (72)

c) To serve as a salty taste of the product (65)

The chemical compositions of sea salts mainly are NaCl, the others such as $MgCl_2$, $CaCl_2$, $MgSO_4$, $CaSO_4$, and KI (65).

4.2 Production of soy sauce

The manufacturing process of fermented soy sauce consists of three major stages including Koji making, brine fermentation (moromi), and refining as shown in Fig 22 (61, 62, 69, 70).

4.2.1 Koji fermentation

Making Koji is a characteristic technique of the orient (63). The word Koji refers to the enzyme preparation produced by growing a mold on steamed rice or other cereal (61) and enzyme convert the carbohydrates and proteins of the raw materials into sugars, peptides, and amino acids (63). The substances converted by the enzymes of Koji become the nutrients of lactic acid bacteria and yeast in the subsequent brine fermentation (63).

In Koji making of Japanese soy sauce, cleaned soybeans or defatted soybean flakes are soaked in water to increase the moisture content (30-45% moisture) (61), and then cooked with steam under pressure (60, 61, 63). Traditionally, the soybeans is steamed or boiled at atmospheric pressure (63). The soybeans become very soft and were dark brown in color due to the residue heat in the cooker (63). Nowadays, soybeans are cooked at a gauge pressure of 6-7 kg/cm^2 pressure (about 170°C) for 20-30 sec (62, 63).

For Koji making of Thai soy sauce, soaked soybeans are cooked with boiling water approximately two hours. Recently, many factories have changed to

cook with steam at 10-13 pounds pressure for one hour. Subsequently, the cooked beans are drained and quickly cooled (61).

Unlike soybeans, wheat is roasted without adding water (63). There are three methods to roast wheat flour: a shallow pan, the most unique and easiest but lowest efficiency; a rotary oven; and a rotary oven with sand which recycles in the oven and is kept separately from the roasted wheat (60, 62, 65). If wheat is insufficiently roasted, its raw starch or β-starch cannot be digested by the mold amylase and becomes white particles in the presscake of mash (62). However, if wheat is overroasted, the protein digestibility decreases and darker color increased (62, 65). The β-starch in wheat kernels must be changed into α-starch by adequate roasting in order to be digested by mold amylase (62).

In the traditionally way of preparing Koji, equal parts of the cooked soybeans and roasted wheat are mixed together and inoculated with 0.1 to 0.2% of pure starter culture mold (*Aspergillus oryzae* or *A. sojae*) in wooden trays with a thickness of 3-5 cm (60, 61, 62, 63). This starter culture is called "Koji starter" or "seed mold" (63). After that, the mixture is kept for 2-3 days in a Koji making room (60, 61) with the temperature between 20-35°C (60). The materials are cooled by hand mixing when their temperature raises to about 35°C or more due to the growth of molds (61, 62).

During incubation period, the temperature, moisture, and aeration are controlled to allow the seed Koji to grow in the mixture, to prevent development of bacteria, and to enhance production of proteolytic and amylolytic enzymes (62). A rather high moisture content (27-37%) is necessary for good mycelial growth and enzyme activity (61, 62). The resulting product (clear yellow to yellowish-green in

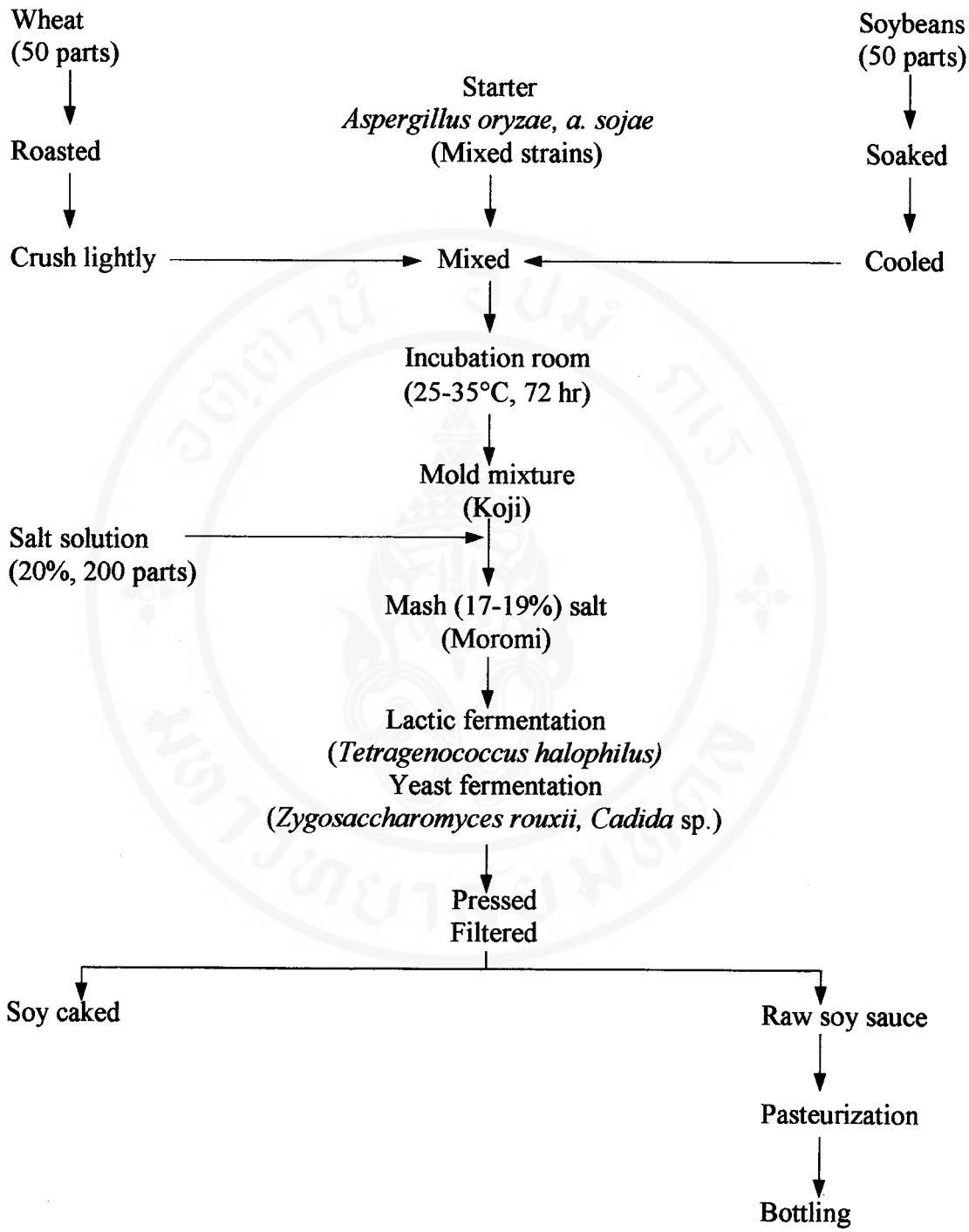


Figure 22: Process for manufacturing of soy sauce (61, 62).

color) is called "Koji", which is a mixture of hydrolytic enzymes and the substrate (60).

In the preparation of Thai soy sauce, the cooked beans are mixed with wheat flour or rice flour. The amount of flour added varies from place to place; generally, a weight of flour about 1/3 to 1/2 of the weight of dry soybeans is used. Some factories use a rotary drum or cement mixer for this mixing step. Nowadays, many factories added 0.1% of a pure spore inoculum of *A. oryzae* into the bean-flour mixture for good quality Koji making (61).

4.2.2 Moromi fermentation

The second step in the manufacture of fermented soy sauce is brine fermentation (63) or moromi fermentation (61) or mash fermentation (60, 62). This fermentation utilizes halophilic lactic acid bacteria and salt-tolerant yeast (63). The brine solution used effectively excludes undesirable microorganisms, but promotes growth of halophilic lactic acid bacteria and salt-tolerant yeast which can withstand high salt concentration (20-25% w/v) (61, 63).

The harvested Koji is mixed with an equal volume or more of saline water containing at least 20% salt in the container, which can be wooden, easthern jars, concrete, or fiberglass (60, 61, 63). The resultant mixture is called the moromi or mash and contains 17-19% w/v sodium chloride (61).

The initial pH value of moromi is 6.7-7.0,and gradually decreases due to the increase of halotolerant lactic acid bacteria, *Pediococcus halophylus* (60, 62, 66). This microorganism produces lactic acid to give a sour taste to soy sauce and the acid plays an important role in making the saltiness milder (61). The pH decreases of the moromi from 6.5 to 5.0 encourages growth of halophilic yeast such as

Zygosaccharomyces rouxii which produce ethanol and other flavoring compounds (61, 63). Recently technique, pure cultures of *P. halophylus* and *S. rouxii* are sometimes added to the moromi to accelerate the alcohol fermentation and to shorten its development time (60, 61, 63).

For moromi fermentation period, in Japanese soy sauce, the moromi is held for one year at ambient temperature or 6-8 months under controlled temperature (63), while the moromi fermentation of Thai soy sauce is carried out under direct sunlight for 1-3 months (61, 66). The moromi is occasionally agitated with compressed air to ensure uniform salt concentration and pH value, to promote microbial growth, and to prevent the growth of too much film-forming yeast on the surface of moromi during the middle and latter stages of fermentation (61, 62).

During the fermentation period, the enzymes from Koji mold hydrolyze most of protein to amino acids and low molecular weight peptides (63). With a few exceptions the amino acids occur in the same amounts and proportions as those in the original Koji mixture. The exceptions include arginine, which is converted into ornithine; tryptophan and cysteine, which are unstable in the fermenting mixture; and tyrosine, which precipitates out due to its low solubility in water (73).

Much of starch is converted into simple sugars, which are fermented primarily to lactic acid and alcohol by lactobacilli and yeast, respectively (63). Yokotsuka (1986) noted that the diversity of these lactobacilli relates to aroma, pH, and color of soy sauce, due to the metabolic roles of organic acids and the presence of sugars and amino acids (Table 3) (62).

Table 3: Various metabolic patterns by Lactobacilli in soy sauce (62).

1. Homofermentation: Glucose → 2 mole lactic acid

2. Heterofermentation: Glucose → 1 mole lactic acid, ethanol, acetic acid, CO_2, H_2, acetone, butanol

3. 67 patterns of metabolic manners for arabinose, lactose, melibiose, and sorbitol

4. Metabolic manners for amino acids and citric acid:

Histidine	→	Histamine + CO_2
Tyrosine	→	Tyramine + CO_2
Arginine	→	Omithine + $2NH_2$ + CO_2
Citric acid	→	Acetic acid + malic acid → Lactic acid + CO_2
Aspartic acid	→	Alanine + CO_2

During the aging period, *Candida* sp. produces various kinds of aroma compounds characteristic of soy sauce, such as 4-ethylguaiacol and 2-phenylalcohol (61). *Candida* sp. is halophylic and grows during the middle and last stage of moromi fermentation (84). This yeast converts glucose or other sugar into volatile alcohol (61, 63).

4.2.3 Refining process

The final process of soy sauce making is refining, which includes pressing or filtering and pasteurizing by heat (61, 63).

Pressing Once the aging of moromi is completed, the raw soy sauce is separated from any undigested soybean-flour mixture that remains by a hydraulic filter press in commercial operations or by a simple mechanical press in a domestic level (60, 61). Sometimes, fresh salt water is added to the press cake and a second fermentation is allowed to proceed for 1-2 months before a second press soy sauce is produced, which is of lower quality than the first one (60, 66). In manufacture, the residue of the moromi fermentation may be used to produce lower grade of soy sauce or soybean paste (61). The final moisture content of the press cake is less than 25% and it is used as animal feed (61, 63).

Pasteurization After pressing, the filtrate or clear-raw soy sauce is heated at 70-80°C in a kettle or in a heat exchanger for 15 min to stop microbial activities, denature enzymes, and coagulate proteins (60, 61, 62, 66). Sometimes a filter aid is added to the pasteurized soy sauce to enhance clarification (60)

The major changes resulting from this heating are the formation of an agreeable flavor and dark brown color, the separation of heat-coagulant substances, an

increase in acidity, clearity, and antiyeast potency, a decrease in the reducing sugar and amino acid content, and the evaporation of volatile compounds (62).

The pasteurization yield benefits as follows (62, 63):

(a) Increased concentration of phenolic compounds such as aldehydes and acetals, mercaptans and mercaptols, organic acids, pyrazines, furfurals, and others, which contribute to flavor and aroma characteristic of soy sauce.

(b) Developed of a reddish brown color.

(c) Improved clearity by precipitating heat-coagulable substance.

(d) Increased resistance to growth of film yeast by production of organic acid and phenolic compounds.

(e) Inactivated most of enzymes.

After clarification of the soy sauce, the clear soy sauce is bottled and marketed. Preservatives such as benzoic acid or propyl- or butyl *p*-hydroxy benzoate are added to the filtered soy sauce during pasteurization (60). In Japan, 0.005% butyl-*p*-hydroxybenzoate or 0.02% sodium benzoate are widely used while in Thai soy sauce; sodium benzoate is used at a concentration not over 0.01% (61).

4.3 Chemical composition of soy sauce

4.3.1 Typical composition

The typical composition of good quality soy sauce contains 1-1.8% total nitrogen, 2-5% reducing sugars, 1-2% organic acids, 2-5% ethanol, 1-1.5% polyalcohol (primarily glycerol), 17-18% sodium chloride, and 4.7-4.8 pH (61, 73). About 45% of the total nitrogen are in the form of simple peptides, 45% in amino acids, and the remaining 10% in ammonium compounds (73).

The major amino acid composition in fermented soy sauce is glutamic acid and aspartic acid. Arginine, lysine, phenylalanine, serine, thereoine, leucine, isoleucine, valine, alanine, and proline are also present in significant quantity (61).

Fermented soy sauce contains the following sugars in terms of %(w/v) of soy sauce: total sugar in terms of glucose 4.45%, mostly are composed of monosaccharide 2.65% (glucose 2.05%, galactose 0.17%, arabinose 0.08%, mannose 0.06%, xylose 0.06%, and unidentified sugar 0.23%); disaccharide 0.65%; and polysaccharide 1.15% (61, 62, 73).

Total organic acid was 0.95% (61) and major organic acid consisted of lactic acid 0.68%, acetic acid 0.16%, citric acid 0.04%, succinic acid 0.05%, and formic acid 0.02% (61, 73). Fukushima (1978) noted that the minor organic components are propionic, pyruvic, isovaleric, *n*-caproic, benzoic, α-ketobutyric, palmitic, oleic, linoleic, and isobutyric acid (73).

4.3.2 Volatile ingredients

Many of the volatile compounds have in Japanese soy sauce are identified as contributors to the fragrance of soy sauce (61, 62). These include 37 hydrocarbons, 32 alcohols, 41 esters, 15 aldehydes, 4 acetals, 19 ketones, 24 acids, 17 phenols, 16 furans, 8 lactones, 6 furanones, 5 pyrones, 27 pyrazines, 7 pyridines, 6 other nitrogenous compounds, 16 sulfur-containing compounds, 4 thiazoles, 3 terpenes, and 3 others (62). The differences in the contents of the major flavor ingredients and various alcohols of Japanese soy sauce are indicated in Table 4.

Table 4: Contents of major flavor ingredients in Japanese soy sauce (62).

compound	content (ppm)
2-Methyl-1-propanol (isobutyl alcohol)	3.07-18.35
1-Butanol (*n*-butyl alcohol)	1.41-11.48
3-Methyl-1-butanol (isoamyl alcohol)	4.47-22.45
3-Hydroxy-2-butanone (acetoin)	5.05-8.44
Ethyl-2-hydroxypropionate (ethyl lactate)	7.35-27.12
Furfuryl alcohol	4.35-10.07
3-(Methylthio)-1-propanol (methionol)	2.60-4.47
2-Phenylethanol	3.71-10.25
4-Hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF)	1.83-5.39
4-Ethyl-2-methyoxyphenol-(4-ethylguaicol) (4-EG)	1.12-3.67
4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(*2H*)-furanone (HEMF)	177.78-418.67
4-Hydroxy-5-methyl-3(*2H*)-furanone (HMMF)	84.54-153.58

Note: See structure at appendix A

Seo et al. (1996) identified 62 flavor compounds in traditional Korean soy sauce. Twenty-two components are also present in the Japanese soy sauce, while forty components have not been reported in the Japanese soy sauce such as bezenemethanol, furfuryl acroloin, ethenylpyrazine, and 3-quinolinamine (75).

A. Organic acids Most of the organic acids found in soy sauce have fairly high threshold values except iso-valeric acid. α-Ketobutyric acid is a strong soy sauce-like aroma with the threshold value equal to 0.04 ppm (62).

B. Alcohols The important alcohols result from their relative threshold values and high proportion (62). Besides ethanol, many kinds of trace amount of alcohols are detected as volatile compounds such as *n*-butanol, isobutanol, isoamyl alcohol, 2-phenyl ethanol, furfuryl alcohol, isopropyl alcohol, and benzyl alcohols (62, 75). Yokotsuka (1986) demonstrated that the amount of furfuryl alcohol increases during pasteurization of soy sauce. Hence, its quantity indicates the degree of pasteurization (62).

C. Esters Yokotsuka (1986) indicated that there are more than forty various types of esters have been isolated from soy sauce. Almost all, except from butyl acetate and isoamyl acetate, are ethyl esters such as ethyl acetate, ethyl propinoate, and ethyl malonate (62). Based on the content of ethyl alcohol and lactic acid, ethyl lactate is predominant among the esters and contributes its fragrance to soy sauce odor (73).

D. Carbonyls and relative compounds The important carbonyl compounds found in soy sauce are α-dicarbonyl compounds, γ-pyrones, 4-hydroxy-3-furanones, alkylcyclopentadiones, and acetals (62).

α-Dicarbonyl compounds, which are produced by Strecker degradation (4, 9, 14), further degrade into other odorous compounds such as acetaldehyde and propionaldehyde from lactic aldehyde (62). Maltol is an important γ-pyrones separated during the heating of soy sauce. 5-Hydroxymaltol and 3-methoxy-2-methyl-4*H*-pyran-4-one were also isolated from soy sauce, although the former has a weak maltol-like flavor while the latter has no aroma (62).

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(*2H*)-furanone (HEMF) has been identified as a sweet-tasting compound, which is the most important flavor, and character impact compound of fermented soy sauce (61, 62, 73, 76). These volatile compounds are believed to be produce by microorganisms during the Koji and moromi fermentation, and through chemical reactions during the pasteurization process (61). Under basic condition, HEMF changes into the orderless compound, 4,4,5-trihydroxy-2-ethyl(or methyl)-5-methyl(or ethyl)-3-tetrahydrofuranone (OX-HEMF) (62).

4-Hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF) and 4-Hydroxy-5-methyl-3(*2H*)-furanone (HMMF) and HEMF (see structure at appendix A) resemble each other in chemical structure, but have different patterns of development (62). Hayashida (1997) suggested that moromi condition, which maximize HMMF concentrations before yeast fermentation will lead to higher HEMF and HDMF concentrations to enhance flavor quality in the final product (77).

E. Phenolic compounds The phenolic compounds were reported to derive for the most part from wheat. The major constituents of this fraction are vanillin, ferulic acid, and vanillic acid, which have been observed the greatest formation during Koji cultivation. Ferulic acid and *p*-hydroxycinamic acid are

metabolized, in the latter half of the period of yeast fermentation, into 4-ethylguaicol (4-EG) and *p*-ethyphenol, respectively (62)

F. Lactones Yokotsuka (1986) reported that eight types of γ-lactones have been identified in Japanese soy sauce: 4-butanolide (γ-butyrolactone), 4-pentanolide (γ-valerolactone), 2-methyl-4-butanolide, 2-pentene-4-olide, 4-hexanolide (γ-caprolactone), 2-methyl-2-buten-4-olide, 2-hydroxy-3,3-dimethyl-4-butanolide, and 5-hydroxy-4-hexanolide (62). The schematic presentation of their chemical structure is presented in appendix A.

G. Others Sulfur-containing compounds such as dimethylsulfide and methyl mercaptan, and terpenes including with pyrazines are trace components presented in soy sauce (73). Pyrazines are formed by the condensation of the aminocarbonyl product of Strecker degradation, followed by oxidation (2). The major pyrazines of soy sauce are 2-methyl-, dimethyl-, ethylmethyl-, and trimethylpyrazine. The flavor of pyrazines is weakened by the weak acidic pH of soy sauce (4.7-4.9), but becomes dominant when pH is neutralized during cooking (62).

4.3.3 Color of soy sauce

The color of soy sauce is also important because it is closely associated with flavor (61, 62). The development of soy sauce color is derived mainly from non-oxidative and non-enzymatic browning reaction, the Maillard reaction (14, 61). Yokotsuka (1986) reported that about 50% of the color of soy sauce are formed during the fermentation and aging of moromi, and the remaining 50% during pasteurization. Both are considered to be due primarily to heat-dependent browning. He also suggested that the sugar involving primarily in the Maillard reaction is a pentose such

as xylose and arabinose although the amount of hexose in soy sauce is from 6-10 times greater than the proportions of pentose (62).

The change in color which occurs in pasteurized soy sauce during storage increases as the result of browning by heat including greatly affected by the action of air and, to a lesser degree, temperature and light (62). The color is relatively stable, when soy sauce is packed in glass bottles. It darkens rather quickly after seal is broken due to oxidation and the Maillard reaction (61). These reactions cause the organoleptic quality of soy sauce to be inferior. The increase of color intensity of pasteurized soy sauce is presumed to proceed as follow: aldose → 3-deoxyosones → color pigments. In amino-carbonyl reactions, 3-deoxyosones are reacted with the excess amount of amino acids instead of being converted into HMF, resulting in the formation of water-soluble pigments.

The color of soy sauce is dependent to the elevation of temperature. The intensity of browning increases 2.3-3 times corresponding to a 10°C elevation of temperature within the range of 50-90°C. Generally, the higher the pH value tends to be, the greater the extent of browning, but within the average range of pH value of soy sauce (4.6-4.9), there is no practical difference in the extent of heat-dependent browning (62).

The effective participation of ascorbic acid, which belongs to reductones, in the oxidative browning reaction was pointed out by Hodge (1953) (4). The non-enzymatic browning of soy sauce that occurs during storage has been attributed to the participation of intermediates of the Maillard reaction such as reductones, Amadori products, and melanoidins (62). The ascorbic acid in soy sauce changes into dehydroascorbic acid with oxidation, which in turn reacts with amino

acids to deepen the color of soy sauce (4, 62). The oxidative browning and the reductone formation, which took place during the heat treatment, were almost proportional to the starting color intensity of pasteurized soy sauce (62).

It is generally acknowledged that deterioration of the soy sauce flavor is related to its oxidative browning. It is more highly correlated with increased darkening (caused by oxidative browning) than with increased color intensity (heightened red color, which occurs with heating). For the mechanism of brown color formation, it is explained in the previous part.

4.4 Safety of soy sauce

Soy sauce mold mostly belongs to the *Aspergillus oryzae*, *A. sojae* (60, 61, 62), and *A. temarii* (60, 62), whereas the mycotoxin (e.g. chratoxins, patulin, sterigmatocystin, islanditoxin, T-2 toxin, cyclopiozonic acid (61) especially alflatoxin-producing molds belong to *A. parasiticus* (60, 61). Although fluorescent compounds are produced by *Aspergillus* molds with Rf value on TLC resembling those of aflatoxins (60, 62), there is no harzard in human consumption of soy sauce (60).

CHAPTER III

MATERIALS AND METHODS

1. Materials and chemicals

Koji, moromi, and soy sauce products were obtained from Thaisang and Vichitrungruang soy sauce factories, while Kikkoman soy sauce was purchased from supermarket.

The 5-hydroxymethyl-2-furaldehyde (HMF) standard, nitric acid (HNO_3), diphenylcarbazone indicator, and phenolphthalein indicator were obtained from Fluka (Buchs, Switzerland). Bovine Serum Albumin (BSA) standard, and concentrated sulfuric acid (38% H_2SO_4) were purchased from Sigma Chemical Co. (MO, USA) and Lab-SCAN Ltd. (Ireland), respectively. Other chemicals used in all experiments were laboratory grade and purchased from Merck (Darmstadt, Germany).

2. Investigation of the Maillard reaction mechanism during storage

2.1 Chemical changes

Thai soy sauce samples, from Thaisang and Vichitrungruang production, were used to study comparing with Kikkoman, a Japanese soy sauce.

Soy sauce samples (30 ml) were incubated at 37°C for three months in Erlenmeyer flasks covered with paraffin and aluminum foil. The samples were withdrawn every two weeks and kept at -20°C for further analysis.

2.1.1 Determination of browning

The browning was determined by monitoring the absorbance at 420 nm (OD 420 nm) in a spectrophotometer (Novaspec®II).

2.1.2 Determination of reducing sugar by dinitrosalicyclic acid assay

The 3,5-dinitrosalicyclic acid (DNS) reagent was prepared by dissolving 0.25 g of DNS and 75 g of Rochelle salt or sodium potassium tartrate ($C_4H_4KNaO_6 \cdot 4H_2O$) in 50 ml of 2 M NaOH (4 g NaOH in 50 ml water) and diluting to 250 ml with water. Samples, D-glucose standard, and the control (0.2 ml) were added with 2.0 ml of the DNS reagent. The mixtures were heated at 100°C for 10 min, then rapidly cooled to room temperature and determined the absorbance at 570 nm (78).

2.1.3 Determination of reactive amino group by 2,4,6-trinitrobenzenesulfonic acid (TNBS)

To 0.05 ml of sample, standard glycine, and the controls were added with 0.1 ml of 0.1% TNBS (1 ml TNBS in 100 ml water) and 2.5 ml of 4% sodium hydrogen carbonate (4 g $NaHCO_3$ in 100 ml water and adjusted pH to 8.4). The solution was allowed to react at 37°C for 90 min. Then 0.1 ml of 1% SDS (1 g SDS in 100 ml water) and 0.1 ml of 1 *N* HCl (0.83 ml conc. HCl in 100 ml water) were added to solubilize the protein and prevent its precipitation, respectively. The absorbance of the solution was read at 340 nm (79).

2.1.4 Analysis of HMF by HPLC

For determination of HMF, the sample was deproteinized with 5% trichloroacetic acid (TCA) by centrifugation at 10,000×g (Sigma 202M, USA).

The supernate was neutralized with 2*N* NaOH before analyzed with an HPLC (Shimazu 6A HPLC set, Japan). The column was 5 mm × 21 mm C_{18}-column (UG 12, Shiseido Corp., Tokyo). The mobile phase was 5% acetonitrile in 0.2% phosphoric acid (H_3PO_4) (see Appendix B), at the pH of 4.2 as the mobile phase. The flow rate was 1 ml/min. The column temperature was 40°C. HMF was detected at 280 nm by using UV-VIS spectrophotometer (SPD-6AV, Shimazu, Japan).

2.1.5 Determination of Amadori product (ARP)

The protein precipitated from deproteinization soy sauce with 5% TCA was redissolved with 0.1 ml of 1 *N* NaOH and 0.9 ml of distilled water, and then analyzed protein and sugar-corporated to protein by Bradford method (80) and phenol-sulfuric acid (78), respectively. The amount of ARP was determined as the ratio of sugar to protein.

Bradford method The dye reagent was prepared by dissolving 200 mg of Coomassie Brilliant blue G-250 (CBB G-250) in 100 ml of 95% ethanol. The dye solution was added with 200 ml H_3PO_4 before diluted to 2 liters with distilled water. Samples (redissolving solution of precipitated protein), BSA standard, and the blank (0.2 ml) were added with 2.0 ml of dye reagent. The solutions were left at room temperature for 20 min, and then read the absorbance at 595 nm.

Phenol-sulfuric acid assays The samples, D-glucose standard, and the blank (0.4 ml) was added with 0.4 ml of 5% phenol reagent (5 g phenol in 100 ml water). The mixtures were rapidly added with 2 ml of concentrated sulfuric acid (38% H_2SO_4) rapidly and directly to the solution surface without allowing it to touch the side of the tube. The solutions were left at room temperature for 10 min, then shaken vigorously and determined the absorbance at 490 nm after 30 min.

2.2 The change of sugars by HPLC

Soy sauce samples were deproteinized by centrifugal ultrafitration at 5,000 Dalton molecular weight cut. The ultrafiltration tube (Millipore, Japan) was washed by centrifugation with 100 µl of distilled water (filtered through 0.45 µm membrane), at 5000×g for 10 min. After that 100 µl of sample was added, and centrifuged at 5000×g for 30 min before submitted onto HPLC.

The conditions of HPLC were 300 mm × 8 mm Ionpak KS-801 column (Shodex, Japan) and distilled water as the mobile phase with the flow rate of 1 ml/min. The column temperature was 60°C and refractive index (RI) detector (refractoMonitor®IV, LDC Analytical, USA) was used to detect the change of sugar profile. D(+)-Xylose, L(+)-arabinose, D(+)-glucose, and sucrose were used as standards.

3. Studying of the effects of metal ions on the Maillard reaction

The Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were used as the sources of Fe^{2+}, Fe^{3+}, and Cu^{2+}, respectively. Each metal ion solution was prepared at the concentration of 7.5 µM and diluted with water to 0.5, 1.0, and 3.0 µM. The metal ion solutions (1 ml) were added to the soy sauce samples (29 ml) obtained from Thaisang factory before covered with paraffin and aluminum foil. The mixtures were incubated at 37°C for 3 months and withdrawn at 0, 2, 4, 6, 8, and 12 weeks. The samples were kept in a freezer (-20°C) for analyses of the Maillard reaction indices (browning, reducing sugars, reactive amino groups, Amadori products, and HMF).

4. Studying the effects of iodine supplementation on the Maillard reaction

The potassium iodide (KI) was prepared at the concentration of 5, 10, 30, and 75 mM, and 1 ml of each these solutions was added to soy sauce samples (29 ml) obtained from Thaisang factory. The solution mixtures were kept at 37°C for 3 months and withdrawn at 0, 2, 4, 6, 8, and 12 weeks. The samples were kept at 37°C for chemical analyses of the Maillard reaction indices.

5. Investigation of the influence of sugar supplementation on the Maillard reaction during storage

Soy sauce obtained from Thaisang factory was added with sugar at various concentrations, i.e. 0, 0.5, and 1.0 M. The mixtures were incubated at 37°C for 3 months with paraffin and aluminum foil cover. The samples were withdrawn at every month and kept at -20°C for analyses of browning, reducing sugars, and reactive amino groups.

The sugar solutions were prepared by dissolving 1.5, 1.8, 3.6, and 3.42 g of xylose, glucose, maltose, and sucrose, respectively, in 5 ml of water and diluted to 10 ml.

6. Elucidation of the Maillard reaction mechanism during moromi fermentation

6.1 Chemical changes during moromi fermentation

6.1.1 Preparation of moromi

Koji and moromi were prepared by the traditional process at Thaisang and Vichitrungruang soy sauce factories. Soybeans were soaked in water for 3-4 hr and cooked with the pressure cooker for 3 hr. The cooked soybean was cooled to

35°C, then mixed with wheat flour at a ratio of 20:3 (w/w). The mixture was inoculated with Koji starter culture (0.1% w/w) and kept in Koji room at 30-35°C for 36 hr. The seed Koji was placed in fiberglass tanks. Brine (20%) was added in the usual ratio of approximately 2.5 liters to 1 kg of Koji. The incubation period for the moromi stage was carried out for 2 months. A total of two containers in each factory were used for the experiment.

The moromi was withdrawn every 5 days at Thaisang factory and every 3 days at Vichitrungruang factory. Each sample was filtered by cotton wool for a separation of macrocolloid such as pieces of Koji and beans. The microcolloid in the filtrate was separated by centrifugation (SORVALL®RC 5C Plus, SLA-1500 rotor) at 15,000×g for 20 min. The supernate was filtered through a 0.45 μM membrane filter (Gelman, USA). The samples were kept at -20°C before determination of the Maillard reaction indices including pH, NaCl, lactic acid, and total soluble protein.

6.1.2 Determination of pH

The pH value of the sample was measured directly with a pH meter (HANNA, instrument 8417).

6.1.3 Determination of NaCl by titration with mercuric nitrate

Salt concentration was measured by titration of 25 ml of appropriately diluted sample with 0.2 M mercuric nitrate or $Hg(NO_3)_2$ (see Appendix B), using 1 ml of 0.1% diphenylcarbazone (see Appendix B) as the color indicator. The end point was determined by the appearance of a permanent dark blue-purple. The concentration of NaCl was calculated by using the following equation:

$$\% \text{ NaCl (w/v)} = \frac{2M_2V_2 \times W \times \text{Dilution factor} \times 100}{V_1 \times 1000}$$

Where V_1 and V_2 are volumes of sample and $Hg(NO_3)_2$ solution, respectively. M_2 is the concentration of $Hg(NO_3)_2$, and W is molecular weight of NaCl. Mercuric nitrate was standardized by titration with NaCl standard before used.

6.1.4 Determination of total acid by titration

Total acidity was determined by titration with 0.1 *N* NaOH (0.2 g NaOH in 50 ml water), using 2 drops of 0.1% phenolphthalein (see Appendix B) as an indicator. Total acidity (as lactic acid) was calculated by using the following equation

$$\% \text{ Total acid (w/v)} = \frac{V_2 \times M_2 \times 90 \times 100}{V_1 \times 1000}$$

Where V_1 and V_2 are volumes of sample and NaOH used, respectively. M_2 is concentration of NaOH. Molecular weight of lactic acid is 90. Sodium hydroxide solution was standardized by titration with 0.1 *N* potassium hydrogen phthalate (KHP; $C_8H_5KO_4$) before used.

6.1.5 Determination of soluble protein

Soluble protein of moromi sample was estimated from the nitrogen content, determined by the Kjeldahl's method (1026 Kjeltec® System: Version 5, Tecator) (see Appendix C).

6.2 Chemical changes during early stage of moromi fermentation

Moromi samples were prepared by Thaisang factory as mentioned before and withdrawn everyday for 10 days. The samples were prepared and analyzed as the previous experiment.

7. Studying the Maillard reaction of pasteurized soy sauce during storage

Raw and cooked soy sauce produced by Thaisang factory was sampled for studying the different mechanism of the Maillard reaction during storage. Raw soy sauce obtained from Vichitrungruang factory was pasteurized in our laboratory by heating the sample to 80°C, and cooled down to room temperature. The difference between cooked soy sauce from both factories was the cooked soy sauce produced by Thaisang factory had been seasoned before pasteurized, while the other was pasteurized without seasoning.

A soy sauce samples (30 ml) were incubated at 37°C for 3 months and were sampled every two weeks. The samples were kept at -20°C for further chemical analyses.

8. Elucidation of the effect of brine concentration on the Maillard reaction during moromi fermentation

The seed Koji prepared by Thaisang factory was placed in the glass bottles. Salt solutions were prepared at the concentrations of 18, 20, 22, and 24% (w/v) NaCl by dissolving 180, 200, 220, and 240 g NaCl in 1 liter of water, respectively. Then, the brine was added into the glass bottle at the ratio of 500 ml salt solution to 250 g of Koji. The incubation period was carried out at room temperature for 20 days. The samples were withdrawn at 0, 1, 2, 3, 4, 5, 10, and 20 days during moromi fermentation.

Moromi sample was filtered through a 0.45 μM membrane and kept at -20°C for analyses of the Maillard reaction indices, i.e. pH, lactic acid, NaCl, and soluble protein.

9. Elucidation of the influence of aeration on the Maillard reaction

Koji obtained from Thaisang factory was fermented in 20% brine with or without aeration. The aeration was done at the flow rate of 20 ml/min for 20 days. The moromi samples were withdrawn at 0, 1, 2, 3, 4, 5, 10, and 20 days, and then filtered with 0.45 μM membrane, kept at -20°C prior to analyses as the previous experiment.

CHAPTER IV

RESULTS

1. Investigation of the Maillard reaction mechanism during storage

1.1 Chemical changes

The chemical changes of soy sauce from Thaisang: (T) and Vichitrungruang: (V) and Kikkoman soy sauce (K) showed the similar pattern of the Maillard reaction progress during prolonged incubation at 37°C for 3 months. Browning of soy sauce significantly increased during storage (Fig 23a). The browning linear equations represented for soy sauce T, V, and K were $y = y_0+ax$ (Table 5), where x was storage time (3-month duration) and y was OD 420 nm. The equation indicated that the rate of browning in soy sauce T was higher than K and V, respectively.

The changes of reducing sugars (RS) and reactive amino groups (RAG) are shown in Fig 23b and 23c, respectively. RS amounts of soy sauce T decreased while those of V and K increased. The correlation equations between RS and storage time of soy sauce T, V, and K are shown in Table 6. This increase came from the seasoning with sugar. The results were confirmed by HPLC in further experiment. For the RAG in each soy sauce, although the changes were not significantly decreased as expected, the RAG tended to decrease during storage. The 5%TCA-precipitated protein (PP) of each soy sauce increased ($P<0.05$) with different rates during storage (Fig 24a).

The PP equation was $y = y_0+ax+bx^2$, where x was storage time and y was the amounts of PP (Table 5). Figure 23d suggested that the rate of PP forming for soy sauce K was higher than those of T and V. When the incubation continued to 6 months, visible precipitate in soy sauce K occurred at the bottom of the flask while no such precipitate occurred in the others.

Amadori products (ARP) were measured in terms of sugar-corporate with PP during prolonged incubation (Fig 23e). The decrease of ARP indicated that there was no accumulation of ARP bound on PP in soy sauce during storage. The correlation equation of ARP over time was $y = y_0+ax+bx^2$, where x and y were storage times (within 3 months) and ARP amounts, respectively (Table 5). HMF was monitored as an intermediate of the Maillard reaction. Figure 23f shows the accumulation of HMF increasing with time in linear manner. These results revealed that HMF occurred in Japanese soy sauce at higher concentration than in Thai soy sauce. Soy sauce T had higher rate of HMF accumulation than soy sauce V (Table 5).

Table 6 reveals total soluble protein, %NaCl, and titratable acidity of each soy sauce. The amounts of total soluble protein and titratable acidity of soy sauce K including the initial brown color indicated that Japanese soy sauce was more concentrated brown color and nutritional value than Thai soy sauce. The chromatograms of HMF in soy sauce T, V, and K were shown in Fig 24, 25, and 26, respectively.

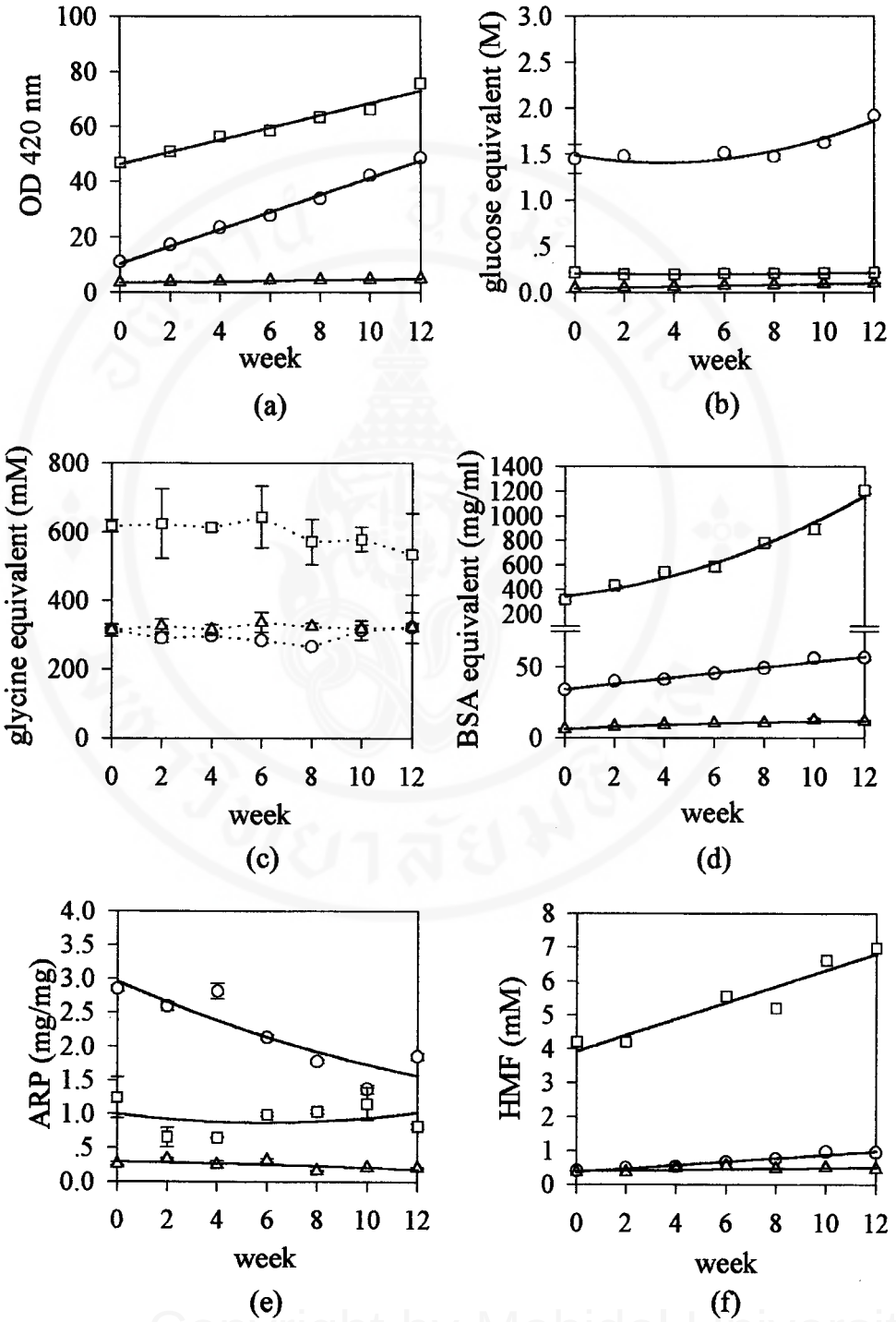


Figure 23: The change of browning (a), RS (b), RAG (c), PP (d), ARP (e), and HMF (f) in soy sauce: Thaisang (○), Vichitrungruang (△), and Kikkoman (□).

Table 5: The regression equations of browning, RS, PP, ARP, and HMF with storage time of all soy sauce.

parameter	soy sauce	equation	r^2
browning	T	$y = 10.64+3.09x$	0.997
(OD 420 nm)	V	$y = 3.66 + 0.116x$	0.991
	K	$y = 46.44 + 2.22x$	0.985
RS	T	$y = 0.149 - 0.0045x + 0.0064x^2$	0.945
(M)	V	$y = 0.047 + 0.053x$	0.995
	K	$y = 0.215 - 0.0035x + 0.0003x^2$	0.758
PP	T	$y = 34.40 + 1.967x + 0.0034x^2$	0.987
(mg/ml)	V	$y = 6.33 + 0.849x + 0.032x^2$	0.971
	K	$y = 346.60 + 22.50x + 3.82x^2$	0.991
ARP	T	$y = 2.97 - 0.160x + 0.0036x^2$	0.882
(mg/mg)	V	$y = 1.001 - 0.045x + 0.0039x^2$	0.254
	K	$y = 0.295 - 0.0036x + 0.0005x^2$	0.639
HMF	T	$y = 0.376 + 0.05x$	0.977
(mM)	V	$y = 0.401 + 0.01x$	0.596
	K	$y = 3.940 + 0.24x$	0.948

Note: x = browning (OD 420 nm); RS (M); PP (mg/ml); ARP (mg/mg) ; and HMF (mM)

y = storage time (weeks) within 3 months

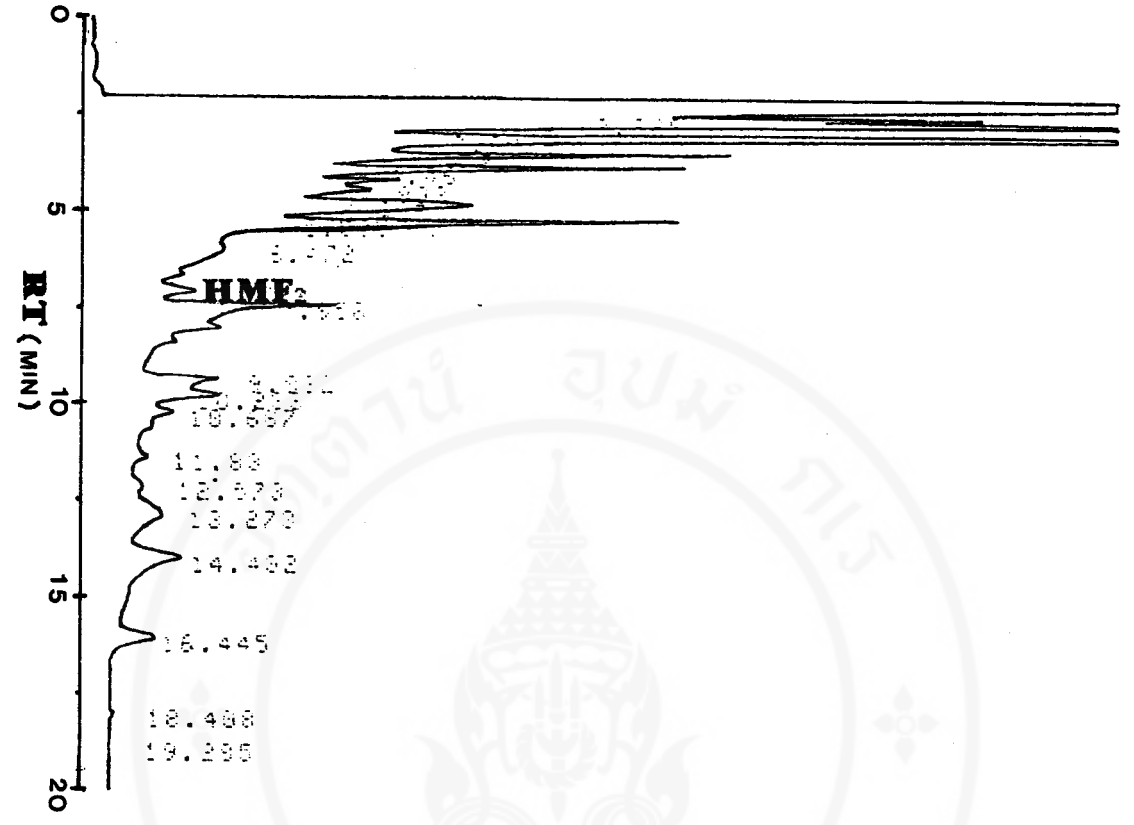


Figure 24: The chromatogram of HMF in soy sauce from Thaisang factory.

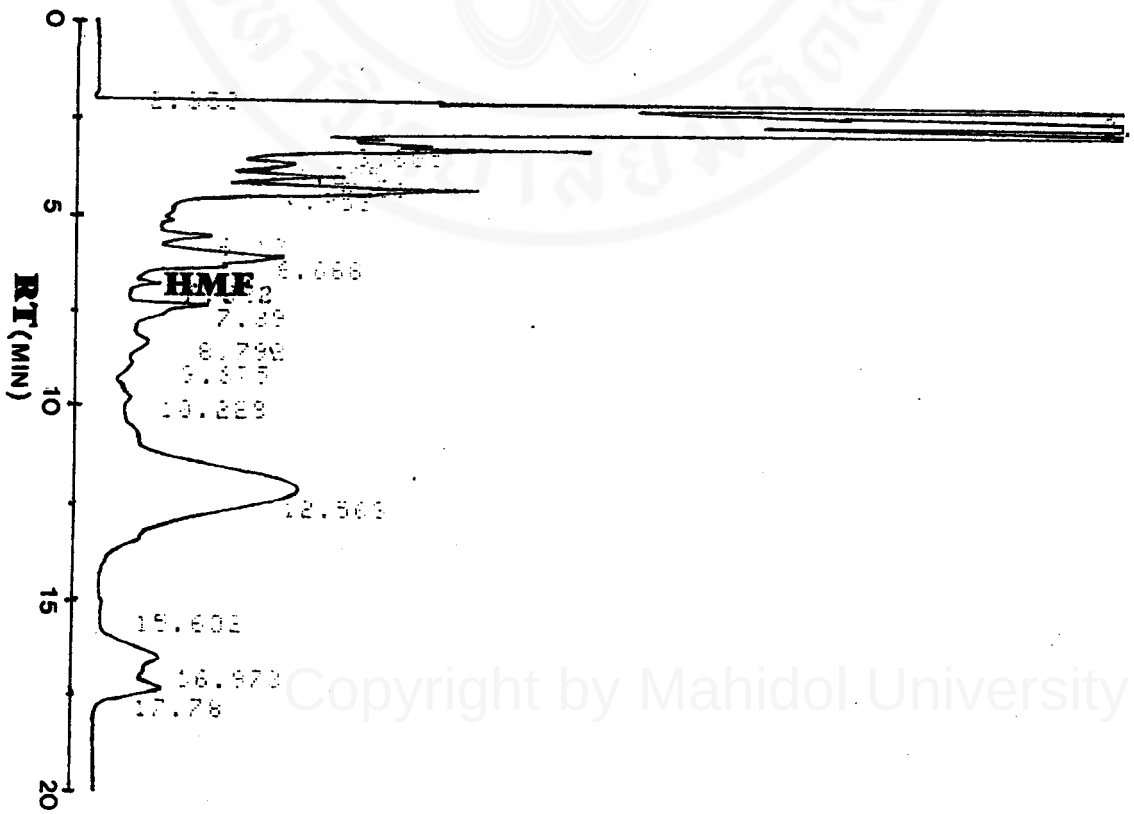


Figure 25: Chromatogram of HMF in soy sauce from Vichitrungruang factory.

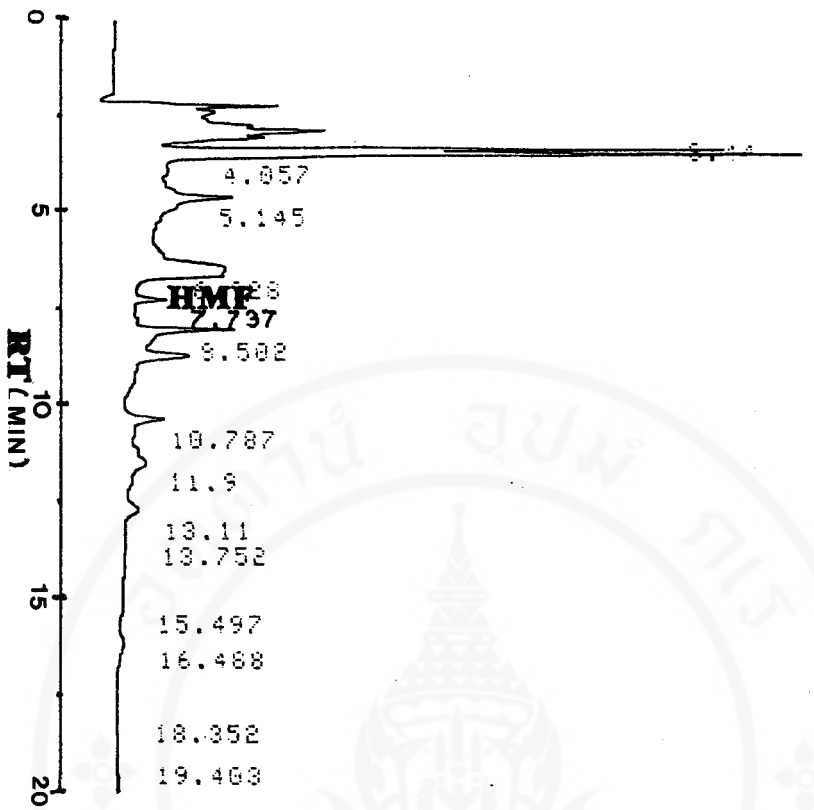


Figure 26: The chromatogram of HMF in Kikkoman soy sauce.

Table 6: Initial brown color, total soluble protein, % NaCl, and titratable acidity in each soy sauce.

soy sauce	T	V	K
initial brown color (OD 420 nm)	11.100	3.600	46.900
total soluble protein (%)	6.186	4.274	11.366
NaCl (%)	29.200	20.590	21.510
titratable acidity (%) (as lactic acid)	0.060	0.030	0.098

1.2 The changes of sugar by HPLC

The concentrations of sugars in soy sauce T, V, and K were measured by using HPLC to confirm the increase of RS in the previous study. The results indicated that reducing sugar increased in linear manner (Table 7) during the prolonged incubation (Fig 27). Soy sauce T and K showed four types of sugar, i.e., xylose, arabinose, glucose, and sucrose. The high concentrations of xylose in soy sauce T resulted from the co-elution of xylose with mannose and galactose. Soy sauce V showed the decrease of sucrose along with the increase of glucose and xylose. Soy sauce K had pentose higher than hexose and disaccharide (arabinose>xylose>glucose>sucrose).

The chromatograms of sugar components in soy sauce are shown in Fig 28. Soy sauce T had high xylose and glucose while soy sauce K had arabinose at the highest. Soy sauce V contained sucrose, glucose, and xylose but not arabinose. The highest sucrose concentration among three samples suggested that sucrose in soy sauce V might be added for seasoning.

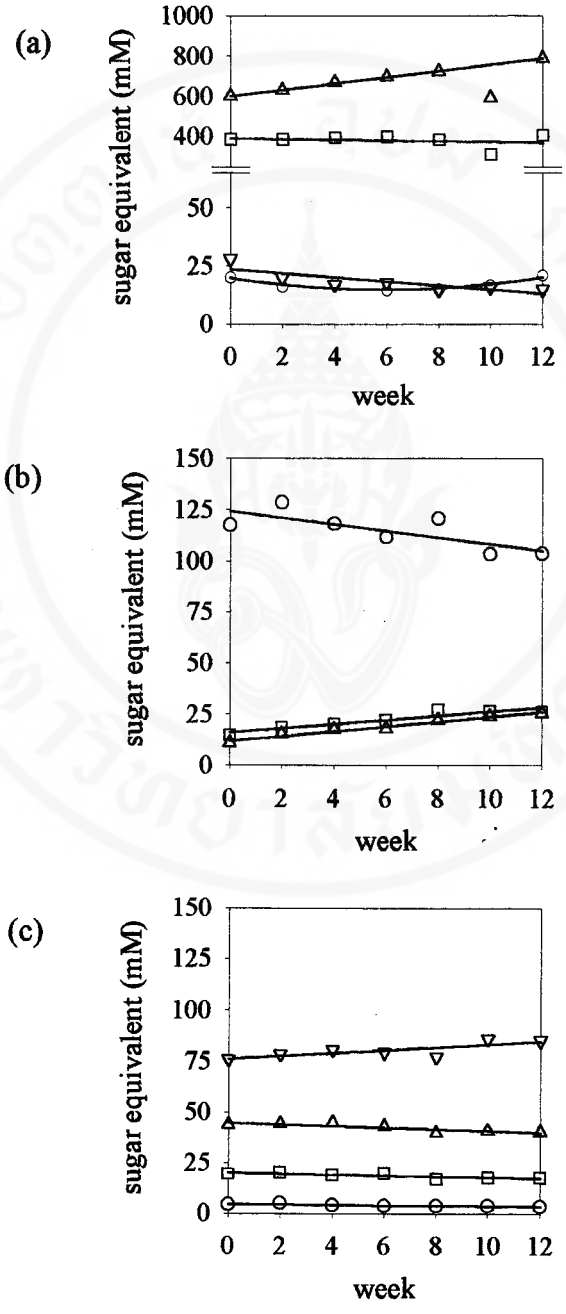


Figure 27: The change of sugars analyzed by HPLC in soy sauce from Thaisang (a), Vichitrungruang (b), and Kikkoman (c) during storage. The standard sugars were sucrose (○), glucose (□), xylose + mannose + galactose (△), and arabinose (▽).

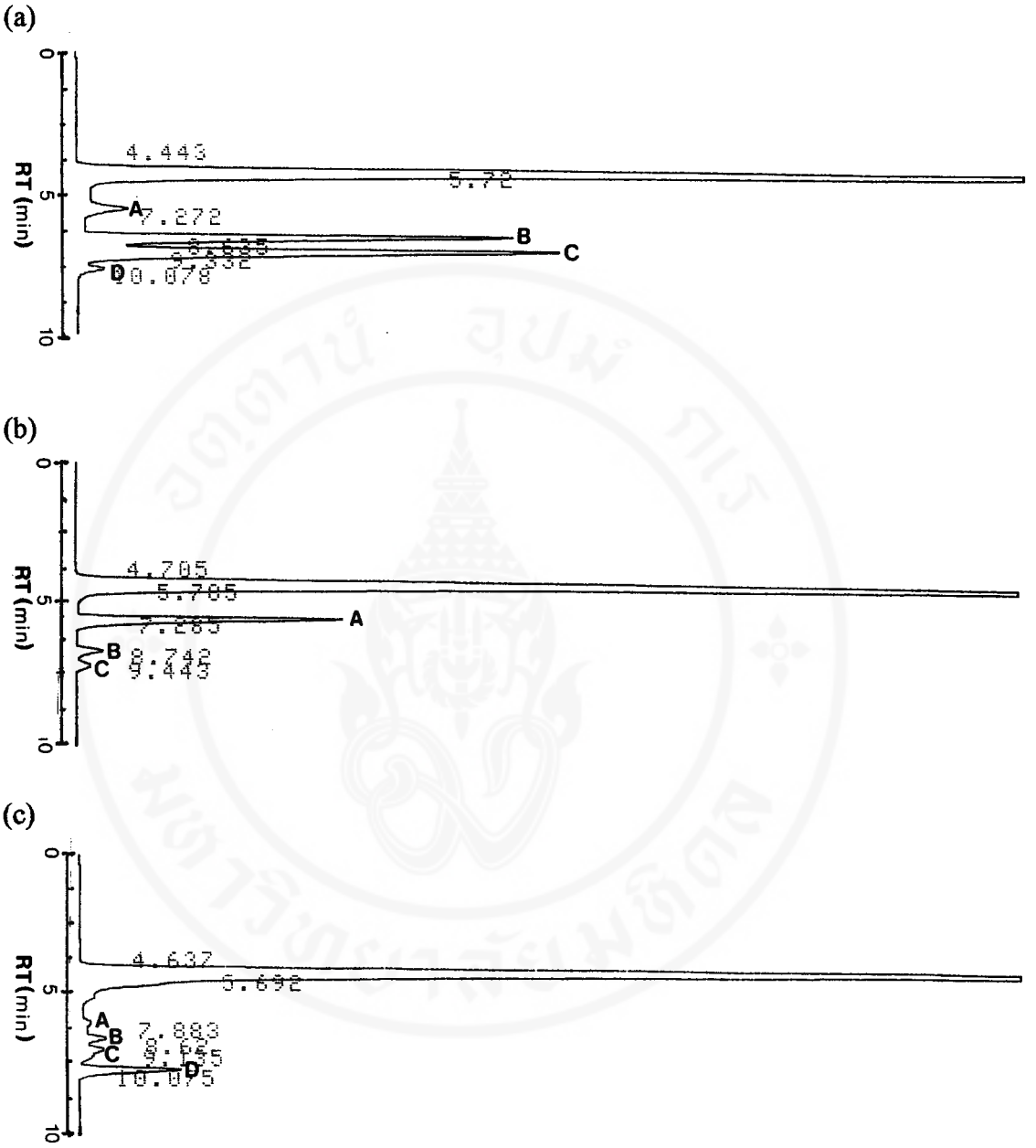


Figure 28: Chromatograms of sugars in soy sauce from (a) Thaisang, (b) Vichitrungruang, and (c) Kikkoman soy sauce. Chromatographic conditions: Ionpak KS-801 column; distilled water as mobile phase; flow rate 1 ml/min at 60°C. RI detector was used to detect sugars (A sucrose, B: glucose, C: co-elute of xylose, mannose, and galactose, and D: arabinose). 3 µl of Ultrafiltrate of each sample was injected to HPLC.

Table 7: The regression equations of sugar contents with storage time of soy sauce T, V, and K.

soy sauce	standard sugar (mM)	equation	r^2
T	sucrose	y = 17.05 + 0.027x	0.047
	glucose	y = 390.57 – 1.857x	0.251
	xylose	y = 600.91 + 15.60x	0.999
	arabinose	y = 23.68 – 0.875x	0.826
V	sucrose	y = 124.27 – 1.592x	0.751
	glucose	y = 16.09 + 1.025x	0.940
	xylose	y = 11.91 + 1.181x	0.985
K	sucrose	y = 4.47 – 0.096x	0.831
	glucose	y = 20.01 – 0.215x	0.757
	xylose	y = 44.71 – 0.399x	0.803
	arabinose	y = 76.09 + 0.715x	0.786

Note: x = sugar contents (mM)

y = storage time (weeks) within 3 months

2. Effects of metal ions on the Maillard reaction

Browning of soy sauce T in the model systems containing metal ions (Fe^{2+}, Fe^{3+}, and Cu^{2+}) at various concentrations (0.5, 1.0, 3.0, and 7.5 μM) was significantly increased with time ($P<0.05$) (Fig 29). The browning equation for all model systems is $y = y_0 + ax$, where x and y are storage time and OD 420 nm, respectively (Table 8). Each model had different rates of browning, however, there was no significantly difference among the concentrations of metal ions in each model.

The RS concentrations in each model were not changed with the incubation period (Fig 30). The RS concentration in model containing Fe^{2+} showed significant difference between concentrations of metal ions, i.e., control>0.5>1.0>3.0>7.5. The model containing Fe^{3+} and Cu^{2+} had no significant difference among the concentrations of metal ions but significant difference from the control. These results demonstrated that the concentration of metal ions in each model affected on the change of RS. The RS equations represented for all model systems were $y = y_0+ax+bx^2$, where x and y were storage time and RS amounts (glucose equivalent), respectively (Table 9). The decrease of RAG in each model were different from control ($P<0.05$) (Fig 31). Models containing Fe^{2+} and Cu^{2+} had lower RAG than control while model containing Fe^{3+} had higher RAG than control. The equation of RAG represented for all models was $y = y_0+ax+bx^2$, where y was the glycine equivalence and x was storage time (Table 10).

The increase of PP and the change of RAG for all models were in the manner of quadratic function (Table 11). PP amount of model containing Fe^{2+} was lower than control and each concentration of Fe^{2+} significantly influenced on the increase of PP

(Fig 32). However, 7.5 μM of Fe^{2+} did not effect on the PP. Regarding models containing Fe^{3+} and Cu^{2+}, their concentrations did not influence on PP

The decrease of ARP bound on PP as a function of time is shown in Fig 33 and the equation represented for the change of ARP was $y = y_0+ax+bx^2$, where x and y were storage time and ARP amounts, respectively (Table 12). Fe^{2+} affected on the ARP but there was no significant difference between their concentrations, while Fe^{3+} and Cu^{2+} did not influence on the amounts of ARP. Fe^{2+} also affected on the increase of HMF (Fig 34) more than Fe^{3+} and Cu^{2+}, resulting in the higher accumulation of HMF than those of control (Table 13). However, the concentrations of metal ions did not affected on the accumulation of HMF. These indicated that types of metal ions affected on the increase of HMF. This experiment did not demonstrate dose-dependence of metal ion.

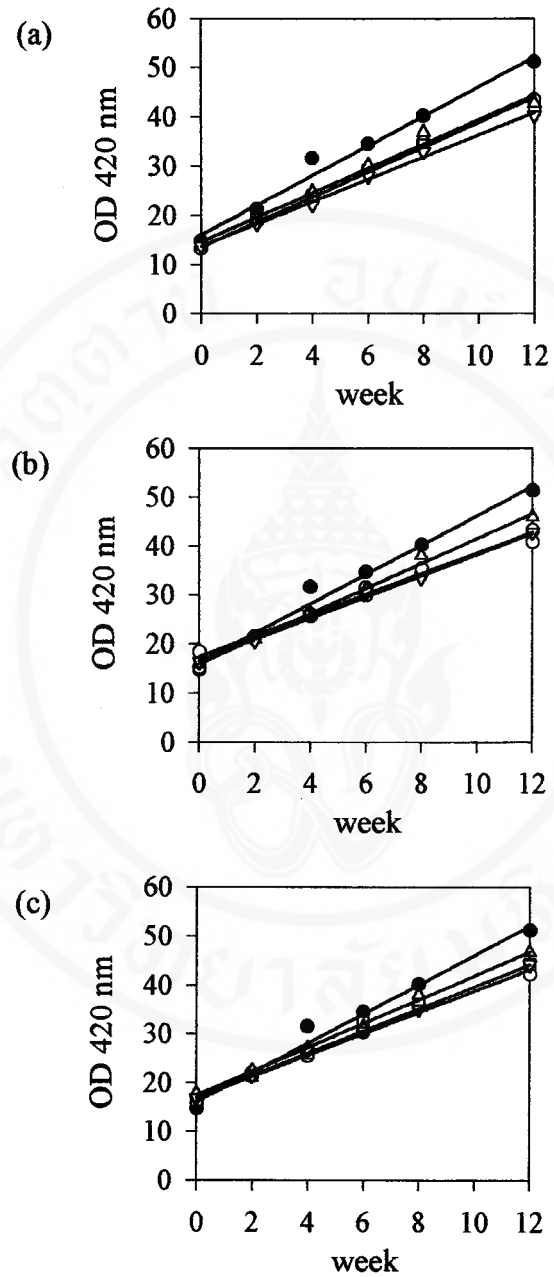


Figure 29: Browning as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △ : 3.0 μM, and ▽ : 7.5 μM).

Table 8: The linear regression equations of browning in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	y = 16.226 + 3.001x	0.991
Fe^{2+}	0.5	y = 13.716 + 2.53x	0.999
	1.0	y = 14.714 + 2.229x	0.997
	3.0	y = 14.801 + 2.478x	0.992
	7.5	y = 13.904 + 2.259x	0.997
Fe^{3+}	0.5	y = 17.470 + 2.115x	0.998
	1.0	y = 16.594 + 2.164x	0.988
	3.0	y = 15.967 + 2.559x	0.997
	7.5	y = 16.544 + 2.189x	0.998
Cu^{2+}	0.5	y = 16.973 + 2.196x	0.995
	1.0	y = 16.594 + 2.164x	0.988
	3.0	y = 17.574 + 2.449x	0.999
	7.5	y = 17.086 + 2.271x	0.999

Note: x = browning (OD 420 nm)

y = storage time (weeks) within 3 months

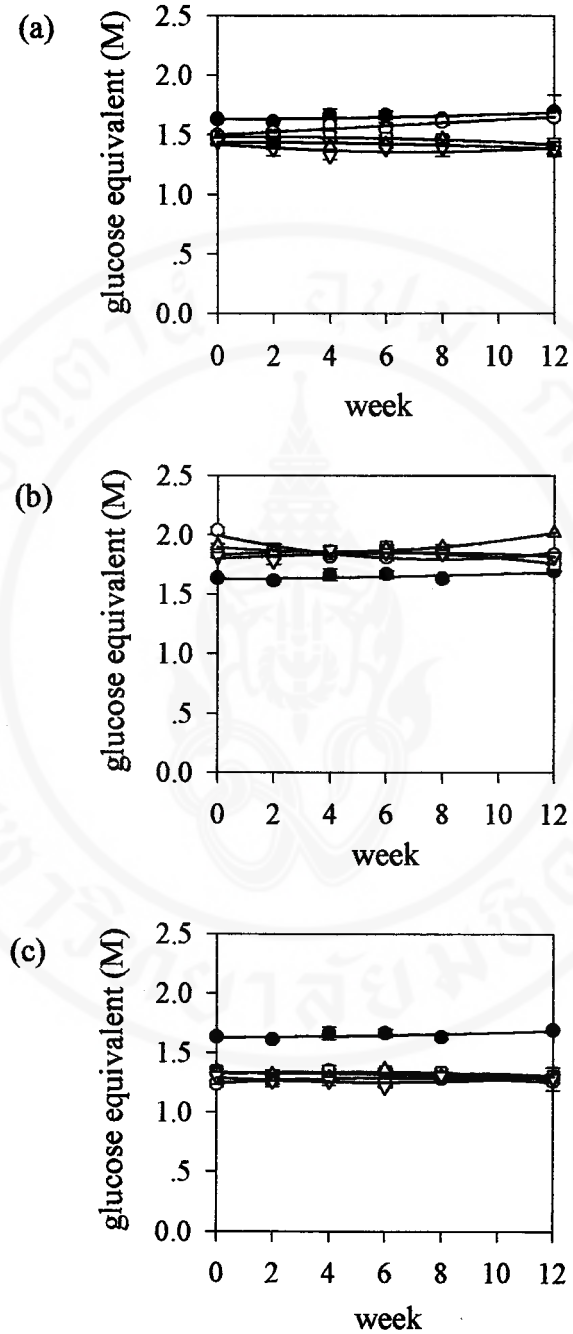


Figure 30: RS as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △ : 3.0 μM, and ▽ : 7.5 μM).

Table 9: The quadratic regression equations of RS in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	$y = 1.630 + 0.002x + 0.0002x^2$	0.697
Fe^{2+}	0.5	$y = 1.499 + 0.0014x - 0.0001x^2$	0.946
	1.0	$y = 1.482 + 0.0025x - 0.0006x^2$	0.992
	3.0	$y = 1.442 + 0.0001x - 0.0004x^2$	0.598
	7.5	$y = 1.427 - 0.0189x + 0.0013x^2$	0.651
Fe^{3+}	0.5	$y = 1.999 - 0.051x + 0.0032x^2$	0.884
	1.0	$y = 1.831 + 0.016x - 0.0018x^2$	0.865
	3.0	$y = 1.899 - 0.020x + 0.0025x^2$	0.986
	7.5	$y = 1.802 + 0.014x - 0.0011x^2$	0.581
Cu^{2+}	0.5	$y = 1.246 + 0.014x - 0.0011x^2$	0.433
	1.0	$y = 1.326 + 0.006x - 0.0006x^2$	0.578
	3.0	$y = 1.333 - 0.001x + 0.0003x^2$	0.540
	7.5	$y = 1.291 - 0.013x + 0.0012x^2$	0.556

Note: x = RS (M)

y = storage time (weeks) within 3 months

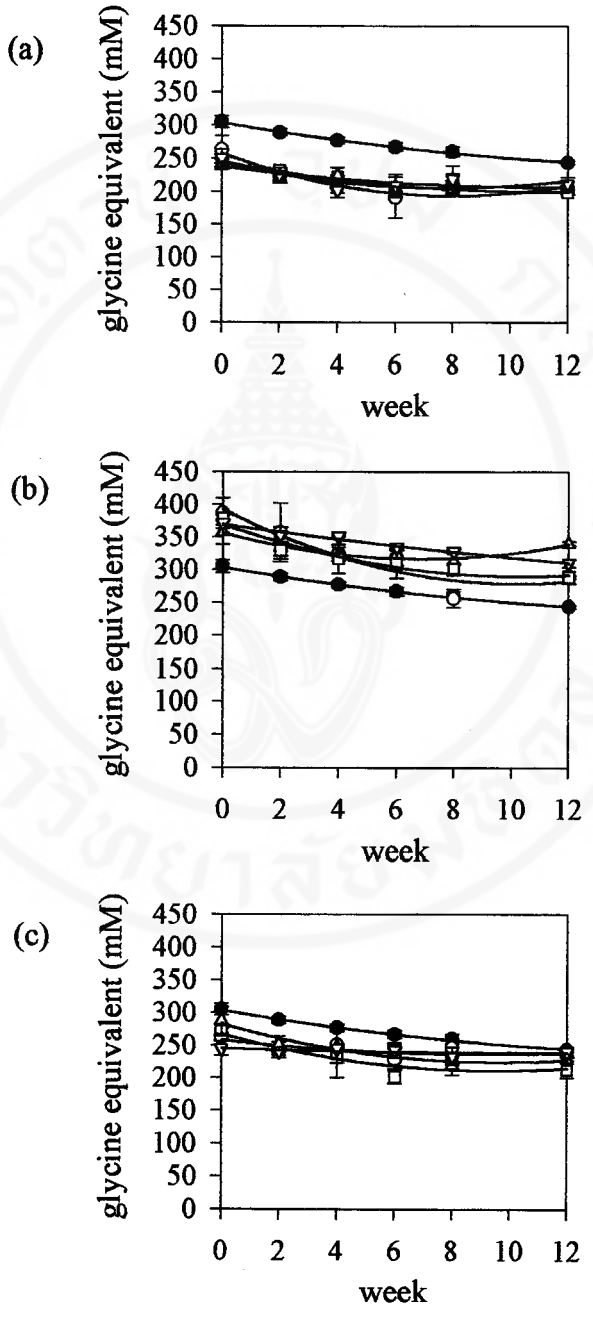


Figure 31: RAG as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △ : 3.0 μM, and ▽ : 7.5 μM).

Table 10: The quadratic regression equations of RAG in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	$y = 304.014-7.348x+0.201x^2$	0.999
Fe^{2+}	0.5	$y = 258.116-16.131x+1.009x^2$	0.956
	1.0	$y = 245.199-8.126x+0.364x^2$	0.979
	3.0	$y = 237.826-5.460x+0.240x^2$	0.891
	7.5	$y = 244.438-9.958x+0.641x^2$	0.850
Fe^{3+}	0.5	$y = 393.089-22.390x+1.099x^2$	0.938
	1.0	$y = 370.297-15.542x+0.753x^2$	0.966
	3.0	$y = 356.710-11.677x+0.853x^2$	0.965
	7.5	$y = 370.036-6.153x+0.101x^2$	0.990
Cu^{2+}	0.5	$y = 258.594-4.755x+0.260x^2$	0.732
	1.0	$y = 268.129-12.109x+0.642x^2$	0.883
	3.0	$y = 283.544-12.560x+0.645x^2$	0.955
	7.5	$y = 245.469-1.258x+0.058x^2$	0.600

Note: x = RAG (mM)

y = storage time (weeks) within 3 months

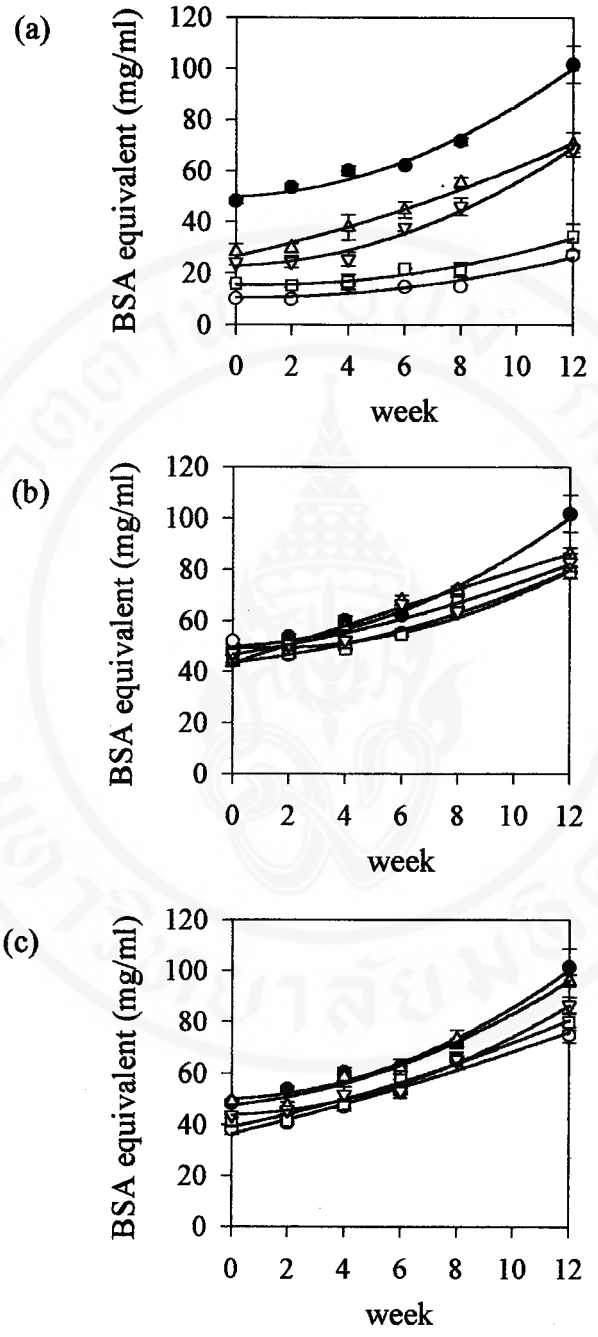


Figure 32: PP as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △ : 3.0 μM, and ▽ : 7.5 μM).

Table 11: The quadratic regression equations of PP in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	$y = 50.036+0.496x+0.310x^2$	0.993
Fe^{2+}	0.5	$y = 10.54+0.0063x+0.109x^2$	0.945
	1.0	$y = 15.607-0.197x+0.144x^2$	0.982
	3.0	$y = 26.801+2.459x+0.104x^2$	0.994
	7.5	$y = 23.008+0.375x+0.292x^2$	0.993
Fe^{3+}	0.5	$y = 49.821-0.618x+0.262x^2$	0.984
	1.0	$y = 43.663+1.280x+0.146x^2$	0.984
	3.0	$y = 43.343+3.840x-0.021x^2$	0.993
	7.5	$y = 46.868+1.745x+0.102x^2$	0.969
Cu^{2+}	0.5	$y = 36.456+2.720x+0.049x^2$	0.986
	1.0	$y = 39.153+2.320x+0.097x^2$	0.988
	3.0	$y = 47.522+1.180x+0.242x^2$	0.993
	7.5	$y = 43.82+0.429x+0.263x^2$	0.995

Note: x = PP (mg/ml)

y = storage time (weeks) within 3 months

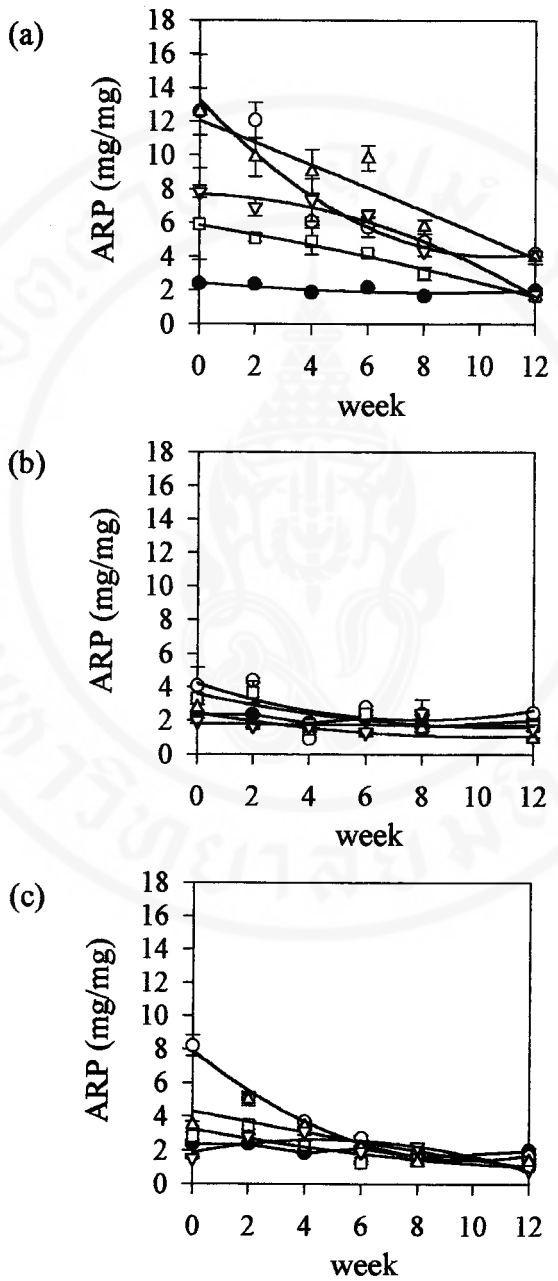


Figure 33: ARP as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △: 3.0 μM, and ▽ : 7.5 μM).

Table 12: The quadratic regression equations of ARP in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	$y = 2.452-0.140x+0.0082x^2$	0.781
Fe^{2+}	0.5	$y = 13.288-1.751x+0.083x^2$	0.950
	1.0	$y = 5.880-0.267x-0.0073x^2$	0.991
	3.0	$y = 12.087-0.649x-0.0028x^2$	0.945
	7.5	$y = 7.692-0.034x-0.0390x^2$	0.980
Fe^{3+}	0.5	$y = 4.252-0.551x+0.0346x^2$	0.652
	1.0	$y = 3.605-0.355x+0.016x^2$	0.835
	3.0	$y = 2.484-0.278x+0.0136x^2$	0.886
	7.5	$y = 1.816-0.009x+0.0001x^2$	0.081
Cu^{2+}	0.5	$y = 7.922-1.312x+0.066x^2$	0.993
	1.0	$y = 3.274-0.298x+0.0093x^2$	0.867
	3.0	$y = 4.343-0.327x+0.0043x^2$	0.830
	7.5	$y = 1.897-0.318x+0.034x^2$	0.840

Note: x = ARP (mg/mg)

y = storage time (weeks) within 3 months

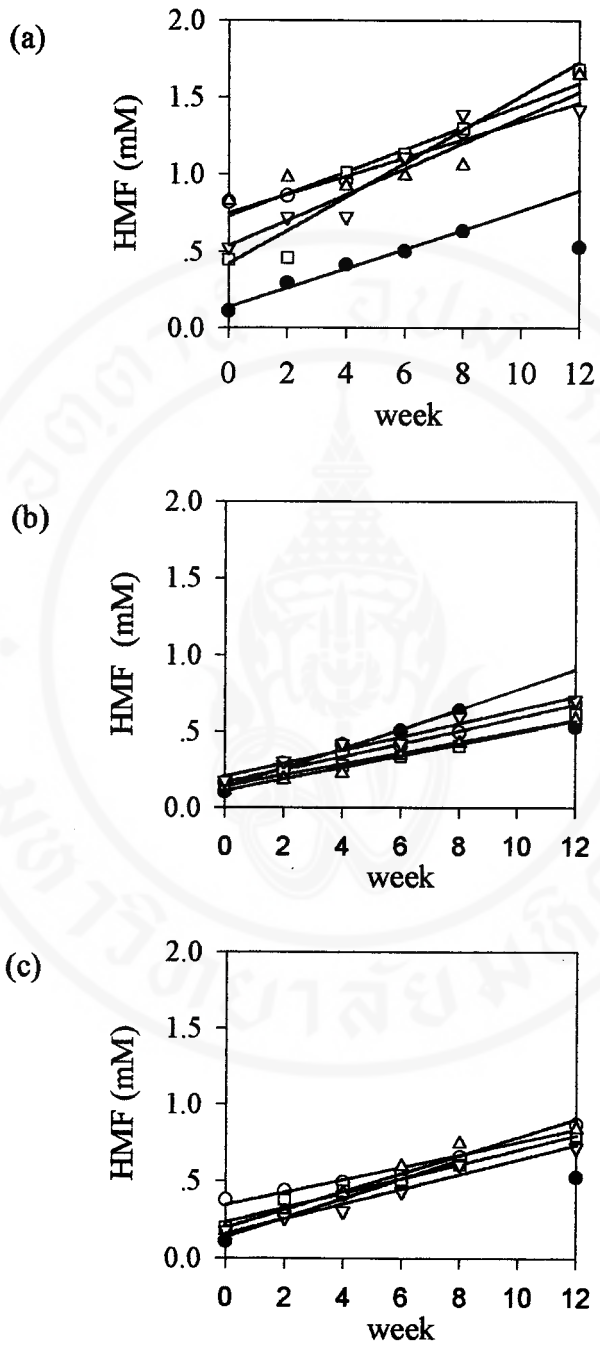


Figure 34: HMF as a function of time in the model system containing; Fe2+ (a), Fe3+ (b), and Cu2+ (c) at various concentrations (● : 0 μM, ○ : 0.5 μM, □ : 1.0 μM, △ : 3.0 μM, and ▽ : 7.5 μM).

Table 13: The linear regression equations of HMF in the model systems containing Fe^{2+}, Fe^{3+}, and Cu^{2+}.

metal ion	supplemented concentration (μM)	equation	r^2
control	0	y = 0.137+0.063x	0.993
Fe^{2+}	0.5	y = 0.734+0.072x	0.981
	1.0	y = 0.423+0.109x	0.973
	3.0	y = 0.754+0.059x	0.878
	7.5	y = 0.541+0.083x	0.947
Fe^{3+}	0.5	y = 0.169+0.042x	0.997
	1.0	y = 0.147+0.036x	0.987
	3.0	y = 0.115+0.038x	0.991
	7.5	y = 0.207+0.043x	0.981
Cu^{2+}	0.5	y = 0.348+0.041x	0.990
	1.0	y = 0.236+0.047x	0.990
	3.0	y = 0.199+0.059x	0.981
	7.5	y = 0.161+0.048x	0.984

Note: x = HMF (mM)

y = storage time (weeks) within 3 months

3. Studying the effects of iodine supplementation on the Maillard reaction in soy sauce

The KI did not significantly influence on browning, RS, RAG, and HMF accumulation (Fig 35a, b, c, and f). Although browning and HMF increased while RAG decreased, these results in each model systems were not significantly different from the control. However, KI affected the accumulation of PP (Fig 35d) and ARP (Fig 35e). Model containing KI showed lower PP than control but the concentration of KI did not significantly affect on PP. The decrease of ARP was higher than control for all concentrations. Using regression equation could fit the correlation of parameter with storage time (3 months). The relationship between browning and HMF was in linear manner ($y = y_0+ax$) while the other parameters were in quadratic manner ($y = y_0+ax+bx^2$) (Table 14).

4. Investigation of the influence of sugar supplementation

The increase of browning in model systems containing xylose was dependent on xylose concentration (Fig 36). The more xylose, the higher browning occurred. However, the increase of browning did not depend on type and concentration of sugars. The amounts of RS relatively constant while RAG decreased (Fig 37 and 38, respectively). However, the changes of RS and RAG were not significant and there was no difference among concentration.

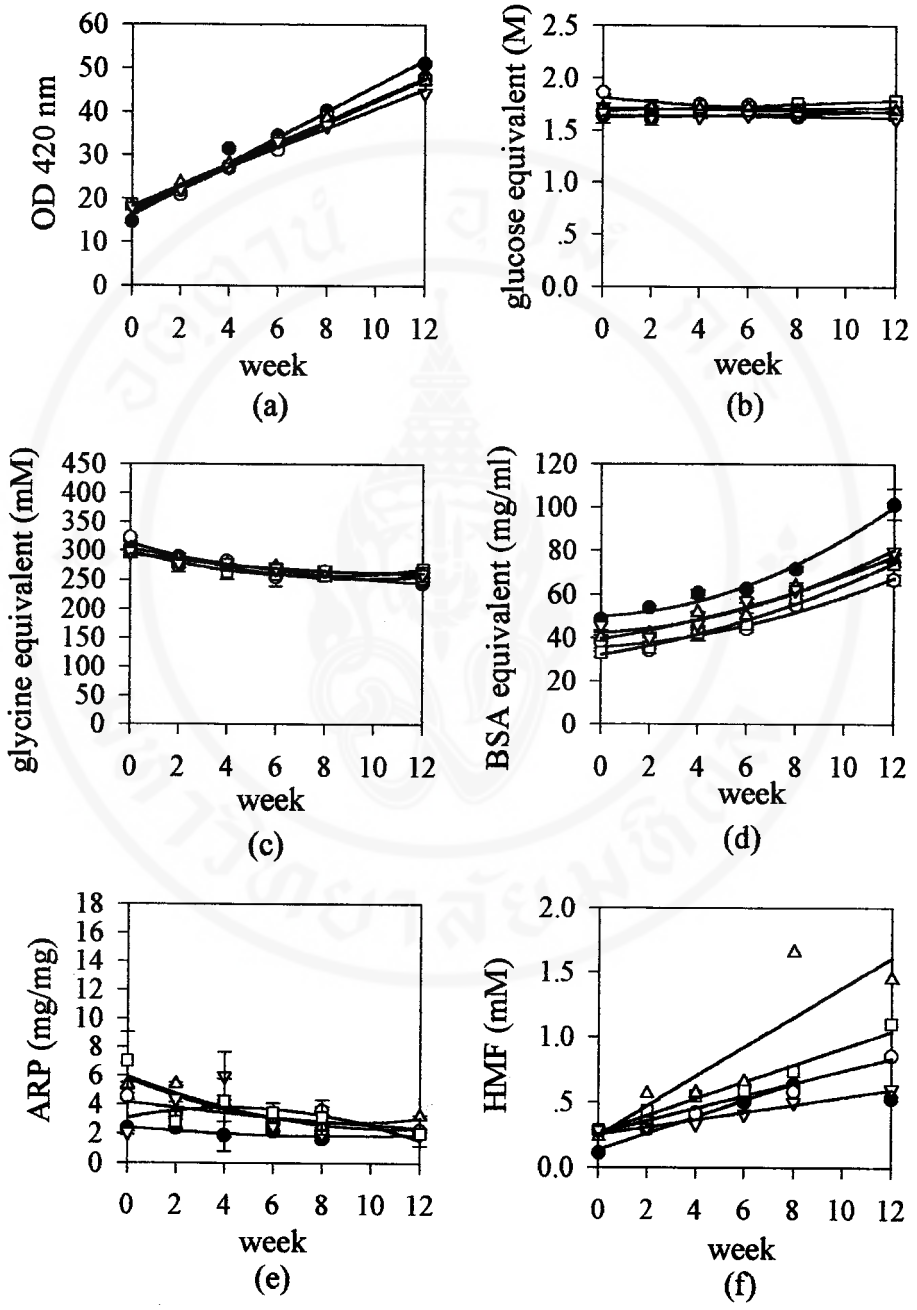


Figure 35: The change of browning (a), RS (b), RAG (c), PP (d), ARP (e), and HMF (f) in model system containing KI at various concentrations (● : 0 μM, ○ : 5 μM, □: 10 μM, △: 30 μM, and ▽: 75 μM).

Table 14: The regression equations of browning, RS, RAG, PP, ARP, and HMF in the model system containing KI at various concentrations.

parameter	supplemented concentration (μM)	equation	r^2
browning (OD 420 nm)	0	$y = 16.226 + 3.001x$	0.991
	5	$y = 16.993 + 2.561x$	0.995
	10	$y = 17.857 + 2.514x$	0.997
	30	$y = 18.279 + 2.457x$	0.999
	75	$y = 18.327 + 2.254x$	0.996
RS (M)	0	$y = 1.630 + 0.0018x + 0.0002x^2$	0.697
	5	$y = 1.816 - 0.024x + 0.0013x^2$	0.669
	10	$y = 1.685 + 0.0079x + 0.0001x^2$	0.892
	30	$y = 1.712 - 0.0029x$	0.837
	75	$y = 1.645 - 0.0001x - 0.0001x^2$	0.463
RAG (mM)	0	$y = 304.01 - 7.348x + 0.201x^2$	0.999
	5	$y = 314.39 - 13.049x + 0.682x^2$	0.912
	10	$y = 298.98 - 10.804x + 0.69x^2$	0.991
	30	$y = 296.10 - 5.713x + 0.224x^2$	0.996
	75	$y = 294.50 - 5.327x + 0.216x^2$	0.961

Table 14: (continued)

parameter	supplemented concentration (μM)	equation	r^2
PP (mg/ml)	0	$y = 50.036+0.496x+0.31x^2$	0.993
	5	$y = 35.75+0.748x+0.160x^2$	0.977
	10	$y = 32.382+1.78x+0.145x^2$	0.995
	30	$y = 39.519+1.805x+0.114x^2$	0.981
	75	$y = 42.42+0.627x+0.214x^2$	0.977
ARP (mg/mg)	0	$y = 2.452 - 0.14x + 0.008x^2$	0.781
	5	$y = 4.189–0.198x+0.0043x^2$	0.739
	10	$y = 5.956–0.642x+0.028x^2$	0.794
	30	$y = 5.80–0.668+0.037x^2$	0.937
	75	$y = 3.106+0.33x–0.038x^2$	0.512
HMF (mM)	0	$y = 0.137 + 0.0063x$	0.993
	5	$y = 0.265 + 0.048x$	0.971
	10	$y = 0.27 + 0.065x$	0.984
	30	$y = 0.248 + 0.114x$	0.873
	75	$y = 0.254 + 0.028x$	0.983

Note: x = browning (OD 420 nm); RS (M); RAG (mM); PP (mg/ml); ARP (mg/mg); and HMF (mM)

y = storage time (weeks) within 3 months

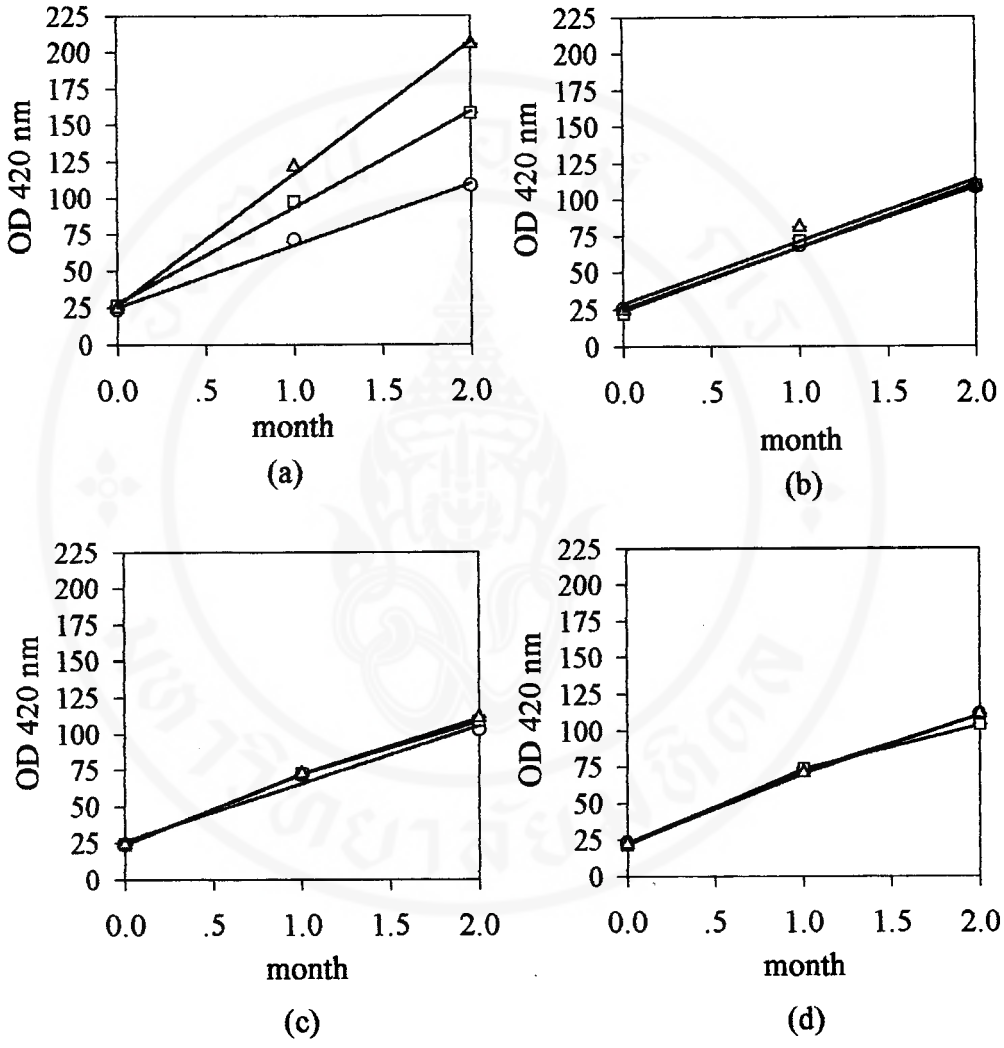


Figure 36: Browning in model system containing; (a) xylose, (b) glucose, (c) maltose, and (d) sucrose at various concentrations (○ : 0 M, □ : 0.5 M, and △: 1.0 M).

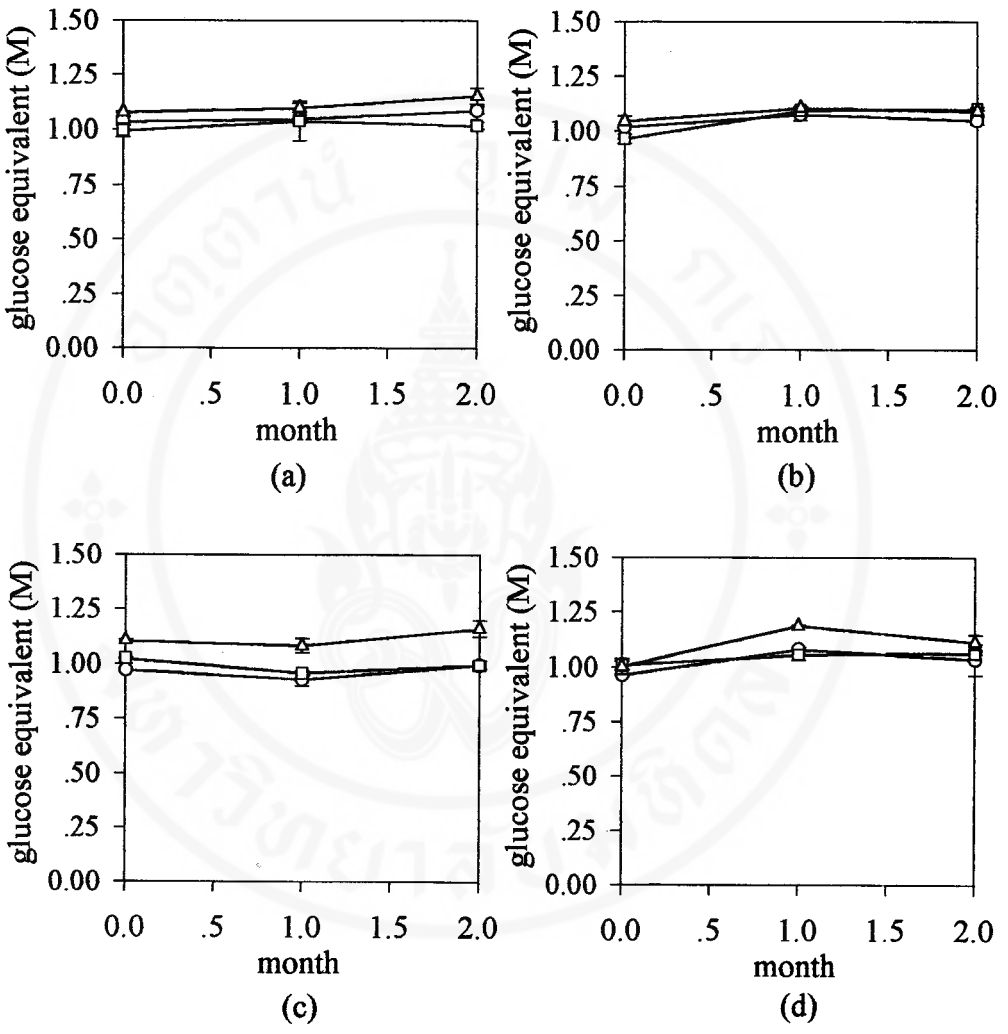


Figure 37: RS in model system containing; (a) xylose, (b) glucose, (c) maltose, and (d) sucrose at various concentrations (○ : 0 M, □ : 0.5 M, and △ : 1.0 M).

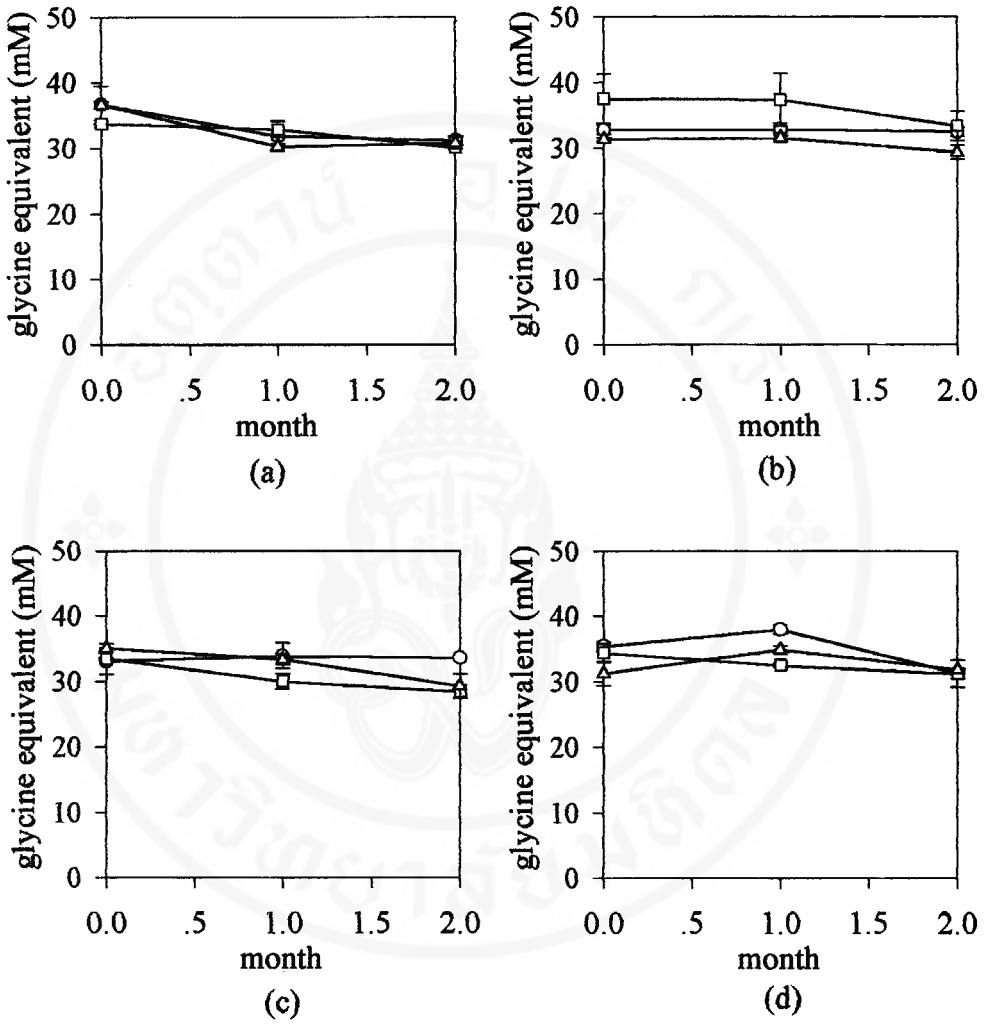


Figure 38: RAG in model system containing; (a) xylose, (b) glucose, (c) maltose, and (d) sucrose at various concentrations (○ : 0 M, □ : 0.5 M, and △ : 1.0 M).

5. Elucidation of the Maillard reaction mechanism during moromi fermentation

Moromi from Thaisang and Vichitrungruang factories developed the similar pattern of the Maillard reaction (Fig 39 and 40). The development of the Maillard reaction could be divided into two stages. At the first 3 days of fermentation (the first stage), the rate of browning increased very rapidly after that the rate decreased, (the second stage). The study of the Maillard reaction during the fist 20 days of moromi fermentation also confirmed those results (Fig 41 and 42). The browning hyperbolic regression equation for moromi from both factories was $y = ax/(b+x)$, where x and y were fermentation time and OD 420 nm, respectively (Table 15).

The changes of RS and RAG are shown in Fig 39b and 39c, respectively. Both RS and RAG including soluble protein (SP) in Fig 40c increased very rapidly during the first stage of fermentation owing to the dissolving of SP and sugar from seed Koji into brine. RS of Thaisang moromi decreased while RS of Vichitrungruang moromi was relatively constant. However, the equation of RS change represented for both moromi was $y = ax/(b+x)+cx$, where x and y are fermentation time and glucose equivalent amount, respectively (Table 15). For both moromi, RAG slightly increased with low rate after the first 3 days due to the remaining the activity of proteolytic enzyme originated from Koji. The hyperbolic regression equation of RAG change was $y = [ax/(b+x)]+ [cx/(d+x)]$, where x was fermentation time (60 days) and y was amounts of RAG as glycine equivalence, respectively (Table 15).

The decrease of PP is shown in Fig 40a. The PP amounts declined as a function of time and its equation represented for both moromi was $y = y_0+ab/(b+x)$, where x, y, and y_0 were fermentation time, amounts of BSA equivalence, and the constant, respectively. This equation also represented for the increase of ARP and the

decrease of pH in which the different rate and correlation coefficient (Table 15). The initial pH of moromi (from both factories) was approximately 6.5 to 7. The pH dropped very quickly to 5.5 in the first 3 days and decreased slowly to 4.9 at the end of fermentation (Fig 40d). The results suggested that the neutral pH enhanced the rate of browning during the first 3 days, then acidic pH could retard the development of browning. The high acidity during the latter stage of fermentation came from the acid produced from microorganisms. The increasing of acidity was ensured by the increasing of titratable acidity (Fig 40e).

During the fermentation, HMF, the important index of the Maillard reaction, increased with respect to the equation: $y = y_0 + ab/(b+x)$ (Fig 39d), where x, y, and y_0 were fermentation time (60 days), amounts of HMF, and the constant, respectively. Moromi from Thaisang illustrated the Maillard reaction during early stage of moromi fermentation (10 days). All of chemical changes are shown in Fig 41 and 42. The results ensured that the browning and other chemical changes occurred rapidly during the first 3 days then occurred with low rate. The regression equations of each parameter with fermentation time (0-9 days) are shown in Table 16.

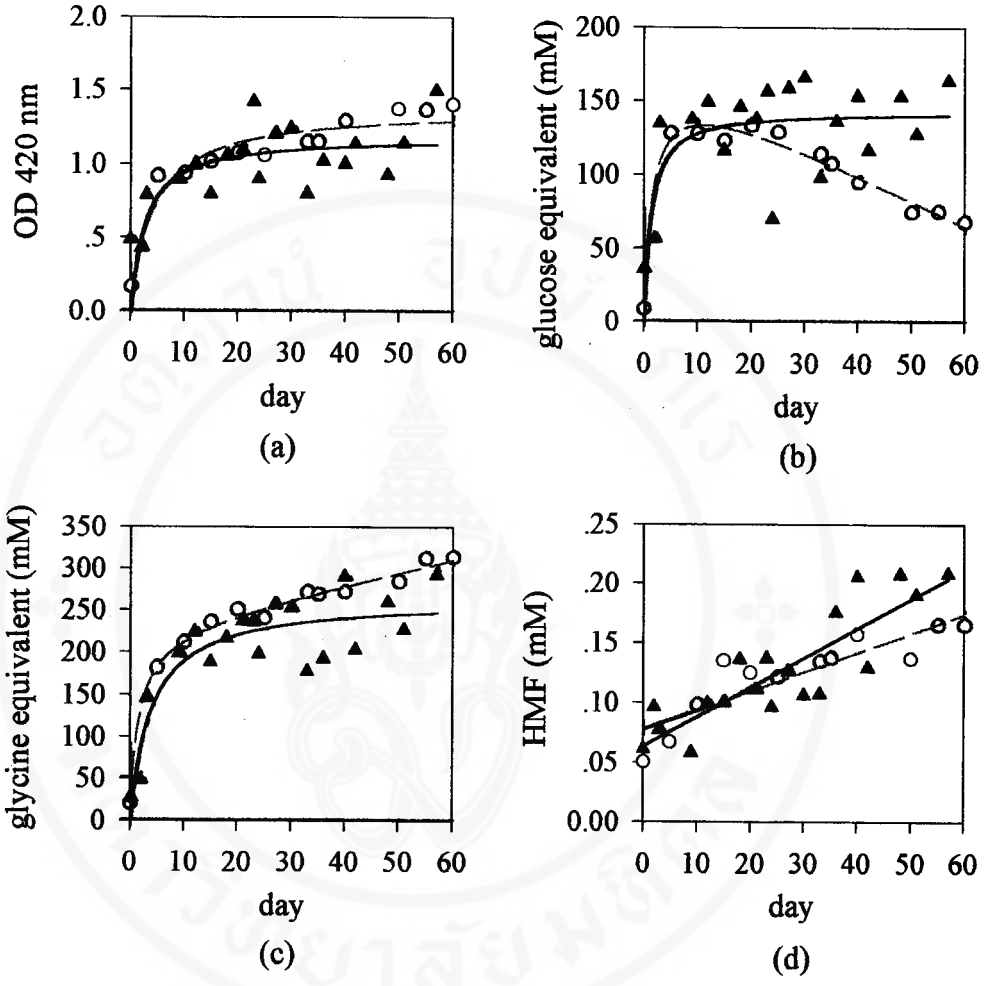


Figure 39: The change of browning (a), RS (b), RAG (c), and HMF (d) during moromi fermentation of soy sauce: Thaisang (○) and Vichitrungruang (▲).

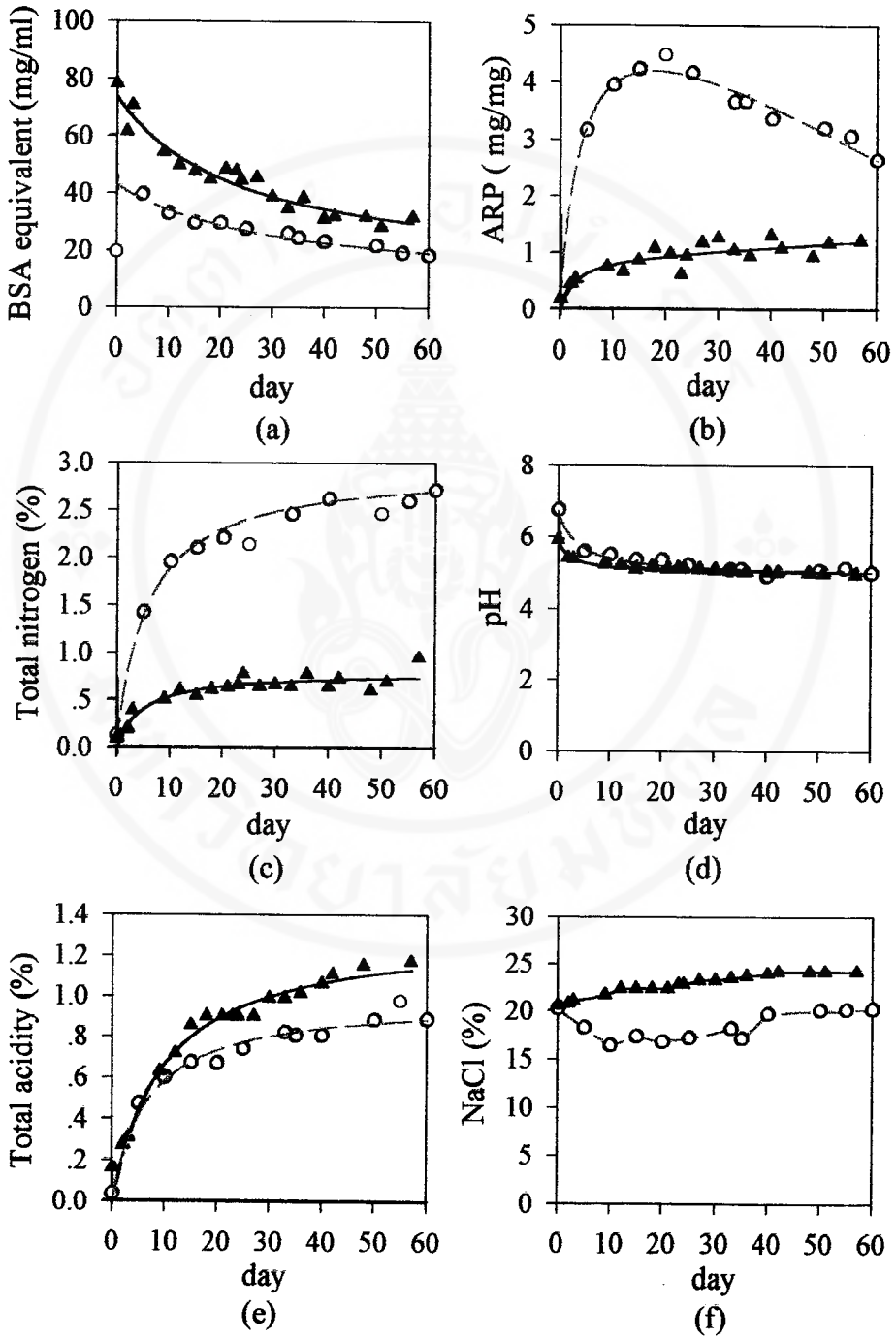


Figure 40: The change of PP (a), ARP (b), SP (c), pH (c), total acidity (e), and % NaCl (f) during moromi fermentation of soy sauce from Thaisang (○) and Vichitrungruang (▲).

Table 15: The regression equations of chemical changes in soy sauce during moromi fermentation (60 days).

parameter	soy sauce	equation	r^2
browning	T	$y = 1.36x/(4.11 + x)$	0.947
(OD 420 nm)	V	$y = 1.185x/(2.553 + x)$	0.632
RS (mM)	T	$y = [174.94x/(1.609+x)] - 1.749x$	0.987
	V	$y = [143.68x/(1.244+x)] - 0.0037x$	0.674
RAG (mM)	T	$y = [227.34x/(1.489+x)] + [1.36*10^{10}x/(9.3*10^{9}+x)]$	0.993
	V	$y = [119.95x/(3.8448+x)] + [143.27x/(3.845+x)]$	0.886
PP (mg/ml)	T	$y =7.148+[(35.77)(30.25)/(30.25+x)]$	0.983
	V	$y =11.329+[(62.48)(23.61)/(23.61+x)]$	0.967
SP (%)	T	$y = 2.955x/(5.675 + x)$	0.953
	V	$y = 0.804x/(4.996 + x)$	0.917
ARP (mg/mg)	T	$y = [6.427x/(4.199 + x)] - 0.056x$	0.965
	V	$y = [0.937x/(2.645 +x)] + 0.0054x$	0.869
HMF (mM)	T	$y = 0.078 + 0.0016x$	0.880
	V	$y = 0.064 + 0.0025x$	0.868
pH	T	$y = 4.917 +[(1.811)(3.65)/(3.65+x)]$	0.984
	V	$y =4.980+[(0.908)(2.483)/(2.483+x)]$	0.982
total acidity	T	$y = 9.849x/(6.809 + x)$	0.984
(%)	V	$y = 1.326x/(9.825 + x)$	0.985

Note: x = browning (OD 420 nm); RS (mM); RAG (mM); PP (mg/ml); SP (%); ARP (mg/mg); HMF (mM); pH; and total acidity (%)

y = fermentation period (days)

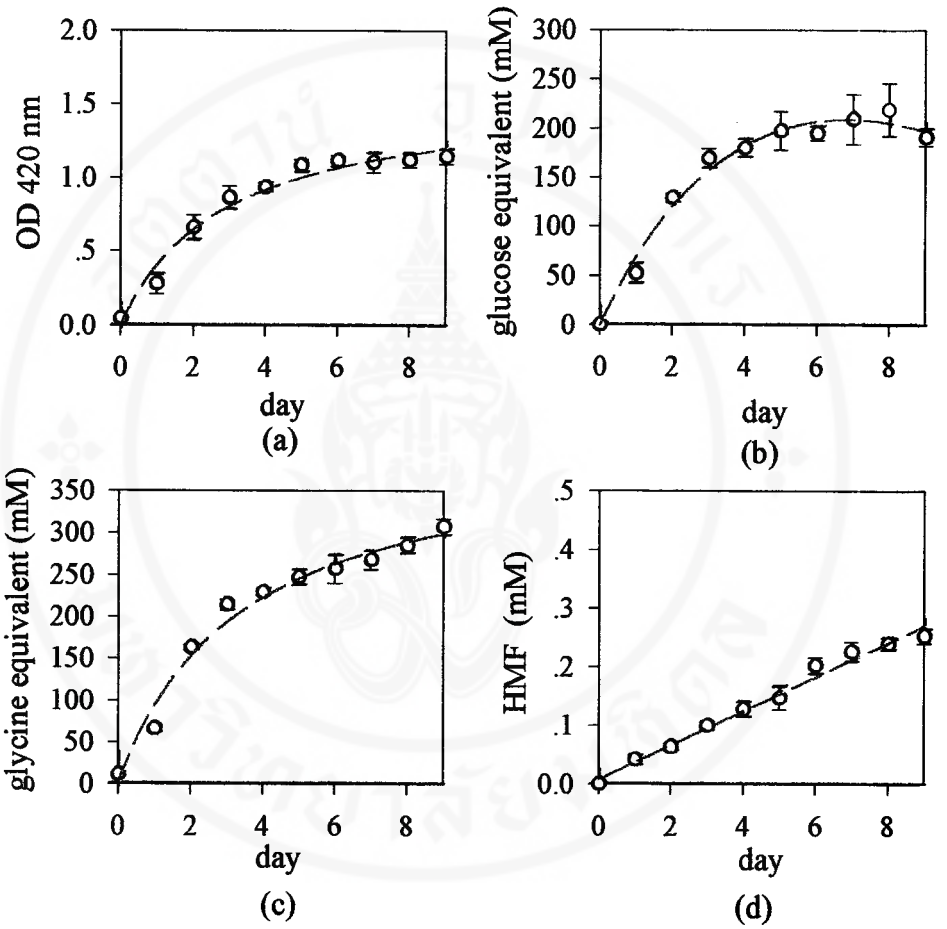


Figure 41: The browning (a), RS (b), RAG (c), and HMF (d) during early stage of fermentation of moromi from Thaisang.

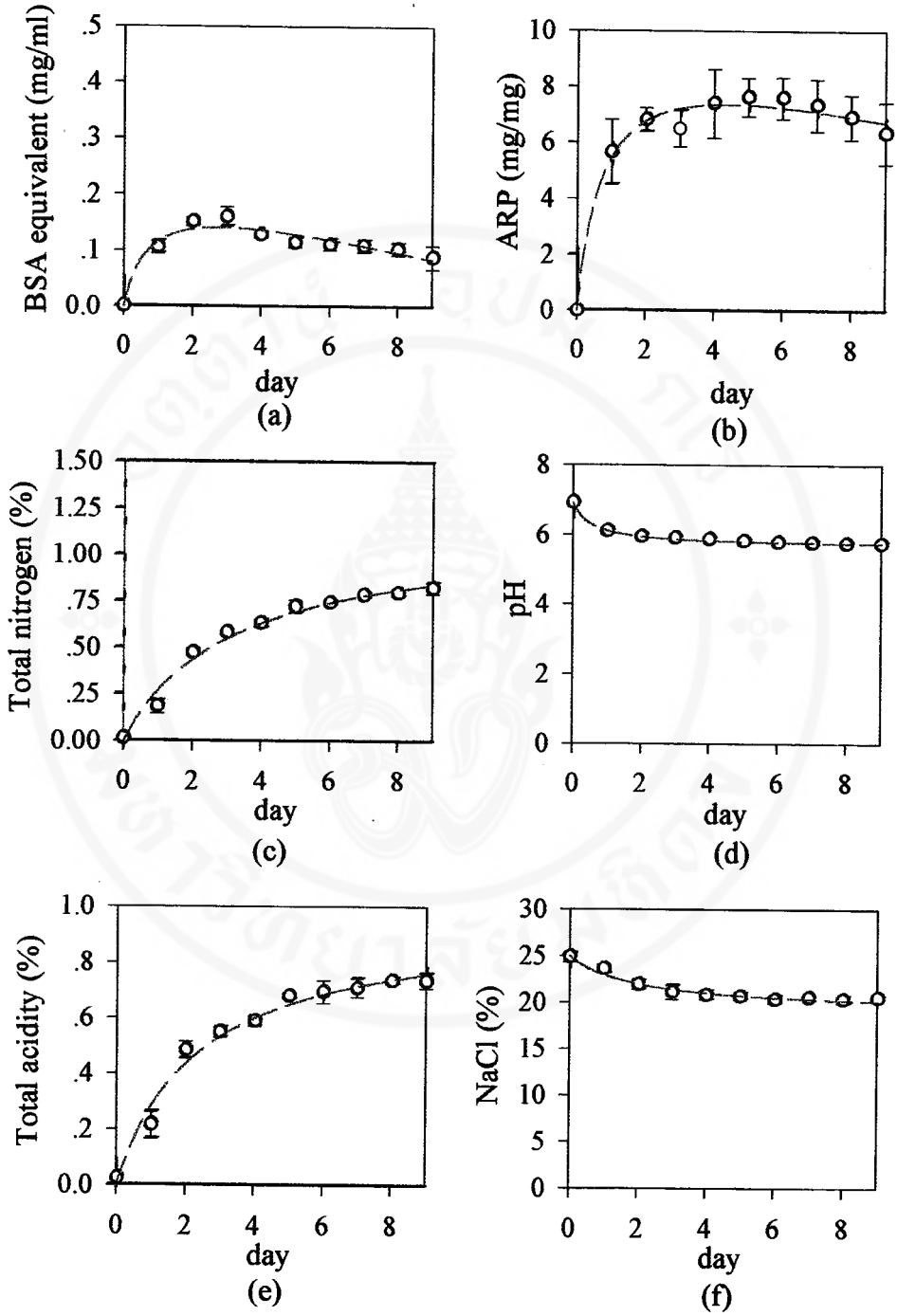


Figure 42: The PP (a), ARP (b), SP (c), pH (d), Total acidity (e), and NaCl (f) during early stage of fermentation of moromi from Thaisang.

Table 16: The regression equations of chemical changes in soy sauce from Thaisang during early stage of moromi fermentation (0-9 days)

parameter	equation	r^2
browning (OD 420 nm)	y = 1.58x/(2.885+x)	0.987
RS (mM)	y = [1502.74x/(11.47+x)] – 51.51x	0.990
RAG (mM)	y =[194.57x/(3.3624+x)]+[216.48x/(3.3623+x)]	0.990
PP (mg/ml)	y =[(0.232)/(0.777+x)] – 0.014x	0.968
SP (%)	y = 1.142x/(3.214 + x)	0.991
ARP	y = [10.143x/(0.761 + x)] – 0.294x	0.990
HMF (mM)	y = 0.0087 + 0.0291x	0.993
pH	y = 5.680 +[(1.238)(0.532)/(0.532+x)]	0.999
total acidity (%)	y = 0.960x/(2.391 + x)	0.991
NaCl (%)	y = 19.087 +[5.901x/(1.961 + x)]	0.983

Note: x = browning (OD 420 nm); RS (mM); RAG (mM); PP (mg/ml); SP (%); ARP (mg/mg); HMF (mM); pH; and total acidity (%)

y = fermentation period (days)

6. Studying the Maillard reaction in pasteurized soy sauce during storage

Raw soy sauce from Thaisang was used to study the Maillard reaction mechanism during storage comparing with cooked soy sauce (pasteurized and seasoned). Raw soy sauce from Vichitrungruang was heated to 80°C for pasteurization without seasoning (pasteurized soy sauce) before used to study.

The brown color development is shown in Fig 43a. The initial browning and rate of browning of cooked soy sauce were higher than that of raw soy sauce up to 2.0-2.5 times. The equations of browning were $y = 1.871+0.268x$ ($r^2= 0.996$) and $y = 4.918+0.908x$ ($r^2= 0.996$) for raw and cooked soy sauce from Thaisang, respectively. Where x and y were storage time (3 months) and OD 420 nm, respectively. For browning of pasteurized soy sauce from Vichitrungruang, the browning increased in linear manner ($y = 1.557+0.398x$, $r^2= 0.999$).

The regression equations showed that the rate of browning of raw soy sauce from Thaisang was relatively closed to that of pasteurized soy sauce from Vichitrungruang because both of them were not seasoned before storage test (Fig 43a). The comparison of the initial browning and regression equation of browning in each model are shown in Table 17.

RS amounts of raw soy sauce from Thaisang decreased during the storage and the equation of decreasing was $y = 78.759–4.444x+0.210x^2$, $r^2= 0.994$ (Fig 43b). The initial RS of cooked soy sauce from Thaisang was higher than that of raw soy sauce approximately 1.67 times. The equations of increasing of RS were $y = 123.153 + 5.981x – 0.084x^2$ ($r^2= 0.999$) and $y=133.986 + 3.242x – 0.231x^2$ ($r^2= 0.935$) for cooked soy sauce from Thaisang and pasteurized soy sauce from Vichitrungruang, respectively. Where x was storage time and y was RS amounts. The RAG amounts of

all models were relatively constant during storage (Fig 43c). RAG of raw soy sauce was lower than cooked soy sauce from Thaisang up to 1.375 times.

During storage, PP amounts of raw soy sauce from Thaisang slightly increased ($y= 39.006 - 1.766x + 0.27x^2$, $r^2 = 0.927$). The initial PP and rate of PP increase in cooked soy sauce from Thaisang was higher than raw soy sauce approximately 2.25 and 10 times, respectively. The PP of cooked soy sauce from Thaisang increased very rapidly during the latter of incubation. The PP in pasteurized soy sauce from Vichitrungruang also increased although the PP was dropped during early incubation (Fig 43d). The equation represented for the PP of cooked soy sauce from Thaisang and pasteurized soy sauce from Vichitrungruang factories were $y = 93.281+1.623x+0.437x^2$.($r^2= 0.992$) and $y = 75.212–5.472x+0.486x^2$ ($r^2= 0.804$), respectively.

The amounts of ARP bound to PP during storage of raw soy sauce was lower than cooked soy sauce from Thaisang, although the initial and final amounts were close to each other (Fig 43e). ARP of cooked soy sauce from Thaisang increased during the first 2 weeks, after that declined, while ARP of raw soy sauce from Thaisang and cooked soy sauce decreased linearly through the incubation. The amounts of ARP in pasteurized soy sauce from Vichitrungruang were lower than raw soy sauce from Thaisang up to 2 times.

These results suggested that the ARP bound to PP not accumulated in the systems. The quadratic regressions of decreasing of ARP in raw and cooked soy sauce from Thaisang and pasteurized soy sauce from Vichitrungruang were $y= 0.873–0.108x+0.0058x^2$ ($r^2= 0.920$), $y= 2.555–0.240x+0.006x^2$ ($r^2= 0.995$), and $y= 0.603–0.064x+0.0038x^2$ ($r^2= 0.921$), respectively.

The change of HMF showing in Fig 43f indicated that HMF in all models increased linearly during storage. The amount of HMF of pasteurized soy sauce from Vichitrungruang was similar to raw soy sauce from Thaisang, while cooked soy sauce from Thaisang had higher HMF. The initial HMF in cooked soy sauce from Thaisang was higher than raw soy sauce approximately 2.67 times. The linear regression equation of HMF in raw and cooked soy sauce from Thaisang including pasteurized soy sauce from Vichitrungruang were $y= 0.159+0.0087x$ ($r^2= 0.997$), $y= 0.420+0.015x$ ($r^2= 0.967$), and $y= 0.065+0.015x$ ($r^2= 0.977$), respectively. For the change of pH, each model showed the slightly decreased during storage from 4.8-5.0 to 4.5-4.6.

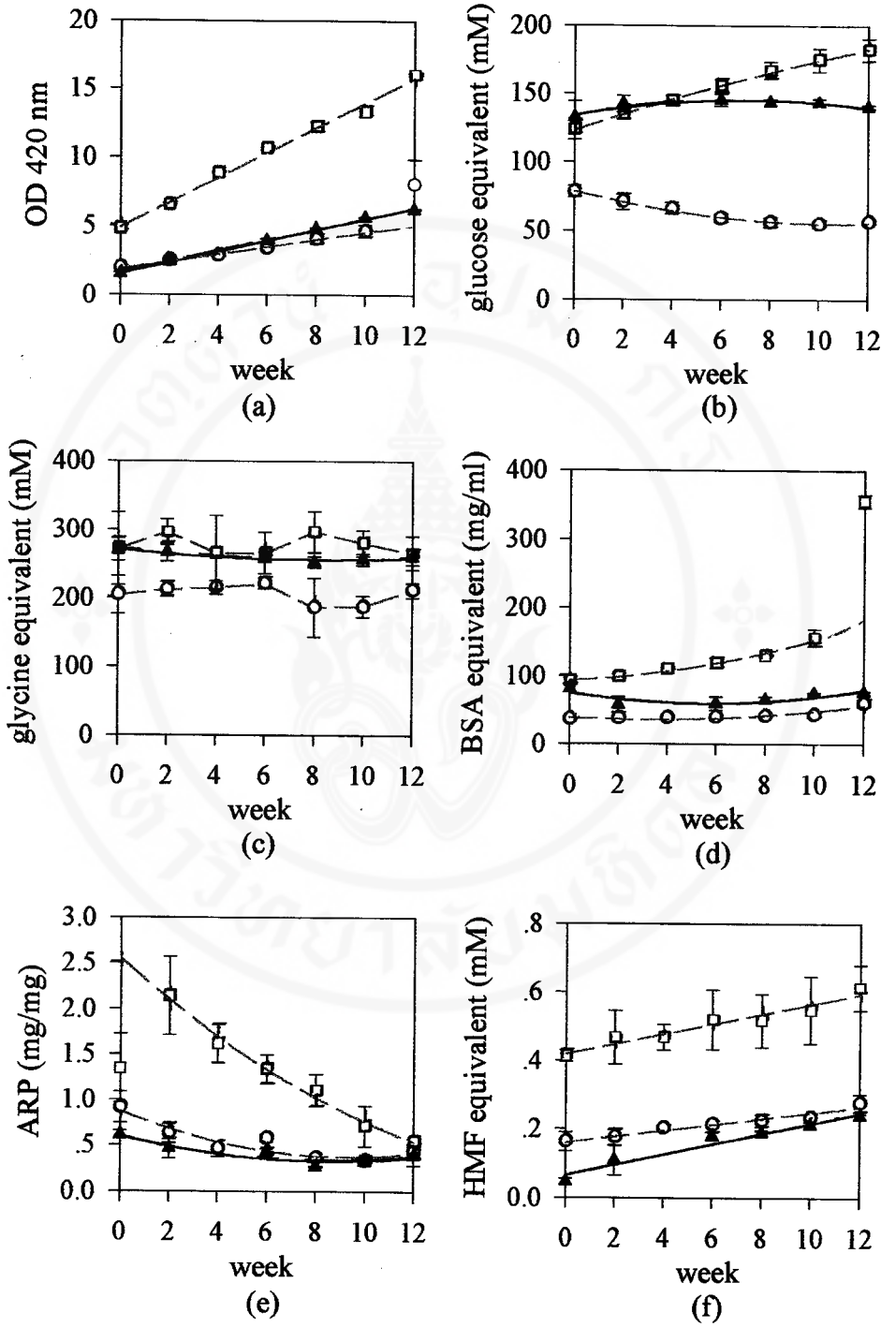


Figure 43: The change of browning (a), RS (b), RAG (c), PP (d), ARP (e), and HMF (f) during storage of raw (○) and cooked (□) soy sauce from Thaisang, and pasteurized (▲) soy sauce from Vichitrungruang.

Table 17: The initial browning and regression equations of browning in pasteurized soy sauce from Vichitrungruang and raw and cooked soy sauce from Thaisang.

soy sauce	initial browning (OD 420 nm)	regression equation	r^2
A	1.478	y = 1.557+0.398x	0.999
B	1.925	y = 1.871+0.268x	0.996
C	4.790	y = 4.981+0.908x	0.996

Note: A is pasteurized soy sauce from Vichitrungruang,

B is raw soy sauce from Thaisang, and

C is cooked soy sauce from Thaisang.

x is browning at OD 420 nm

y is storage time (weeks) within 3 months

7. Elucidation the effect of brine concentration on the Maillard reaction during moromi fermentation

Koji from Thaisang factory was fermented with 18, 20, 22, and 24% brine for studying the effect of % brine on the Maillard reaction. During moromi fermentation, there was film yeast and tyrosine crystals occurred at the 3^{rd}-4^{th} day. Both of them occurred at 24% brine higher than 22, 20, and 18% brine, respectively. The chemical changes (browning, RS, RAG, SP, PP, and pH) as shown in Fig 44, 45 and their regression equations with time-course that showed the same pattern as their changes during moromi fermentation (Table 18). All results showed the same changed during the first 3 days without difference ($P>0.05$) among the different brine concentrations. The exception is the increase of PP in 24% brine system was higher than others since the early stage of fermentation. After the first 3 days, there was significant difference of PP amounts between each brine concentration. The % NaCl in the system decreased during the first 3 days and then slightly increased before remained constant during fermentation. During the first 3 days, brine level in the fermented bottle slightly decreased through evaporation and diffusion into seed Koji as the expansion of seed Koji observed. Then brine level slightly increased due to the release some water from seed Koji.

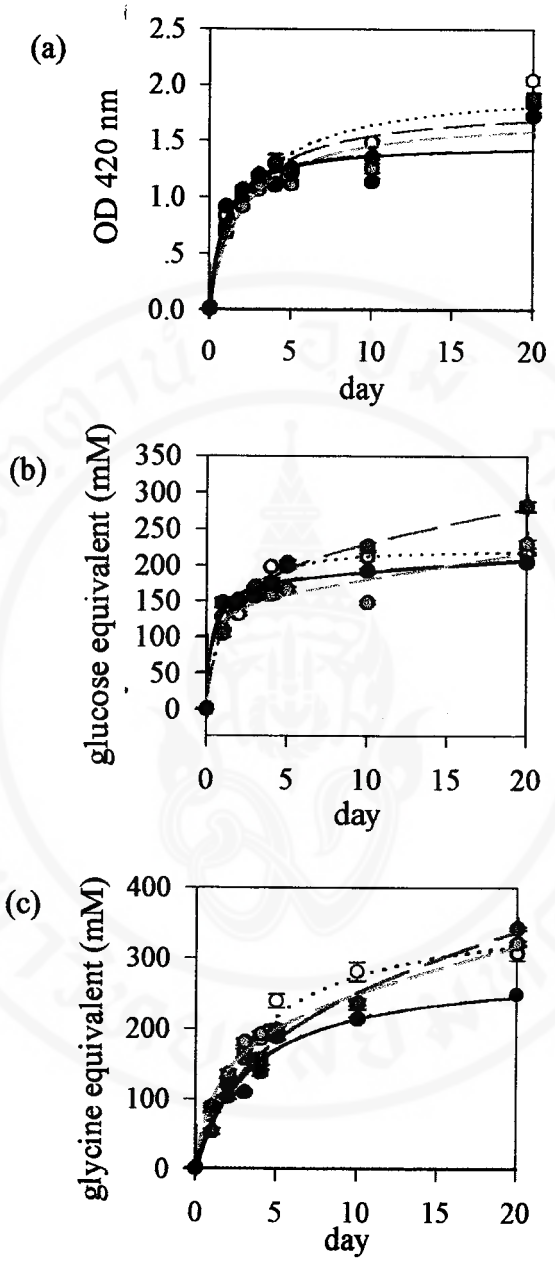


Figure 44: The change of browning (a), RS (b), and RAG (c) during moromi fermentation at various concentrations (○: 18%,◎: 20%,◉: 22%, and● : 24%).

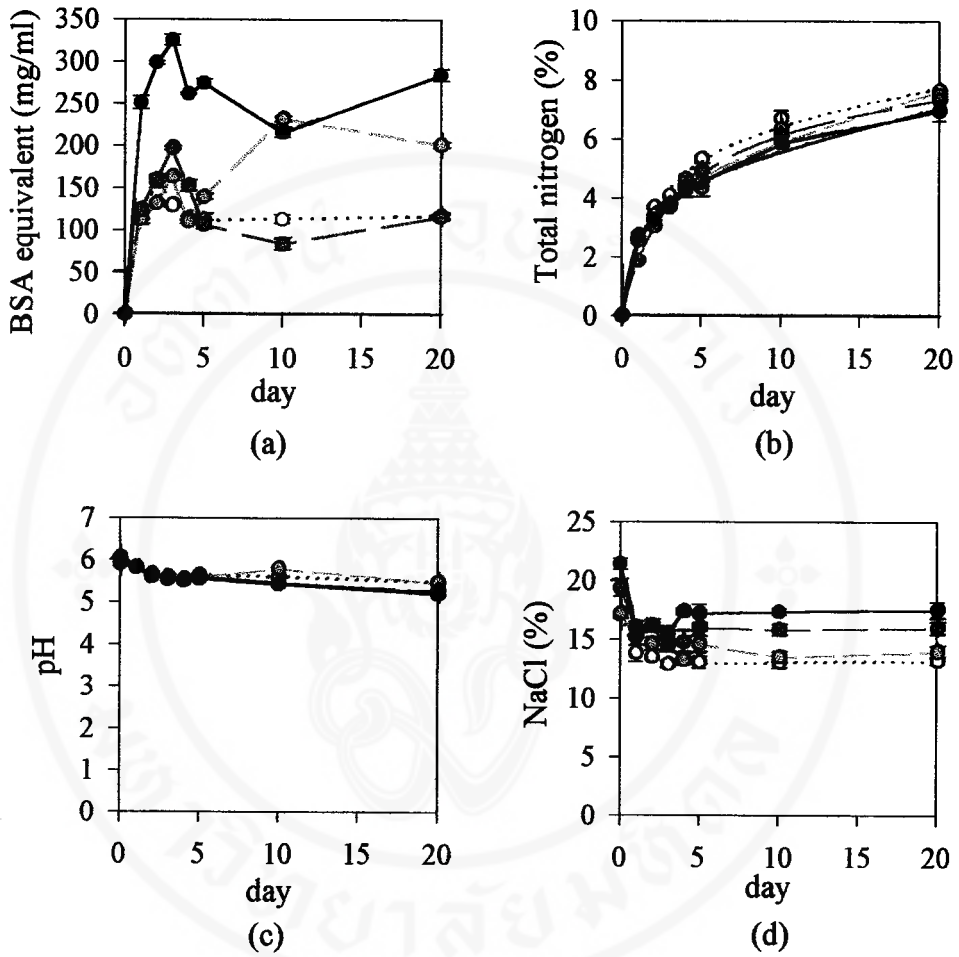


Figure 45: The change of PP (a), SP (b), and pH (c), and NaCl (d) during moromi fermentation at various brine concentrations (○: 18%, ◍: 20%, ●: 22%, and ●: 24%).

Table 18: The regression equations of browning, RS, RAG, SP, and pH of moromi at various concentrations.

parameter	% brine	equation	r^2
browning (OD 420 nm)	18	$y = 2.007x/(2.238+x)$	0.962
	20	$y = 1.739x/(1.943+x)$	0.960
	22	$y = 1.829x/(1.823+x)$	0.976
	24	$y = 1.480x/(0.835+x)$	0.945
RS (mM)	18	$y = [245.558x/(1.173+x)]-0.711x$	0.987
	20	$y = [149.379x/(0.289+x)]+3.542x$	0.973
	22	$y = [211.369x/(1.055+x)]+3.926x$	0.998
	24	$y = [183.561x/(0.349+x)]+1.338x$	0.986
RAG (mM)	18	$y = [173.399x/(3.5866+x)] + [198.474x/(3.5866+x)]$	0.994
	20	$y = [220.894x/(1.448+x)] + [1.314*10^{10}x/(2.379*10^{9}x)]$	0.996
	22	$y = [250.108x/(3.223+x)] + [9.114*10^{9}x/(1.486*10^{9}+x)]$	0.993
	24	$y = [15.034/(x-0.613)] + [293.899x/(4.949+x)]$	0.985

Note: x = browning (OD 420 nm); RS (mM); and RAG (mM)

y = fermentation period (days) within 20 days

Table 18: The regression equations of browning, RS, RAG, SP, and pH of moromi at various concentrations (continued).

parameter	% brine	equation	r^2
SP (%)	18	y = 8.649x/(2.997 + x)	0.995
	20	y = 8.49x/(3.470 + x)	0.995
	22	y = 8.660x/(3.887 + x)	0.999
	24	y = 7.550x/(2.720 + x)	0.987
pH	18	y = 12.964+[(4.112)(0.235)/(0.235+x)]	0.991
	20	y = 13.531+[(3.173)(0.739)/(0.739+x)]	0.934
	22	y = 15.485+[(3.918)(0.079)/(0.079+x)]	0.905
	24	y = 5.163+[(0.867)(2.900)/(2.900+x)]	0.978

Note: x = SP (%); and pH

y = fermentation period (days) within 20 days

8. Elucidation of the influence of aeration on the Maillard reaction

Koji was fermented at 20% brine with or without aeration. The results showed the same pattern as the previous studied, that is, the reaction occurred very rapidly in the first 3 days and then occurred slowly (Table 19). Browning of both model systems similarly increased in the linear manner ($P>0.05$) (Fig 46a). RS amounts of both systems increased without significant difference in the first stage, after that, RS in the aeration system decreased (Fig 46b). The aeration also effected the RAG, PP, SP, and pH after the 3rd day (Fig 46c and 47a-d, respectively). The protein precipitation was affected by aeration (Fig 47a). The aeration caused the decreased of PP, although the PP increased during the first 3 days.

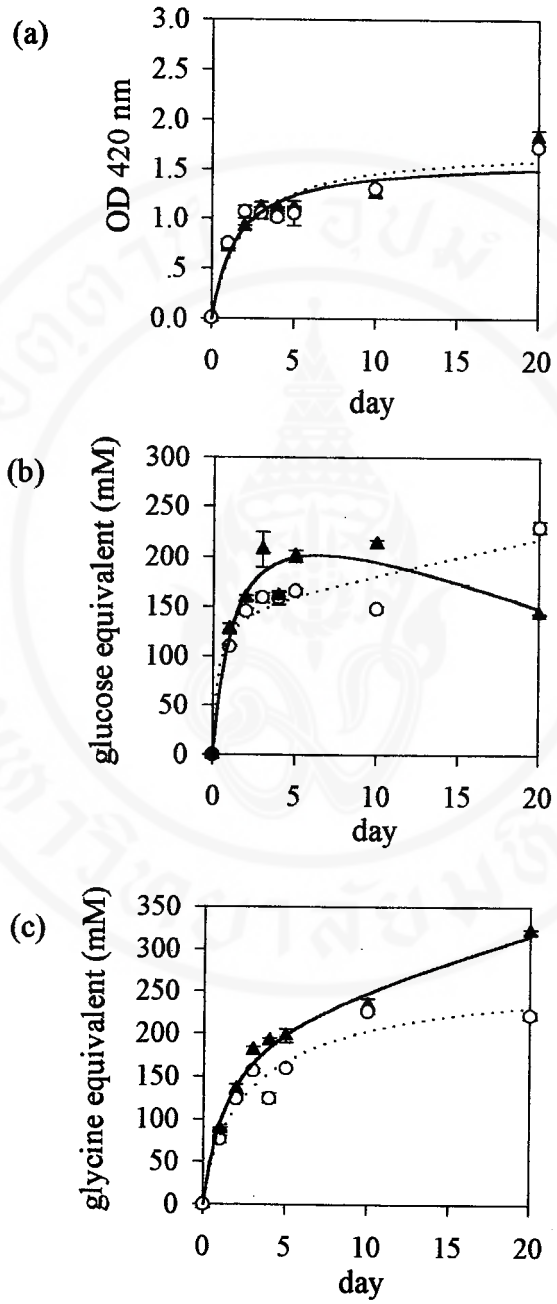


Figure 46: The change of browning (a), RS (b), and RAG (c) in moromi (20%) with (○) or without (▲) aeration during moromi fermentation.

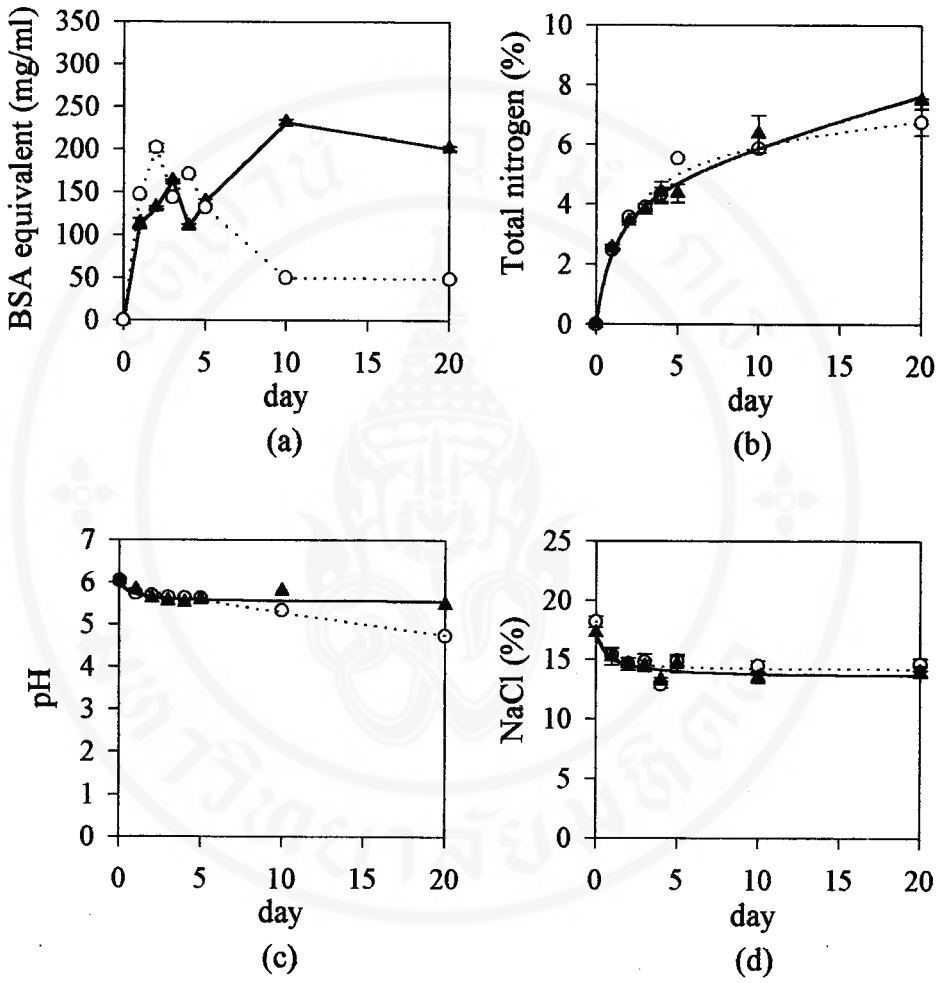


Figure 47: The change of PP (a), SP (b), pH (c), and NaCl (c) in moromi (20%) with (○) or without (▲) aeration during moromi fermentation.

Table 19: The regression equations of browning, RS, RAG, SP, and pH of moromi at 20% brine concentration with (+) or without (–) aeration.

parameter	condition	equation	r^2
browning (OD 420 nm)	+	$y = 1.739x/(2.238+x)$	0.960
	–	$y = 1.607x/(1.511+x)$	0.954
RS (mM)	+	$y = [149.379x/(0.289+x)] + 3.542x$	0.973
	–	$y = [289.271x/(1.271+x)] - 6.193x$	0.968
RAG (mM)	+	$y = [220.894x/(1.448+x)] + [1.314*10^{10}x/(2.379*10^{9}x)]$	0.996
	–	$y = [75.430x/(0.641+x)] + [195.791x/(4.778+x)]$	0.996
SP (%)	+	$y = 8.486x/(3.470+x)$	0.985
	–	$y = 7.398x/(2.288+x)$	0.991
pH	+	$y = 13.531+[(3.173)(0.739)/(0.739+x)]$	0.934
	–	$y = 14.134+[(4.036)(0.307)/(0.307+x)]$	0.897

Note: x = browning (OD 420 nm); RS (mM); RAG (mM); SP (%); and pH

y = fermentation period (days)

CHAPTER V

DISCUSSION

From the preliminary experiment, the changes of browning, RS, RAG, HMF, PP, and ARP were analyzed as the Maillard reaction indices. These chemical changes in each soy sauce during prolonged incubation for 3 months at 37°C showed the similar progress pattern of the Maillard reaction (Fig 23). The rate of browning reaction in soy sauce T was higher than those of in soy sauce K and V, although the initial brown color in soy sauce K was the highest. These results indicated that the initial brown color in each soy sauce product came from the manufacturing and it did not mainly effect on the progress of browning during storage.

The reason for the higher initial brown color in soy sauce K, which was Japanese soy sauce, than others (Thai soy sauce) came from the long aging period of moromi fermentation. In Japanese soy sauce, the moromi fermentation is held for one year (59, 61) while it is carried out for 1-3 months in Thailand (61, 73). Moreover, the long fermentation means the extent of protein hydrolysis. Yong and Wood (1977) illustrated that the extent of protein hydrolysis is the most important factor governing the quality of soy sauce due to the nutritional value depending on the protein and peptide hydrolyzing enzymes of Koji molds (65). These caused the higher RAG in soy sauce K than others did.

While each testing systems was controlled in temperature and time, reactants and other reactive intermediates were the remaining factors to be investigated in the

progress of the Maillard reaction. From Fig 23b, the levels of RAG remained constant during storage and soy sauce K had the highest amount of RAG. The accumulation of HMF through storage test in soy sauce K was higher than soy sauce T and V, respectively (Fig 23f), while browning in soy sauce T occurred faster than soy sauce K. Those results suggested that the change of RAG and HMF could not be used to predict the progress of the browning reaction. The accumulation of HMF was also observed as the index for the Maillard reaction in multi sugars and amino acids model systems, which were storage at 55, 65, and 75°C over 10 days, by Gogos et al (14). They also suggested that HMF level was often used as a quality indicator in processed food (14).

During storage, the amounts of ARP on PP in soy sauce T decreased while remained constant in others (Fig 23e). This result indicated that the ARP decreased when the browning occurred. Ge and Lee (1997) reported that in a tryptophan-glucose model system, ARP amounts reach the maximum before appreciable amounts of brown color developed (79). The decrease of ARP in soy sauce during storage came from the conversion to other intermediate products. As a result, the change of ARP could not be used to predict the progress of the Maillard reaction due to the less correlation between the change of ARP and browning. The correlation coefficients (r^2) between the change of ARP and browning for soy sauce T, V, and K were 0.054, 0.103, and 0.201, respectively.

The change of PP could be predicted the progress of the browning reaction. The correlation coefficients (r^2) between PP and browning for soy sauce T, V, and K were 0.765, 0.667, and 0.924, respectively. The high r^2 indicated that PP amounts increased concurrently with the increase of browning. The precipitation of protein

might come from cross-link of high molecular weight peptides or proteins. Yaylayan and Huyghues-Despointes (1994) reported that the presence of RS *in vivo* could promote protein-bound cross-linking structures and modification of their functional property (14). The higher r^2 between PP and RS for soy sauce T (r^2= 0.614) indicated that the excess RS influenced on the accumulation of PP.

The amounts of RS during storage did not decrease as expected, especially in soy sauce T and V. On contraries, RS increased and such increase was ensured by HPLC analysis. Four types of sugar, i.e. arabinose, xylose, glucose, and sucrose were used as the standards, while Fukushima (1989) reported that mostly of monosaccharide in soy sauce are glucose, xylose, galactose, arabinose, and mannose (73). Glucose in soy sauce comes from two sources; wheat starch (73) and the fragmentation of sucrose, which was added into raw soy sauce for seasoning, during pasteurization. Sasaki and Nunomura (1993) reported that hexoses and pentoses in soy sauce converted from starch and the other carbohydrates, which are remained from the consumption of moulds during Koji fermentation (81).

According to the review of Chean (1991), he reported that the sugar level in soy sauce significantly increases after pasteurization (65). Furthermore, other monosaccharide (xylose, galactose, arabinose, and mannose) derived from soy bean and wheat polysaccharides, while sucrose comes from the seasoning before pasteurization. The amounts of sucrose adding were different in each soy sauce. However, the sugar levels in Thai soy sauce was higher than soy sauce K, while soy sauce K had higher RAG than soy sauce T and V.

From Fig 27a, the xylose levels in soy sauce T was relatively high. Such results may be caused from the resolution of HPLC-column (Ionpak KS 801). This

column can not well separate xylose, mannose, and galactose. When the sugar standard of xylose, mannose, and galactose were co-injected onto HPLC, the chromatogram apparently showed only one peak, which was higher height than the chromatogram from submitted one standard. Although this peak depicted xylose, mannose, and galactose, this peak reflected increasing of xylose. Xylose, which is pentose, is known to undergo browning faster than hexose (3). Yokotsuka (1986) reported that the sugar involved primarily in the Maillard reaction of soy sauce is a pentose such as xylose and arabinose, although the amount of hexose, which is mainly glucose, is for 6 to 10 times greater than the proportion of pentose (62).

In soy sauce T and V, the amounts of xylose and glucose, which were reducing sugar, increased while sucrose decreased (Fig 27a-b). These results ensured the increase of RS in the previous studied. However, the loss of arabinose in soy sauce T and the loss of xylose in soy sauce K indicated that arabinose and xylose were responsible for the browning in soy sauce T and K, respectively.

Fig 27b showed the decrease of sucrose together with the increase of xylose and glucose. The results demonstrated that the disaccharide was hydrolyzed into monosaccharide. However, the change of sucrose, which is the disaccharide of glucose and fructose, to be xylose (5C sugar) were questioned. The increase of xylose might be came from the increase of mannose or galactose (6C sugar) because xylose, mannose, and galactose were eluted in the same time.

According to sucrose as the disaccharide of glucose and fructose, the hydrolysis of sucrose should be the mixture of glucose and fructose. However, fructose was not detected in soy sauce. These results supported the results of Sasaki

and Nunomura (1993) that fructose in soy sauce was undetectable due to its trace amounts (81). Thus, fructose possibly changed into other products.

To investigate the influence of sugar supplementation on the increase of RS during storage, soy sauce T was added xylose, glucose, maltose, and sucrose at various concentrations. Xylose and glucose represented for monosaccharide with 5C and 6C, respectively. Both of them were reducing sugar. Maltose and sucrose represented for reducing and non-reducing disaccharide, respectively.

The progress of brown color in each model was not significantly different, except the model containing xylose (Fig 37). These results suggested that pentose participated in the Maillard reaction and caused more browning than hexose and disaccharide. This phenomenon also ensured the report of Labuza and Baisier (3). Although RS slightly increased and RAG decreased, both reactants could not be used to predict the progress of the Maillard reaction. In this experiment, since the sugar in soy sauce was in excess, the influence of sugar supplementation could not be clearly seen. To clarify this point, sugars should be added before pasteurized as seasoning and tested for storage study.

As in any chemical reaction, many factors influence on the on going of the Maillard reaction. Several reports revealed that metal ions accelerate the development of Maillard reaction products, especially in latter stage of the reaction (1, 3, 43, 44). The metal ions in soy sauce might be came from the contamination from the container during aging or pasteurization. Soy sauce T was added Fe^{2+}, Fe^{3+}, and Cu^{2+} at various concentrations to study the effect of metal ions on the progress of the Maillard reaction during storage. The chemical changes showed no significantly difference between each concentration of metal ions, except the effect of Fe^{2+} on the accumulation of PP

(Fig 32a) and the loss of ARP (Fig 33a). The higher Fe^{2+}, the more accumulation of PP. The results ensured the effect of metal ion on the Maillard reaction products in the previous studies of O'Brien and Morrissey (43) and Hayase et al. (44). As shown in Fig 30, the initial RS in the model systems differ from that of in control system due to the limitation of the monitoring of RS by dinitrosalicyclic acid (DNS) assay. Chaplin and Kennedy (1994) reported that non-carbohydrate reducing agents such as metal ions interfere the DNS assay (78).

The supplementation of potassium iodide (KI) into soy sauce showed the similar results as the studies in the effect of metal ions. KI influenced on the lower accumulation of PP during storage than control and there was no significantly different between each concentration. These results indicated that KI was able to use as iodine supplementary for soy sauce. Moreover, KI could reduce the loss of protein owing to preventing the formation of protein. However, the study of both metal ion and KI on the progress of browning failed to show dose-dependence because there were no significant difference in each concentration.

To investigate the beginning of browning and the Maillard reaction in soy sauce, moromi T and V were collected for analyzing the chemical changes during fermentation. The development of browning during moromi fermentation could be divided into 2-stage consecutive mechanism. The first stage takes approximately 3 days and the browning occurred very rapidly. After the first 3 days of fermentation, the second stage takes the long time till the end of fermentation. In this stage, the browning progressed slowly. As shown in Fig 39b and 39c, the amounts of RS and RAG, which are the important reactants for the Maillard reaction, increased during the

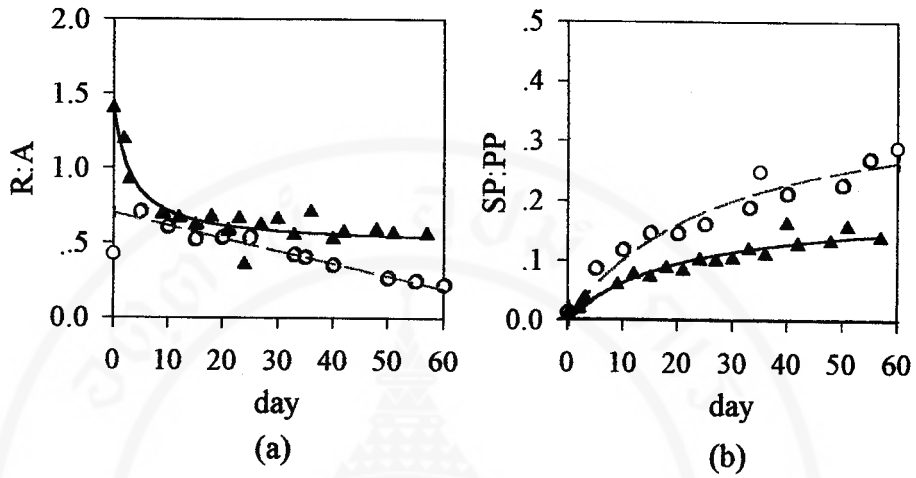


Figure 48: The change in ratio of RS and RAG (R:A) (a) and the ratio of SP and PP (SP:PP) (b) of moromi T (○) and moromi V (▲).

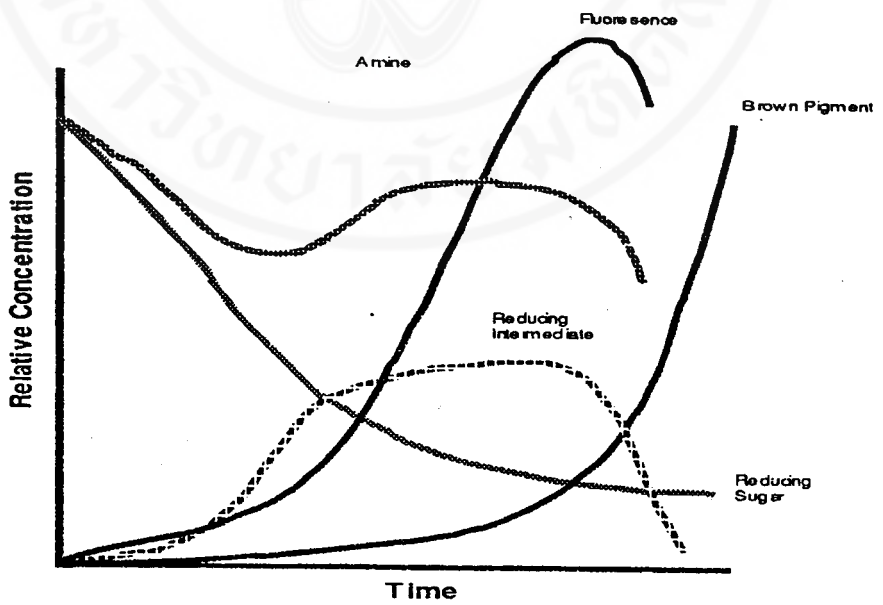


Figure 49: Concentration of reacting and formed species as a function of time during the Maillard reaction (83).

fermentation. These results indicated that the reactants were in excess in both moromi T and V.

The exception was the decrease of RS during the second stage in moromi T. The RS decreased while browning and RAG increased. These results indicated that the Maillard reaction was not the main cause for the loss of RS and both RS and RAG were in excess for developing the brown color in moromi. The decrease of RS resulted from the uptaking sugar by yeast. The increase of RAG while browning progress implied that the change of RAG could not be used to predict the progress of the Maillard reaction.

The ratio of RS and RAG (R:A) during fermentation of both moromi were different (Fig 48a). The R:A ratio of moromi T increased rapidly during the first stage while it decreased in moromi V. After that, the R:A ratio constantly decreased in both moromi. The regression equations of R:A ration in moromi T and V were $y = 0.702 - 0.0085x$ (r^2=0.984) and $y = 0.584 + 0.825^{-0.22x}$ (r^2=0.948), respectively. The R:A ratio in moromi T was lower than that of in moromi V. Renn and Sathe (1977) reported that browning in excess amino acid system is higher than the limit-amino acid system (38). Hence, the high amino acid in moromi T than moromi V might be one of the factors causing the faster browning in moromi T than moromi V.

During moromi fermentation, the amounts of PP in both moromi T and V decreased (Fig 40a), showing the loss of high molecular weight protein in the system. The extent of fermentation produced lower molecular weight peptides due to protein digestion of protease and peptidase resulting from Koji (59, 60, 71). Therefore, the change of SP:PP ratio in moromi T and V increased during moromi fermentation indicating the lower molecular weight of soluble protein (Fig 48b). The hyperbolic

regression equation of the SP:PP ratio were $y = 0.388x/(28.015+x)$, $r^2=0.964$ and $y = 0.195x/(20.588+x)$, $r^2=0.877$ for moromi T and V, respectively. Where x is fermentation time (60 days) and y is SP:PP ratio. The higher SP:PP ratio of moromi T than moromi V implied the higher activity of proteolytic enzyme in moromi T.

During the first stage of fermentation, the pH value of moromi T and V dropped very quickly from 6.5-7.0 to 5.5 because of the organic acid production (mainly in lactic acid) of microorganisms (59, 61). In the second stage, the higher lactic acid production, the lower pH. The pH dropped to 4.9-5.0 and yeast growing replaced the lactic acid bacteria (59). The salt tolerant yeast responsible for the alcoholic fermentation and conversion of glucose or other sugars into volatile alcohol (59, 60). The pH also influences on the RS through the mutarotation reaction (38).

Since only the acyclic form of sugar with free aldose group can react with amino group to undergo the Maillard reaction (3), the acidic pH could retard the rate of browning owing to the less acyclic form of RS than basic pH. Therefore, the model systems are studied in basic solution to accelerate it (82). In Fig 41a and 42d, brown color occurred slowly and pH slightly decreased since the 4^{th} day. Hence, these results suggested that pH approximately 5.8-5.9 could be retarded the development of brown color in moromi. The decrease of pH value during the second stage of moromi fermentation partially caused the lower rate of browning.

The accumulation of ARP (Fig 40b) in moromi T and V related to the change of RS (Fig 39b). The level of ARP increased rapidly then declined in moromi T, while ARP was accumulated through the fermentation in moromi V. However, amounts of ARP in moromi T were higher than moromi V. These results suggested that the decrease of RS in moromi caused the less sugar-bound on PP. The higher ARP in

moromi T also indicated that moromi T had higher initial intermediate to form brown pigment higher than moromi V. Thus, moromi T brown faster than moromi V, although the ARP in moromi T was higher than moromi V. These results indicated that PP in moromi T bound with sugar to form ARP more than that of in moromi V.

The change of total acidity showed higher acidity in moromi V than moromi T (Fig 40e) because moromi V was agitated during fermentation. The agitation in moromi V caused the better growth of microorganisms and produced more acidity than moromi T, which was not agitated. The high acidity in moromi V resulted the slightly lower pH than moromi T. Hence, brown color formation in moromi T was higher than moromi V.

According to the calculation of browning rate by using the browning equations, the browning rate of moromi T and V dropped from 0.365 to 0.0014 and 0.464 to 0.0009, respectively. The rate of accumulation of HMF was 0.0025 and 0.0016 for moromi T and V, respectively. The rate of HMF increased in moromi V was higher than moromi T approximately 1.563 times, although the brown color in moromi T has higher than moromi V.

After the moromi fermentation was finished, the raw soy sauce T was filtered the seed Koji out and leave to precipitate the tiny particle for few days. Raw soy sauce was collected before it was seasoning and pasteurized by the factory. After the pasteurization and bottling, cooked soy sauce was sampling again while raw soy sauce from Vichitrungrunag factory was picked to filtrate and pasteurize without seasoning in laboratory. Raw and cooked soy sauce T (from Thaisang factory) and pasteurized soy sauce V (from Vichitrungruang factory) were incubated at 37°C for 3 months for studying the Maillard reaction mechanism during storage.

The chemical changes during storage of raw soy sauce T and pasteurized soy sauce V continued from the change during moromi fermentation, while cooked soy sauce T was different due to the seasoning and pasteurization.

The initial browning of cooked soy sauce T was higher than others about 2.5 times (Fig 43a). The rate of browning derived from the regression equation was highest to lowest in the following order: cooked soy sauce T (0.9076 OD/week), pasteurized soy sauce V (0.398 OD/week), and raw soy sauce T (0.268 OD/week). The rate of browning of cooked soy sauce T was higher than pasteurized soy sauce V and raw soy sauce T approximately 2.280 and 3.387 times, respectively.

As shown in Fig 43b, RS amounts in cooked soy sauce T increase (4.977 mM/day) as occurred in the first experiment implying the excess RS in the system. Baisier and Labuza (1992) studied the effect on increasing sugar concentration at constant amine concentration (47). The further study of Labuza ensured this finding (83). They explained that the loss of amine during the early reaction is the first order reaction, then amine increase owing to the recycle step of bound amine into free amine (Fig 49). After that, amine remained constant reaching a no-loss period (47, 83). The constant RAG might be came from the on going of the loss of amine during no-loss period or there is amine excess in soy sauce.

PP of cooked soy sauce T increased with higher rate than pasteurized soy sauce V and raw soy sauce T (Fig 43d) due to the modification or cross-linking of amino acid residues of protein as reviewed by Fukushima (63). Despite the heat treatment did not make immediately precipitation. Heat treatment may generate reactive carbonyl compounds. These carbonyl compounds could modify the protein and

enhance PP formation. The seasoning and pasteurization also influenced on the accumulation of HMF and the loss of ARP in cooked soy sauce T.

Almost of chemical changes of cooked soy sauce T were higher than raw soy sauce T, which are similar change as pasteurized soy sauce V. These results indicated that not only the pasteurization caused for raising of brown color in soy sauce manufacture, but the seasoning also involved in increasing the RS amounts in the system and enhanced browning.

As shown in Fig 40b, there was fluctuation of % NaCl during the fermentation of moromi T, while the % NaCl of moromi V increased. Therefore, the effect of brine concentration on the Maillard reaction during moromi fermentation were studied by fermenting Koji from Thaisang factory with various brine concentrations for 20 days. Browning, RS, RAG, SP, PP, and pH were analyzed as the chemical changes. The results showed the same pattern of changes as in the previous studies, that is, the fermentation could be divided into the first and the second stage due to the rate of reaction. However, all of results had no difference between concentration of brine. It implied that the brine concentration in this study did not apparently influence the progress of browning. Obviously, the slightly difference of browning, RS, and RAG occurred during the late of fermentation (10^{th}-20^{th} day). Hence, the extent of fermentation period more than 20 days might showed the difference change of browning, RS, and RAG between each concentration.

However, the brine concentration influenced the change of PP. The model containing 24% brine showed the highest PP amounts. This might come from the more solubility of high molecular weight protein in high brine concentration, and less of those soluble proteins were further digested to free amino acid.

Further experiment investigated the influence of aeration on the Maillard reaction during moromi fermentation (20 days). Koji from Thaisang factory was fermented in 20% brine with or without aeration. The aeration was controlled 20 ml/min constantly through the fermentation. The chemical changes showed the similar pattern as the previous studied. The aeration did not effect the browning due to no difference between both systems (with or without aeration). These results indicated that oxidative browning was not mainly involved in the browning development during moromi fermentation. During the first stage of fermentation, the change of RS, RAG, and PP in each model system were not significantly different. After that, amounts of RS, RAG, and PP in aeration system were lower than that of in non-aeration system. The aeration also enhanced the growth of yeast, which was uptaking sugar for growing and caused the reducing of RS during the second stage of fermentation.

Although the aeration act as agitator providing the sufficient oxygen to promote the growth of yeast, a too frequent agitation makes moromi too sticky and promotes lactic acid fermentation and suppresses yeast fermentation (60). Therefore, lactic acid bacteria converted reducing sugar including with the conversion of citric acid contained in the soybean to lactic acid (61). These caused the declined of RS together with pH in aeration model lower than non-aeration model during the second stage of fermentation.

The agitation by compressing air also turbid the system. Not only the free amino acid but also peptides were interfered by aeration. Nevertheless, the aeration did not effect on the soluble protein during 20-day fermentation (Fig 47b), although Yokotsuka (1986) found the higher total nitrogen solubility by agitation (62). The

extent fermentation period could provide the more data that could support or oppose the Yokotsuka suggestion.

All the results suggested that the brown color development in soy sauce began from moromi fermentation and increased rapidly when it was seasoned and pasteurized. Hence, the conventional method of heat treatment (heated to 80°C, then left to reduce its temperature to room temperature) might be changed to other method such as high temperature, short time (HTST) for preventing the brown color formation. Because HTST could be avoided the long holding of soy sauce in high temperature condition. Moreover, moromi could be agitated sometimes through fermentation to prevent the growth of yeast and the agitation did not affect the brown color development.

CHAPTER VI

CONCLUSION

1. The browning reaction development in moromi of Thai soy sauce was a 2-stage consecutive mechanism. The browning occurred rapidly in the first stage during the first 3 days of fermentation and then occurred slowly in the second stage. The amounts of HMF as the Maillard reaction index increased linearly through the fermentation. These indicated that the Maillard reaction probably not play an important role of brown color during the first stage of fermentation but play as the major role during the second stage. The pH was also a factor, which limited the on going of the Maillard reaction, because the low pH could retard the rate of browning. Since RS and RAG were in excess in moromi, they were not limiting factor for the Maillard reaction.

2. In storage test of soy sauce products, seasoning and pasteurization were the main effect on the high accumulation of HMF and 5% TCA-precipitated protein. The increase of HMF could be used as the Maillard reaction index during storage test. The heat treatment generated the reactive carbonyl compounds resulting from the sugar fragmentation stage of the Maillard reaction. The seasoning also supplied sugar and carbonyl compounds to soy sauce, resulting in the increase of RS during storage test. The HPLC analysis of sugar levels ensured the increase of RS and the results indicated that arabinose and xylose were responsible for the progress of the Maillard reaction in Thai soy sauce and Japanese soy sauce, respectively.

3. The difference of brine concentration (18, 20, 22, and 24%) did not effect on browning development during the first 20 days of moromi fermentation. Only the model system containing 24% brine gave the higher 5% TCA-precipitated protein.

4. The aeration not only compressed the air into the model but also agitated the system. Despite aeration did not affect on the chemical changes during the first 3 days of fermentation, after that, it influenced on the turbidity, decrease of pH value, brown color formation, and less reducing sugar and amino acid in the model system.

5. The existence of metal ions (Fe^{2+}, Fe^{3+}, and Cu^{2+}) in soy sauce from Thaisang did not directly effect on the progress of the Maillard reaction during storage. However, Fe^{2+} influenced on the higher accumulation of 5%TCA-precipitated protein and the loss of Amadori products during storage.

5. The KI supplementary at various concentrations into soy sauce caused the less precipitation of protein with 5% TCA without dose-dependence but it did not influence the progress of browning. The results implied that KI is able to use as iodine supplementary in soy sauce and does not cause browning effect.

REFFERENCES

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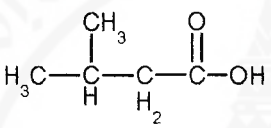
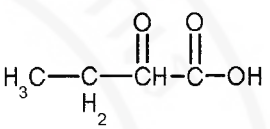
APPENDIX A

STRUCTURES OF VOLATILE COMPOUNDS

1. Organic acids

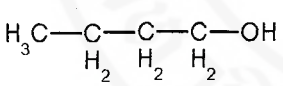


iso-valeric acid

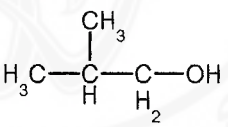


ketobutyric acid

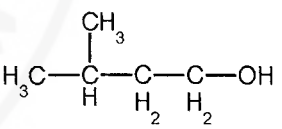
2. Alcohols



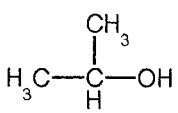
1-butanol
(*n*-butyl alcohol)



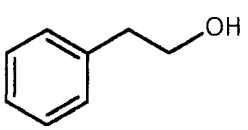
2-methyl-1-propanol
(isobutyl alcohol)



3-methyl-1-butanol
(isoamyl alcohol)



isopropyl alcohol



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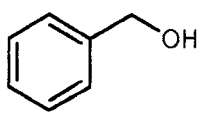
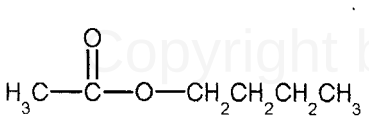


benzyl alcohol

3. Esters



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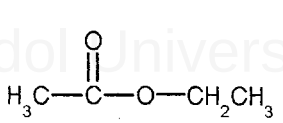

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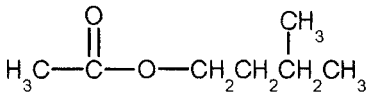
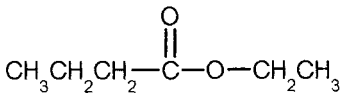
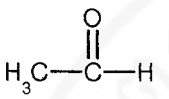


isoamyl acetate

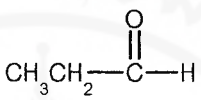


ethyl propionate

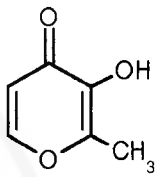
4. Carbonyl and relative compounds



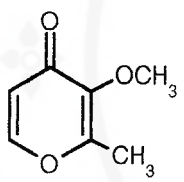
acetaldehyde



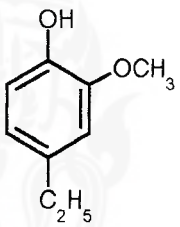
propionaldehyde



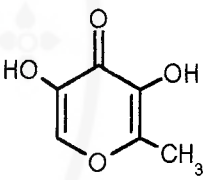
maltol



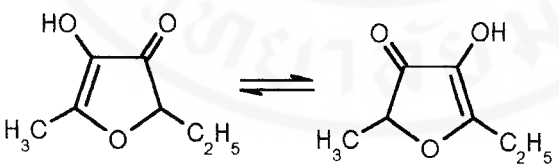
3-methoxy-2-methyl-4H-pyran-4-one



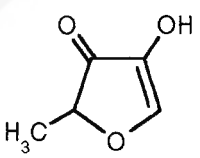
4-EG



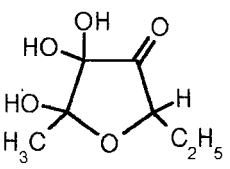
5-hydroxymaltol



HEMF



HMMF



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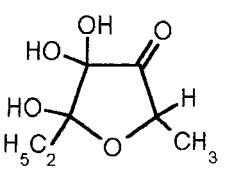
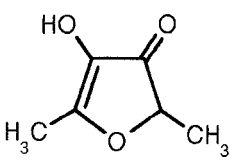
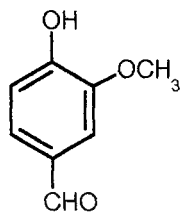


OX-HEMF

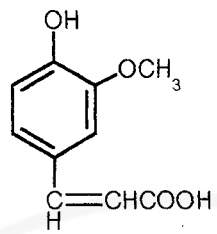


HDMF

5. Phenolic compounds



vanillin



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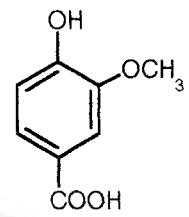
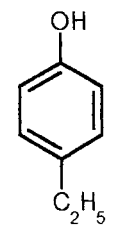
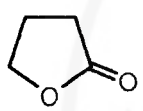


vanillic acid

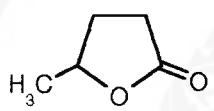


p-ethylphenol

6. Lactones



4-butanolide



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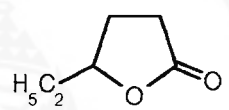
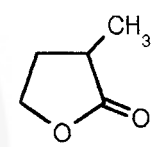
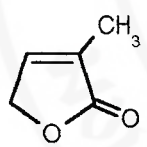


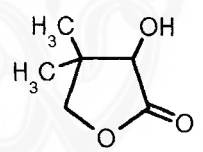
4-hexanolide



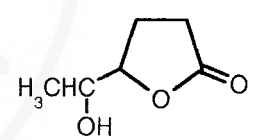
2-methyl-4-butanolide



2-methyl-2-buten-4-olide

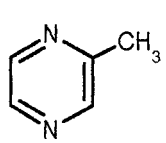


2-hydroxyl-3,3-dimethyl-1,4-butanolide

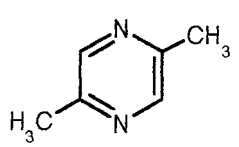


5-hydroxyl-4-hexanolide

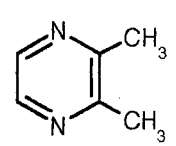
7. Others



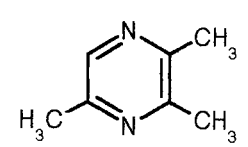
2-methylpyrazine



or



dimethylpyrazine



trimethylpyrazine

APPENDIX B

THE PREPARATION OF REAGENTS

1. Mobile phase for HPLC

Two ml of phosphoric acid (H_3PO_4) was added to distilled water, and then filtered through 0.45 μM membrane before added 50 ml of acetonitile. The mixture was degassed for 10 min by stirring under vacuum condition.

2. Mercuric nitrate solution, 0.2 M $Hg(NO_3)_2$

The solution of mercuric nitrate was prepared by dissolving 34 g $Hg(NO_3)_2$ in 400 ml distilled water which containing 10 ml of 2 M HNO_3 and filled up with distilled water to 500ml. The mercuric nitrate solution was standardized with standard salt solution of 0.1 N NaCl.

3. Color indicator

a) 0.1% Diphenylcarbazone was performed by dissolving 0.1 g of diphenylcarbazone in 100 ml of 95% ethanol and stored in a dark bottle.

b) 0.1% Phenolphthalein was performed by dissolving 0.1 g of phenolphthalein in 10 ml of 95% ethanol and made up the volume to be 100 ml with distilled water.

APPENDIX C

THE DETERMINATION OF TOTAL NITROGEN ACCORDING TO KJELDAHL'S METHOD

The following text describes a step by step procedure for Kjeldahl analysis by using Tecator Kjeltec Systems.

1. One ml of soy sauce sample and blank in digestion tube were added 0.8 g of $CuSO_4 \cdot 5H_2O$ and 7 g of K_2SO_4 (alternatively Na_2SO_4), and then carefully added 15 ml of concentrated H_2SO_4.

2. Attach the exhaust system to the digestion tubes in the rack and set the water aspirator to full effect.

3. Load the rack with exhaust into a preheated digestion block (420°C).

4. After 5 minutes, turn down the water aspirator until the acid fumes are just contained within the exhaust head and continue to digest for 40 minutes. The samples are clear with a blue/green solution.

5. Remove the rack of tubes with exhaust still in place and put in the stand to cool for 20 min. Then carefully add 30 ml of distilled water to the tubes.

6. Add 25 ml of 4% boric acid (400 g of boric acid in hot distilled water 1 liter) to a receiver flask with 2-3 drops of bromocresol green/methyl red indicator solution. Then place the receiver flask into the distillation unit and raise the platform so that the distillate outlet is submerged in the receiver solution.

7. Place the digestion tube in the distillation unit and close the safety door and dispense 50 ml of 40 % NaOH (800 ml NaOH with 200 ml distilled water) into the tube.

8. Open the steam value on the distillation unit and distil for approximately 4 min. At the end of distillation, the receiver solution in the distillate flask will now be green and contain 150 ml of the solution.

9. Titrate the distillates with 0.1 N HCl (8.3 ml of concentrated HCl in distilled water 1 liter), which standardized with Na_2CO_3 before used, until the pink color occurs.

The volume of 0.1 N HCl used to reach the end point is used to calculate the concentration of total nitrogen in the sample by the following equation:

$$\% \text{ Total nitrogen (w/v)} = \frac{(T-B) \times N \times 14.007 \times 100}{\text{Volume of sample (ml)}}$$

Where T is titration volume for sample (ml), B is titration volume for blank (ml), and N is normality of acid to 4 places of decimal.

BIOGRAPHY

NAME	Miss Roungdao Maungma
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INSTITUTIONS ATTENDED	Thammasat University, 1993-1996: Bachelor of Science (Food science and technology)
	Mahidol University, 1997-1999: Master of Science (Biotechnology)
RESERCH GRANT	Partially supported by Thailand Research Foundation (Grant No. PDF 22/2541) and Faculty of Science, Mahidol University.