FACTORS AFFECTING PROPERTIES AND ANTIOXIDANT CAPACITIES OF ROSELLE (*HIBISCUS SABDARIFFA* L.) EXTRACTS AND THEIR APPLICATION IN MEAT PRODUCTS

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ABSTRACT

A growing trend in food industry is the development of functional foods or foods from nature. The roselle calyces have attractive color and contain bioactive compounds associated with a number of health benefits that have been confirmed by scientific evidences. Three experiments were conducted to determine: i) the physicochemical and antioxidant properties of roselle extracts prepared by two extraction methods (OG and NG), ii) the antioxidant capacity of roselle extract in Chinese-style sausage as affected by sweeteners and iii) the ability of roselle extract in reactive nitrogen species (RNS) scavenging *in vitro* and nitrite scavenging in meat products.

The properties OG and NG roselle extracts were evaluated under different pH (3.0 and 4.0) with or without sucrose (20 %) and heat treatment (50 to 70 °C). The Delphinidin 3-sambubioside (Dp 3-sam) and Cyaniding 3-sambubioside (Cy 3-sam) were identified as major and minor anthocyanins found in both OG and NG roselle extracts respectively. Significant loss of pH and temperature-dependent anthocyanin pigments were observed in all roselle extract model samples. In addition, results indicated that the highest pigment extraction efficiency was found in the NG roselle at pH 3.0 than at pH 4.0 and then OG roselle. It can be concluded that nano-grinding may favor the extraction of pigments but lead to their serious degradation during storage. *In vitro* antioxidant activities of the OG and NG roselle extracts were evaluated by ferrous ions chelating activity (FICA), trolox equivalent antioxidant capacity (TEAC) and ferric thiocyanate (FTC). Overall results showed that NG samples tended to exhibit higher antioxidant activities for all assay methods compared to the OG samples.

The effect of different sweeteners including sucrose, lactitol, maltitol and xylitol on the physicochemical property and oxidative stability of Chinese-style sausage (CSS) with addition of

roselle anthocyanin extracts (RAE) were evaluated. Xylitol added CSS showed lower moisture content and water activity compared to other sweetener evaluated. No serious changes of color and texture parameters were observed for all CSS samples during 28 day storage. The RAE treated CSS with sucrose addition showed the greatest TBARS values during storage, while the samples with sugar alcohols had significantly lower values. The xylitol can only promote the antioxidant activity of RAE in prevention of lipid oxidation in CSS. Xylitol addition at 16.6 % in the CSS resulted in the sausage with quality characteristics similar to the control CSS. However, xylitol at higher concentration (21.6 %) could cause the pro-oxidant activity in the RAE treated CSS. The sensory evaluation by Quantitative descriptive analysis (QDA) and 7-point hedonic scale revealed a significant higher panel preference for RAE treated CSS with xylitol addition compared to the sucrose added samples and the control CSS (sucrose without RAE). Results also proved the potential use of 16.6 % xylitol with 0.3 % RAE in CSS that ensured the overall quality of the products. It can be concluded that xylitol is a promising alternative sweetener in CSS especially when RAE is used as natural antioxidant.

The ability of RAE in scavenging of reactive nitrogen species (RNS) compared to anthocyanins from black carrot and grape was investigated. These anthocyanin samples exhibited concentration and pH dependent in nitrite scavenging activity. At pH 3.0, the activity increased from 15 to 80 % when the concentration of the anthocyanins increased from 0.1 to 1.0 mg/ml. The nitrite scavenging activity was dramatically decreased when the pH of the reaction system increased from pH 3.0 to 9.0. In addition, the concentration dependent activity was also observed for the nitric oxide scavenging and inhibition of peroxynitrite induced oxidation of Evan-blues dyes. The potency of RNS scavenging activity for the anthoycyanins tested was in the order: grape > roselle > black carrot.

The RAE was then evaluated for its capacity in nitrite reduction in meat products including Vienna pork sausage and traditional Thai fermented pork (Nham). The residual nitrite in RAE treated (0.3 %) Vienna pork sausage with initial 125 and 250 ppm nitrite reduced to 65 and 168 ppm, respectively after refrigerated storage for 24 days. On the other hand, residual nitrite in all Nham samples rapidly decreased > 90 % of the initial nitrite level after 3 days of fermentation at room temperature. Degradation kinetics of nitrite in Nham was the first-order kinetics. The lactic acid fermentation enhanced the reduction of residual nitrite in Nham.

Overall results revealed that roselle calyces can be a good source of anthocyanins with strong antioxidant activity and can potentially be used as natural antioxidant in meat products.

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LIST OF ABBREVIATION

ACN	Anthocyanins
OG	“Original-grinding roselle extracts
Ng	“Nano-grinding roselle extracts
Cy	Cyanidin
Pg	Pelargonidin
Mv	Malvidin
Pt	Petunidin
Pn	Peonidin
Dp	Delphinidin
FICA	Ferrous ions chelating ability
TEAC	Trolox equivalent antioxidant capacity
FTC	Ferric thiocyanate antioxidant assay
TBARS	Thiobarbituric acid reactive substances
TEP	1,1,3,3 tetramethoxypropane
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
$NaNO_2$	Sodium nitrite
$ONOO^-$	Peroxynitrite
NO^-	Nitric oxide
HNO_2	Nitrous acid
N_2O_3	Dinitrogen trioxide
RO_2^-	Peroxyl radicals
CSS	Chinese-style sausage
BHA	Butyric hydroxyanisole
RAE	Roselle anthocyanin extracts
IC_{50}	inhibitor concentration which results in 50% inhibition of activity

CHAPTER 1

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L) is commonly known as "kra-jeab" in Thai, calyx of roselle contains various antioxidants, such as anthocyanin, quercetin, L-ascorbic acid and proto-catechuic acid (Rice-Evans et al., 1996). Roselle anthocyanins are natural food colorant (E163) that has been approved on positive lists issued by Food and Drug Administration in the US, EU and Japan (Socaciu, 2008). Many biological activities of anthocyanins have been extensively reported, such as antihypertensive and cardioprotective agents, hepatoprotector, inhibitor against porcine pancreatic α-amylase and antioxidant capacity (Rice-Evans et al., 1996).

Anthocyanin pigments, in a model system, are highly affected by pH and solution system. They are in equilibrium of color (cationic) and/or colorless (pseudobase) structure. Moreover, other factors such as temperature, light, oxygen and hydrogen are also important in the stability of anthocyanins. In foods system, at high temperature, high sugar concentration, pH or ascorbic acid and others additives may affect the rate of anthocyanins destruction. Another factor that can give negative effect on anthocyanin stability is metal reactivity as anthocyanins form stable complex with tin (Sn), copper (Cu) and iron (Fe) (Patras et al., 2010).

Over the past decade, the use of a small particle has been studied extensively in biomedical and pharmaceutical application (Chen et al., 2006; Salata, 2004). By reducing particle size, the nanotechnology can contribute to improve the properties of bioactive compounds, such as delivery properties, solubility, prolonged residence time in the gastrointestinal tract and efficient absorption through cells (Sozer and Kokini, 2009). In recent report, Morris (2007) has shown that nanoparticle of carotenoids can be dispersed in water, allowing them to be added to fruit drinks providing improved bioavailability. However, no scientific finding has been reported on physicochemical properties of anthocyanains extracted from nano-particle plant materials compared to those prepared from traditional ground particles.

Nowadays, consumers' demand for natural products, as well as their concern over commonly use of synthetic antioxidants, suggests that it is important to identify functional natural antioxidants to use in meat and meat products. Lipid and protein oxidation is important to the meat industry because it is one of the major causes of quality deterioration which undergo auto-oxidation before meat is cooked. Lipid and protein oxidation can impart negative effects on

sensory attributes such as color, texture, odor and flavor, as well as negatively impacting the nutritional quality of the products (Nunez de Gonzalez et al., 2008; Raghavan and Richards, 2007). In the food industry, the most common approach to control oxidative rancidity is through the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propylgallate (PG) (Jayathilakan et al., 2007). However, the uses of these synthetic antioxidants at high concentration are undesirables in terms of toxicological effects (Sebranek and Bacus, 2007). Moreover, a list of some synthetic antioxidant seems incompatible with functional claims. Based on these reasons, food processors are seeking for natural antioxidants to replace the synthetic ones.

Roselle anthocyanins have been reported to be a good antioxidant (in terms of antilipoperoxidant activity) in ethnic meat products including Chinese-style sausage and pork chips with sucrose content lower than 10 %. However, high concentrations of sucrose (> 10 %) have been shown to negatively affect the antilipoperoxidant capacity of roselle anthocyanins when incorporated at 0.3 % (w/w) in Chinese-style sausage (Pinsirodom, 2008). In addition, when the xylitol was used to replace sucrose in Chinese-style sausage, the roselle anthocyanins efficiently exhibited antilipoperoxidant capacity. It is, therefore, of interest to further investigate whether different types of sugar alcohols will affect the antioxidant activity of roselle anthocyanins in the same manner.

The use of sugar alcohols as an alternative sweetener in meat products has not yet been reported elsewhere. The interest in application of sugar alcohols in food products is not only due to their low calorie and ability to improve blood sugar control, but also their excellent hydroxyl radical ($HO^{\bullet}$) scavenging capacity and *in vitro* inhibition of diazocompound-induced erythrocyte damage (den Hartog et al., 2010). Thus, sugar alcohols would be a promising sweetener for meat products especially when roselle anthocyanins will be incorporated as anti-lipid oxidation agent (Pinsirodom, 2008).

In the case of processed meat such as ham, bacon, frankfurter, bologna and others that are typical cured by addition of sodium/potassium nitrite or nitrate, which are used to control the growth of food born pathogens, provide cured meat flavor and color and extent shelf-life of products (Sebranek and Bacus, 2007). However, the concern about the potentially adverse health implications of the breakdown products of nitrate, i.e. nitrites and nitrosamines, have led the health authorities to set upper limits for nitrate in meat products and regulate the use of nitrate and nitrite as food additives. Thus, the reduction of the residual nitrite could be an acceptable

alternative to reduce the intake of nitrite in processed meats. The use of nitrate and/or nitrite has fallen under scrutiny due to their potential toxicological effects (Nunez de Gonzalez et al., 2008; Naveena et al., 2008). Plant extracts high in phenolic contents such as tomato and citrus co-products have been reported to be effective nitrite reduction agent (Østerlie and Lerfall, 2005; Viuda-Martos et al., 2009). It is possible to use the roselle anthocyanins extract to retard lipid and protein oxidation and at the same time reduce residual nitrite of meat products during storage. Successful application of roselle anthocyanins in meat products may enhance the value of roselle, thus expanding the utilization of the roselle as an inhibitor against lipid and protein oxidation and nitrite reduction agent.

The aim of this dissertation is to assess factors (the particle size of roselle extract, pH, sucrose and temperature) that can influence the physicochemical properties and antioxidant capacities of roselle extracts preparing from different extraction of dried roselle calyxes in model solution. The application of roselle extracts as natural antioxidant and nitrite residual reduction agent in meat products will also studied.

The objectives of this study were:

1 To determine physicochemical and antioxidant properties of roselle extracts prepared by original-grinding and nano-grinding method.

2 To evaluate antioxidant capacity of roselle anthocyanin extracts on lipid and protein oxidation as affected by sucrose and different sugar alcohols in Chinese-style sausage.

3 To investigate the ability of roselle anthocyanin extracts in scavenging of reactive nitrogen species in model system and evaluates their effect on nitrite reduction in meat products.

CHAPTER 2

LITERATURE REVIEWS

2.1 Roselle (*Hibiscus sabdariffa* Linn.)

2.1.1 Introduction

Roselle (*Hibiscus sabdariffa* Linn.) is a shrub belonging to the family-Malvaceae. It is thought of native to Asia (India to Malaysia) or Tropical Africa. In English, roselle is called as Rozelle, Sorrel, Red sorrel, Jamaica sorrel, Indian sorrel, Guinea sorrel, Sour-sour, Queensland jelly plant, Jelly okra, Lemon bush and Florida cranberry. In Thai, it is called Krajeab or Krajeab-dang and it is known by these names in the pharmaceutical and food-flavoring.

The species *H. sabdariffa* comprises a large number of cultivated types which, on the basis of their growth habit or end use, are classified broadly under two varieties, *H. sabdariffa* var. *sabdariffa* and *H. sabdariffa* var. *altissima Wester*. Roselle is cultivated in various parts of Nakhon Pathom, Pathum Thani, Nonburi, Suphanburi, Samutsakorn, Pijit, Kanjanaburi, Ratchaburi, Rayong and Nakornnayok during July to September. The propagation is done by seeds or by rooting shoot cuttings. The edible fleshy calyces are collected after 15 to 20 days of flowering. Rest of the crop is left in the field until seeds are ready for threshing. The calyces can be dried and stored in air-tight container.

2.1.2 Phytochemistry of roselle

The leaf is reported to contain protein, fat, carbohydrate, fibre, ash, calcium, phosphorus, iron, thiamine, β-carotene, riboflavin, niacin and ascorbic acid. The calyces are rich in acid and pectin (Wong et al., 2002). Analysis of calyces has shown the presence of crude protein and minerals such as iron, phosphorus, calcium, manganese, aluminium, magnesium, sodium and potassium. Mucilage, calcium citrate, ascorbic acid, gossypetin and hibiscin chlorideare also present in calyces.

The seeds contain protein (18.8 to 22.3 %), fat (19.1 to 22.8 %) and dietary fibre (39.5 to 42.6 %). The seeds were found to be a good source of minerals like phosphorus, magnesium, calcium, lysine and tryptophan. Seed oil is rich in unsaturated fatty acids (70 %), of which linoleic acid constituted 44 %. The physicochemical analysis of the fresh calyces and leaves are given in Table 2.1 and phytochemicals present in the various parts of the plant are presented in Table 2.2.

Table 2.1 Physicochemical constituents of the fresh calyces and leaves of roselle

Constituents	Calyces (fresh)	Leaves (fresh)
Moisture	9.2 g	86.2 %
Protein	1.145 g	1.7-3.2 %
Fat	2.61 g	1.1 %
Fibre	12.0 g	10 %
Ash	6.90 g	1 %
Calcium	12.63 mg	0.18 %
Phosphorus	273.2 mg	0.04 %
Iron	8.98 mg	0.0054 %
Carotene	0.029 mg	-
Thiamine	0.117 mg	-
Riboflavin	0.277 mg	-
Niacin	3.765 mg	-
Ascorbic Acid	6.7 mg	-

Source: Shivali and Kamboj (2009)

Table 2.2 Phytochemicals of roselle

Part of the plant	Chemical constituents
Flower	Carbohydrates, arabinans, mannose, sucrose, thiamin, xylose, mucilage, niacin, pectin, proteins, fat, arabinogalactans, rhamnogalacturans, riboflavin, β-carotene, phytosterols, citric acid, ascorbic acid, fruit acids, maleic acid, malic acid, hibiscic acid, oxalic acid, tartaric acid, (+)-allooxycitronic acid-lactone, allohydroxycitric-acid, glycolic acid, utalonic acid, protocatechuic acid, cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-xyloglucoside, delphinidin, delphinidin-3-glucoside, delphinidin-3-sambubioside, delphinidin-3-xyloglucoside, delphinin, gossypetin, gossypetin-3-glucoside, hibiscetin, hibiscin, hibiscitrin, sabdaretin, sabdaritrin, fibre, resin, minerals and ash.

Table 2.2 Phytochemicals of roselle (cont.)

Leaf	α-Terpinyl acetate, anisaldehyde, *β*-carotene, *β*-sitosterol, *β*-D-galactoside, *β*-sitosteryl benzoate, niacin, fat, isoamyl alcohol, iso-propyl alcohol, methanol, 3-methyl-1-butanol, benzyl alcohol, ethanol, malic acid, fibre and ash.
Seed	Starch, cholesterol, cellulose, carbohydrates, campesterol, *β*-sitosterol, ergosterol, propionic acid, pentosans, pelargonic acid, palmitoleic acid, palmitic acid, oleic acid, myristic acid, methanol, malvalic acid, linoleic acid, sterculic acid, caprylic acid, formic acid, stearic acid, cis-12,13-epoxy-cis-9-octadecenoic acid, isopropyl alcohol, isoamyl alcohol, ethanol, 3-methyl-1-butanol, fibre and minerals.
Fruit	α-Terpinyl acetate, pectin, anisaldehyde, ascorbic acid, calcium oxalate, caprylic acid, citric acid, acetic acid, ethanol, formic acid, pelargonic acid, propionic acid, isopropyl alcohol, methanol, benzyl alcohol, 3-methyl-1-butanol, benzaldehyde and minerals.
Root	Tartaric acid and saponin.

Source: Shivali and Kamboj (2009)

Roselle is implicated in many biological activities that may impact positively on human health (Ali, 2004). Their use for therapeutic purposes has long been supported by epidemiological evidence, but only in recent years some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been verified by controlled *in vitro*, *in vivo*, or clinical research studies (Ali, 2004).

1) Antioxidant activity. The antioxidant and free radical scavenging effects of extract obtained from dried roselle petals were investigated (Tsai et al., 2002; Tsai and Huang, 2004). Moreover, the fractions of the ethanol extract (chloroform soluble fraction and ethyl acetate soluble fraction) of dried flower showed scavenge hydrogen peroxide and inhibitory effects on superoxide anions radicals $\left(O_2^-\right)$ (Farombi and Fakoya, 2005).

2) Anticancer. Anthocyanins can cause cancer cell apoptosis, especially in HL-60 cells (Chang et al., 2005). Anti-oxidative activity of anthocyanins was evaluated by their effects on LDL oxidation in cell free system and anti-apoptotic abilities in RAW 264.7 cells (Chang et al., 2006a). The study showed that anthocyanins of this plant may be used to inhibit LDL oxidation and oxLDL-mediated macrophage apoptosis, serving as a chemopreventive agent. Inhibitory

effect of protocatechuic acid on tumour promotion in mouse skin demonstrated that protocatechuic acid possesses potential as a cancer chemopreventive agent against tumour promotion (Tseng et al., 1998).

3) Other activities. Delphinidin 3-sambubioside, a anthocyanins isolated from the dried calyces of *H. sabdariffa* can induce a dose-dependent apoptosis in human leukemia cells (HL-60) as characterized by cell morphology, DNA fragmentation, activation of caspase 3, 8 and 9, and inactivation of poly(ADP)ribose polymerase (Hou et al., 2005). Ethanol and aqueous extracts of its calyces possess antipyretic activity in experimental animals (Reanmongokol and Itharat, 2007).

Investigation of the anti-inflammatory activity showed that its extract had no effect on rat paw edema but had an inhibitory effect on yeast induced pyrexia and a significant effect on the hot plate reaction time. Polysaccharides from its flowers can stimulate proliferation and differentiation of Human Keratinocytes (Brunold et al., 2004). The study also showed that raw polysaccharides and all acidic fractions cause a strong induction of proliferation of human keratinocytes while the neutral polymers were ineffective.

Antibacterial activity of gossypetin isolated from *H. sabdariffa* was carried out and results revealed that the activity may be due to polyphenolic nature of the flavonoid gossypetin (Mounnissamy et al., 2002). Investigation on nootropic acitivity of its calyces in mice indicated that the extract of calyces might prove to be useful memory restorative agent in the treatment of dementia seen in elderly which may be due to its anti-acetylcholinesterase property (Joshi and Parle, 2006).

2.2 Anthocyanins

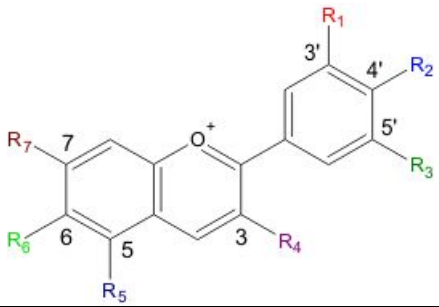
2.2.1 Introduction

Anthocyanins are a class of flavonoids which are prominent in many colored plants. The intense absorption of anthocyanins at visible wavelengths of light imparts color (orange, red, and blue) to plant tissues including flowers, vegetables, and fruits. As a result of their intense colors, they have a history of use as dyeing agents and food additives. There are 18 common base anthocyanidin (aglycone) species which differ in their patterns of hydroxylation and methylation (Figure 2.1). In addition, there are well over 600 glycosides and a rapidly expanding list of identified acylated derivatives (Delgado-Vargas and Paredes-López, 2003). In plants, anthocyanins occur in glycosylated forms, generally linked with glucose, galactose, arabinose, rhamnose, xylose, or fructose. The sugar moiety is most often found on the 3 or 5 position, but

can also occur at the 7, 3', or 5' positions. Cyanidin, delphinidin, and pelargonidin are the most common anthocyanins in nature with cyanidin glycosides reportedly present in nearly 90 % of all fruits (Prior, 2003). Contributing to the colorful appearance of fruits, vegetables, and flowers, anthocyanins help them to attract animals, leading to seed dispersal and pollination.

2.2.2 Distribution and content of anthocyanins in fruits and vegetables

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom. They are accumulated in fruit such as blackberry, red and black raspberries, blueberries, bilberries, cherries, currants, blood orange, elderberries, grapes, and vegetables such as roselle, red onion, radish, red cabbage, eggplant, purple sweet potato. Although they are accumulated mostly in flowers and fruits, but are also present in leaves, stems and storage organs (Delgado-Vargas and Paredes-López, 2003). Among different plants or even cultivars in the same plant, the total anthocyanins content varies considerably, affected by genes, light, temperature, and agronomic factors. In general that level of anthocyanins in fruits is much higher than in vegetables (Table 2.3).



Anthocyanidin	R1	R2	R3	R4	R5	R6	R7	main color
Cyanidin	-OH	-OH	-H	-OH	-OH	-H	-OH	magenta
Delphinidin	-OH	-OH	-OH	-OH	-OH	-H	-OH	purple, blue
Pelargonidin	-H	-OH	-H	-OH	-OH	-H	-OH	orange, salmon
Malvidin	$-OCH_3$	-OH	$-OCH_3$	-OH	-OH	-H	-OH	purple
Peonidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	-OH	magenta
Petunidin	-OH	-OH	$-OCH_3$	-OH	-OH	-H	-OH	purple
Rosinidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	$-OCH_3$	red

Figure 2.1 Basic structures of anthocyanins (flavylium cation)

Source: Delgado-Vargas and Paredes-López (2003)

Mean distribution of the six most common anthocyanidins in the edible parts of plants is as follow: Cyaniding; Cy (50 %), Pelargonidin; Pg (12 %), Peonidin; Pn (12 %), Delphinidin; Dp (12 %), Petunidin; Pt (7 %), and Malvidin; Mv (7 %) (Kong et al., 2003). The three non-methylated anthocyanidins; Cyanidin, Delphinidin and Pelargonidin are the most widespread in nature. Most species contain a limited number of anthocyanins pigments (apples, plums, pears), but in some cases-like red grapes, may contain a mixture of more than 20 pigments (Clifford, 2000). Overall, cyanidin aglycone occurs in about 90 % of fruits, and is the most frequently appearing aglycone (Prior, 2004) (Tables 2.4).

Table 2.3 Total anthocyanins content in selected common fruits and vegetables

Food source	Anthocyanins content in mg per 100 g	Literature sources
Açaí	320	Schauss et al., 2006
Blackcurrant	130-400	Clifford, 2000
Black raspberry	589	Clifford, 2000
Chokeberry	1,480	Bridle and Timberlake, 1997
Cherry	350-400	Eder, 2000
Eggplant	750	Clifford, 2000
Roselle	340	Chumsri et al., 2008
Orange, blood	200	Clifford, 2000
Onion, red	up to 250	Franke et al., 2004
Purple corn	1,640	Lieberman, 2007
Purple sweet potato	17-30	Brown, 2006
Red cabbage	25	Wu et al., 2006
Red grape	888	Eder, 2000; Clifford, 2000
Red wine	24-35	Clifford, 2000
Red radish	110-600	Giusti et al., 1998
Strawberries	127-360	Clifford, 2000

Source: Adapted from Horbowicz et al. (2008)

Anthocyanins in fruits and vegetables are naturally presented in glycosylated forms. However, many of the analytical procedures convert the glycosides into aglycones and thus results in Table 2.4 are reported as aglycones. Therefore results summarized in this table is after

conversion the glycoside values into aglycone forms based on molecular weight to make data consistent across the database.

In plants, the following three classes of anthocyanidin glycosides are common: 3-monoglycosides, 3-diglycosides, and 3,5-diglycosides. 3-Glycosides occur about two and half times more frequently than 3,5-diglycosides (Kong et al., 2003). Considering that glucoside form is the most abundant comparing to other glycosides, cyanidin 3-glucoside is the most widespread anthocyanins in nature (Prior, 2004; Kong et al., 2003).

Table 2.4 Content of main anthocyanidins in selected fruits and vegetables

Species	Milligram per 100 gram						Literature sources
	Cy	**Pg**	**Mv**	**Pt**	**Pn**	**Dp**	
Banana						7.4	Harnly et al., 2006
Black bean			6.5	9.6		12.0	Franke et al., 2004 Wu et al., 2006
Bilberry	112.6		54.4	51.1	51.1	161.9	Kähkönen et al., 2003
Blueberries	17.0		61.4	26.4	11.4	47.4	Franke et al., 2004
Egg plant						13.8	Wu et al., 2006
Radish		25.7					Harnly et al., 2006 Wu et al., 2006
Red bean	1.2	2.4					Wu et al., 2006
Red cabbage	72.9					0.1	Wu et al., 2006
Red onion	6.2				1.2	2.3	Franke et al., 2004 Wu et al., 2006 Arabbi et al., 2004
Red grape	1.5	1.1	34.7	2.9	2.1	3.7	Franke et al., 2004 Wu et al., 2006
Roselle	25 (%)					75 (%)	Wong et al., 2002
Strawberries	2.0	31.3				0.3	Franke et al., 2004 Wu et al., 2006

Source: Adapted from Horbowicz et al. (2008)

2.2.3 Extraction and determination of anthocyanins

The most widely used solvents for the extraction of anthocyanins are aqueous solutions of acetone, methanol, and ethanol. The extraction of anthocyanins is the first step in determination of total as well as individual anthocyanins in any type of plant tissue. Extraction procedures have generally involved the use of acidic solvents, which denature the membranes of cell tissue and simultaneously dissolve pigments. The acid tends to stabilize anthocyanins, but it may also change the native form of the pigment in the tissue by breaking associations with metals, co-pigments, or other factors. Extraction with solvents containing hydrochloric acid may result in pigment degradation during concentration, and is one of the reasons why acylations with aliphatic acids had been overlooked in the past (Rodriguez-Saona and Wrolstad, 2001).

The qualitative and quantitative determination of anthocyanins in plant extracts can be achieved by a variety of classical (spectrophotometric) or contemporary methods – HPLC coupled with a various types of mass spectrometers or NMR apparatus.

Spectrophotometric measurements. As was described earlier, anthocyanins absorption spectra are dependent of pH. At pH equal or below than 2, anthocyanins solutions show two maxima of absorption, first in the ultraviolet region (approximately 260 to 280 nm) and second in the visible region (approximately 415 and 490-540 nm). The wavelengths of these absorbance peaks can differ slightly by a few nanometers among various anthocyanins, depending on structure of each anthocyanins. The maximum of absorption at 520 to 540 nm in the visible region is the most common wavelength used in the spectrophotometric measurement of total anthocyanins (Giusti and Wrolstad, 2001).

Spectrophotometric procedures, however, are unsatisfactory for precise characterization of the particular anthocyanins present in plant materials. Variability in the sample extraction media (water, acidic water, acidic organic solvent, etc.), chemical properties of anthocyanins, and co-pigmentation of anthocyanins with other secondary metabolites present in the sample solution can cause an inaccurate correlation between absorbance and actual anthocyanins concentration (Bridle and Timberlake, 1997). Moreover, the spectrophotometric method does not provide any specificity as far as the molecular identity of the anthocyanins composition present in the material is concerned.

Analysis of anthocyanins and anthocyanidins by HPLC. HPLC separation combined with diode array detection (DAD) is the most common contemporary method for qualitative and quantitative analysis of anthocyanins (Durst and Wrolstad, 2001). Single wavelength detection is

performed at range 520 to 540 nm, while data acquisition between 200 and 800 nm is useful when DAD detection is available. Although UV-visible spectra of anthocyanins are quite similar, a tentative identification of compounds can be achieved by comparison with the spectra of reference compounds.

Reversed-phase (RP) columns: 4.6 mm (internal diameter) and 100 to 300 mm (length), are the mostly used in HPLC analysis of anthocyanins. Columns are usually maintained at ambient temperature, and elution systems are binary, using aqueous acidified solvents such as acetic acid, perchloric acid, or formic acid in an organic solvent such as methanol or acetonitrile (Zhang et al., 2004). In the reversed-phase system, the more polar phenolic acids elute first, followed by the 3, 5-diglycosylated anthocyanins, 3-monoglycosylated anthocyanins, aglycones, and last the acylated anthocyanins. Anthocyanidins elution order on a reversed-phase column is delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Durst and Wrolstad, 2001). The retention of glycosides in comparison to that of the aglycones is decreased by the presence of sugars, with diglucosides preceding monoglucosides. Subsequently, acylation increases the retention time of anthocyanins.

2.2.4 Stability of anthocyanins

Many factors, such like the pH, temperature, light, presence of other phenolic compounds, enzymes, metal ions, sugars, ascorbic acid, and oxygen have impact on the stability of anthocyanins. In aqueous solution, anthocyanin undergoes structural transformations that are pH-dependent (Figure 2.2) (Delgado-Vargas and Paredes-López, 2003). It has been found that four major anthocyanins forms exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone. At pH < 2, anthocyanins exist primarily in the form of the red flavylium cation. Process of a flavylium salt salvation in a slightly acidic or neutral aqueous solution resulted in formation of neutral or quinonoidal bases. Hydration of the flavylium cation gives the colorless carbinol pseudobase at pH values ranging from 3 to 6. This can further equilibrate to an open form, the colorless chalcone pseudobase.

Processing and storage under low temperature can improve the stability of anthocyanins. Temperature has been reported to induce a slow destruction of the pigments. When temperature is increased, anthocanins can be transformed into unstable formation of chalcone, and the chalcone is further degraded to brown products.

Light is usually deleterious to anthocyanins components. Acylated anthocyanins are less affected by light, with only slight difference on pigment stability when exposed to light as

compared to stored in the dark (Giusti et al., 1999). Oxygen and hydrogen peroxide can easily oxidize anthocyanins, and this mechanism is often accelerated by the presence of ascorbic acid. The negative impact of ascorbic acid on anthocyanins has been of great concern because of the universality of ascorbic acid in fruit and vegetable juices (Delgado-Vargas and Paredes-López, 2003). The interaction of ascorbic acid and oxygen may be mediated by H_2O_2 because the mechanism of ascorbic acid oxidation produces peroxide, and peroxide is known to destroy anthocyanins.

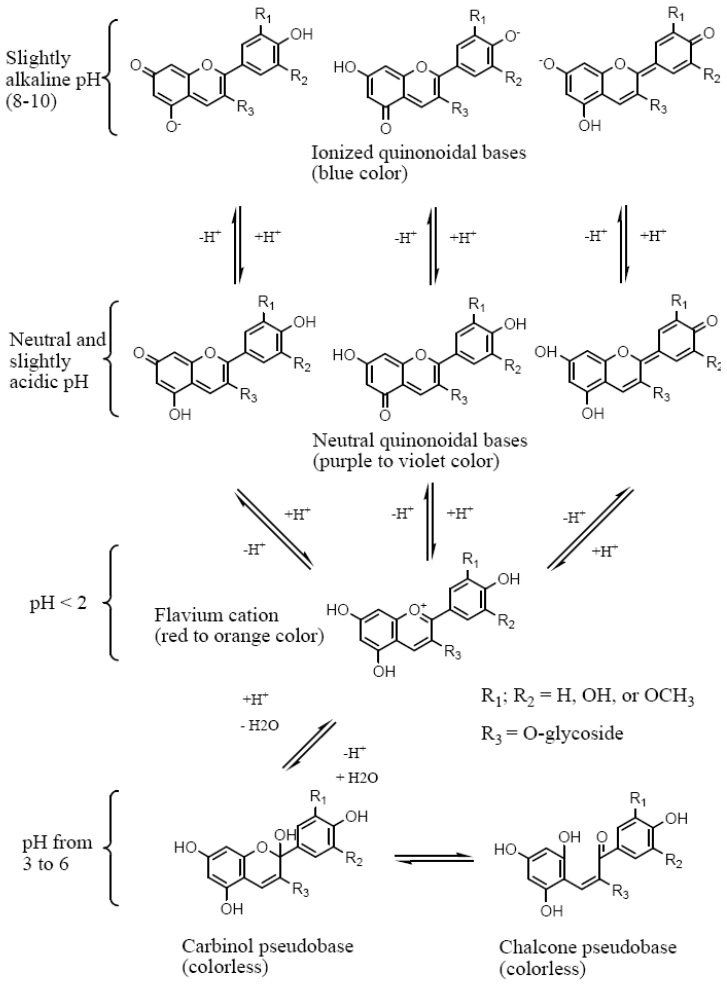


Figure 2.2 Inter-conversion pathways of various forms of anthocyanins in acidic aqueous medium

Source: Delgado-Vargas and Paredes-López (2003)

Increased sugar level may affect the rate of anthocyanins destruction. It was found that sucrose addition improved the color characteristics of frozen strawberries (Wrolstad et al., 1990). Tsai et al. (2004) reported, according to the degradation index (DI), half life and activation energy

of anthocyanin degradation, that sucrose is a good anthocyanin protector. In another study, the anthocyanins stability of grape, elderberry and blackcurrant extracts was lower in all sucrose (10 %) added systems as compared to the control at pH values of 3, 4 and 5, whereas the browning index did not change with addition of sugar (Malien-Aubert et al., 2001). Addition of 20 g/L of sucrose to a drink model system (pH 3) containing red cabbage and grape extracts did not influence the thermal and photo stability of the anthocyanins (Duhard et al., 1997).

The mechanism could be associated with the inhibition of enzymatic activities of phenolooxidase and peroxidase. Anthocyanins are very reactive toward metals, and form stable complexes with tin, copper, and irons. For instance, cyanidin 3-glucoside forms a stable colored complex in the presence of aluminum ions at pH 5.5.

2.2.5 Bioactivity of anthocyanins

In recent years, numerous studies have suggested that anthocyanins are protective against many chronic degenerative diseases (Prior, 2004; Kong et al., 2003). For the most part, the beneficial qualities of anthocyanins have been primarily attributed to their antioxidant capacities (Kong et al., 2003). Cancer is generally believed to be initiated in part by oxidative mechanisms acting upon genetic materials such as DNA and RNA. The oxidation of macromolecules such as lipids and proteins may result in altered cellular processes associated with cancer development and progression (Rao and Agarwal, 2000). Anthocyanins have been observed to prevent oxidation of these structures in vitro (Rice-Evans et al., 1996; Hou, 2003, Kong et al., 2003) and a growing body of evidence now suggests a possible association between anthocyanins and cancer prevention (Hou, 2003). Furthermore, an increased level of oxidation in the blood is assumed to be associated with cardiovascular disease (CVD). Elevated oxidation in the serum/plasma results in a variety of reactions including the oxidative modification of LDL particles, initiation of inflammation, activation of nitric oxide synthase (NOS), increased platelet aggregation, and increased foam cell production (Parthasasrathy et al., 2001). Recently, anthocyanins has been observed to reduce serum oxidation in the postprandial hyperlipidemic state (Kay and Holub, 2002; Kong et al., 2003) and to enhance the resistance of LDL to oxidative modification (Natella et al., 2002). The antioxidant characteristics of anthocyanins have been associated with a variety of properties including free radical scavenging, chelation of trace metals, and inhibition of lipid peroxidation and DNA oxidation. Additionally, anthocyanins consumption has been observed to directly increase the hydrophilic and lipophilic antioxidant capacity of the blood serum/plasma in many investigations (Seeram and Nair, 2002).

Antioxidant activity. Anthocyanins are highly reactive radical scavengers in various *in vitro* environments. Anthocyanins not only scavenge radicals, but through their ability to bind heavy metals such as iron, zinc, and copper, also prevent the formation of radicals (Rice-Evans et al., 1996; Rao and Agarwal, 2000). Anthocyanins may also exert antioxidant abilities through the protection or enhancement of endogenous antioxidants or through the induction of antioxidant enzymes such as glutathione-*S*-transferase (GST) and superoxide dismutase (SOD) (Ross and Kasum, 2002).

Structural characteristics effecting antioxidant activity. The structural characteristics responsible for the antioxidant effect of anthocyanins are generally associated with the number of free hydroxyls around the pyrone ring, greater number of hydroxyls showed greater antioxidant capacity. The antioxidant capacity of a polyphenolic is dictated not only by the number of free hydroxyls, but also by the basic structural orientation of the compound. The ring orientation will determine the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical and the ability of the compound to support an unpaired electron. The conjugation of the anthocyanins ring structure is also important. The C_2-C_3 double bond of the C-ring is consistently associated with a higher antioxidant capacity, reportedly having a stabilizing effect on the phenoxy radical (Middleton et al., 2000; Zheng and Wang, 2003). The positioning of hydroxyls in relation to one another is also a very important determinant of the antioxidant capacity of anthocyanins. Hydroxyl groups in close proximity, such as the ortho-hydroxyls of the B-ring, appear to greatly enhance the antioxidant capacity of the anthocyanins (Zheng and Wang, 2003) in experimental (*in vitro*) models; however, the availability of the highly reactive ortho-hydroxyls in a biological system (*in vivo*) has yet to be established. Conceptually, this site on the B-ring could form bonds with many compounds within biological fluids thus inhibiting the ability of this reactive site to participate in oxidation, metal chelation, or protein binding *in vivo*.

Glycosylation and antioxidant capacity. Anthocyanins are found in plants in glycosylated forms. Glycosylation is reported to influence the antioxidant capacity of anthocyanins/flavonoids (Seeram and Nair, 2002). It is generally stated in the literature that glycosylation decreases the antioxidant capacity of anthocyanins by reducing free hydroxyls and metal chelation sites; however, contradictory results have been reported (Kähkönen and Heinonen, 2003). It is important to note that the effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed such as aqueous-soluble or lipid-soluble phases.

Glycosylation diminishes the antioxidant capacity of the anthocyanins in an artificial membrane system by decreasing the number of free hydroxyls and metal chelation sites. More importantly, glycosylation will decrease the accessibility of the flavonoids to membranes as a result of the increased polarity (increased water-solubility) associated with the sugar moiety. The physiological relevance of this effect has not been sufficiently established *in vivo*. Aglycones are less water-soluble and therefore have an increased partitioning into the lipid-soluble phase of the artificial membrane system. One would assume that the increased antioxidant capacity of anthocyanidins (aglycones) in this environment would therefore be partly a result of the increased lipid solubility of the aglycones over the glycosides. Conversely, other assay systems such as the oxygen radical absorbance capacity (ORAC) assay (Wang et al., 1997), the ferric reducing assay, and certain lipid oxidation models (Kähkönen and Heinonen, 2003) have found some glycosides to have higher antioxidant capacities than their respective aglycones. Therefore, the *in vitro* effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed (aqueous-soluble or lipid-soluble phase). Additionally, since anthocyanins aglycones have not been identified in the blood or urine, the physiological relevance of the antioxidant capacity of aglycones in the circulation is questionable. This being said, as anthocyanins glycosides are generally believed to be cleaved by colonic microflora, the aglycones could have physiological relevance within the colon with the glycosides having more systemic relevance. It is clear that the respective *in vivo* antioxidant capabilities of the anthocyanins aglycones versus their glycoside derivatives require further investigation.

Effect of pH on antioxidant activity. Anthocyanins exist in equilibrium in a variety of protonated, deprotonated, and hydrated forms. These range from colored quinonoid forms, to the flavylium ion, and to colorless hemi-acetal forms (Kähkönen and Heinonen, 2003). The expression of the predominant form is generally pH dependent. There is little evidence regarding the effect of pH on the biological activity of these compounds. Although in spite of the loss of color of anthocyanins at physiological (pH 7), evidence presented by Narayan et al. (1999) suggests that anthocyanins glycosides retain their antioxidant activity.

Compartmentalization and antioxidant activity. Results of trials aimed at determining the link between antioxidant consumption, antioxidant status, and oxidative-associated disease have been inconsistent (Willett, 2000). Although anthocyanins have shown promise in many *in vitro* antioxidant models, it has yet to be established if these compounds can reach their target of suspected action and if high enough concentrations are attained to elicit a biologically significant

response. Youdim et al. (2000) have shown evidence of the incorporation of anthocyanins into cells and cell membranes. In a cell culture experiment, using human aortic endothelial cells, cyanidin glycosides from the elderberry were observed to be incorporated into both the plasma membrane and cytosol. The cells containing anthocyanins were determined to have significant protection against oxidation induced by reactive oxygen species. Subsequently, Bagchi et al. (2004) reported the cellular uptake of berry anthocyanins by endothelial cells. Although uptake was indicated in these studies, the mechanism by which anthocyanins enter intracellular compartments has yet to be determined.

2.3 Reactive oxygen and nitrogen species

2.3.1 Production of reactive oxygen and nitrogen species

A free radical is by definition an atom or a molecule which contains an unpaired electron in its outer orbit. Radicals are therefore highly reactive and can consequently cause harmful effects on cell structures. Free radicals and other oxygen-related reactive compounds, such as singlet oxygen $\left({}^1O_2\right)$ and hydrogen peroxide $\left(H_2O_2\right)$ are collectively termed reactive oxygen species (ROS). Although ${}^1O_2$ and H_2O_2 are not radicals, the oxidizing ability of ${}^1O_2$ is increased due to its altered spin, whereas H_2O_2 can form ROS in the presence of transition metals (Fenton reaction). Oxides of nitrogen, i.e. nitric oxide (NO) and nitrogen dioxide $\left(NO_2\right)$ molecules fall into the definition of free radicals, since they possess odd numbers of electrons (Wiseman and Halliwell, 1996).

Reactive oxygen species are generated during normal aerobic metabolism in the reaction where molecular oxygen $\left(O_2\right)$ is reduced to water (a four-electron transfer) by cytochrome oxidase, about 1 to 3 % is reduced to ROS, especially O_2^- (one electron) and H_2O_2 (two electrons) (Figure 2.3). The intermediates of this reaction are superoxide anion $\left(O_2^{\bullet -}\right)$, hydrogen peroxide and hydroxyl radical $\left(OH^\bullet\right)$. The most reactive and harmful form of these oxygen radicals is the hydroxyl radical (Halliwell and Gutteridge, 1999; Davis et al., 2001).

The production of $^\bullet OH$ radicals is difficult to demonstrate in vivo because of their extremely high reactivity as compared with that of O_2^- radicals (lifetime of 2 to 4 μs) and H_2O_2 (lifetime of 1 ms). The $^\bullet OH$ radicals can be formed from H_2O_2 by Fenton reactions that are catalyzed by traces of redox-active metals, like iron and copper, and, therefore, the concentrations of free “catalytic” metals in cells tend to approach zero (Halliwell and Gutteridge, 1999).

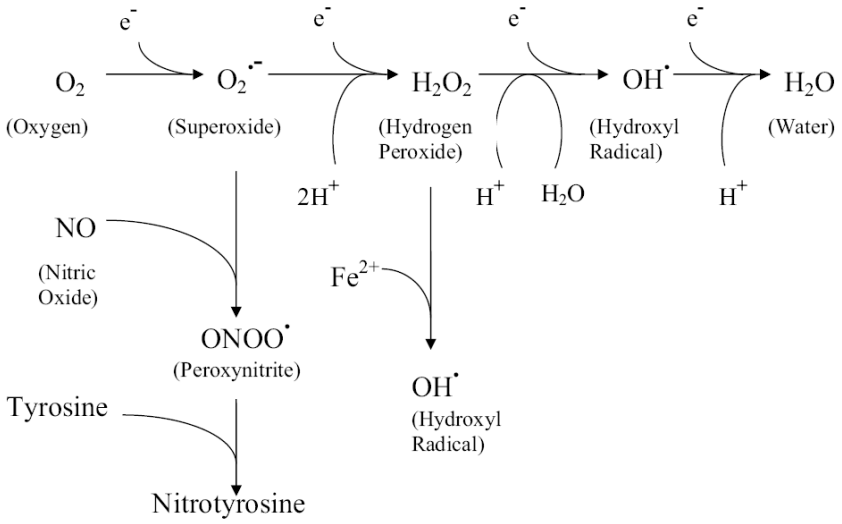


Figure 2.3 The formation of reactive oxygen species and nitric oxide

Source: Halliwell and Gutteridge (1999), Davis et al. (2001)

2.3.2 Sources and chemistry of reactive oxygen species

Reactive oxygen species (ROS) are those produced directly or indirectly from molecular oxygen (Halliwell and Gutteridge, 1999). Thus, superoxide $\left(O_2^{\bullet -}\right)$, produced by electrons "leaking" from the electron transport chain of oxidative metabolism or from the oxidative burst of activated phagocytes, is a major contributor of ROS *in vivo* and as much as 2 kg may be produced in the human body per year. This is not necessarily damaging because this species is not very reactive and "superoxide dismutase" (SOD) enzymes exist which convert superoxide to molecular oxygen and hydrogen peroxide. Hydrogen peroxide is more reactive but can also be degraded to harmless products by enzymes such as catalase. If the production of these species exceeds the ability to catalyze their degradation then damage may occur. Both superoxide and hydrogen peroxide are frequently regarded as poorly reactive. However, they may be involved in the production of more reactive species.

Hydrogen peroxide may form more damaging ROS such as hydroxyl radical either by reaction with superoxide (Haber-Weiss chemistry) or transition metals such as copper and iron. Hydroxyl radical is so reactive that it will attack whatever biological molecule exists nearby, initiating free radical chain reactions. Hydroxylation of aromatic structures has been used to probe the involvement of hydroxyl radical in protein damage *in vivo* (Pennathur et al., 2001). Hydrogen peroxide is also utilized by myeloperoxidase to produce hypochlorous acid which is responsible for chlorinating and, indirectly, nitrating tyrosine residues of proteins (van Dalen et al., 2000).

Hypochlorous acid also behaves directly as an oxidising agent producing p-hydroxyphenylacetaldehyde from tyrosine (Hazen et al., 1996).

The ability of superoxide to produce highly reactive species is exemplified by its reaction with nitric oxide to produce peroxynitrite. The consequences of this are discussed later since peroxynitrite is derived from nitric oxide and is therefore generally regarded as a reactive nitrogen species. Despite this, it will be shown that peroxynitrite is capable of exhibiting ROS-like reactivity through the production of reactive intermediates which behave like the hydroxyl radical, and through the formation of dityrosine.

Free radical chain reactions initiated by ROS may propagate through lipid compartments via the initial formation of a lipid radical $\left(L^{\bullet}\right)$. This will give rise to lipid peroxides $\left(LOO^{\bullet}\right)$ through the reaction of the lipid radical with oxygen. This species is a major contributor to free radical chain reactions in lipid structures as it effectively abstracts hydrogen atoms from nearby lipids, especially bis-allylic hydrogens of polyunsaturated fatty acids, to form a second lipid radical and a lipid hydroperoxide $\left(LOOH\right)$. The relatively unstable peroxide bond may initiate further free radical chain reactions via formation of alkoxyl radicals $\left(LO^{\bullet}\right)$ thus magnifying the effects of the initiating radical.

2.3.3 Sources and chemistry of reactive nitrogen species

The term reactive nitrogen species (RNS) describes an array of compounds which are typically higher oxidation products of nitric oxide and are derived from nitric oxide either directly (peroxynitrite) or indirectly (nitrylchloride) through the activation of a secondary nitric oxide product to a reactive tertiary product. Therefore, various reactions of nitric oxide with other species have been proposed to give rise to the RNS *in vivo*. Perhaps the most widely studied is peroxynitrite $\left(ONOO^{-}\right)$, a potent oxidizing agent which can react with a wide range of biological molecules.

Although various figures are quoted (Goldstein and Czapski, 1995), its rate of production from nitric oxide and superoxide is known to approximate a diffusion controlled reaction rate and thus peroxynitrite is thought to be rapidly formed wherever concurrent production of nitric oxide and superoxide occurs. Evidence indicates that peroxynitrite is sufficiently stable to diffuse large distances on a cellular scale, until it might reach a target molecule such as protein or DNA (Squadrito and Pryor, 1998).

The exact reaction mechanisms of peroxynitrite are not clear although the products derived from its breakdown exhibit behavior similar to both RNS and ROS such that the products

of the reaction of peroxynitrite with biological molecules may not reflect its nitric oxide origins. It is commonly expected that a RNS imparts a nitrogenous modification upon a target molecule but this may not always be the case. For example, peroxynitrite has been shown to produce both 3NT and dityrosine depending upon the concentration or flux of peroxynitrite used such that at low flux, dityrosine is formed and at higher flux 3NT is formed (Pfeiffer et al., 2000; Goldstein et al., 2000). This apparent biphasic reaction mechanism of peroxynitrite has important implications in the study of RNS-induced damage *in vitro*. Specifically, it indicates that experiments examining peroxynitrite should mimic its *in vivo* production. Many experiments have used bolus addition of synthetic peroxynitrite to an experimental solution to investigate nitration/anti-nitration effects. This is not satisfactory since, if peroxynitrite is produced through the reaction of nitric oxide with superoxide *in vivo*, then experiments using a simultaneous generator of $^{\bullet}NO$ and $O_2^{\bullet -}$ or independent generators of $^{\bullet}NO$ and superoxide should be used. This has implications for the experimentally derived product profile of peroxynitrite. The observations of Goldstein et al. (2000) and Jourd'heuil et al. (2001) indicate also that the exact flux of $^{\bullet}NO$ and $O_2^{\bullet -}$ can influence the degree of nitration versus oxidation produced by peroxynitrite suggesting that where chemical production of nitric oxide and superoxide is used, the ratio of the two reactants can influence the results obtained. Thus, the chemistry associated with peroxynitrite is extremely complex and renders it virtually impossible to propose a standard set of experimental conditions which might mimic a heterogeneous *in vivo* situation.

Other factors can influence the product profile of peroxynitrite through the formation of intermediates which follow alternative reaction mechanisms. Peroxynitrite is thought to produce reactive tertiary products through the interaction with other species present *in vivo* such as carbon dioxide. This is thought to produce an intermediate nitrosoperoxycarbonate species which favors nitration of targets due to a decomposition mechanism which produces nitrogen dioxide and the carbonate radical. Alternatively, protonation of peroxynitrite yields peroxynitrous acid which is unstable and spontaneously decomposes into poorly described intermediates which behave mostly like hydroxyl radical. Figure 2.4 shows the species thought to be produced from peroxynitrite. This could explain dityrosine and aromatic hydroxylation products which are also frequently observed following treatment with peroxynitrite. Peroxynitrite can also rapidly react with other biological targets such as glutathione and glucose to produce meta-stable intermediates which release nitric oxide. This could represent a means of scavenging peroxynitrite *in vivo* but would have to compete with carbon dioxide.

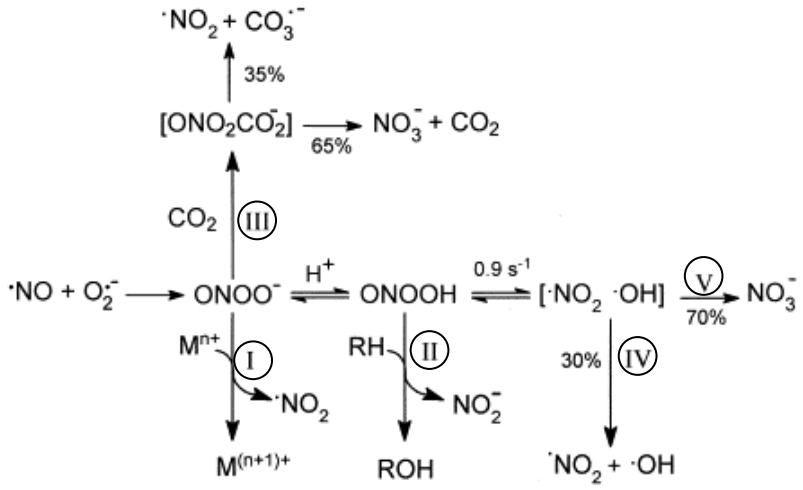


Figure 2.4 Peroxynitrite reaction pathways. Numbers I to V indicate possible fates of peroxynitrite:

i) direct reactions include the one-electron oxidation of transition metal centers (Fe, Mn, Cu)

ii) the two-electron oxidation with a target substrate (RH)

iii) the formation of nitroso-peroxocarboxylate

iv) that rapidly decomposes to secondary radicals in 35% yield. Peroxynitrous acid undergoes homolysis at 0.9 s^{-1} to yield free radicals in 30% yields

v) or rearrange to nitrate

Source: Radi et al. (2001); Uppu et al. (1996)

Although peroxynitrite has been widely studied, very little evidence exists for its production *in vivo*. It is a generally accepted principle that peroxynitrite is formed where nitration of tyrosine can be demonstrated (Halliwell, 1997). A study by Linares et al. (2001) has taken steps to remedy this by comparing mouse strains which are resistant or susceptible to *Lieshmania amazonensis* infection. In the presence of low and unchanged levels of peroxidase, control of the parasite required induction of nitric oxide synthase and nitration of parasite proteins was observed. Although nitrogen dioxide could conceivably act to nitrate tyrosine residues in these experiments, the rise in tyrosine nitration was palleled by an increase in tyrosine hydroxylation suggestive of the mixed reaction mechanisms of peroxynitrite. Nitration of tyrosine is not a specific marker of peroxynitrite (Halliwell, 1997). Many species are capable of nitrating tyrosine and other targets.

Nitrite, an oxidation product of nitric oxide, is present at low to sub-micromolar levels in biological fluids and is quite unreactive unless it becomes protonated to form nitrous acid

(HNO_2). Nitrous acid is able to nitrate phenolic structures and is used in organic synthesis for this purpose. Therefore, nitrous acid could potentially contribute to nitration *in vivo*. However, the requirement for acidic conditions perhaps eliminates this species as a contributor to overall nitration of endogenous targets since sufficiently low pH levels are unlikely *in vivo* (Halliwell, 1997).

2.4 Meat Curing

Historically, meat curing was a practice primarily done to preserve meat. As meat curing progressed, the definition was understood as the addition of salt, sugar, spices and nitrate or nitrite for aiding in flavor and preservation properties. As time passed, various spices and flavorings were added to achieve distinctive product and brand flavor characteristics. Today, meat curing is utilized to achieve consumer demands for products that have unique sensory characteristics and convenience attributes associated with cured meats. Meat curing has traditionally been associated with processed meats for the purpose of altering the color, texture, flavor, safety and shelf life characteristics which makes these products unique from other meat products (Pegg and Shahidi, 2000).

The curing process is a dynamic, complex and still not fully understood system of reactions, meat pigment changes, chemical state alterations as well as an entire host of secondary reactions. Meat curing results in a vast variety of processed meat products that are available to consumers. Variations in raw materials, formulations and processing technologies and techniques lead to the immense amount of different cured products that are manufactured and available to consumers today. For whole muscle type products, curing can be accomplished by various methods such as submersion of cure in brines or pickles, injection of cure containing brine or direct addition of dry cure for dry cured type products. Curing comminuted products is most commonly accomplished by direct addition of cure during the grinding, mixing, chopping or by other comminuting processes.

2.4.1 The curing reaction

Although nitrates were first discovered as curing agents, research findings have demonstrated that the role of nitrate is to serve as a source of nitrite for curing reactions Pegg and Shahidi (2000).

The chemistry of nitrite curing is indeed complex and in many cases not clearly understood. The term nitrite is generically used to describe both the anion, nitrite and the neutral nitrous acid. Nitrite curing has been often associated with the production of potentially dangerous

compounds formed from nitrosating species of nitrite. In examining the chemistry of meat curing, Pegg and Shahidi (2000) revealed that nitrite itself is not the primary nitrosating species or reactive compound. It was further revealed by Pegg and Shahidi (2000) and Sebranek and Fox (1985) that one of the derivatives, nitrous acid (HNO_2), actually can form nitrosating (*N*-nitroso producing) compounds which are the compounds involved in potential nitrosamine formation. These nitrosating compounds enter into a number of complex reactions which, in the end, yield nitrosylmyochromagen. The pK_a of a compound represents the acid dissocation constant, the strength of the acid and the ability of the compound to donate protons for affecting reactions. Nitrite has a pK_a of 3.36. These authors further explained that since the pH of meat (approximately 5.5 to 6.5) is clearly above the pK_a of HNO_2, the concentration of HNO_2 in cured meat is therefore extremely low. It is believed that the main reactive species of HNO_2 in meat is the anhydride of HNO_2 which is dinitrogen trioxide (N_2O_3). The dinitrogen trioxide reacts with reductants that are naturally found in muscle as well as any ones added such as ascorbates. This compound can readily form nitroso compounds.

Reducing reactions can increase the nitric oxide production which can then form coordinate-covalent complexes of nitric oxide with the heme pigments of meat. These complexes, nitrosylmyoglobin, nitroyslhaemoglobin and dinitrosylhaemochrome, form the red and pink colors of cured meats. The amount of nitrous oxide produced during curing is dependent on pH, temperature and time.

Beyond the reactions explained, there are also many other complex reactions that can and do occur. A lower pH will increase the conversion of nitrous acid to nitric oxide. When nitrite is added to comminuted meat, a browning effect occurs due to properties of nitrite acting as a strong heme oxidant. Myoglobin and oxymyoglobin are oxidized to metmyoglobin by nitrite. Through the series of already mentioned, complex reactions involving the reduction of nitrite to nitrous acid, the intermediate pigment, nitrosylmetmyoglobin, is formed. Nitrosylmetmyoglobin is not a stable pigment and therefore autoreduces in the presence of both endogenous and exogenous reductants to form a more stable nitric oxide myoglobin or nitrosylmyoglobin. Upon thermal processing, the globin portion denatures and detaches from the iron atom. The resulting pigment formed from the thermal processing is the stable nitrosylmyochromogen or nitrosylhemochrome. For the use of nitrate in a curing system, an additional step of the conversion of nitrate to nitrite is necessary. This step is normally accomplished by the bacterial reduction of nitrate to nitrite (Pinotti et al., 2001). Bacterial reduction can be accomplished by microorganisms found in the

natural flora of meat or by intentional addition of microorganisms with nitrate reducing properties (Sanz et al., 1997, 1998).

Pegg and Shahidi (2000) examine the importance of salt or sodium chloride in meat curing summarized that chloride ions could actually help catalyze nitrosation reactions. Additionally, salt levels used in the curing process are generally not high enough to provide complete preservation but at levels typically used in conjunction with nitrite, salt does offer preservative effects.

2.4.2 Residual nitrite

When nitrite is added to meat systems, it reacts chemically or is bound to components such as protein. Heat during thermal processing serves to speed up these reactions. After normal manufacturing processes, the amount of detectable nitrite is usually only approximately 10 to 20 % of the initial added amount when analytically measured (Reis et al., 2009; Pariza, 1997). These levels of nitrite or also called residual nitrite decline over the storage life of cured meat products until they are often non detectable.

In a comprehensive study of nitrite and cure accelerator levels for cooked sausages and dry and semi-dry sausages, the nitrite safety reported “highly variable” residual nitrite and cure accelerator levels not only within product categories but also between specific types of sausages (beef, pork and poultry) (Keeton et al., 2009). They reported that generally, 25 to 50 % of added nitrite remained in the product during 24 to 48 hours after processing. Purchasing and analyzing local retail commercial cured bacon, sliced ham, and wieners and analyzing for nitrite revealed residual nitrite in bacon to range between 1 and 15 ppm, sliced ham to range between 3 and 9 ppm (Cassens, 1997)

2.4.3 Factors affecting levels of residual nitrite

There are several factors that can affect the amount of residual nitrite found in cured, cooked meat products. Those factors can include non-meat ingredients added in conjunction with nitrite or nitrate such as cure accelerators, physical processing procedures. The factors can result in physical nitrite loss during processing, packaging characteristics such as packaging type and method, and storage parameters such as storage time and storage temperature. The use of cure accelerators such as erythorbates and ascorbates are the largest factor that contribute to the levels of residual nitrite found in cured meat products.

In a review by Pegg and Shahidi (2000), the importance of pH on the residual nitrite levels was discussed. A small pH decrease, as low as 0.2 pH units, during product manufacture

can result in a doubling in the rate of color formation due to more favorable nitrite-myoglobin interactions. Due to these more favorable reactive conditions, subsequent lower residual nitrite concentrations were found. Research by Kilic et al. (2001) supported these statements. In their investigations on the influence turkey meat had on residual nitrite in cured meat products, the authors reported treatments with higher pH values to also had higher residual nitrite levels.

Different raw materials and non-meat ingredients can also contribute to pH and the effects pH has on residual nitrite levels. Kilic et al. (2002) found a relationship between residual nitrite and pH values. Including poultry meat in treatments was found to alter overall product pH and thus affect residual nitrite levels. The addition of phosphates that altered product pH was also found to affect residual nitrite levels. Kilic et al. (2001) conversely found that heating temperature during thermal processing affected residual nitrite concentration by lowering levels as temperatures increased. Pérez-Rodríguez et al. (1996) indicated that the length of thermal processing can cause different depletion rates of residual nitrite.

Storage time at 14 days resulted in approximately a 90 % reduction of the ingoing nitrite levels that ranged between 30 and 300 ppm nitrite. Levels of nitrate remained comparable to ingoing levels up to approximately 21 days when frankfurters were stored at 7 ºC. At 27 ºC storage temperatures, approximately one-half of initial ingoing nitrate levels were present at 14 days storage. This was believed to be due to the microbial activity at higher storage temperatures on the conversion of nitrate to nitrite. Ahn et al., (2000) support the storage time effects but also noted packaging effects. Sausage samples stored in vacuum packages had lower residual nitrite values than samples stored in aerobic conditions. The authors believed this phenomenon was caused by the environment being in the reduced state thus allowing the conversion of nitrite to nitric oxide and the lower residual nitrite levels found.

2.5 Oxidation and antioxidant strategies

2.5.1 Lipid oxidation

Regardless of microbial spoilage, lipid oxidation is the main factor reducing the quality of meat and meat products. Though lipid oxidative reactions contribute to certain desirable quality attributes such as the development of a pleasant flavors in cooked meats (Kanner, 1994), the overall effect of lipid oxidation is negative leading to adverse effects on sensory traits, nutritional value and healthiness of muscle foods (Morrissey et al., 1998).

Mechanism of lipid oxidation. The overall mechanism of fatty acid oxidation is generally a free radicals process including initiation, propagation and termination stages. The first

step of lipid oxidation involves the removal of a hydrogen atom from a methylene carbon in a fatty acid (RH) to generate fatty acyl (R^-) and peroxyl radicals (RO_2^-). The initiation step needs to be catalysed and the role of iron in promoting the generation of species capable to abstract a hydrogen atom from an unsaturated fatty acid has been described (Gray et al., 1996). The radicals previously generated propagate the chain reaction to other fatty acids which are consequently oxidized (Figure 2.5). The reaction between fatty acids and peroxyl radicals lead to the formation of new peroxyl radicals and lipid hydroperoxides $(ROOH)$. Radicals and hydroperoxides (primary lipid oxidation products) are finally decomposed to generate stable molecules with small molecular weight (secondary lipid oxidation products) such as hydrocarbons, aldehydes, ketones, acids, esters, lactones and a large variety of nitrogen and sulphur containing compounds (Morrissey et al., 1998).

Initiation Fatty acyl (R^-) and radicals formation (RO_2^-)

Propagation
$$\begin{cases} R^\bullet + O_2 \rightarrow RO_2^\bullet \\ RO_2^- + RH \rightarrow ROOH + R^\bullet \end{cases}$$

Termination
$$\left.\begin{cases} 2R^\bullet \\ R^\bullet + RO_2^\bullet \\ 2RO_2^\bullet \end{cases}\right\} \quad \text{(Stable products)}$$

Figure 2.5 Mechanism of lipid oxidation

Source: Morrissey et al. (1998)

As far as muscle foods contain unsaturated fatty acids and pro-oxidant components are prone to suffer oxidative reactions. Between the muscle lipid fractions, the higher sensitivity of the polar lipid fraction to oxidation is mainly explained by the facts that polar lipids from cellular membranes contain a higher proportion of unsaturated fatty acids and are in close relationship with oxidation promoters located in the aqueous phase of the muscular cell (Gandemer, 2002). As opposed to the neutral lipid fraction, the polar fraction is primarily responsible for lipid oxidation in muscle foods. Though muscle tissues have endogenous antioxidant mechanisms to control lipid oxidation in vivo such as antioxidant enzymes and lipid-soluble tocopherols and β-carotenes (Chan and Decker, 1994), their effectiveness is largely diminished with increasing time post-mortem. In fact, the third phase of lipid oxidation in muscle foods frequently occurs during handling, processing, storage and cooking processes.

Factors influencing lipid oxidation. As partially described above, there are several aspects affecting the occurrence and intensity of lipid oxidative reactions in muscle foods. The amount and characteristics of the muscle lipids and the presence of pro-oxidant (*i.e*. iron, sodium chloride) and antioxidant (*i.e*. tocopherols, sodium ascorbate and nitrite) factors are known to be largely influential (Gray et al., 1996; Morrissey et al., 1998).

The amount and composition of muscle lipids largely determine the oxidative stability of a muscle food. Sasaki et al. (2001) reported significant positive correlations between fat content and lipid oxidation suggesting that the higher amount of total lipids, the higher substrate to undergo oxidative reactions. The sensitivity of fatty acids to oxidative reactions increases with the number of double bounds and therefore, poly-unsaturated fatty acid (PUFA) is more prone to be oxidized than mono-unsaturated fatty acid (MUFA) or saturated fatty acid (SFA) (Morrissey et al., 1998). High levels of PUFA in muscle foods have been previously associated with high oxidative instability during meat cooking and subsequent storage (Cortinas et al., 2005). As described above, amongst the meat components, the role of iron and heme pigments in the promotion of lipid peroxidation has been well established (Kanner, 1994). Iron, free and protein bound, heme and non-heme, oxidized or reduced has the ability to promote the oxidation of unsaturated fatty acids in meat but the relative contribution of each chemical form has not been assigned (Gray et al., 1996). The mechanisms by which iron could promote the initiation of lipid oxidative reactions can be classified in three types of reactions:

i) a direct initiation by higher valence state iron $\left(Fe^{3+}\right)$ or by reactive oxygen species (ROS) produced by a metal autoxidation process

ii) an indirect initiation by hypervalent iron complexes such as those in heme protein and porphyrin compounds and

iii) an indirect initiation-propagation of lipid oxidation through the decomposition of preformed hydroperoxides into peroxyl radicals

In addition to the intrinsic pro- and antioxidant components of the muscle itself, a number of extrinsic factors influence lipid oxidation in muscle foods. The manufacture of meat products involves the addition of non-meat ingredients and the application of technological processes that can modify the oxidation status of the muscle foods.

Meat cooking enhances the development of oxidative reactions since the reaction between molecular oxygen and muscle lipids increases with increasing temperatures (Monahan, 2000). In fact, cooking process leads to a increase lipid oxidation in muscle foods and the

development of the ‘warmed-over’ flavor of refrigerated cooked meats. The acceleration of lipid oxidation following cooking has been attributed to heat-induced changes in muscle components including disruption of cellular compartmentalization and exposure of membrane lipids to a pro-oxidative environment, thermal activation or release of catalytic free iron from myoglobin and thermal inactivation of antioxidant enzymes. The intensity of lipid oxidation is dependent on the cooking temperature since the formation of maillard reaction products with antioxidant activity at temperatures above 100 °C, would inhibit the development of oxidative reactions to some extent. The manufacture of meat products includes a number of technological processes such as cutting or mincing which involves some physical disruption which leads to the exposure of muscle lipids to the pro-oxidative environment (Monahan, 2000). The vacuum packaging and modified atmosphere packaging successful strategies to minimize lipid oxidation in raw and cooked meats (Kingston et al., 1998).

The use of certain additives enhances the susceptibility of muscle lipids to oxidative reactions. Sodium chloride is commonly used in meat products to reduce water activity and inhibit microbial spoilage and contributes to saltiness. Sodium chloride can promote lipid oxidation possibly through displacement of iron from heme proteins. On the other hand, sodium nitrite exhibit antioxidant effect in cured meats and several mechanisms have been proposed including:

i) formation of stable complex with heme pigments

ii) chelation of free iron released from heme pigments following heating or

iii) stabilization of unsaturated fatty acids

Phosphates are widely used in comminuted cooked meat products to increase water binding capacity and also act as antioxidants through metal chelating.

Other antioxidant strategies are also deliberately used to inhibit the adverse effect of lipid oxidation in muscle foods including the modification of the muscle lipid characteristics through dietary means and the direct addition of synthetic and natural antioxidants.

2.5.2 Protein oxidation

The major concerns regarding the occurrence of oxidative processes in muscle foods are related to the adverse effect of those on certain quality traits. Whereas the undesirable oxidative changes in muscle foods have been extensively studied, the precise mechanisms of alteration have not been accurately identified (Xiong, 2000). For instance, color, flavor and texture changes during refrigerated storage of meat coincide with the development of lipid oxidation and in many

cases, the oxidation of unsaturated fatty acids have been highlighted as the main cause of these adverse changes. Some other changes in muscles such as those related to the loss of texture-forming ability and water holding capacity have been associated to a loss of protein functionality though no exact mechanism has been elucidated yet (Xiong, 2000).

Though recent studies of protein oxidation in biomedical sciences have been shown on the mechanisms by which extra-cellular and membrane proteins can be affected by ROS leading to adverse biological effects (Stadtman, 2001), hardly any work devoted to the study of protein oxidation in muscle foods has been carried out. Recent studies on model and food systems have pointed out that the oxidative damage of proteins leads to alterations in gelation, emulsification, viscosity and solubility (Wang and Xiong, 1998). However, about protein oxidation in muscle foods concerning the precise chemical mechanism of protein oxidation, the characterization of the protein oxidation products, the adverse effects on meat quality and the effectiveness of different antioxidant strategies against protein oxidation.

Mechanism of protein oxidation. In muscle foods, the occurrence of protein oxidation can be linked to any of the pro-oxidant factors traditionally associated to lipid oxidation. Therefore, proteins can be oxidized by similar oxidation promoters capable to oxidize unsaturated fatty acids such as heme pigments, transition metal ions and various oxidative enzymes (Xiong, 2000). In the presence of oxidizing lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation, including initiation, propagation and termination stages (Figure 2.6). Theoretically, all amino acids are susceptible to be oxidized by free radicals and non-radical ROS but, actually, some of them are particularly vulnerable, with the cysteine and other amino acids with reactive side chains (sulphydryl, amino group, imidazole or indole ring) being the most susceptible to undergo oxidative reactions (Xiong, 2000).

The attack of ROS on muscle proteins leads to the loss of sulphydryl groups and the generation of carbonyl compounds. The generation of carbonyls (aldehydes and ketones) has been highlighted as one of the most relevant changes in oxidized proteins. Protein carbonyls can be generated via four possible pathways (Xiong, 2000):

i) direct oxidation of amino acid side chains

ii) fragmentation of the peptide backbone

iii) reactions with reducing sugars and

iv) binding non-protein carbonyl compounds

Nevertheless, the deamination reaction is considered the most common way of protein carbonyls formation.

Initiation	$RH \rightarrow R^{\bullet}$
Propagation	$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet}$
Hydrogen abstraction	$RO_2^{\bullet} + P \rightarrow ROOH + P^{\bullet}(-H)$
Addition	$RO_2^{\bullet} + P \rightarrow ROOP^{\bullet}$
Complex	$ROOP^{\bullet} + P + O_2 \rightarrow RO_2PO_2P^{\bullet}$
Polymerization	$P-P^{\bullet} + P^{\bullet} + P \rightarrow P-P-P^{\bullet} + P-P-P$

Figure 2.6 Mechanism of protein oxidation (RH: fatty acid; P: Protein)

Source: Xiong (2000)

Howell et al. (2001) have demonstrated that ROS can cause meat protein polymerization by formation of protein-protein cross-linked derivatives by the following mechanisms:

i) by the oxidation of cysteine sulphydryl groups to form disulphide linkages

ii) by the complexing of two oxidised tyrosine residues

iii) by the interactions of an aldehydes group in one protein with the amine group of a lysine residue in another protein

iv) by the crosslinking of two amine groups (lysine residues) in two different proteins through a dialdehyde (i.e. malondialdehyde) and

v) by the condensation of protein free radicals

Finally, peptide scission can take place concurrent with formation of polymers. According to Stadtman (2001) free radicals can abstract a hydrogen atom from the α-carbon of a polypeptide backbone leading to the generation of new radicals which finally undergo the peptide bond scission. The protein degradation products include those that contain the reactive carbonyl groups described above.

2.6 Nanotechnology

2.6.1 Introduction

The starting point must be to identify what is meant by the nanoscale. One widely used definition is a size range with a lower limit of approximately 1 and an upper limit of 100 nanometres. In respect of impacts on human health and/or the environment, there is no good scientific evidence in favor of either the lower or upper limit. Based on their origin, three types of

nanoscale materials (natural, by-products of human activity, engineered or manufactured) can be distinguished. Since the nanotechnologies are only concerned with the third category, further attention will be confined to this type. An engineered or manufactured 'nanomaterial' is a categorization of a material by the size of its constituting parts. It may be considered to include biological materials that are commonly used and processed and thus can be considered to be "engineered" or "manufactured" in the food and pharmaceuticals industry. Therefore, a modification of the definition might be necessary for regulatory purposes for sector uses such as food/feed and pharmaceuticals. Development of a more suitable definition depends on an understanding of the key physico-chemical and biological properties that influence the adverse effects of nanomaterials.

2.6.2 Relevant physicochemical properties

Size. This aspect has not surprisingly had the greatest attention to date. There is sufficient evidence that reduction of size at the nanoscale results in changes in some properties of the material as a consequence for example of the increase in surface-to-volume ratio. These nano specific properties raise concerns on their potential for harm to humans and the environment. Based on the likelihood of exposure and uptake by biological organisms a particular focus of attention from a risk assessment view point is required for those nanomaterials that either exist as, or may be converted to nanoparticles (3 dimensions in the nanoscale) or nanofibres, nanorods or nanotubes (two dimensions in the nanoscale).

Surface properties and chemical reactivity. The chemical reactivity increases with increasing surface area. This property may or may not be associated with an increase in biological activity or toxicity. The design of nanomaterials often includes the application of coatings and other means of modifying surface properties. Nanoparticles have the potential to generate free radicals and active oxygen. This is an important property in view of the favored theory regarding the toxicity of nanoparticles that they mediate at least some of their effect through the generation of active oxygen.

Solubilisation and other changes. Like other particulate matter, nanomaterials can:

i) be solubilized or degraded chemically

ii) form agglomerates or stable dispersions depending on solvent chemistry and their surface coating and/or

iii) have the ability to react with proteins (Linse et al., 2007).

Behaviour in biological systems. It is too early in the development of the nanotechnologies to identify general rules that can confidently be applied to predicting the risk from individual products other than the focus of concern should nanomaterials that have two or three dimensions in the nanoscale. Consequently all aspects of the life cycle from the production phase to the waste treatment at the end of the life cycle of nanomaterial products need to be considered.

Exposure and toxicokinetic aspects. It appears that for some types of nanoparticles size may be a limiting factor for absorption across the intestinal wall whereas for others similar absorption occurs up to 500 nanometres. From studies using metal particles it appears that there is increasing distribution among body organs with diminishing particle size following oral administration to rodents. Inhalation studies indicate that there is also the potential for uptake across the lung. So far it has not been possible to identify the key characteristics of nanoparticles that influence the extent of uptake nor those that facilitate persistence.

Hazard aspects. It cannot be assumed that a nanomaterial will necessarily have different hazard properties compared to its constituents, nor is it the case that nanoparticles of comparable size will have similar toxicity. Rather some may be virtually not toxic while others are clearly toxic. Although most of the existing toxicological and ecotoxicological methods for hazard identification are likely to be appropriate, they may not be sufficient to address all the hazards of nanomaterials. A particular concern with some in vitro techniques for example is whether they are able to take up the nanoparticles.

2.6.3 Main applications and potential benefits

Recent advances in nanosciences and nanotechnologies have led to a lot of interest in the control and manipulation of material properties at the nano-scale. The new materials, products and applications derived from nanotechnologies are anticipated to bring lots of improvements to the food and related sectors, impacting agriculture and food production, food processing, packaging, distribution, storage and developments of innovative products. A number of recent reports have identified the current and short-term projected applications of nanotechnologies for food and related sectors (Chaudhry et al., 2008). The main driving principle behind these developments seems to be aimed at enhancing uptake and bioavailability of nano-sized nutrients and supplements, and improving taste, consistency, stability and texture of food products (Chaudhry et al., 2008). A major area for current nanotechnology applications in the food sector is for food packaging. The new nanoparticle-polymer composites can offer a number of

improvements in mechanical performance as well as certain functional properties, such as antimicrobial activity to protect the packaged foodstuffs. Food packaging applications of nanotechnologies are estimated to make up the largest share of the current and short-term predicted nano-food market (Chaudhry et al., 2010).

Other main applications relate to health-food sector, where nano-sized supplements and nutraceuticals have been developed to enhance nutrition, and to improve health and well-being. Compared to this, most applications relating to the mainstream food and beverage areas are at the R&D stage, and only a few products are currently available. These applications include development of nano-structured (also termed as nano-textured) food materials. This relates to processing foodstuffs to develop nano-structures and stable emulsions to improve consistency, taste and texture attributes. Nano-textured foodstuffs can also enable a reduction in the use of fat. A typical product of this technology would be a nano-textured ice cream, mayonnaise or spread, which is low-fat but as "creamy" as the full-fat alternative. Such products would offer 'healthy' but still tasteful food products to the consumer. Examples include ongoing research and development (R&D) in Taiwan and Japan on development of micronized starch, cellulose, wheat and rice flour, and a range of spices and herbs for herbal medicine and food applications (FAO, 2010).

Another area of application relates to the use of nano-sized additives in food products. The main claimed benefits include better dispersibility of water-insoluble additives (colors, flavors, preservatives and supplements) in food products without the use of additional fat or surfactants. This is also claimed to enhance taste and flavor due to the enlarged surface areas of the nano-sized additives, and enhance absorption and bioavailability in the body compared with conventional bulk forms. Currently available examples include vitamins, antioxidants, colors, flavors, and preservatives. Also developed for use in food products are nano-sized carrier systems for nutrients and supplements. These are based on nanoencapsulation of the substances in the form of liposomes, micelles, or protein based carriers. These nano-carrier systems are used to mask the undesirable taste of certain additives and supplements, or to protect of from degradation during processing. The nano-encapsulated nutrients and supplements are also claimed for enhanced bioavailability, antimicrobial activity, and other health benefits. An example application, currently under research and development, is that of a mayonnaise which is composed of an emulsion that contains nano-droplets of water inside. The mayonnaise would

offer taste and texture attributes similar to the full fat equivalent, but with a substantial reduction in the fat intake of the consumer (Chaudhry et al., 2010; FAO, 2010).

Certain inorganic nano-sized additives are also finding applications in health food area. Example of these include transition metals (silver, iron), alkaline earth metals (calcium, magnesium), and non metals (selenium, silica). The use of inorganic nano-additives is claimed for enhanced tastes and flavors due to enlarged surface areas. An example is nano-salt, the use of which would give more salt particles on a product (chips/crisps) and allow the consumer to taste the salt more when added at a lower level. Food supplements in this category are also claimed for enhanced absorption and improved bioavailability compared with conventional equivalents (Chaudhry et al., 2010; FAO, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Raw material

3.1.1 Roselle calyces (Dr. Green, Thailand)

3.1.2 Sucrose (Mitr Phol Sugar Corp., Ltd)

3.1.3 Sugar alcohol

3.1.3.1 Xylitol-crystalline (Danisco, Denmark)

3.1.3.2 Maltitol syrup powder MU90G (Ueno, Japan)

3.1.3.3 Lactitol-monohydratecrystalline (Danisco, Denmark)

3.1.4 Anthocyanin

3.1.4.1 Grape skin (AC 12 WSP, Christian Hansen)

3.1.4.2 Black carrot (ColorFruit Carrot 12 WSP, Christian Hansen)

3.1.5 Chinese-style sausage ingredients (e.g. Chinese herb, seasoning etc)

3.1.6 Thai pork fermented “Nham” ingredients (e.g. salt, sugar, sticky rice etc.)

3.1.7 Vienna sausage ingredients (e.g. seasoning etc)

3.1.8 Collagen casing (Nippi casing, Japan)

3.1.9 Nylon/PE vacuum bag (PA/LLDPE/LDPE), Thickness 80 micron

3.2 Reagents

Rosell anthocyanin extraction

3.2.1 Ethanol 95% (commercial grade)

3.2.2 Hydrochloric acid (Sigma-Aldrich, USA)

Anthocyanins determination

3.2.3 Sucrose (Sigma-Aldrich, USA)

3.2.4 Sodium phosphate, dibasic (J.T. Baker, Austria)

3.2.5 Citric acid, monohydrate (J.T. Baker, Austria)

3.2.6 Acetaldehyde (Sigma-Aldrich, USA)

3.2.7 Sulfur dioxide (Sigma-Aldrich, USA)

3.2.8 Acetronitrile (Merck, Germany)

3.2.9 Acetic acid (Merck, Germany)

3.2.10 Phosphoric acid (Merck, Germany)

Ferrous Ions Chelating Ability (FICA) assay

3.2.11 Ferric chloride (Sigma-Aldrich, USA)

3.2.12 Ferrozine (Sigma-Aldrich, USA)

3.2.13 Methanol (Merck, Germany)

Trolox Equivalent Antioxidant Capacity (TEAC) assay

3.2.14 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trolox® (Sigma-Aldrich, USA)

3.2.15 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic Acid) Diammonium Salt; ABTS (Sigma-Aldrich, USA)

3.2.16 Potassium persulfate (Merck, Germany)

Ferric thiocyanate (FTC) assay

3.2.17 Ammonium thiocyanate (Sigma-Aldrich, USA)

3.2.18 2,6-Di-tert-butyl-4-methylphenol; BHT (Sigma-Aldrich, USA)

3.2.19 Linoleic acid (Sigma-Aldrich, USA)

3.2.20 Tocopherols (Sigma-Aldrich, USA)

3.2.21 Ferric chloride (Sigma-Aldrich, USA)

3.2.22 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.23 Ethanol 95% (commercial grade)

Thiobarbituric acids reactive substtance (TBARS)

3.2.24 Sulfanilamide (Sigma-Aldrich, USA)

3.2.25 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.26 2-thiobarbituric acid (Sigma-Aldrich, USA)

Protein oxidation (carbonyl) by 2,4-dinitrophenylhydrazones (DNPH)

3.2.27 Potassium chloride (Sigma-Aldrich, USA)

3.2.28 Trichloroacetic acid (Merck, Germany)

3.2.29 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.30 2,4-dinitrpphenylhydrazine (DNPH) (Merck, Germany)

3.2.31 Ethanol 95% (commercial grade)

3.2.32 Ethyl acetate (Sigma-Aldrich, USA)

3.2.33 Sodium phosphate, dibasic (Sigma-Aldrich, USA)

3.2.34 Guanidine hydrochloride (Sigma-Aldrich, USA)

3.2.35 Bovine serum albumin (BSA) (Merck, Germany)

Nitrite scavenging activity

3.2.36 Sodium nitrite (Sigma-Aldrich, USA)

3.2.37 Sulfanilamide (Sigma-Aldrich, USA)

3.2.38 *N*-(1-naphthyl)-ethyline diamine hydrochloride (Sigma-Aldrich, USA)

3.2.39 Phosphoric acid (Merck, Germany)

3.2.40 Ascorbic acid (Merck, Germany)

3.2.41 Butylated hydroxyanisole (BHA) (Merck, Germany)

Nitric oxide scavenging activity

3.2.42 Sodium nitroprusside (Sigma-Aldrich, USA)

3.2.43 Sulfanilamide (Sigma-Aldrich, USA)

3.2.44 *N*-(1-naphthyl)-ethyline diamine hydrochloride (Sigma-Aldrich, USA)

3.2.45 Naphthylethylenediamine dihydrochloride (Sigma-Aldrich, USA)

Peroxynitrite scavenging activity

3.2.46 Nitric acid (Sigma-Aldrich, USA)

3.2.47 Hydrogenperoxide 30% (Sigma-Aldrich, USA)

3.2.48 Monopotassium phosphate (Sigma-Aldrich, USA)

3.2.49 Dipotassium hydrogen phosphate (Sigma-Aldrich, USA)

3.2.50 Evans blue (Sigma-Aldrich, USA)

3.2.51 Manganese dioxide (Sigma-Aldrich, USA)

3.2.52 Potassium chloride (Sigma-Aldrich, USA)

3.2.53 Sodium chloride (Sigma-Aldrich, USA)

3.2.54 Diethykenetriaminepentaacetic acid (Sigma-Aldrich, USA)

3.2.55 Sodium hydroxide (Sigma-Aldrich, USA)

3.2.56 Sodium nitrite (Sigma-Aldrich, USA)

3.3 Equipments

3.3.1 Nano (grinding and dispersing) machine (JBM-BD35, Taiwan)

3.3.2 Transmission electron microscopy (TEM)

3.3.3 High performance liquid chromatography (Hitachi, Japan)

3.3.4 pH meter couple with Mettler LE438 probe (Mettler Toledo, delta 320, USA)

3.3.5 pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany)

3.3.6 Centrifuge (Harmle Z233 MK-2, Germany)

3.3.7 Centrifuge (Hettich Universal 16, Germany)

3.3.8 UV-Vis spectrophotometer (Hitachi U-2001, Japan)

3.3.9 UV-Vis spectrophotometer (Shimadzu UV-1601, Japan)

3.3.10 Colormeter (ZE 2000, Nippon Denshoku, Japan)

3.3.11 Colormeter (Minolta CR-400, Japan)

3.3.12 Water activity meter (AquaLab™ Series3TE, USA)

3.3.13 Rotary evaporator unit (RII, Büchi Rotavapor, USA)

3.3.14 Homogenizer (Ultra-Turrax® T25Bbasic, Germany)

3.3.13 Texture analyzer, TA-XT2i plus (Stable Micro Systems, U.K.)

3.3.15 Meat grinder (SevenFive, Thailand)

3.3.16 Stuffer (Thailand)

3.3.17 Tray dryer (Patch663, Thailand)

3.3.18 Vacuum packing machine (Alpha-Pack, Thailand)

3.3.19 Convention oven (MR-1302, Mamaru, France)

3.4 methods

3.4.1 Determination of physicochemical and antioxidant properties of roselle extracts prepared by original-grinding extraction and nano-grinding extraction method

Comparative studies on physicochemical and antioxidant properties of roselle extracts prepared by original-grinding and nano-grinding extraction were done. The model solution with different pH, temperature and sucrose concentration was used to evaluate the properties of the roselle extract samples.

3.4.1.1 Sample preparation

1) Roselle calyxes

Fresh roselle calyxes were purchased from Peasant Association of Area Taitung, Taiwan. The calyces were dried at 40 °C using hot air oven until a_W ~0.48. Dried calyxes were ground using household blender.

2) Roselle extraction

In this study two methods were used to extraction roselle calyxes;

1) ground roselle calyxes was soaked in ethanolic solution (1.5 M hydrochloric acid in ethanol) for 24 hr, the sample from this extraction refers to “OG”, and

2) ground roselle calyxes was extracted in ethanolic solution coupled with nano machine with set speed at 2000 rpm for 1 hr. Zirconium oxide (ZrO_2) was used as grinder materials. The extract obtained by this procedure refers to “NG”.

The roselle extracts, OG and NG, were evaporated at 30 °C until the acidified ethanol was removed. The extracts were made up to 10 ml with acidified ethanol and stored at -25 °C until further analyzed.

3) Model solutions

Citric acid-Na_2HPO_4 (Mcllvaine) buffer solution was used (see Appendix B1) to prepare a model solution of roselle extracts by dissolving the extracts (OG and NG) in the buffer solution at different pH (pH 3.0 and 4.0) with or without sucrose (0 % and 20 % w/w) and heated at 50, 60, and 70 °C.

3.4.1.2 Analytical methods

1) Color determination

Color of roselle extract solutions were measured using a Colormeter (ZE 2000, Nippon Denshoku, Japan). The absorbance of the roselle extract sample was read at 420, 520, and 700 nm using 1 cm quartz cell by a UV-vis spectrophotometer (U-2001, Hitachi, Japan). Samples were also placed in an optical glass cell and L, a, b and chroma were measured in triplicate. The color density (CD; Equation 1) and degradation index (DI; Equation 2) of samples diluted with pH 3.0 or pH 4.0 buffer were measured.

$$Color\ density\ (CD) = \left(A_{520nm} - A_{700nm}\right) + \left(A_{420nm} - A_{700nm}\right) \quad (1)$$

$$Degradation\ Index\ (DI) = \frac{A_{420nm}}{A_{\lambda_{max}}} \quad (2)$$

2) Co-pigmented, monomeric and polymeric anthocyanins

Co-pigmented (Equation 3), monomeric (Equation 4) and polymeric anthocyanins (Equation 5) were determined according to Tsai and Huang (2004). Briefly, 10 % (v/v) acetaldehyde was added to sample. The mixture was allowed to stand for 45 min at room temperature before measuring A_{520nm} (A^{acet}). To another sample, 5 % (w/v) SO_2 was added, and A_{520nm} was measured (A^{SO_2}). The A_{520nm} was also measured directly from the sample. This reading was multiplied by 10 to give the $A^{extract}$. From these readings, the percent distribution of the various forms of anthocyanins can be calculated as:

$$Copigmented\ anthocyanins\ (\%) = \frac{A^{acet} - A^{extract}}{A^{acet}}\ x\,100 \quad (3)$$

$$Monomeric\ anthocyanins\ (\%) = \frac{A^{extract} - A^{so_2}}{A^{acet}}\ x\,100 \quad (4)$$

$$Polymeric\ anthocyanins\ (\%) = \frac{A^{so_2}}{A^{acet}}\ x\,100 \quad (5)$$

3) Antioxidant capacities

The antioxidant activities of roselle extracts were evaluated by various *in vitro* assays, including the ferrous ion chelating activity (FICA) which was measured according to the method of Chang et al. (2006b) (see Appendix B3), antioxidant activity by trolox equivalent antioxidant capacity (TEAC) was measured according to the method of Arts et al. (2003) (see Appendix B4), and inhibitory activity toward linoleic acid peroxidation by ferric thiocyanate (FTC) was measured according to the method of Manian et al. (2008) (see Appendix B5).

4) HPLC anthocyanin analysis

The roselle anthocyanin solutions were analyzed using HPLC with a UV-vis (Tsai and Huang, 2004). Briefly, the separation of anthocyanins was accomplished on a reverse phase C_{18} column (250 mm × 4.6 mm i.d.; Hitachi High-Technologies Corporation, Japan). In direct analyses, mobile phase were A, 5 % acetic acid and B, acetonitrile. The gradient condition was 0-10 min, 100 % A; 10 to 40 min, 90 to 60 %; and back to 100 % after 45 min. Other chromatographic conditions were as follows: flow fate 1 ml/min, 20 μL injections and the detection wavelength was 280 nm (Hitachi UV-vis detector; Hitachi high-technologies Corporation, Japan).

3.4.1.3 Statistical analysis

Mixed model factorial experiments were used in this study. The statistical analysis of the data was carried out using SPSS software for the analysis of variance (ANOVA) in determining significant differences at a confidence level at 95 % (p<0.05). Variable means were compared by Duncan's multiple rang test.

3.4.2 Evaluation of antioxidant capacity of roselle extracts on lipid and protein oxidation as affected by sucrose and different sugar alcohols in Chinese-style sausage

This objective is intended to evaluate the effect of sucrose and different sugar alcohols on anti-lipid and anti-protein oxidation capacity of roselle extracts in Chinese-style sausage.

3.4.2.1 Effect of sucrose and different sugar alcohols on antioxidant capacity of roselle anthocyanin extracts

1) Materials

Roselle extract: Roselle extract was prepared by soaking ground roselle calyces in 95 % ethanol for 24 hr. The extract is then evaporated at 30 °C until the ethanol is removed.

The extract was kept in brown glass vial with screw cap and stored at -25 °C until further analysis.

Sugar alcohols: Sugar alcohols including Lactitol-monohydratecrystalline (Danisco, Denmark), Xylitol-crystalline (Danisco, Denmark) and Maltitol syrup powder MU90G (Ueno, Japan) were used to replace sucrose in the sausage formulation.

2) Chinese-style sausage preparation

Chinese-style sausage was prepared as described by Pinsirodom (2008). Briefly, fresh lean pork and back fat were purchased from CP Fresh mart (Bangkok, Thailand). The lean pork and pork back fat were trimmed out of connective tissue. They were ground separately through a 4 mm grinder plate (SevenFive, Thailand), and then kept at 4 °C for 2 hr. The mixture of lean pork (65 %) and pork fat (16 %) were formulated to contain 0.3 % roselle extracts. All other ingredients were added: 0.2 % potassium nitrite (prague powder), 0.1 % Chinese five-spice blend, 0.3 % monosodium glutamate, 1.80% salt and 16.6 % sucrose (see Appendix A1). The sausage mixtures were stuffed into collagen casing (Nippi casing, Japan), linked into 10 cm length and then dried at 60 °C for 24 hr. Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (29±1 °C) and analyzed every week for 4 weeks. To compare the effect of sugar alcohols on lipid and protein oxidation in the products, sugar alcohols were used to replace for sucrose at the level of 16.6 % (w/w) based on the control formula weight. Three replicates with batches were conducted in this study.

3) Physical properties

Moisture content: The moisture content of samples were determined using the oven method at 105±1 °C (AOAC, 2000)

Water activity: Water activity (a_W) of the ground samples were measured by a Water activity meter (AquaLab™ Series3TE, USA).

pH: Each 1 g from samples each treatment was blended by homogenizer (Ultra-Turrax® T25Bbasic, Germany) with 10 ml distilled water for 1 min, then pH was determined using pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany).

Color: Color parameters were measurement by a colormeter (as CIE L*, a*, b*). The color measurements of Chinese-style sausage were performed at room temperature. Total color difference (TCD; Equation 6), Hue angle (Equation 7), chroma (saturation index; Equation

8), and browning index (BI; Equation 9) was calculated using Hunter L*, a*, and b* values (Bozkurt and Bayram, 2006) as:

$$TCD = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (6)$$

$$Hue\ angle = \arctan(b/a) \quad (7)$$

$$Chroma = \sqrt{a^2 + b^2} \quad (8)$$

$$BI = \frac{[100\ x\ (X - 0.31)]}{0.17} \quad (9)$$

$$\text{Where: } X = \frac{(a + 1.75\ x\ L)}{(5.645\ x\ L + a - 3.012\ x\ b)}$$

4) Antioxidant capacities

Lipid oxidation measurement: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) (Min et al., 2009). Results were expressed as mg MDA per kg of sausage (see Appendix B6).

5) Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.2.2 Effect of sugar alcohol concentration on antioxidant capacity of roselle extracts

In this study, a sugar alcohol was selected (from 3.4.2.1) according to the physical properties and oxidative stability of the sausage samples. The Chinese-style sausages were then prepared with varied concentrations of the sugar alcohol. All samples were compared with Chinese-style sausage containing 16.6 % (w/w) sucrose as a control sample.

1) Materials

Roselle extract: Preparation was done as described in 3.2.1.1

Sugar alcohol: Type of sugar alcohol was selected from the results in experiment 3.4.2.1

2) Sausage preparation

Chinese-style sausage was prepared as described earlier (3.4.2.1.2). To compare the effect of sugar alcohol concentration on lipid and protein oxidation in the products, the sugar alcohol was used to replace for sucrose at the level of 11.6, 16.6 and 21.6% (w/w) based on the control formula weight (see Appendix A2). Three replicates with batches were conducted in this study. Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (30±1 °C) and analyzed every week for 4 weeks.

3) Physical properties: Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

4) Antioxidant capacities

Lipid oxidation measurement: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) method (Min et al., 2009). Results were expressed as mg MDA per kg of sausage (see Appendix B6).

Protein Carbonyl Content: Protein oxidation in the sausage was determined by measuring the formation of protein carbonyls by converting them to 2,4-dinitrophenylhydrazones (DNPH) and the derivatives were measured spectrophotometrically according to method described by Viljanen et al. (2005) (see Appendix B7).

5) Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.2.3 Effect of sugar alcohol on texture profile and sensory qualities of Chinese-style sausage during storage

In this study, the Chinese-style sausage sample (from 3.4.2.2) was selected by the consideration of physical properties and as lipid oxidation (TBARS) and protein carbonyl by DNPH method.

1) Materials

Roselle anthocyanins extract: Preparation was done as described in 3.2.1.1

Sugar alcohol: The sugar alcohol was selected from the results in experimental 3.4.2.2

2) Sausage preparation

Chinese-style sausage was prepared as described in experiment 3.4.2.1 number 2). To compare the effect of sucrose and sugar alcohol on texture profile analysis and sensory quality of the products, sucrose was added at 16.6 % (w/w) in the control sample and the level of sugar alcohol added was selected from the result in experiment 3.4.2.2 (see Appendix A3). Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (30±1 °C) and 4 °C and measured every week for 4 weeks.

3) Physical properties: Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

4) Texture profile analysis: Texture profile analysis (TPA) was applied as described by Bourne (2002) using a TAXT2i texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe P/25 to determine hardness, cohesiveness, adhesiveness, gumminess, chewiness and springiness and a reversible probe to determine the maximum cutting force and the cutting work. The cutting samples are approximately 1.5 cm long and 2.5 cm wide which will be compressed twice to 50 % of their thickness. The following parameters are defined: hardness (H) = maximum strength required to achieve compression; area of the first compression (A_1) = total energy required for the first compression; area of the second compression (A_2) = total energy required for the second compression, adhesiveness = area under the abscissa after the first compression; springiness (S) = height the sample recovers between the first and second compression; cohesiveness $\left[(C)=\frac{A_2}{A_1}\right]$; gumminess $[(G)=HxC]$; chewiness $[(ch)=SxG]$.

5) Sensory analysis: Sensory evaluations were following the procedure of Chambers and Wolf (1996) and conducted at days 0, 7, 14, 21 and 28 of storage times. The Chinese-style sausages were evaluated by an experienced 25-members panel consisting of master and PhD students, aged from 25 to 35, were selected from the Agro-Industry, KMITL. Panelists were chosen on the basis of previous experience in consuming traditional Chinese-style sausage. Sausages were first cooked in a convention oven (Mamaru, MR-1302, France) at 150 °C for 20 min. Cooled at room temperature (30 °C), then sliced into 3 mm thick pieces and served on plastic dishes covered with plastic films and coded with three-digit random number. The method performed to describe the organoleptic of the Chinese-style sausage was inspired by a

Quantitative descriptive analysis (QDA) was carried out as described by García et al (2010) and Pérez-Cacho et al. (2005). The attributes of this evaluated including appearance, odor, taste, mouth feel and overall acceptability (see Appendix C1). The panelists evaluated sausage over structure scales of 15 cm delimited at both ends. The sensory attributes use in this study including color, odor, taste, texture and overall acceptance were determined using 1-7 point hedonic scale, with 1, 4 and 7 representing extremely dislike, neither like nor dislike and extremely like, respectively (see Appendix C2).

6) Statistical analysis

A completely randomized experimental design (CRD) was used for texture profile analysis and randomized completely block design (RCBD) was used for sensory analysis. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was be determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.3 Investigation of the ability of anthocyanins in scavenging of reactive nitrogen species in model system and evaluation of their effect on nitrite reduction in meat products.

These studies were intended to evaluate the ability of roselle extracts in scavenging of reactive nitrogen species (RNS) comparing to the anthocyanins from different sources, and their effect on the nitrite reduction in Vienna sausage and traditional Thai fermented sausage (Nham).

3.4.3.1 Materials

Natural anthocyanins: Roselle anthocyanin extracts was prepared following the method in 3.2.1.1. Anthocyanins from various sources; grape skin (AC 12 WSP, Christian Hansen) and black carrot (ColorFruit Carrot 12 WSP, Christian Hansen) were obtained from The Each Asiatic (Thailand) Public Company Limited and used for the comparative study.

3.4.3.2 Reactive nitrogen species (RNS) scavenging capacities

Nitrite scavenging activity: This assay was carried out as described by Kang et al. (2006) and Saha et al. (2004). The reaction mixtures were prepared in pH 3.0, 6.0 and 9.0 (see Appendix B1 and B2) measured using a UV-vis spectrophotometer at 540 nm (see Appendix B8).

Nitric oxide scavenging activity: Nitric oxide scavenging activity was measured spectrophotometrically as described by Govindarajan et al. (2003). The sample was reacted with Griess solution, the absorbance of reaction mixtures were measured using a UV-vis spectrophotometer at 546 nm (see Appendix B9).

Peroxynitrite ($ONOO^-$) scavenging activity: The peroxynitrite scavenging activity was measured according to the method of Küçük et al. (2007). The absorbance of the color mixtures after incubated at 25 °C were measured using UV-vis spectrophotometer at 611 nm (see Appendix B10).

All RNS scavenging tests were performed three times. Ascorbic acid, Butylated Hydroxyanisole (BHA), and/or Gallic acid were used as a positive control.

3.4.3.3 Nitrite reduction in meat products

Meat products preparation: Preparation of meat products including Vienna sausage (see Appendix A4) and Nham (traditional Thai fermented pork) (see Appendix A5) were followed the method described by Heinz and Hautzinger (2007) and by Swetwiwathana *et al*. (2007), respectively.

The Vienna sausage and Nham were produced under typical processing conditions, with 0.3 % of roselle extracts and two levels of sodium nitrite, 0 and 125 mg/kg (125 ppm), the highest permitted level for use in meat products by Thai Industrial Standards Institute (TISI 3540-2006; TSI 1219-2004).

The Vienna sausage mixtures were stuffed into collagen casing (Nippi casing, Japan), linked into 10 cm length and dried at 60 °C for 1 hr, then cook in hot water (65±3 °C) for 45 min. After cooled, Vienna sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at refrigerated temperature (4±1 °C) and analyzed every 3 days for 24 days. For the traditional Thai fermented pork sausage "Nham", the 100 g of mixtures were stuffed into 2.5 cm diameter polyethylene (PE) bag and tied with thread. After that, the samples were kept at room temperature (30±1 °C) and analyzed every day for 7 days.

1) Physical properties:

Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

2) Antioxidant capacities

Lipid oxidation determination: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) method (Min et al., 2009). Results were expressed as mg MDA per kg of sample (see Appendix B6).

Nitrite determination: Sodium nitrite in the samples was measured according to the method of Liu et al. (2010). The residual nitrite of all samples was reacted with the Griess

solution. Absorbance values of each sample were measured using a UV-vis spectrophotometer at 540 nm (see Appendix B11).

3.4.3.4 Kinetic degradation of nitrite in traditional Thai fermented pork

The traditional Thai fermented pork "Nham" was produced, with or without 0.3 % of roselle extracts and positive control (BHA 200 ppm) and three levels of sodium nitrite; 125, 250 and 500 ppm (see Appendix A6). Briefly, minced pork was mixed thoroughly with roselle extracts following salt and sodium nitrite for 1 min. Then ground garlic and sugar were added and mixed for 1 min. Cooked sticky rice was mixed additionally for 2 min, and then minced pork lard was added and mixed for 2 min. The 100 g mixture was stuffed into 2.5 cm diameter polyethylene (PE) bag and tied with thread. After that, the samples were kept at room temperature (30±1 °C) and analyzed every day for 4 days.

3.4.3.4.1 Chemical properties:

pH: The pH was measured after homogenization (Ultra-Turrax® T25Bbasic, Germany) with distilled water at a ratio of 1:10 using pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany)**.**

Total acidity (as lactic acid): Ten gram samples were homogenized with 90 ml distilled water. Titratable acidity was determined as % lactic acid by titrating with 0.1 M NaOH using phenolphthalein as an indicator (AOAC. 2000).

Nitrite determination: Sodium nitrite in products was measured according to the method of Liu et al. (2010). The nitrite residual of samples were reacted with the Griess solution. Absorbance values of each sample were measured using a UV-vis spectrophotometer at 540 nm (see Appendix B11).

Kinetics of nitrite degradation: The degradation kinetics of most biological materials of food system follows the first-order (Equation 10) reaction (Villota and Hawkes, 2007). The first-order reaction rate constant (k) (Equation 10) and half-life time ($t_{1/2}$) values (Equation 11) were expressed by the following equations:

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \tag{10}$$

$$t_{1/2} = -\ln 0.5k^{-1} \tag{11}$$

Where C_o is the concentration of sodium nitrite at zero time, C_t is the concentration of sodium nitrite at time t (hour) of fermentation time, k is the first-order kinetic constant.

3.4.3.5 Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Determination of physicochemical and antioxidant properties of roselle extracts prepared by original-grinding and nano-grinding

This study was to determine the effect of original-grinding (OG) and nano-grinding (NG) on the physicochemical property and antioxidant activity of roselle extracts. The comparison of the physicochemical property and antioxidant activity of roselle extracts were investigated when prepared under different model systems of pH (pH 3.0 and 4.0), with or without sucrose (20 % by weight) and temperature (50, 60 and 70 $^{\circ}$C). Results from the experiment were described below.

4.1.1 Physicochemical properties of roselle extracts prepared by original-grinding and nano grinding

1) Particle size of NG roselle

The particle size of NG roselle determined by transmission electron microscopy (TEM) is presented in Figure 4.1. As seen in the figure, the primary (a) and secondary (b) particle size of NG roselle were around 100 nanometers. These confirmed the nano-particle size of the roselle in this study.

2) The spectra of OG and NG roselle extracts

The spectra curves from 400 to 700 nm of roselle extracts from OG and NG in different systems with or without sucrose are presented in Figure 4.2. Roselle extracts from OG and NG were red color solution, roselle extracts in all systems showed maximum peak at 520 nm and no absorption band longer than 525 nm. This result agreed with that reported by Tsai et al. (2005) and Giusti and Wrolstad (2003); the maximum absorbance of roselle anthocyanins and anthocyanins from other sources, was between 520 to 525 nm. Results from this experiment also showed that spectral characteristics of roselle extracts were dependent on pH and roselle particle size. At the same pH, the NG roselle extract solution showed higher absorbance at λ_{max} tan the OG roselle extract solution, indicating the hyperchromic effect (ΔA_{max}). The occurrence of hyperchromic effect in the model systems may be affected by copigmentation of anthocyanins with phenol and other compounds (Boulton, 2001). Moreover, absorbance of OG and NG roselle extracts solution increased with the decreasing pH of the solution. These results similar to those

reported by Gauche et al. (2010) in that the maximum absorbance of grape skin extracts increased when pH of solution decreased as revealed by hyperchromic effect.

3) HPLC profile of anthocyanins in OG and NG roselle extracts

The separation and identification of anthocyanins from roselle extracts were performed according to the procedure described by Tsai and Huang (2004). In this study, only two anthocyanins were detected in OG and NG samples (Figure 4.3). Anthocyanins (peak a and peak b) were identified by matching their retention times to those of the anthocyanins present in an authentic sample of roselle extract, as described previously (Hong and Wrolstad, 1990; Wong et al., 2002; Tsai et al., 2002). The anthocyanins were expressed as percentage based on total peak area. The relative percentages of delphinidin-3-sambubioside (peak a) and cyanidin-3-sambubioside (peak b) in OG and NG roselle extracts were 82.33 and 84.46 % and 17.67 and 15.54 %, respectively. Results of this study showed that delphinidin-3-sambubioside and cyanidin-3-sambubioside were the major anthocyanins found in roselle extracts and this corresponded to those reported previously by Hong and Wrolstad (1990), Wong et al. (2002) and Tsai et al. (2002).

As the particle size decreases, surface area and surface group reactivity such as hydrophilic, hydrophobic and catalytic groups will increase (Chen et al., 2006; Nickols-Richardson, 2007). Since the particle size of NG sample (100 nanometers) were much smaller than that of OG sample (more than 1000 nanometers, data not shown), the larger surface area in NG roselle resulted in the greater efficiency of anthocyanin pigment extraction and higher intensity of redness compared to the OG roselle extracts. Figure 4.3, confirmed that the NG roselle extract contained higher anthocyanin content.

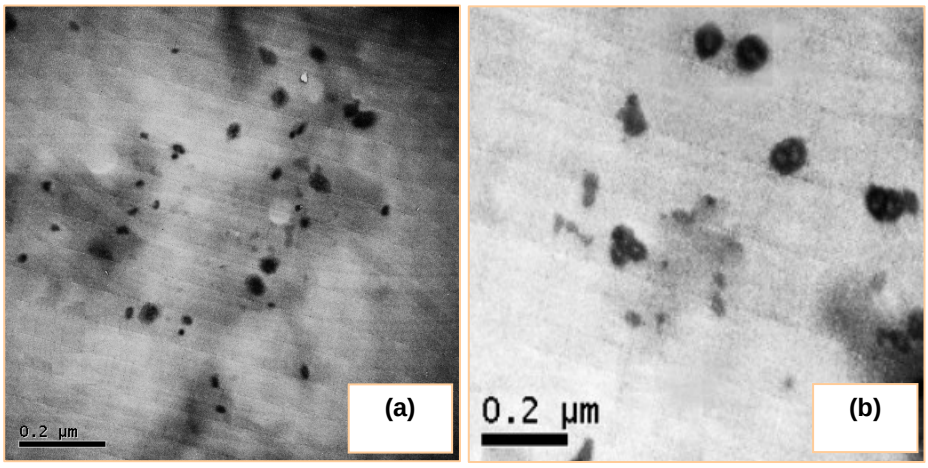


Figure 4.1 Transmission electron micrographs (TEM) of roselle extracts with primary particle size (a) and secondary particle size (b) after nano-grinding

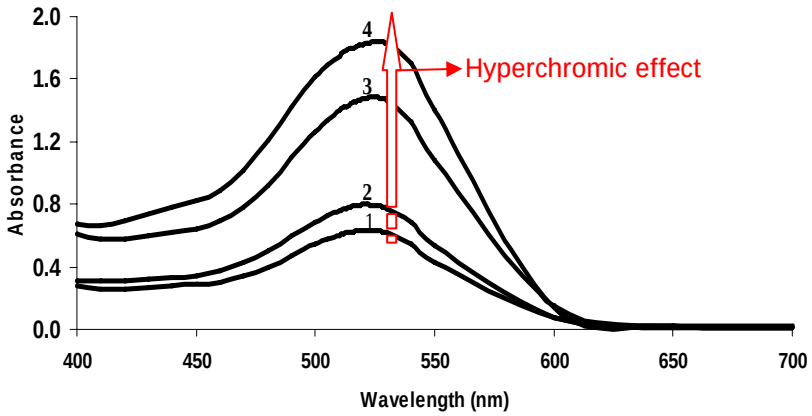


Figure 4.2 The UV-spectrum of roselle extracts in different pH citrate-phosphate buffer solution, 1 = OG in buffer pH 4.0, 2 = OG in buffer pH 3.0, 3 = NG in buffer pH 4.0 and 4 = NG in buffer pH 3.0

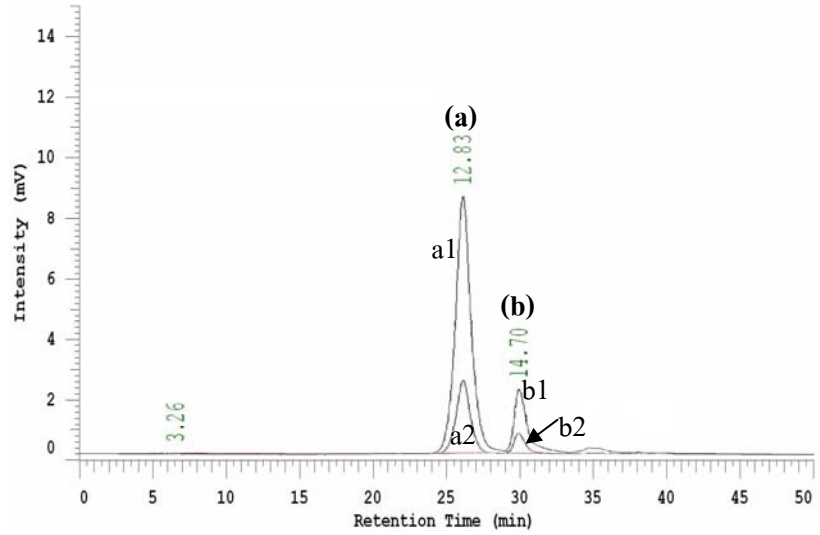


Figure 4.3 HPLC chromatogram of anthocyanins in roselle extracts (a) Delphinidin 3-sambubioside (Dp 3-sam) and (b) Cyaniding 3-sambubioside (Cy 3-sam); (a1) Dp 3-sam of NG roselle, ((b1) Cy 3-sam of NG roselle, (a2) Dp 3-sam of OG roselle and (b2) Cy 3-sam of OG roselle

4.1.2 Thermal stability of anthocyanins in OG and NG roselle extracts in different model systems

Thermal stability of the anthocyanins in roselle extracts (OG and NG) was determined by heating 20 ml of the roselle extracts in citrate-phosphate buffer solution pH 3.0 and 4.0 with and without sucrose (20 %) in screw capped test tube in an incubator at 50, 60 and 70 °C for 388, 199

and 72 hrs, respectively, following by cooling at room temperature for at least 7 min. Visuals color (L, a, b) were measured by colormeter. Anthocyanin contents, pigment retention and kinetic of roselle anthocyanin degradation were analyzed based on the absorbance at 420, 520 and 700 nm. Antioxidant activities of OG and NG roselle extracts were measured by ferrous ion chelating ability (FICA), Trolox equivalent antioxidant capacity (TEAC) and ferric thiocyanate capacity (FTC). Results are presented below.

4.1.2.1 Change of visual color of OG and NG roselle extracts solution

To investigate visual colors of roselle extract solution, it is necessary to measure color as well as pigment concentration (Wrolstad et al., 2005). The visual colors (L, a, b) of OG and NG roselle extracts in model system (pH 3.0 and 4.0 with and without sucrose) after treated at 50, 60 and 70 °C are presented in Table 4.1. After heat treatment, a-value of OG and NG roselle extract solution at pH 3.0 and 4.0 with or without sucrose significantly decreased and the lowest a-values were observed after heating at 70 °C. The decrease of a-value could be attributed to the degradation of anthocyanins at high temperature resulting in color fading (increased L-value and decreased a-value) during heat treatment. Consequently, the color of roselle extract solution became browning at higher temperature which in accordance with previously reported results on roselle anthocyanins after heat treatment at 30-70 °C (Tsai and Huang, 2004; Tsai et al., 2005). Slightly increasing of b-value was observed in all roselle extract solution after heat treatments excepted for NG roselle extract sample at pH 3.0 (with or without sucrose). However, OG and NG roselle extract solution for all heat treatments exhibited the same trend of increased L-values and decreased a-values. These results were in agreement with those of Tsai et al. (2005) who evaluated the thermal stability of roselle anthocyanins in buffer solution pH 3.0 at the temperature ranged between 50 to 70 °C. A significant increase of L-values and decrease of a-values was observed in relation with increased temperature. In addition, Tseng, et al. (2006) suggested that the thermal degradation of anthocyanins could be occur via two mechanism; 1) hydrolysis of the C_3 linkage to form the more labile aglycone, and 2) hydrolytic opening of the pyrilium ring to form a substituted chaconne, which degrades to brown insoluble compounds of a polyphenol.

Table 4.1 Visual colors of OG and NG roselle extracts in model systems at pH 3.0 and 4.0 after heating at different temperatures

	Roselle model system without sucrose			Roselle model system with sucrose		
	L-value	a-value	b-value	L-value	a-value	b-value
pH 3.0						
OG	71.16±0.58^{d}	48.03±0.95^{a}	5.51±0.38^{d}	71.44±1.23^{d}	48.41±0.56^{a}	5.70±0.19^{d}
OG/50C	91.00±0.56^{c}	9.59±0.81^{b}	9.58±0.68^{c}	90.61±0.01^{c}	8.91±0.00^{c}	9.06±0.01^{c}
OG/60C	96.09±0.29^{a}	10.04±1.41^{b}	10.53±0.08^{b}	95.83±0.01^{a}	9.34±0.00^{b}	10.46±0.01^{b}
OG/70C	94.77±0.36^{b}	5.48±0.23^{c}	12.48±0.04^{a}	93.81±0.00^{b}	2.27±0.01^{d}	12.52±0.00^{a}
NG	48.81±0.87^{d}	71.90±0.77^{a}	17.91±0.95^{c}	51.58±0.57^{d}	71.20±0.77^{a}	20.44±0.53^{b}
NG/50C	78.63±0.75^{c}	13.60±0.35^{b}	19.23±0.67^{b}	78.26±0.02^{c}	18.92±0.01^{b}	18.61±0.01^{c}
NG/60C	87.72±0.01^{a}	3.67±0.01^{c}	21.10±0.01^{a}	85.75±0.00^{a}	6.56±0.15^{c}	22.19±0.01^{a}
NG/70C	82.18±0.03^{b}	1.53±0.12^{d}	14.92±0.09^{d}	82.15±0.02^{b}	4.42±0.01^{d}	20.53±0.00^{b}
pH 4.0						
OG	73.46±0.73^{b}	42.00±0.66^{a}	4.24±1.32^{d}	73.87±0.16^{d}	41.65±0.97^{a}	4.09±0.46^{d}
OG/50C	95.00±0.77^{a}	2.25±0.51^{b}	10.08±0.53^{c}	93.71±0.01^{c}	1.93±0.01^{b}	10.43±0.01^{c}
OG/60C	94.70±0.02^{a}	1.58±0.01^{b}	11.72±0.01^{b}	96.36±0.01^{a}	1.42±0.01^{c}	10.61±0.01^{b}
OG/70C	94.43±0.84^{a}	1.24±0.05^{c}	13.98±0.53^{a}	94.39±0.01^{b}	0.69±0.01^{d}	12.44±0.00^{a}
NG	52.81±0.69^{d}	68.84±0.84^{a}	14.85±0.75^{d}	55.99±0.77^{d}	69.37±0.98^{a}	12.57±0.83^{d}
NG/50C	78.96±0.65^{c}	13.94±0.62^{b}	19.06±0.61^{c}	80.99±0.01^{c}	12.28±0.01^{b}	21.84±0.00^{b}
NG/60C	87.72±0.01^{b}	3.67±0.01^{c}	21.10±0.01^{a}	86.67±0.00^{a}	4.84±0.01^{c}	23.87±0.01^{a}
NG/70C	90.34±0.39^{a}	0.86±0.45^{d}	20.68±0.16^{b}	84.20±0.02^{b}	1.63±0.01^{d}	20.82±0.00^{c}

Values are means ±standard deviations; *n=3*. Means within columns and treatment group with different letters are significantly different ($p<0.05$).

OG = original-grinding roselle extracts, OG/50C = original-grinding roselle extracts treated at 50°C, OG/60C = original-grinding roselle extracts treated at 60°C, OG/70C = original-grinding roselle extracts treated at 70°C, NG/50C = nano-grinding roselle extracts treated at 50°C, NG/60C = nano-grinding roselle extracts treated at 60 °C, NG/70C = nano-grinding roselle extracts treated at 70°C.

4.1.2.2 Anthocyanins in OG and NG roselle extracts

As seen earlier in Figure 4.2 and 4.3, roselle extracts by nano-grinding method exhibited higher in pigment concentration and anthocyanin contents. This suggested that nano-grinding had better extraction efficiency of pigments from roselle. Moreover, heat treatments can cause a discoloration of the red color (as seen in Table 4.1), leading to a decreased in monomeric anthocyanins and further heat of increased temperature will cause an increase in polymeric anthocyanin (Tsai and Huang, 2004). In addition, high temperature is though to induce hydrolysis of glycoside bonds in anthocyanin molecules, leading to the formation of unstable aglycones which degrade rapidly in aqueous system (Dao et al., 1998). The present of sugar, ascorbic acid and their degradation products are also known to decrease anthocyanin stability via oxidation reaction and might enhance the formation of large polymer pigments (Krifi et al., 2000).

Therefore, a comparison of the OG and NG roselle extract solution can e used to evaluate the effect of roselle particle size on anthocyanin degradation. According t the data shown in Table 4.2, it is clear that total monomeric anthocyanins decreased and total polymeric anthocyanins increased in all roselle extract samples as the heating temperature increased. There was a tendency for the NG roselle extract solution at pH 3.0 and 4.0 with or without sucrose to have slightly lower total monomeric anthocyanins and higher total polymeric anthocyanins after heating compared to the OG roselle extract solution. The results indicated that the OG roselle extracts were more stable in terms of anthocyanin degradation.

Considering the effect of sucrose, there was no obvious tendency for the influence of sucrose on the anthocyanin degradation, since most of the model samples with or without sucrose contained about the same amount of total monomeric and polymeric anthocyanins. However, the increase of polumeric anthocyanins as a function of temperature observed in this study are in agreement with the reports of Garzon and Wrolstad (2002) and Aurelio et al. (2008).

The anthocyanin degradation in OG and NG roselle extract solution under various heat treatment conditions were confirmed by HPLC analysis (see Appendix D1-D4). Overall results suggested that the NG roselle extracts were likely less stable under heat treatments. This may be due to their higher surface area, heat sensitivity and susceptibility to oxidation (Tsai et al., 2011). The results entirely support the previous conclusion that heating temperature has a considerable impact on the stability of monomeric anthocyanins.

4.1.2.3 Influence of model system and heat treatment on degradation index for OG and NG roselle extract solution

Degradation index (DI) is a ratio between absorbance at 420 nm and at 520 nm of the anthocyanin solution. As the roselle anthocyanin solution is heated, an intensity of red color decreases and A_{520} also decreases. Further heating will cause an increase in browning and A420; therefore, the ratio of A_{420} and A_{520} represents the degradation index (Tsai et al., 2005).

Figure 4.4 shows the effects of pH, temperatures and sucrose on the degradation indexes of anthocyanins in OG and NG roselle extract solution. Results clearly showed that anthocyanins in OG and NG roselle extracts were more stable at pH 3.0 either with or without sucrose (20 %) than at pH 4.0 during heating at 50 to 70 °C. The degradation indexes of all samples were also greater at the higher heating temperature. In addition, roselle anthocyanins tended to have lower stability in the present of sucrose, which can be seen from the greater slope of the curves in Figure 4.4b and 4.4d. Although the effect of sucrose on the stability of roselle anthocyanins was not clear in the previous data (Table 4.2), results in Figure 4.4 suggest that sucrose might enhance the anthocyanin degradation. Similar results were also reported by Kopjar et al. (2009) for the effect of sucrose on the stability of blackberry anthocyanins.

In contrast, Wrolstad et al. (1990) reported that increasing of sucrose concentration to about 20 % could promote the stability of anthocyanins in frozen strawberry during storage. The inconsistence in the effect of sucrose on stability of anthocyanin suggesting the multiple factors can influence the stability of anthocyanins, including their structure and composition, concentration and type of sugar (Delgado-Vargas and Paredes-López (2003).

In contrast to the previous results (Table 4.2); which revealed the slightly higher stability of OG roselle anthocyanins then the NG roselle anthocyanins, the data from degradation index (Figure 4.4) did not show obvious trend of stability difference between OG and NG roselle anthocyanins during heat treatments.

Table 4.2 Changes of total monomericic and polymeric anthocyanins (%) in OG and NG roselle extract solution at pH 3.0 and 4.0 after heating at 50 to70 °C

	Roselle model system without sucrose			Roselle model system with sucrose		
	Mono[a]	Poly[b]	Copig[c]	Mono[a]	Poly[b]	Copig[c]
pH 3.0						
REx	99.68±0.00[a]	0.32±0.01[g]	nd	99.64±0.01[a]	0.37±0.01[g]	nd
OG/50C	23.60±0.00[d]	76.40±0.01[d]	nd	23.85±0.02[d]	76.16±0.01[d]	nd
OG/60C	30.22±0.00[b]	70.33±0.04[f]	nd	29.08±0.08[b]	72.04±0.07[f]	nd
OG/70C	18.45±0.02[f]	81.84±0.03[b]	nd	18.44±0.10[f]	82.03±0.12[b]	nd
NG/50C	23.45±0.00[e]	76.55±0.00[c]	nd	23.18±0.02[e]	76.83±0.02[c]	nd
NG/60C	28.90±0.00[c]	71.88±0.04[e]	nd	27.79±0.06[c]	73.03±0.15[e]	nd
NG/70C	16.97±0.02[g]	83.35±0.05[a]	nd	17.10±0.05[g]	83.24±0.08[a]	nd
pH 4.0						
REx	99.63±0.01[a]	0.39±0.01[g]	nd	99.52±0.01[a]	0.43±0.01[g]	nd
OG/50C	22.80±0.01[d]	77.24±0.03[d]	nd	22.57±0.03[d]	77.43±0.03[d]	nd
OG/60C	28.53±0.00[b]	72.74±0.00[f]	nd	26.91±0.10[b]	73.23±0.20[f]	nd
OG/70C	15.25±0.07[g]	85.55±0.09[a]	nd	18.44±0.10[f]	83.49±0.21[b]	nd
NG/50C	21.62±0.00[e]	78.37±0.03[c]	nd	19.93±0.13[e]	80.34±0.08[c]	nd
NG/60C	26.28±0.07[c]	74.18±0.18[e]	nd	26.39±0.02[c]	73.58±0.05[e]	nd
NG/70C	16.06±0.03[f]	84.47±0.00[b]	nd	16.13±0.11[g]	84.34±0.05[a]	nd

Values are means ±standard deviations; *n=3*, Means within columns and pH group with different letters are significantly different ($p < 0.05$), nd = not detected.

Mono[a] = monomeric anthocyanins (%), Poly[b] = polymeric anthocyanins (%) and Copig[c] = co pigmented (%), nd = not detected, REx = roselle extracts (OG and NG), OG/50C = original-grinding roselle extracts treated at 50°C, OG/60C = original-grinding roselle extracts treated at 60°C, OG/70C = original-grinding roselle extracts treated at 70°C, NG/50C = nano-grinding roselle extracts treated at 50°C, NG/60C = nano-grinding roselle extracts treated at 60°C, NG/70C = nano-grinding roselle extracts treated at 70°C.

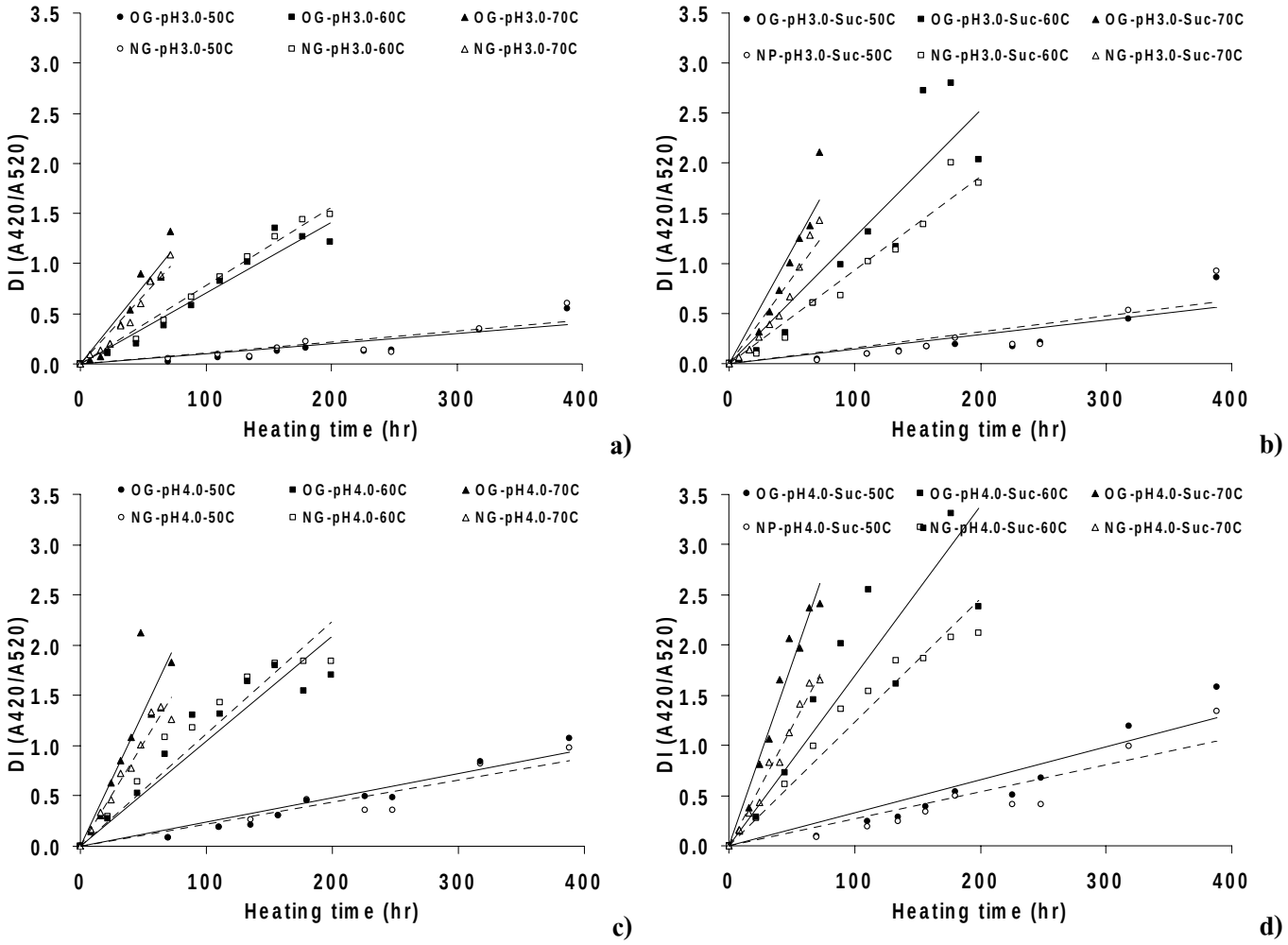


Figure 4.4 Degradation indexes of OG and NG roselle extracts in model solution at pH 3.0 (a) pH 3.0 with 20 % sucrose (b) and OG and NG roselle extracts in model system at pH 4.0 (c) pH 4.0 with 20 % sucrose (d) during heating at 50, 60 and 70 °C

4.1.2.4 Degradation kinetics of anthocyanins in OG and NG roselle extracts

Figure 4.5 illustrates the thermal degradation curves of anthocyanins from OG and NG roselle extracts and the kinetic parameters derived from the data in this figure are shown in Table 4.3. It is well accepted that the thermal degradation kinetics of anthocyanins follow the first-order reaction (Falcão et al., 2008; Kırca et al., 2007; Wang, and Xu, 2007).

The results from this study are in agreement with those reports which confirmed the first-order reaction of roselle anthocyanin degradation. Overall results in Table 4.4 indicted clearly rate of roselle anthocyanin degradation in all samples increased with the increase of temperature from 50 to 70 °C which can be seen from the decrease in half-life ($t_{1/2}$) and the increase in rate constant (k) and Q_{10}.

The samples at pH 3.0 showed higher activation energy (E_a), $t_{1/2}$ and lower k values compared to the samples at pH 4.0, suggesting the higher stability of roselle anthocyanins at the lower pH. The E_a for anthocanin degradation also revealed the higher rate of degradation for anthocyanins in NG samples compared to those in the OG samples. Again, no obvious effect of sucrose on the stability of roselle anthocyanins could be drawn from kinetic parameters.

The Q_{10} value of the anthocyanns from OG and NG roselle extracts reached a value of 1.0 to 3.4 and 1.0 to 2.8, depending on the pH and given temperature interval. Asafi (2004) reported the Q_{10} values of anthocyanins from pomegranate and sour cherry which were 2.1 to 2.7 and 2.3 to 2.7, respectively. These Q_{10} values indicated the greater anthocyanin degradation at higher temperature.

The E_a values for anthocyanins from OG and NG roselle extracts were 83.36 to 70.78 kJ/mole and 79.78 to 59.33 kJ/mole in buffer solution with or without sucrose at pH 3.0 and 4.0, respectively. These E_a values are similar to the E_a values reported by Reyes and Cisneros-Zevallos (2007) at 72.49, 66.70, 75.03 and 81.34 kJ/mole for the degradation of anthocyanins in purple-flesh potato, red-flesh potato, grape and purple carrot, respectively.

Kinetic parameters obtained from this study revealed clearly that roselle anthocyanins from the OG extract was more stable than those from the NG extract.

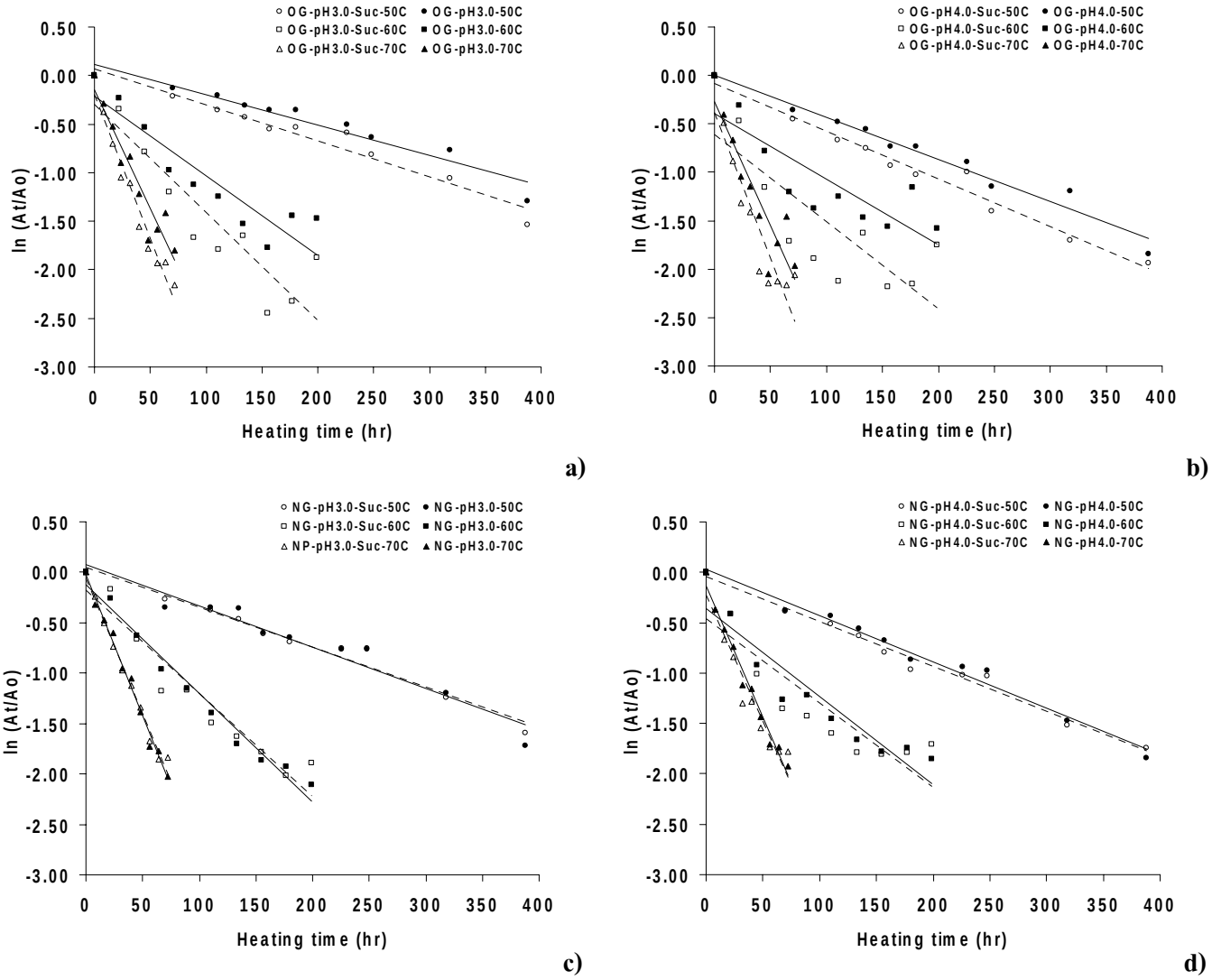


Figure 4.5 Thermal degradation of anthocyanins from OG roselle extract in model solution at pH 3.0 (a) pH 4.0 (b) and from NG roselle extract in model solution at pH 3.0 (c) pH 4.0 (d) during heating at 50 to 70 °C

Table 4.3 Kinetic behavior for the thermal degradation of anthocyanins in OG and NG roselle extract solution

Roselle system*	Temp (°C)	*k* (h)	$t_{1/2}$ (h)	Q_{10} (h)	E_a (kJ/mole)	r^2
	50	0.0033	208.33		83.36	0.8968
OG/3.0	60	0.0074	93.80	2.22		
	70	0.0250	27.74	3.38		
	50	0.0040	175.45		81.02	0.9009
OG/3.0/suc	60	0.0094	73.52	2.39		
	70	0.0299	23.19	3.17		
	50	0.0044	156.20		79.78	0.9669
NG/3.0	60	0.0106	65.59	2.38		
	70	0.0281	24.69	2.66		
	50	0.0041	169.02		79.15	0.9649
NG/3.0/suc	60	0.0095	73.11	2.31		
	70	0.0255	27.13	2.69		
	50	0.0048	145.99		70.78	0.6882
OG/4.0	60	0.0079	87.36	1.67		
	70	0.0273	25.43	3.44		
	50	0.0050	138.70		73.43	0.7348
OG/4.0/suc	60	0.0088	78.85	1.76		
	70	0.0287	24.20	3.26		
	50	0.0048	145.97		59.33	0.7667
NG/4.0	60	0.0093	74.49	1.96		
	70	0.0267	25.98	2.87		
	50	0.0045	154.58		65.73	0.8453
NG/4.0/suc	60	0.0086	80.63	1.92		
	70	0.0247	28.04	2.88		

k is the reaction rate constant of anthocyanin at heating time (h), $t_{1/2}$ is the half life (h), Q10 is temperature coefficient (for every 10°C rise of temperature the rate is doubled) and *Ea* is the activation energy (kJ/mole).

OG/3.0 = original-grinding roselle extracts in buffer pH 3.0, OG/3.0/suc = original-grinding roselle extracts in buffer pH 3.0 with 20 % sucrose, NG/3.0 = nano-grinding roselle extracts in buffer 3.0, NG/3.0/suc = nano-grinding roselle extracts in buffer 3.0 with 20 % sucrose, OG/4.0 = original-grinding roselle extracts in buffer pH 4.0, OG/4.0/suc = original-grinding roselle extracts in buffer pH 4.0 with 20 % sucrose, NG/4.0 = nano-grinding roselle extracts in buffer 4.0, NG/4.0/suc = nano-grinding roselle extracts in buffer 4.0 with 20 % sucrose.

4.1.3 Comparison of antioxidant activity in OG and NP roselle extracts

The antioxidant activities of OG and NG roselle extracts after heat treatments (50 to 70 °C) at pH 3.0 and 4.0 with or without sucrose were assayed by ferrous ions chelating ability (FICA), trolox equivalent antioxidant capacity (TEAC) and ferric thiocyanate capacity (FTC) and results are shown in Table 4.4 and Figure 4.6.

Ferrous ion chelating ability of the roselle extracts significantly decreased with the increase of heating temperatures. The NG roselle extracts generally exhibited a higher ability in chelating of ferrous ions than the OG roselle extracts. However, no obvious trend for the effect of pH and sucrose on the FICA of roselle extracts. The OG samples at pH 3.0 showed higher percentage of FICA after exposure in the solution with the present of sucrose; while the NG samples at pH 4.0 opposite effect of sucrose on the FICA.

The ABTS radical cation scavenging activity of the roselle extracts was evaluate by the TEAC values. As seen in Table 4.4, significantly higher TEAC values were observed in NG roselle extracts at both pH 3.0 and 4.0. The effect of heating temperature on the TEAC values of roselle extracts was not pronounced, although the average values were decreased after heat treatments. Results indicated that the antioxidant activity detected by TEAC method might not only relate to the content of anthocyanins, but might also relate to the products from degradation of component in the reaction mixture and/or from maillard reaction (Yilmaz and Toledo, 2005). It has been shown that maillard reaction products are capable in contributing as reducing agents, metal chelating and radical scavengers (Kim and Lee, 2010).

The ferric thiocyanate (FTC) method was used to evaluate the ability of roselle extracts in the inhibition of linolecic acid peroxidation. This method was used to measure the amount of peroxide during lipid peroxidation. Peroxide will react with ferrous chloride and form ferric ion. The ferric ion formed a complex with ammonium thiocyanate. The substance is red in color, which had a maximum absorbance at 500 nm. All samples of roselle extracts showed high antioxidant activity measured by FTC method. No obvious effects of heating temperature, pH sucrose and grinding method were observed (Table 4.4). However, roselle extracts exhibited strong activity on the inhibition of linoleic acid peroxidatin which similar to the reference antioxidant, ascorbic acid, Trolox and BHA (Figure 4.6)

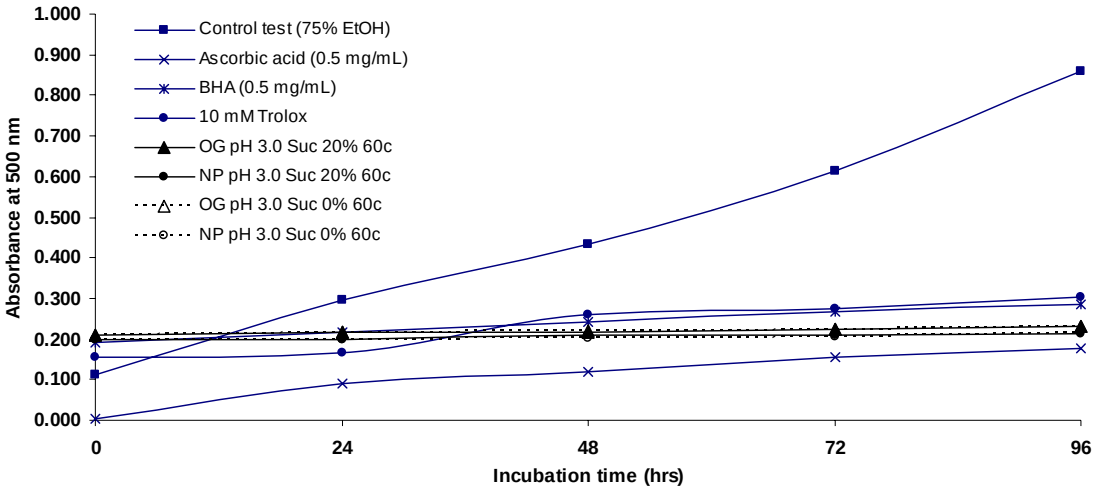


Figure 4.6 Antioxidant properties determined by the ferric thiocyanate method for OG and NG roselle prepared in model solution at pH 3.0 with sucrose after heating at 60 °C, ascorbic acid, BHA and Trolox® was used as positive references

Table 4.4 Antioxidant activity of roselle anthocyanin extracts determined by FICA[1], TEAC[2] and FTC[3] method

Model system	Temperature (°C)	FICA[1] (%)	TEAC[2] (mM Trolox)	FTC[3] (%)
pH 3.0				
OG	-	10.63±0.83[a]	0.37±0.03[a]	97.83±0.97[a]
	50	10.12±0.77[a]	0.15±0.05[c]	95.38±0.31[b]
	60	6.52±0.75[b]	0.26±0.01[b]	97.36±0.91[a]
	70	3.62±0.78[c]	0.29±0.03[b]	94.53±0.85[b]
OG/Suc	-	21.23±0.96[a]	0.69±0.02[a]	96.97±1.20[a]
	50	15.10±0.52[b]	0.48±0.02[b]	93.56±0.08[b]
	60	11.77±0.75[c]	0.40±0.01[c]	96.79±0.62[a]
	70	9.94±1.02[d]	0.46±0.02[b]	91.97±0.72[c]
NG	-	18.54±1.84[a]	1.34±0.02[a]	97.78±0.79[a]
	50	9.93±0.58[b]	1.19±0.02[c]	93.51±0.20[c]
	60	5.63±0.24[c]	1.01±0.01[d]	97.84±0.46[a]
	70	2.71±0.24[d]	1.27±0.02[b]	94.92±0.23[b]
NG/Suc	-	13.88±0.93[a]	1.60±0.06[a]	97.87±1.32[a]
	50	10.74±0.55[b]	1.41±0.06[b]	94.76±0.15[b]
	60	7.06±1.36[c]	1.26±0.01[c]	98.28±1.13[a]
	70	8.19±0.60[c]	1.44±0.02[b]	92.58±0.79[c]
pH 4.0				
OG	-	10.93±0.97[a]	0.42±0.03[a]	97.27±0.43[a]
	50	10.93±0.64[a]	0.25±0.03[c]	93.64±0.28[b]
	60	7.87±0.81[b]	0.20±0.03[d]	97.54±1.03[a]
	70	5.01±0.83[c]	0.30±0.04[b]	94.23±1.18[b]
OG/Suc	-	12.17±0.80[a]	0.77±0.02[a]	97.75±0.88[a]
	50	12.36±0.88[a]	0.48±0.02[c]	94.80±0.23[b]
	60	9.22±0.47[b]	0.57±0.04[b]	98.13±0.43[a]
	70	4.30±1.08[c]	0.57±0.05[b]	90.45±0.79[c]
NG	-	26.87±1.79[a]	1.32±0.02[a]	96.90±0.62[a]
	50	23.18±0.95[b]	1.14±0.03[c]	94.98±0.98[b]
	60	12.88±0.27[c]	1.11±0.01[c]	97.58±1.34[a]
	70	10.18±0.28[d]	1.21±0.04[b]	93.79±0.72[b]
NG/Suc	-	18.57±1.99[a]	1.96±0.03[a]	95.95±1.03[a]
	50	12.17±1.03[b]	1.80±0.05[b]	95.60±0.13[a]
	60	12.46±0.63[b]	1.80±0.02[b]	95.54±0.32[a]
	70	11.97±0.18[b]	1.68±0.02[c]	93.71±0.15[b]

Values are means ±standard deviations; *n=3*, Means within columns and treatment group with different letters are significantly different ($p<0.05$).

FICA[1] = Ferrous ions chelating ability (% inhibition). TEAC[2] = Trolox® equivalence antioxidant capacity, FTC[3] = Ferric thiocyanate method (Antioxidant activity in linoleic acid emulsion system).

OG = original-grinding roselle extracts, OG/suc = original-grinding roselle extracts in buffer with 20 % sucrose, NG = nano-grinding roselle extracts and NG/suc = nano-grinding roselle extracts in buffer with 20 % sucrose.

4.1.4 Conclusion

Roselle extracts prepared from NG had a profound influence on visual color, color density anthocyanin contents and antioxidant properties. Anthocyanin pigment was directly related to the color intensity of the roselle extracts. A_{520} of solution was enhanced by the anthocyanin extracts by nano-grinding extraction. However, NG and OG roselle extracts had similar levels of monomeric anthocyanins. NG roselle extracts was more capability than OG roselle extracts in term of chelating ability (FICA) and antioxidant capacity (TEAC). However, NG and OG roselle were found similar higher in peroxidation inhibition.

The above results indicate the roselle extracts prepared by nano-grinding are good quality in physiochemical and antioxidant properties. However, the pigment in NG roselle extracts sample was much less stable during thermal treatment. Therefore, further process at low temperature and pH without sugar is necessary for NG roselle extracts when developing functional food in order to maintain their functional ingredients-anthocyanins.

4.2 Evaluation of antioxidant capacity of roselle anthocyanin extracts on lipid and protein oxidation as affected by sucrose and different sugar alcohols in Chinese-style sausage

In this objective, the experiments were divided into 3 parts: i) effect of sucrose and different sugar alcohols on antioxidant capacity of roselle anthocyanin extracts, ii) effect of sugar alcohol concentration on antioxidant capacity of roselle anthocyanin extracts, and iii) effect of sugar alcohol on texture profile and sensorial qualities of Chinese-style sausage during storage.

4.2.1 Effect of sucrose and different sugar alcohols on antioxidant capacity of roselle anthocyanin extract in Chinese-style sausage

Background of this study

Sugar alcohols (polyols), are a group of low calorie, carbohydrate-based sweeteners. polyols deliver the taste and texture of sugar with about half the calories. They are used as a food ingredient, often to replace sugar, cup for cup, in many sugar-free and low-calorie foods. Recently, xylitol have been shown to enhance antioxidative capacity of roselle anthocyanin extracts in Chinese-style sausage (Pinsirodom, 2008). Experiment was intended to evaluate the effect of sucrose and difference sugar alcohols (lactitol, maltitol and xylitol) at the same concentration (16.6 %) on anti-lipid oxidation capacity of roselle anthocyanin extracts (RAE) in Chinese-style sausage (CSS). Physicochemical properties including moisture content, water activity, pH, color parameters and oxidative stability of the CSS samples storage were evaluated. Results are presented below.

4.2.1.1 Physicochemical properties of RAE treated Chinese-style sausage with different sugars

1) Moisture content, water activity and pH

Table 4.5 shows moisture content, water activity and pH values of RAE treated CSS with different sweeteners during storage compared to the control (sucrose added without RAE). Moisture contents of all CSS samples were within the range considered as acceptable values (22.26 %) according to the standard for Chinese-style sausage from pork (TISI, 103-2003) which designates moisture content of CSS not higher than 29 %. The water activity of all sausage samples (0.70 to 0.78) also corresponded to a typical intermediate moisture food (Rao, 1997). It is interesting that RAE treated CSS with xylitol addition exhibited lower moisture content and water activity compared to the control and other sugars added samples.

Considering pH values, all RAE treated CSS tended to be slightly lower compared to the control sausage. This was due to the crude RAE contained other organic acids (Wong et al., 2002) that could reduce the pH of the CSS.

Although the physicochemical properties studied were significantly different ($p<0.05$) between some treatment, no serious changes of moisture content, water activity and pH during the storage were observed for the control and all RAE treated CSS.

Table 4.5 Means for moisture content, water activity and pH of RAE treated Chinese-style sausage with different sweeteners during storage at 30±1 °C

Parameters	Storage time (days)				
	0	7	14	21	28
Moisture content (%)					
Control	24.86±0.53^{a}	23.50±0.33^{c}	24.34±0.19^{b}	25.58±0.27^{b}	24.54±0.17^{b}
Suc/RAE	24.93±0.50^{a}	25.56±0.19^{a}	25.10±0.22^{a}	27.77±0.24^{a}	25.11±0.32^{a}
Lac/RAE	25.00±0.10^{a}	23.08±0.14^{d}	25.16±0.17^{a}	25.01±0.17^{c}	23.99±0.20^{c}
Mal/RAE	24.10±0.12^{a}	24.55±0.16^{b}	24.54±0.20^{b}	25.01±0.34^{c}	24.07±0.18^{c}
Xyl/RAE	23.10±1.41^{b}	22.38±0.25^{e}	22.90±0.40^{c}	23.62±0.29^{d}	22.84±0.38^{d}
Water activity					
Control	0.777±0.001^{a}	0.779±0.001^{a}	0.779±0.002^{a}	0.780±0.001^{a}	0.759±0.002^{b}
Suc/RAE	0.776±0.001^{b}	0.776±0.001^{b}	0.777±0.001^{b}	0.776±0.001^{b}	0.765±0.001^{a}
Lac/RAE	0.776±0.001^{b}	0.776±0.001^{b}	0.776±0.001^{b}	0.777±0.001^{b}	0.756±0.001^{c}
Mal/RAE	0.766±0.001^{c}	0.766±0.001^{c}	0.763±0.001^{c}	0.767±0.001^{c}	0.755±0.001^{d}
Xyl/RAE	0.710±0.001^{d}	0.706±0.001^{d}	0.708±0.002^{d}	0.708±0.001^{d}	0.699±0.001^{e}
pH					
Control	6.11±0.01^{a}	6.16±0.01^{a}	6.15±0.01^{a}	6.14±0.01^{a}	6.16±0.01^{a}
Suc/RAE	5.88±0.01^{d}	5.92±0.01^{c}	6.06±0.04^{b}	6.02±0.01^{b}	6.01±0.01^{b}
Lac/RAE	5.89±0.01^{d}	5.90±0.01^{d}	6.01±0.02^{c}	5.99±0.01^{c}	5.96±0.01^{c}
Mal/RAE	5.90±0.01^{c}	5.92±0.01^{c}	6.04±0.01^{c}	5.99±0.01^{c}	5.97±0.01^{c}
Xyl/RAE	5.97±0.01^{b}	5.96±0.01^{b}	6.02±0.01^{c}	6.02±0.01^{b}	6.01±0.01^{b}

Values represent means±SD, n=6. Means within columns and parameter group with different letters (a, b, c, d, e) are significantly different ($p<0.05$).

Control = Sucrose without RAE, Suc/RAE = 16.6 % Sucrose with 0.3 % RAE, Lac/RAE = 16.6 % Lactitol with 0.3 % RAE, Mal/RAE = 16.6 % Maltitol with 0.3 % RAE and Xyl/RAE = 16.6 % Xylitol with 0.3 % RAE.

2) Color parameters

Color parameters of all RAE treated CSSs with difference sweeteners and control samples significantly changed (p<0.05) during storage (Figure 4.7a, b, c). Lightness (L*-value) and yellowness (b*-value) gradually increased over time in all samples (Figure 4.7a). At day 28, higher L*-value and b*-value were observed in the Lac/RAE than other samples and control, while higher a*-value were found in control sample. The discoloration of CSSs during storage was affected by the addition of different sweeteners since the total color difference (TCD; $\Delta_{E_{0-28}}$) values in Lac/RAE were significantly lower than in the Suc/RAE, Mal/RAE, Xyl/RAE and control samples. The color changes described in this study agree with those previously reported in relation to the discoloration of Chinese-style sausage and cooked sausage during storage (Tan et al., 2006; Jo et al., 2000). Based on the color parameters measured, Lac/RAE sample exhibited different color characteristics compared to the other CSS samples and control. Lac/RAE sample was lighter and yellowish (Figure 4.7b) compared to Mal/RAE and Xyl/RAE. Among the RAE treated CSS samples, the Xyl/RAE and Suc/RAE showed a similar redness (Figure 4.7a). However, the control (without RAE) sample exhibited the higher redness (higher a*-value) and closer to the true red axis (lower hue value) (Figure 4.7c) compared to that of all RAE treated CSS. Surprisingly, RAE addition did not promote the redness of CSS. This may be due to the small amount of RAE (0.3 % by wt.) was used and the predominant heme pigment and iron content supplied by meat (Lombardi-Boccia et al., 2002), which is the main ingredient (65 %) (see Appendix A1) were responsible for color characteristics of the CSS.

According to the browning index (BI), Lac/RAE and Mal/RAE CSS showed higher BI at day 0 and over storage time compared to the control and Suc/RAE and Xyl/RAE samples (Figure 4.8) Although BI value of all CSS samples tended to increase during storage, Xyl/RAE was the only sugar alcohols added CSS that showed similar BI value as the control and Suc/RAE samples.

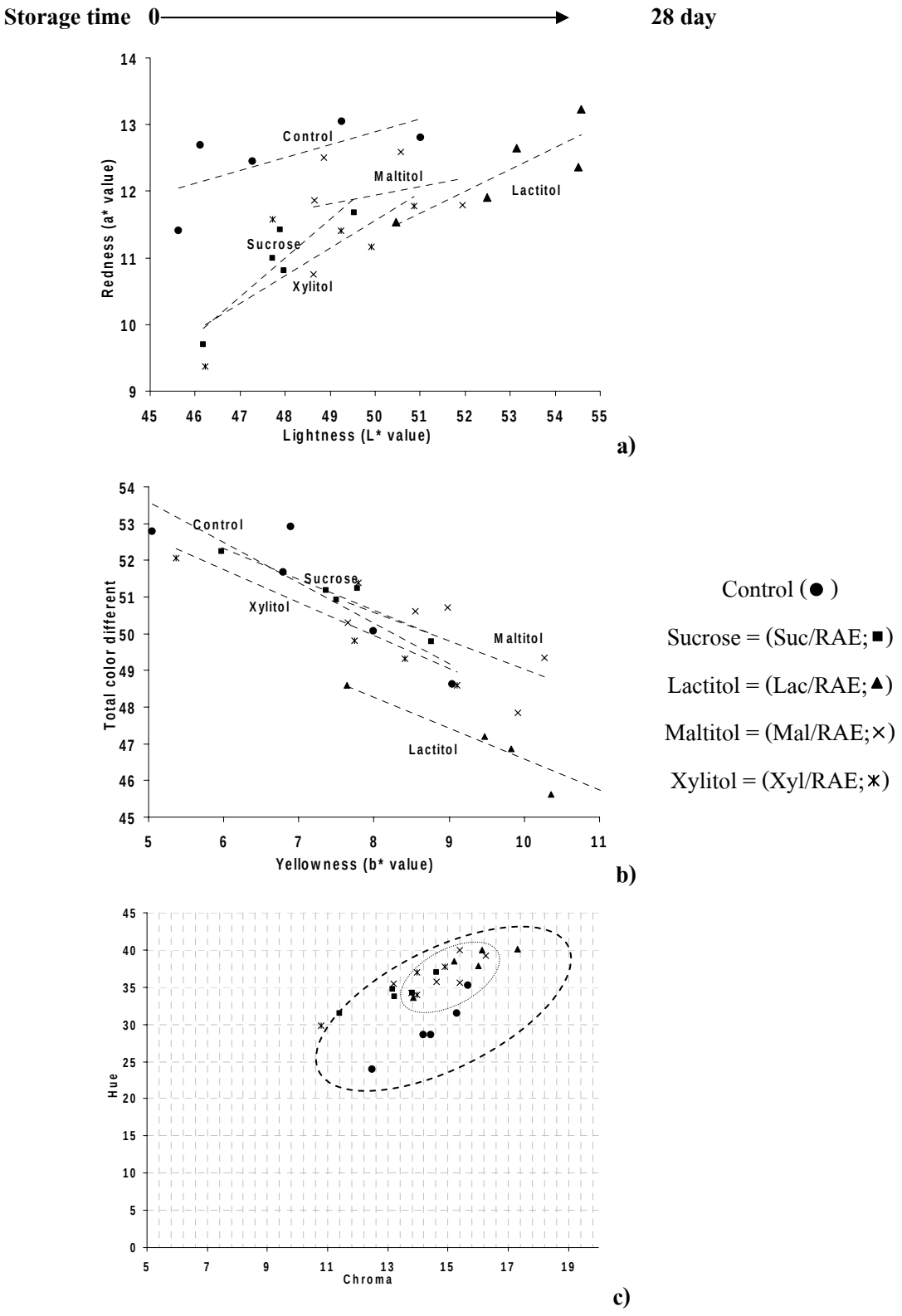


Figure 4.7 Changes of color parameters of Chinese-style sausage as affected by sweeteners during storage at 30±1 °C, a) figure plotted between lightness vs redness , b) yellowness vs total color difference and c) chroma vs hue, n=6. Average coefficient of variation (CV) = 10.25 %

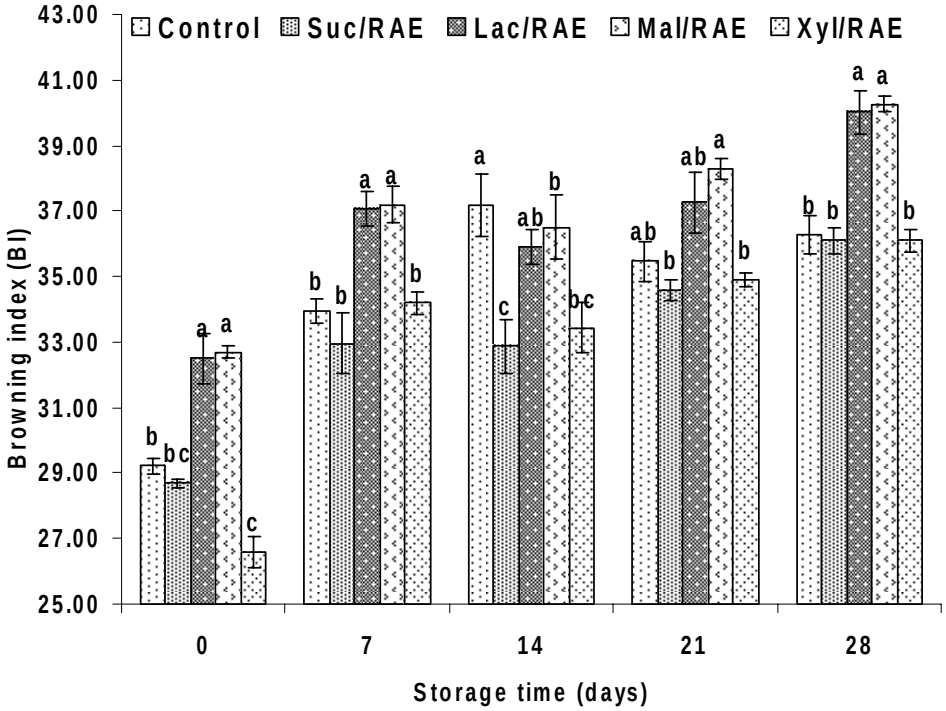


Figure 4.8 Changes of browning index of Chinese-style sausage as affected by sweeteners during storage at 30±1 °C. Bars with different letters are significantly different ($p<0.05$), n=6. Average coefficient of variation (CV) = 10.54 %

4.2.1.2 Lipid oxidation by TBARS value of RAE treated Chinese-style sausage with different sugars

Changes of TBARS values during storage of RAE treated CSS with different sweeteners comparing to the control sample (sucrose without RAE) are shown in Figure 4.9. The addition of different sugars showed significant effect on oxidative stability of RAE treated CSS. Results also indicated that the ability of RAE to prevent lipid oxidation in CSS was dependent of the type of sugar added. RAE treated CSS with sucrose had much higher TBARS values compared to the samples with sugar alcohols addition. During storage, the values of TBARS increased from 0.35 to 1.27, from 0.35 to 1.06, and from 0.10 to 0.20 mg MDA/kg CSS in the control sample, Suc/RAE and sugar alcohols/RAE, respectively. Pinsirodom (2008) has reported the effect of sucrose concentration on antioxidant activity of RAE in Chinese-style sausages and pork-chips. The author suggested that high concentration of sucrose (up to 20 % by weight) accelerated lipid oxidation in RAE treated CSS and pork chips. This may explain the higher TBARS values in the CSS sample when sucrose was used as sweetener in this experiment. The

reason of pro-oxidant behavior of RAE in the present of sucrose observed in CSS is not understood. Tinsley and Bockian (1959) reported that the increasing concentration of glucose, sucrose and fructose resulted in increased on the rate of degradation of anthocyanins (as pelagonidin3-glucoside). Considering the effect of sugar alcohols in the enhancement of antioxidant ability of RAE it is obvious that addition of sugar alcohols in RAE treated CSS resulted in significant lower TBARS values of samples with those sugar alcohols compared to the control and sucrose added samples even at the beginning of the storage (day zero) suggested that sugar alcohols themselves might possess the antioxidant property in addition to that of the RAE. den Hartog et al. (2010) have reported the antioxidant activity of sugar alcohol, their excellent hydroxyl radical ($HO^{\bullet}$) scavenging capacity and in vitro inhibition of diazocompound-induced erythrocyte damage. However, *in vitro* tests for antioxidant activities of xylitol at different concentration by diphenylpicrylhydrazyl (DPPH), ferric thiocyanate (FTC), and ferrous reducing activity of plasma (FRAP) method did not show any positive results (data not shown). The effect of sugar alcohol on oxidative stability of RAE treated CSS will be further discussed in the next experiment.

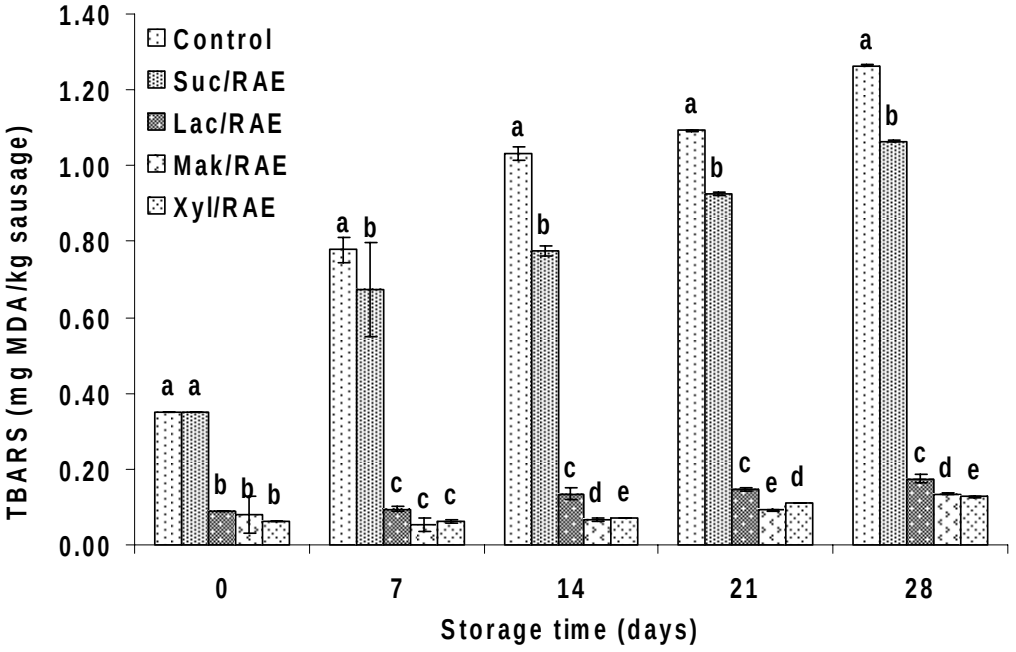


Figure 4.9 Changes of TBARS values of RAE treated Chinese-style sausage with different sweeteners during storage at 30±1 °C. Bars with different letters is significantly different ($p< 0.05$), n=6

4.2.1.3 Conclusion

Sucrose can cause negative effect on the antioxidant activity of RAE when incorporated in the CSS as sweetener. Sugar alcohols would be a promising alternative sweetener that exhibits no adverse effect on the antilpo-peroxidant capacity of the RAE treated CSS products. According to the physicochemical properties oxidative stability of the CSSs and economical stand point, xylitol would be a suitable sugar; among the three sugar alcohols studied, to be used as a replacer for sucrose when the RAE is incorporated as a natural antioxidant in CSS. Therefore, xylitol was selected for the further study in the next experiment.

4.2.2 Effect of sugar alcohol concentration on antioxidant capacity of roselle anthocyanin extracts

Background of this study

In this part, the effect of xylitol concentration on oxidative stability of RAE treated CSS was focused. Xylitol concentration was varied at 11.6, 16.6 and 21.6 % (by weight) with the same level of 0.3 % RAE in CSS. The CSS samples were compared with three control samples: Control 1 referred to CSS with 16.6 % sucrose and 0.3 % RAE, Control 2 referred to CSS with 16.6 % sucrose and no RAE, Control 3 referred to CSS with 16.6 % xylitol and no RAE. The lipid oxidation by TBARS assay and protein carbonyl by total carbonyl content assay were major assays used to monitor the effect of xylitol concentration on antioxidant capacity of RAE in CSS product during storage. Some physicochemical properties were also determined to confirm the quality of CSS when xylitol was used to replace the sucrose.

4.2.2.1 Physicochemical properties of RAE treated CSS with different concentration of xylitol

1) Moisture content, water activity and pH

The analysis of the moisture content, water activity and pH of RAE treated CSS with different xylitol concentration and control sample are presented in Table 4.6. Moisture content was observed in the range of 21 to 23 % and 23 to 26 % in xylitol sausage samples (RAE treated CSS with different xylitol concentration and control 3) and control sausage samples with sucrose addition (control 1 and control 2), respectively. However, no significant difference were found between control 1 (Suc-16.6/RAE) and Xyl-11.6/RAE, which had similar content of moisture thought out the storage time. The highest water activity (0.86 to 0.89) was observed in control 1 and control 2, while the lowest (0.75 to 0.78) was found in the Xyl-21.6/RAE sample. Increasing xylitol concentration resulted in the reduced water activity in all RAE treated CSS samples. This was agreed with the moisture contents of the samples observed. There was a tendency of decreasing pH in CSS when higher concentration of xylitol was added and lower pH value was found in all samples of RAE treated CSS. However, no serious change of moisture content, water activity and pH during storage was observed in all CSS samples.

2) Color parameters

Figure 4.10 shows color parameters including lightness (L*-value), redness (a*-value), total color different (TCD) and browning index (BI) of RAE treated CSS with difference xylitol concentration compared to the control samples. In general, all CSS samples exhibited

similar color parameters measured, although there was a tendency for the decreasing value of lightness and the increasing value of redness, TCD and BI in RAE treated CSS when the higher concentration of xylitol was added. In addition, the BI value increased from about 30 to 40 and slightly increased of redness was also observed for all CSS samples after 28-day storage. Results from this study indicated that xylitol can be used to replace sucrose in RAE treated CSS without any undesirable color characteristic comparing to the control CSS.

Table 4.6 Means for moisture content, water activity and pH of RAE treated Chinese-style sausage with different xylitol concentration during storage at 30±1 °C

Parameters	Storage time (days)				
	0	7	14	21	28
Moisture content (%)					
Control 1	25.49±0.16^{a}	24.32±0.16^{b}	23.56±0.23^{b}	23.38±0.21^{bc}	23.25±0.26^{b}
Control 2	24.94±0.16^{b}	24.72±0.14^{a}	26.15±0.22^{a}	25.63±0.23^{a}	26.03±0.16^{a}
Control 3	23.17±0.08^{d}	23.80±0.15^{c}	21.22±0.12^{d}	23.24±0.14^{c}	22.18±0.34^{d}
Xyl-11.6/RAE	25.54±0.14^{a}	24.72±0.30^{a}	23.38±0.23^{bc}	23.56±0.18^{b}	23.52±0.21^{b}
Xyl-16.6/RAE	24.03±0.18^{c}	23.54±0.18^{d}	23.17±0.11^{c}	22.38±0.31^{e}	21.95±0.35^{d}
Xyl-21.6/RAE	22.56±0.18^{e}	23.43±0.11^{d}	21.45±0.25^{d}	22.75±0.27^{d}	22.94±0.15^{c}
Water activity					
Control 1	0.88±0.00^{a}	0.86±0.00^{a}	0.85±0.00^{b}	0.85±0.00^{b}	0.84±0.00^{b}
Control 2	0.86±0.00^{b}	0.86±0.00^{a}	0.89±0.00^{a}	0.87±0.00^{a}	0.87±0.00^{a}
Control 3	0.82±0.00^{d}	0.82±0.00^{b}	0.80±0.00^{d}	0.82±0.00^{c}	0.81±0.00^{d}
Xyl-11.6/RAE	0.84±0.00^{c}	0.82±0.00^{c}	0.83±0.00^{c}	0.87±0.00^{a}	0.83±0.00^{c}
Xyl-16.6/RAE	0.82±0.00^{d}	0.79±0.00^{d}	0.80±0.00^{e}	0.81±0.00^{d}	0.80±0.00^{e}
Xyl-21.6/RAE	0.78±0.00^{e}	0.78±0.00^{e}	0.75±0.00^{f}	0.77±0.00^{e}	0.78±0.00^{f}
pH					
Control 1	5.80±0.01^{c}	5.83±0.01^{d}	5.85±0.01^{c}	5.82±0.01^{c}	5.80±0.01^{b}
Control 2	5.94±0.01^{a}	5.97±0.01^{b}	5.96±0.01^{a}	5.95±0.01^{a}	5.96±0.01^{a}
Control 3	5.95±0.01^{a}	5.98±0.01^{a}	5.94±0.01^{b}	5.91±0.01^{b}	5.96±0.01^{a}
Xyl-11.6/RAE	5.80±0.01^{c}	5.90±0.01^{c}	5.84±0.01^{d}	5.83±0.01^{c}	5.80±0.01^{b}
Xyl-16.6/RAE	5.82±0.01^{b}	5.83±0.01^{d}	5.75±0.01^{e}	5.75±0.01^{d}	5.79±0.01^{c}
Xyl-21.6/RAE	5.81±0.01^{b}	5.82±0.01^{d}	5.72±0.01^{f}	5.72±0.01^{e}	5.78±0.01^{c}

Values represent means±SD, n=6. Means within columns and parameter group with different letters (a, b, c, d, e) are significantly different ($p<0.05$).

Control 1 = 16.6 % Sucrose with 0.3 % RAE, Control 2 = 16.6 % Sucrose without RAE, Control 3 = 16.6 % Xylitol without RAE, Xyl-11.6/RAE = 11.6 % Xylitol with 0.3 % RAE, Xyl-16.6/RAE = 16.6 % Xylitol with 0.3 % RAE and Xyl-21.6/RAE = 21.6 % Xylitol with 0.3 % RAE.

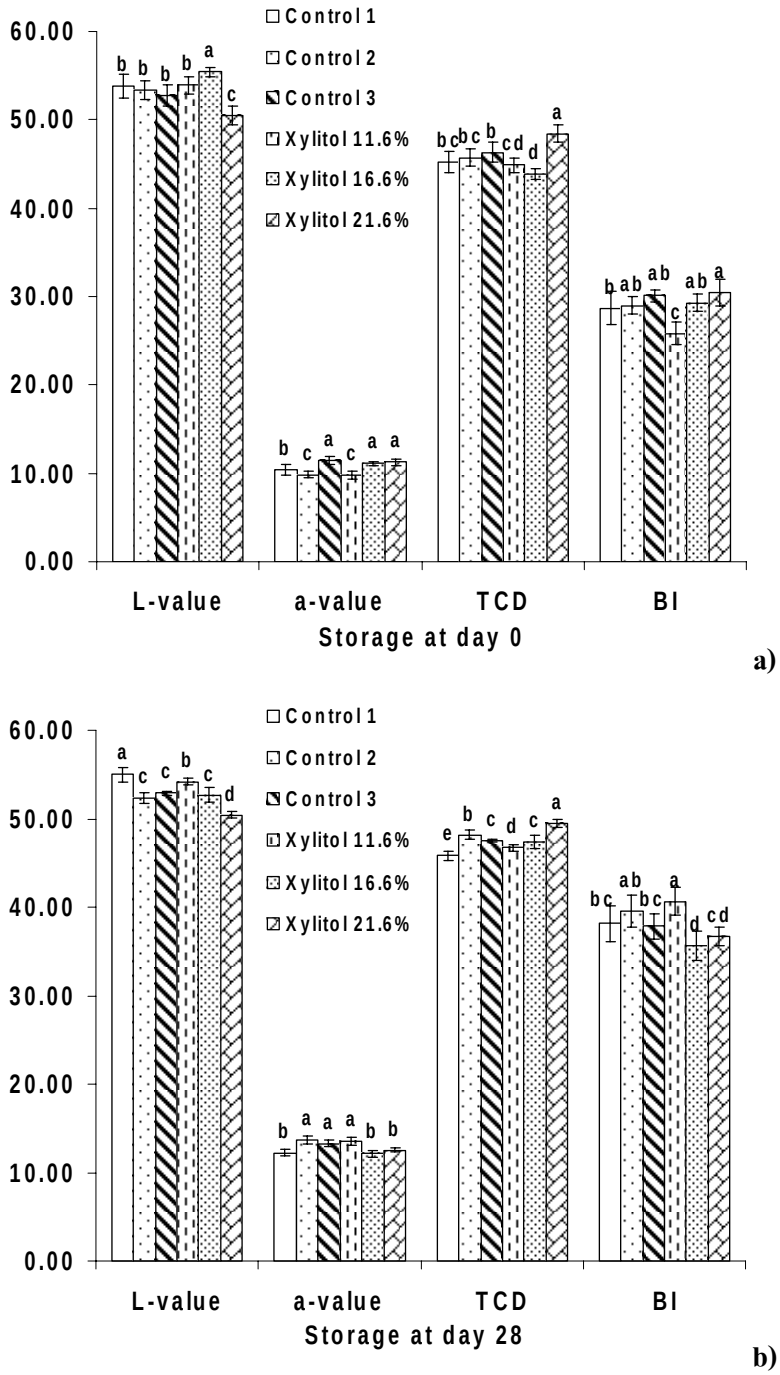


Figure 4.10 Changes of color parameters of RAE treated Chinese-style sausage as affected by different xylitol concentration during storage at 30±1 °C. Bars with different letters are significantly different ($p<0.05$), n=6

Control 1 = 16.6 % Sucrose with 0.3 % RAE, Control 2 = 16.6 % Sucrose without RAE, Control 3 = 16.6 % Xylitol without RAE, Xyl-11.6/RAE = 11.6 % Xylitol with 03 % RAE, Xyl-16.6/RAE = 16.6 % Xylitol with 0.3 % RAE and Xyl-21.6/RAE = 21.6 % Xylitol with 0.3 % RAE.

4.2.2.2 Oxidative stability of RAE treated Chinese-style sausage with different xylitol concentration

1) Lipid oxidation by TBARS values

Changes of TBARS values during storage of RAE treated CSS with different xylitol concentration comparing to the control samples is shown in Figure 4.11a. Results obviously showed that control 1 which referred to RAE treated CSS with 16.6 % sucrose had the highest TBARS values and the value increased throughout the storage period. The higher TBARS value of control 1 comparing to the control 2 (16.6 % sucrose without RAE) clearly indicated the effect of sucrose on pro-oxidant behavior of RAE and confirmed the previous finding reported by Pinsirodom (2008). In addition, the significantly lower TBARS value observed in the control 3 (16.6 % xylitol without RAE) comparing to the control 2 (16.6 % sucrose without RAE) also obviously indicated that xylitol exhibited anti-lipid oxidation property in CSS. The antioxidant ability of sugar alcohols in prevention of lipid oxidation in meat products has not yet been reported elsewhere. As mentioned earlier in experiment 4.2.1.2, *in vitro* assays using common methods such as diphenylpicrylhydrazyl (DPPH), ferric thiocyanate (FTC), and ferrous reducing activity of plasma (FRAP) could not detect positive antioxidant activity of xylitol (data not shown). This may be due to the different matrix of CSS and reaction mixture solution.

Considering the RAE treated CSS with different xylitol concentration, positive effect of xylitol on the enhancement of antioxidant capacity of RAE was observed. However, the CSS sample with 21.6 % xylitol exhibited less oxidative stability comparing to the samples with 11.6 % and 16.6 % xylitol. This might be due to the pro-oxidant behavior of RAE as affected by too high concentration of xylitol similar to the case of sucrose as mentioned before. However, the much lower TBARS values of CSS with xylitol addition even at 21.6 % comparing to the control 1 indicated the positive effect of xylitol to enhance the ability of RAE in prevention of lipid oxidation.

2) Protein oxidation by total protein carbonyl contents

Results from the analysis of the oxidative deterioration of protein during storage of RAE treated CSS with different xylitol concentration and control samples is shown in Figure 4.11b. The content of protein carbonyl significantly increased during storage of control 1 (from 0.3 to 1.7 nM carbonyl/mg protein), control 2 (from 0.30 to 1.30 nM carbonyl/mg protein), control 3 (from 0.50 to 1.35 nM carbonyl/mg protein), Xyl-11.6/RAE (from 0.30 to 1.20 nM carbonyl/mg protein), Xyl-16.6/RAE (from 0.25 to 1.10 nM carbonyl/mg protein) and Xyl-

21.6/RAE (from 0.30 to 1.15 nM carbonyl/mg protein). Resulted showed that RAE was not as affect in prevention of protein oxidation in CSS as in prevention of lipid oxidation. However, effect of xylitol concentration on the ability on RAE to inhibit protein oxidation in CSS was similar to the lipid oxidation. Addition of xylitol in RAE treated CSS at 16.6 % seemed to be the suitable concentration in prevention of both lipid and protein oxidation.

Generally, the oxidation of lipid in meat products as accessed by TBARS values could reflect sensory quality of the products (Liu et al., 2010; Ferrari and Torres, 2002). Gray et al. (1996) reported that rancid flavor was initially detected in cooked pork muscle with TBARS values around 2 mg MDA/kg depended on the product formulation.

Protein oxidation is considered to link to lipid oxidation of meat products. In a present of oxidizing agents, protein oxidation is involved by free radical chain reaction similar to those occur in lipid oxidation, which includes initiation, propagation and termination (Morrissey et al., 1998; Xiong, 2000; Monahan, 2000). In this study, lipid and protein oxidation as determined by TBARS and total protein carbonyl content showed a considerable correlation with $r=0.784$ ($p<0.001$). This result confirmed that protein oxidation in meat products would occur easily under the condition that promotes lipid oxidation. In addition, the breakdown of the heme molecule and releasing of iron from porphyrin ring might cause a chain of oxidative deterioration of protein in meat products (Carlsen et al., 2005).

The use of anthocyanins as an inhibitor of lipid oxidation in the meat products has been reported by parinyapatthanaboot and Pinsirodom (2010) Bozkurt and Belibagli (2009) and Karabacaka and Bozkurt (2008). Recent study also reported the effectiveness of berry phenolics as inhibitors of protein oxidation in liposome model system (Viljanen et al., 2004). The results in this study were agreement with those findings. In addition, the activity of RAE could have been affected by the initial oxidation state of the CSS to which it was added. In the system with high oxidative instability, the activity of RAE could be decreased since RAE can be oxidized and the oxidation products could act as pro-oxidant promoting oxidative reaction (Huang and Frankel, 1997). In this study, the higher oxidative instability of RAE treated CSS formulated with 16.6% sucrose and 21.6 % xylitol as observed from the lipid and protein oxidation intensity could be the reason that caused pro-oxidant activity of the RAE in CSS.

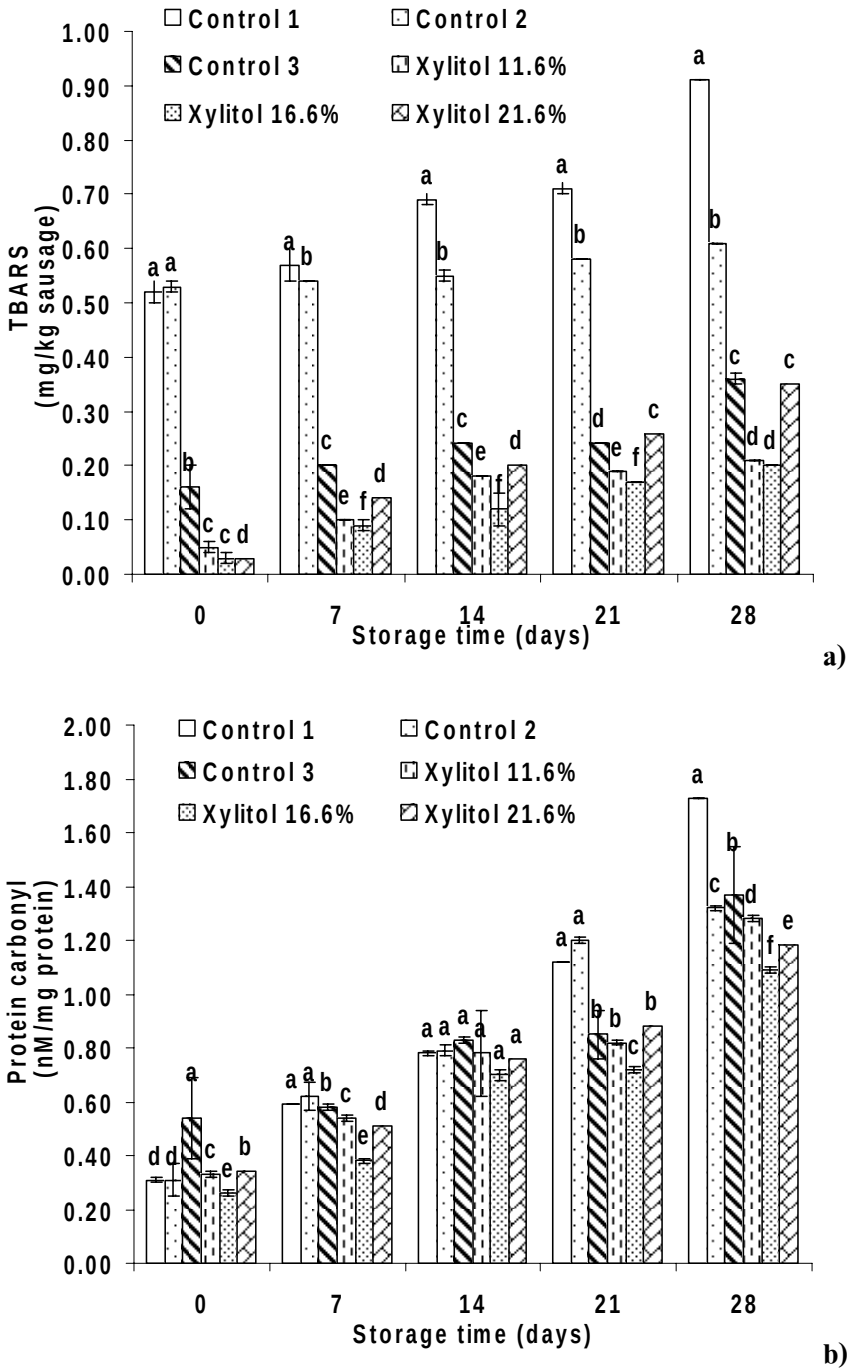


Figure 4.11 Effect of different xylitol concentration on a) lipid oxidation by TBARS values and b) protein carbonyl of Chinese-style sausage during 0-28 day storage at 30±1 °C. Storage time (day) with different letters is significantly different ($p< 0.05$), *n=6*. Average coefficient of variation (CV) = 2.47 % and 5.12 %, respectively

Control 1 = 16.6 % Sucrose with 0.3 % RAE, Control 2 = 16.6 % Sucrose without RAE, Control 3 = 16.6 % Xylitol without RAE, Xyl-11.6/RAE = 11.6 % Xylitol with 0.3 % RAE, Xyl-16.6/RAE = 16.6 % Xylitol with 0.3 % RAE and Xyl-21.6/RAE = 21.6 % Xylitol with 0.3 % RAE

4.2.2.3 Conclusion

It can be concluded that sucrose had negative effect on the ability of RAE to prevent lipid and protein oxidation of CSS. On the other hand, xylitol can efficiently promote the oxidative stability of CSS when RAE was used as natural antioxidant. Although too high concentration (*i.e.* 21.6 % in this study) of xylitol might cause pro-oxidant activity of RAE in CSS, addition of xylitol to replace sucrose at 16.6 % resulted in CSS with similar and acceptable quality compared to the original CSS.

4.2.3 Effect of xylitol on texture profile and sensorial qualities of Chinese-style sausage during storage

Background of this study

The data obtained from experiment 4.2.2, the 16.6 % xylitol showed a good capacity to enhanced oxidative stability of RAE and remained the original quality of CSS. In this part, 16.6 % xylitol was selected and used in RAE treated CSS. Texture profile analysis and sensory were used to confirm the physicochemical and sensory quality of RAE treated CSS with xylitol and sucrose added and control (commercial formula CSS) during storage.

4.2.3.1 Physicochemical properties of Chinese-style sausage

1) Moisture content, water activity and pH

As seen in Table 4.7, RAE treated CSS with xylitol sample (Xyl/RAE) showed significantly lower moisture content and water activity throughout the storage time compared to the Suc/RAE and control samples. Results indicated that addition of xylitol to replaced sucrose can reduce the moisture content and water activity of CSS. These might be an advantage for the CSS in terms of microbiological stability. Similar to the previous experiment, slightly lower pH values were observed in RAE treated CSS due to the acidic components in the RAE. However, it was unlikely that this slightly lower pH would cause detectable sour taste in the CSS.

2) Color parameters

Changes of color parameters during storage of the RAE treated raw CSS with the addition of sucrose and xylitol compared to the control sample are showed in Table 4.8. The Xyl/RAE sample tended to have lower values for most of color parameters at the beginning of storage (day zero). However, the value slowly increased and became no significant different compared to the Suc/RAE and control sample at the end of the storage. In general, the redness (a*-value) can represent a good characteristics of meat products. Surprisingly, addition of RAE did not enhance the redness of the CSS. This may be due to the small amount of RAE (0.3 % by weight) was used in CSSs. Overall results indicated that no serious changes of the color parameters were observed during storage of RAE treated CSS with xylitol addition compared to the sample with sucrose and the control.

Table 4.7 Means for moisture content, water activity and pH of RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C

Parameters	Storage time (days)				
	0	7	14	21	28
Moisture content (%)					
Control (Suc)	26.00±0.13^{a}	27.45±0.15^{a}	29.23±0.22^{a}	27.53±0.62^{a}	27.3±00.57^{a}
Suc/RAE	26.17±0.20^{a}	27.57±0.14^{a}	27.71±0.49^{b}	25.20±0.28^{b}	26.48±0.17^{b}
Xyl/RAE	23.73±0.13^{b}	22.99±0.18^{b}	24.74±0.51^{c}	24.04±0.10^{c}	23.86±0.31^{c}
Water activity					
Control (Suc)	0.82±0.00^{a}	0.84±0.00^{b}	0.84±0.00^{a}	0.85±0.00^{a}	0.83±0.00^{b}
Suc/RAE	0.82±0.00^{a}	0.85±0.00^{a}	0.83±0.00^{b}	0.83±0.00^{b}	0.84±0.00^{a}
Xyl/RAE	0.77±0.00^{b}	0.77±0.00^{c}	0.78±0.00^{c}	0.78±0.00^{c}	0.77±0.00^{c}
pH					
Control (Suc)	6.24±0.01^{a}	6.22±0.01^{a}	6.22±0.01^{a}	6.22±0.01^{a}	6.22±0.01^{a}
Suc/RAE	6.04±0.01^{b}	6.04±0.01^{b}	6.04±0.01^{b}	6.05±0.01^{b}	6.06±0.01^{b}
Xyl/RAE	6.01±0.01^{c}	6.00±0.01^{c}	6.00±0.01^{c}	6.01±0.01^{c}	6.00±0.01^{c}

Values represent means±SD, n=6. Means within columns and parameters group with different letters are significantly different ($p<0.05$)

Control (Suc) = 16.6 % Sucrose without RAE, Suc/RAE = 16.6 % Sucrose with 0.3 % RAE and Xyl/RAE = 16.6 % Xylitol with 0.3 % RAE.

3) Texture parameter analysis

The results of instrumental texture profile analysis (TPA) of the roasted CSS samples are shown in Figure 4.12. From the literatures, hardness, gumminess and chewiness were likely to be important parameters to reflect texture properties of meat products such as Chinese-style sausage (Lin and Chao, 2001), dry fermented sausage (Herrero et al., 2007), fish sausage (Rahman et al., 2007) and rae and cooked meat (Huidobro et al., 2005). As seen in Figure 4.12, the beginning of storage, hardness and gumminess were significantly higher in the Suc/RAE compared to the Xyl/RAE and control roasted sample, while no significantly different in chewiness. Slightly changed of hardness, gumminess and chewiness were found, but significantly detectable difference in Suc/RAE, Xyl/RAE and control for each storage time. At the end of storage, Suc/RAE and Xyl/RAE roasted sample showed similar values in hardness and gumminess but significant lower compared to the control. However, chewiness had no significant difference among CSS samples.

Table 4.8 Changes of color parameters of RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C

Parameters	Storage time (days)				
	0	7	14	21	28
Lightness (L*)					
Control (Suc)	51.31±0.61^{a}	50.01±0.77^{a}	49.60±1.68^{a}	51.27±1.37^{a}	48.65±0.86^{b}
Suc/RAE	48.98±1.47^{b}	49.33±1.09^{a}	48.07±1.48^{a}	47.97±1.04^{b}	49.72±0.7^{a}
Xyl/RAE	48.86±0.95^{b}	49.72±0.98^{a}	48.36±0.85^{a}	50.54±1.48^{a}	49.38±0.78^{ab}
Redness (a*)					
Control (Suc)	12.66±0.33^{a}	12.11±0.78^{a}	12.64±0.53^{a}	13.10±0.30^{a}	13.00±0.73^{a}
Suc/RAE	11.92±0.56^{b}	12.03±0.40^{a}	11.38±0.23^{b}	11.34±0.91^{c}	12.66±0.51^{a}
Xyl/RAE	10.35±0.72^{c}	11.38±1.01^{a}	11.03±0.45^{b}	12.24±0.65^{b}	12.77±0.72^{a}
Yellowness (b*)					
Control (Suc)	7.96±0.72^{a}	7.26±0.61^{a}	8.25±0.48^{a}	8.42±0.82^{b}	7.67±1.33^{a}
Suc/RAE	6.89±0.49^{b}	6.44±0.30^{ab}	6.79±0.43^{b}	7.16±0.99^{a}	8.03±0.68^{a}
Xyl/RAE	5.51±0.68^{c}	6.14±1.10^{b}	6.32±0.71^{b}	7.99±0.38^{ab}	7.75±0.86^{a}
TCD					
Control (Suc)	48.16±0.49^{b}	49.20±0.58^{a}	49.93±1.47^{a}	48.28±1.11^{b}	50.76±0.74^{a}
Suc/RAE	50.07±1.34^{a}	49.68±1.03^{a}	50.91±1.47^{a}	50.94±0.81^{a}	49.74±0.73^{b}
Xyl/RAE	49.72±1.04^{a}	49.17±0.82^{a}	50.49±0.96^{a}	48.75±1.31^{b}	50.02±0.86^{ab}
hue					
Control (Suc)	32.10±1.76^{a}	30.94±1.71^{a}	33.13±0.71^{a}	32.66±2.31^{a}	30.43±4.66^{a}
Suc/RAE	30.04±1.53^{b}	28.19±1.43^{b}	30.79±1.16^{b}	32.15±2.01^{a}	32.36±2.66^{a}
Xyl/RAE	27.98±1.72^{c}	28.15±2.60^{b}	29.75±2.07^{b}	33.17±1.16^{a}	31.19±1.90^{a}
Chroma					
Control (Suc)	14.97±0.65^{a}	14.12±0.90^{a}	15.09±0.69^{a}	15.59±0.63^{a}	15.14±0.95^{a}
Suc/RAE	13.77±0.64^{b}	13.65±0.37^{a}	13.25±0.40^{b}	13.41±1.26^{b}	15.01±0.49^{a}
Xyl/RAE	11.73±0.93^{c}	12.94±1.38^{a}	12.72±0.69^{b}	14.62±0.69^{a}	14.94±1.01^{a}
BI					
Control (Suc)	34.19±1.89^{a}	32.65±1.87^{a}	36.08±1.15^{a}	35.86±1.36^{a}	35.91±3.36^{a}
Suc/RAE	32.22±1.33^{a}	31.07±0.84^{ab}	31.86±1.87^{b}	32.73±3.15^{b}	35.50±1.73^{a}
Xyl/RAE	26.85±2.85^{b}	29.22±3.59^{b}	30.04±2.65^{b}	34.22±1.10^{ab}	35.23±3.14^{a}

Values represent means±SD, n=6. Means within columns and parameter group with different letters are significantly different ($p<0.05$)

Control (Suc) = 16.6 % Sucrose without RAE, Suc/RAE = 16.6 % Sucrose with 03 % RAE and Xyl/RAE = 16.6 % Xylitol with 0.3 % RAE.

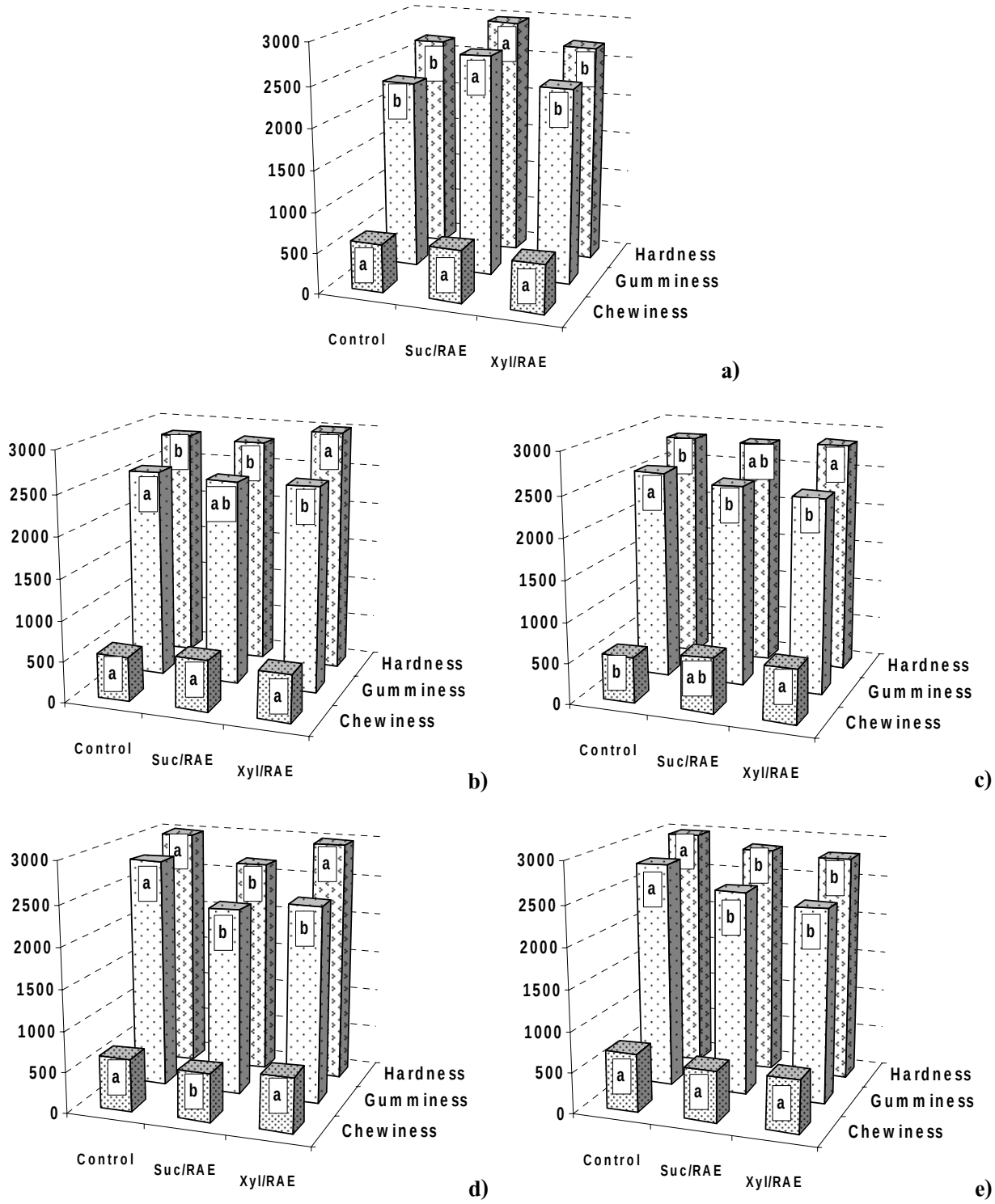


Figure 4.12 Change of hardness (N), gumminess (N) and chewiness (N*mm) of roasted RAE treated Chinese-style sausage with sucrose or xylitol addition during storage for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days at 31±1 °C. Within parameter, bars with different letters are significantly different ($p<0.05$), *n=12*. Average coefficient of variation (CV) = 10.78 %

4.2.3.2 Sensory evaluation of RAE treated CSS with sucrose or xylitol addition

The experienced sensory panels were used to evaluate the sensory characteristics by quantitative descriptive analysis (QDA) and a 7-point hedonic scale of CSS samples during storage.

1) Quantitative descriptive analysis (QDA) evaluation

The following discriminants of sensory profile of color, odor; normal (CSS flavor) and rancid (oxidation), taste; sweetness and texture; hardness, gumminess and juiciness were evaluated using a 15 cm line anchored on the left side with the lowest intensity of each attribute and the right side with the highest intensity was used. The mean results of sensory profiles of CSS during storage are presented in Figure 4.13.

Color. As seen in Figure 4.13, the sensory scores of internal color for all roasted CSS samples during storage fell into the range of 7-10 which indicated the moderate red color. The internal color of Xyl/RAE sample tended to get slightly higher scores throughout the storage times compared to the Suc/RAE and the control CSS. The result were not corresponded to the redness (a*-value) of raw CSS sample measured by colormeter (Table 4.8). This may be due to the fact that cooked (by roasted in this study) and raw CSS exhibited different color characteristics.

Odor. All roasted samples of CSS showed the same quality in terms of normal odor especially at the first week of storage, although the Xyl/RAE tended to get higher sensory scores at the longer storage times. The better sensory quality of the Xyl/RAE roasted sample was also observed for the rancid odor as the lower scores were received for this attribute. These results were actually corresponded to the oxidative stability data measured by TBARS and total protein carbonyl content in the previous study (Figure 4.11). This finding confirmed the advantage of xylitol to enhance the ability of RAE in prevention of lipid oxidation in CSS.

Sweetness. All roasted CSS samples showed similar moderate sweet taste (sensory score ranging from 6.2-8.1) (Figure 4.13). This result indicated that the replacement of sucrose with xylitol in CSS at the same concentration (16.6 % by weight) showed about the same sweetness intensity. This was not surprising, since sucrose and xylitol have same sweetness (Maguire, 2006).

Texture and overall acceptability. According to the data in Figure 4.13, roasted Xyl/RAE sample tended to have higher sensory scores for the texture attributes including

hardness, gumminess and juiciness compared to the Suc/RAE and control CSS samples. Comparing to the instrumental values of texture parameters measured by the texture analyzer (Figure 4.12) no obvious correlation could be made. Greater hardness and gumminess of Xyl/RAE sample could be due to the lower moisture content and water activity (Table 4.7). Throughout the storage times, the sensory scores for the hardness, gumminess and juiciness of Xyl/RAE ranged from 8 to 10, 9 to 10.3 and 8.5 to 10, respectively. This values were no serious different compared to the Suc/RAE or even he control sample at the beginning and the end of storage. Moreover, the overall acceptability of the Xyl/RAE CCS was always highest over the storage period.

2) Sensory evaluation by 7-point hedonic scale

Figure 4.14 illustrates the sensory evaluation of CSS samples during storage using 7-point hedonic scale method. The sensory scores 1, 4 and 7 represented “extremely dislike”, “neither like nor dislike” and “extremely like”, respectively. Obviously, the RAE treated CSS with xylitol (Xyl/RAE) tended to get significantly higher scores for all attributes including color, odor, taste, texture and overall liking compared to the Suc/RAE and control. This suggested that xylitol could improve overall acceptance of RAE treated CSS. Although the sensory scores of Xyl/RAE sample for all attributes were slightly lower after 21 days of storage, the values ranged from 5-6 meaning that the sample was still acceptable as “slightly like” to “moderately like”. It can be concluded that RAE treated CSS with xylitol addition was still accepted, when stored room temperature (31±1 $^{\circ}$C) in vacuum packing for 28 days.

4.2.3.3 Correlation between sensory and instrumental texture value

According to the data obtained from the texture profile analysis by texture analyzer (Figure 4.12) and from sensory evaluation using QDA method (Figure 4.13) of the CSS samples. Correlation was determined between the instrumental values and sensory scores. Correlation coefficience (r) for the hardness and gumminess of the CSS samples were 0.324 ($p<0.01$) and 0.349 ($p<0.01$) respectively. Results indicated that the instrumental values were not directly correlated to the sensory scores for the texture parameters of the CSS. Beside the nature of instrumental texture analysis and sensory evaluation, the difference of sample temperature (60 $^{\circ}$C for sensory test and room temperature for instrumental measurement) would be one of the reasons that contributed to the non correlation observed.

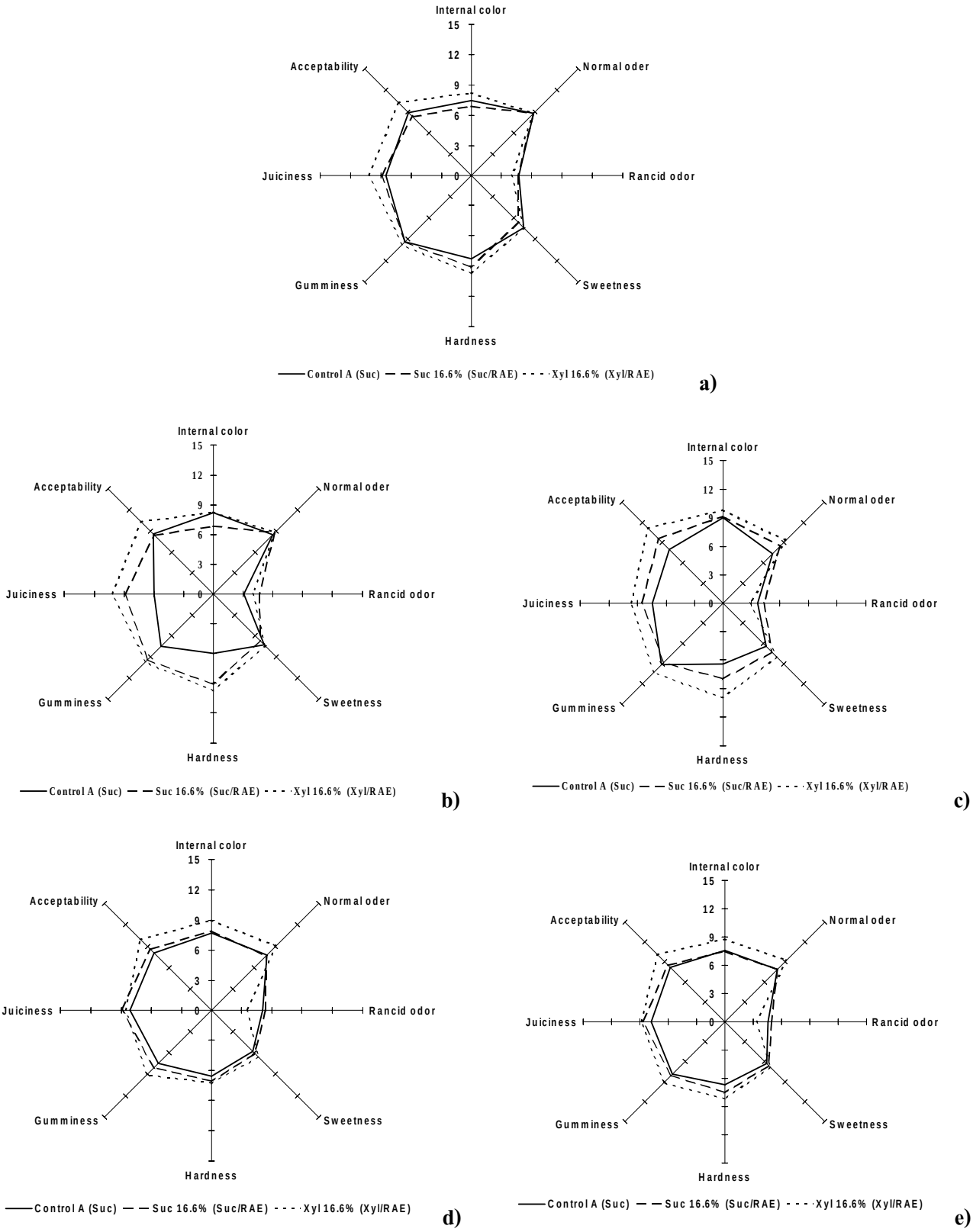


Figure 4.13 Quantitative descriptive analysis of roasted RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days

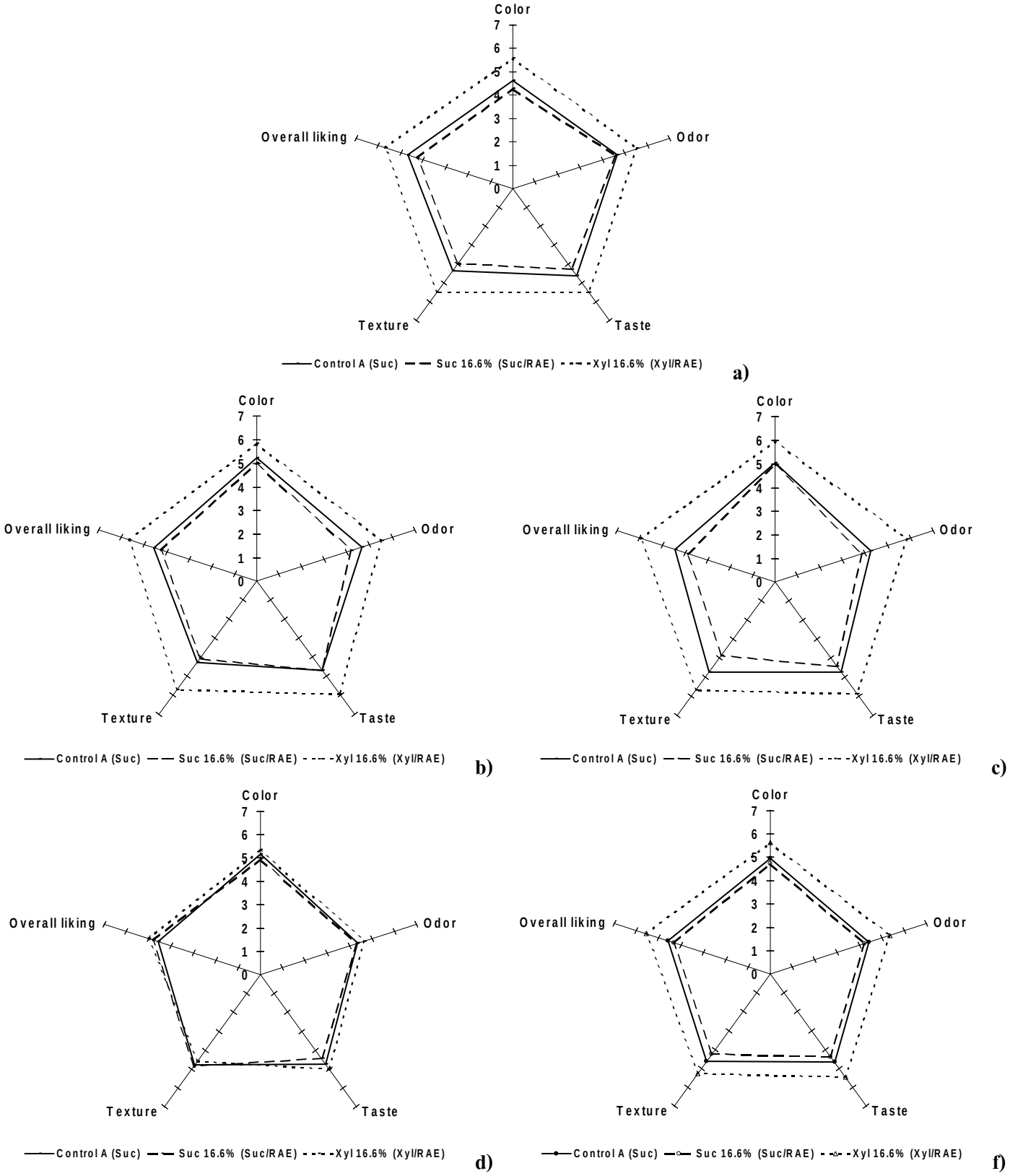


Figure 4.14 Sensory evaluation by 7-point hedonic scale of RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days

4.2.3.4 Conclusion

The use of xylitol at 16.6 % by weight in RAE treated CSS with vacuum packed were proved to be effective in controlling both physicochemical and sensory qualities at least for 28 days at 31±1 °C. It can be concluded that xylitol can be used as an alternative sweetener in CSS without unacceptable overall qualities. Moreover, in case of RAE is used as natural antioxidant, replacement of sucrose by xylitol in the CSS will result in better oxidative stability and sensory quality.

4.3 Effect of roselle anthocyanin extracts on *in vitro* scavenging of reactive nitrogen species and on nitrite reduction in meat products.

Background of this study

Besides the reactive oxygen species (ROS), reactive nitrogen species (RNS) have been known to involve in oxidative deterioration of food products and other biological systems. Sodium or potassium salts of nitrate and nitrite are also commonly used as food additive in meat products, although they potentially generate carcinogenic substances. As a consequence, plant materials and/or plant extracts have been of interest to be used as alternative natural antioxidants. Previously reported in this study, rosele anthocyanin extracts (RAE) was found to be effective in inhibition f the development of lipid and protein oxidation in Chinese-style sausage (CSS). In this part, the ability of RAE to scavenging RNS including nitrite, nitric oxide and peroxynitrite was investigated. In addition, residual nitrite reduction in two different models of meat products by RAE was also evaluated.

4.3.1 Reactive nitrogen species scavenging capacity of RAE

4.3.1.1 Nitrite scavenging activity

The nitrite scavenging activity of RAE comparing to the anthocyanins from black carrot and grape and positive controls (BHA and vitamin C) were studied and results are presented in Figure 4.15. Anthocyanins from all plant sources studied and positive controls exhibited a concentration and pH dependent nitrite scavenging activity. The activities were greater at the higher concentration but at the lower pH. All anthocyanin samples showed similar activity in scavenging of nitrite and also similar to that of vitamin C. In addition, greater activities were found for the three anthocyanin samples compared to BHA.

The effect of pH can be clearly observed form the IC_{50} values which refer to the concentration of the tested sample at which the nitrite scavenging activity equals to 50 %. The IC_{50} of roselle anthocyanin extracts reduced about 7 times, when pH of the reaction mixtures decreased from pH 9.0 to pH 3.0. Similar results could also be seen for all other samples and positive controls. This findings are in agreement with previous studies reported that nitrite scavenging activities of the ethanol extracts of bamboo oil were > 90 % and > 50 % at pH 1.2 and 3.0, respectively and were even lower at pH 4.2 and 6.0 (Choi et al., 2008). More specifically, the nitrite scavenging activity of green tea extracts (Bae and Lee, 2010), *Sonchus oleraceus* L. extracts (Yin et al., 2007) and citrus peel powder (Kang et al., 2006) decreased with the increase of pH and the activity was greatest at pH lower than 3.0. The influence of pH on the nitrite

scavenging activity of RAE was also confirmed in the model of meat products and will be discussed later.

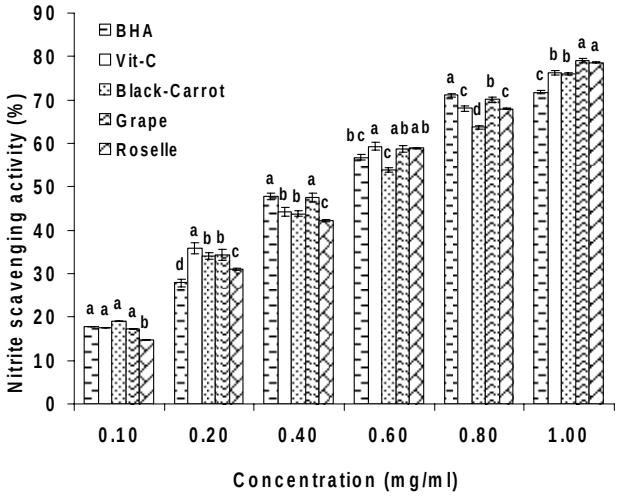
4.3.1.2 Nitric oxide scavenging activity

Nitric oxide is a free radical product in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases (Pacher et al., 2007). The nitric oxide scavenging activity of anthocyanins from black carrot, grape and roselle is presented in Figure 4.16, BHA and Vitamin C was used as reference compounds. The higher activities were found in all anthocyanin samples with IC_{50} 113 and 120 μl/ml. While, BHA and Vitamin C showed IC_{50} 151.89 and 165.99 μl/ml, respectively. In addition, at 180 μl/ml, the percent inhibitions of nitric oxide for all anthocyanin samples were 70 to 75 %, whereas those of BHA and vitamin C were 52 and 56 %, respectively. Among the anthocyanin samples studied, RAE exhibited slightly higher nitric oxide scavenging activity compared to anthocyanins prepared from black carrot and grape.

4.3.1.3 Peroxynitrite scavenging activity

The peroxynitrite scavenging activity of the tested samples was determined according to the Evan blue bleaching assay which measures the peroxynitrite degradation. As seen in Figure 4.17, the anthocyanins from black carrot had slightly lower activity compared to those from grape and roselle extracts at the concentration 20 to 200 μl/ml. Generally, the ability of all anthocyanin samples in scavenging of peroxynitrite was similar to that of standard gallic acid.

The peroxynitrite scavenging property of anthocyanin especially pelargonidin, has been previously reported by Tsuda et al. (2000). Moreover, the activity of anthocyanins in perxynitrite scavenging at pH 7.4 decreased in the following order: delphinidin > cyaniding ≈ petunidin > malvidin ≈ (-)-catechin > peonidin > pelargonidin (Rahman et al., 2006; Muselik et al., 2007). Black carrot and grape mainly consist of cyaniding-based pigments, while roselle consists of mostly delphinidin. This indicates that roselle anthocyanin extract (RAE) would be a good source of anthocyanins with strong peroxynitrite scavenging activity. However, results in Figure 4.17 did not clearly show the strong scavenging activity of RAE compared to anthocyanins from black carrot and grape. This could be due to RAE used in the experiment was a crude ethanolic extract prepared from the roselle, while the anthocyanins from black carrot and grape were commercial samples.



BHA (IC_{50} 2.51±0.12 mg/ml)

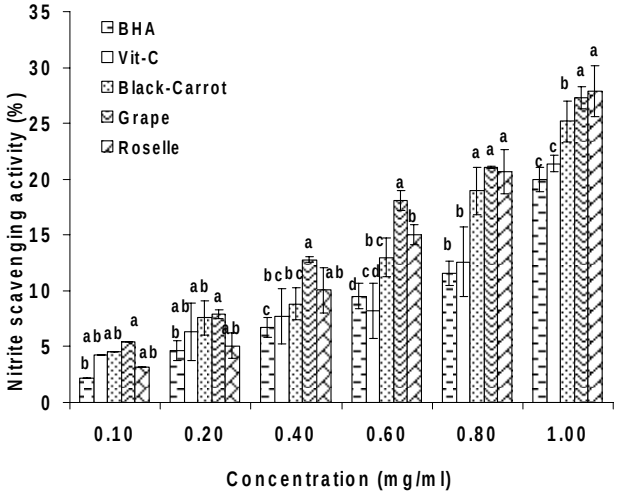
Vitamin C (IC_{50} 0.66±0.01 mg/ml)

Black carrot (IC_{50} 0.66±0.01 mg/ml)

Grape (IC_{50} 0.63±0.01 mg/ml)

Roselle (IC_{50} 0.64±0.01 mg/ml)

a)



BHA (IC_{50} 0.70±0.10 mg/ml)

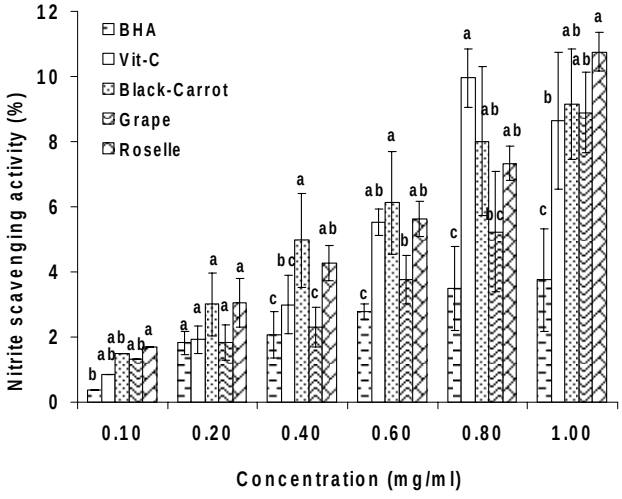
Vitamin C (IC_{50} 2.34±0.07 mg/ml)

Black carrot (IC_{50} 1.99±0.14 mg/ml)

Grape (IC_{50} 1.83±0.06 mg/ml)

Roselle (IC_{50} 1.79±0.05 mg/ml)

b)



BHA (IC_{50} 14.01±3.64 mg/ml)

Vitamin C (IC_{50} 5.80±0.22 mg/ml)

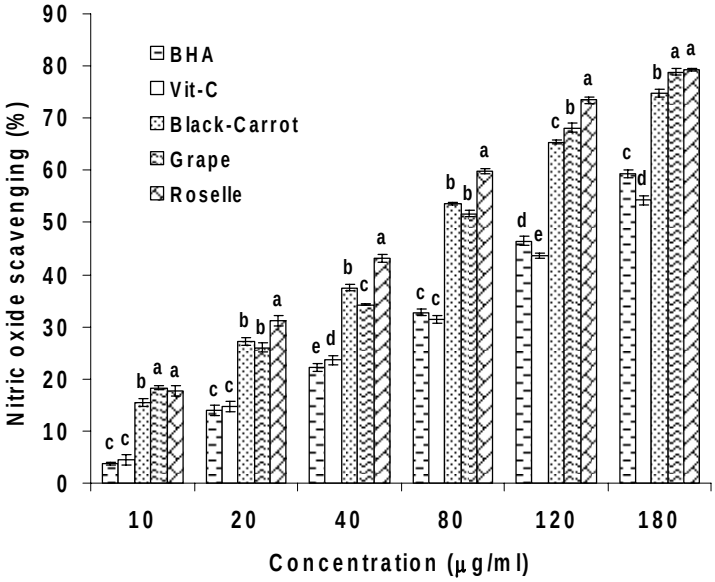
Black carrot (IC_{50} 5.58±0.97 mg/ml)

Grape (IC_{50} 5.65±0.45 mg/ml)

Roselle (IC_{50} 4.67±0.39 mg/ml)

c)

Figure 4.15 Nitrite scavenging activity of anthocyanins from black carrot, grape and roselle extracts and the standard BHA and ascorbic acid. The data represent the percentage nitrite scavenging at a) pH 3.0, b) pH 6.0 and c) pH 9.0. Bars with different letters are significantly different ($p<0.05$), n=6. The IC_{50} represents the concentration of a sample test that is required for 50 % scavenging of nitrite in vitro



BHA (IC_{50} 151.89±1.81 µl/ml)

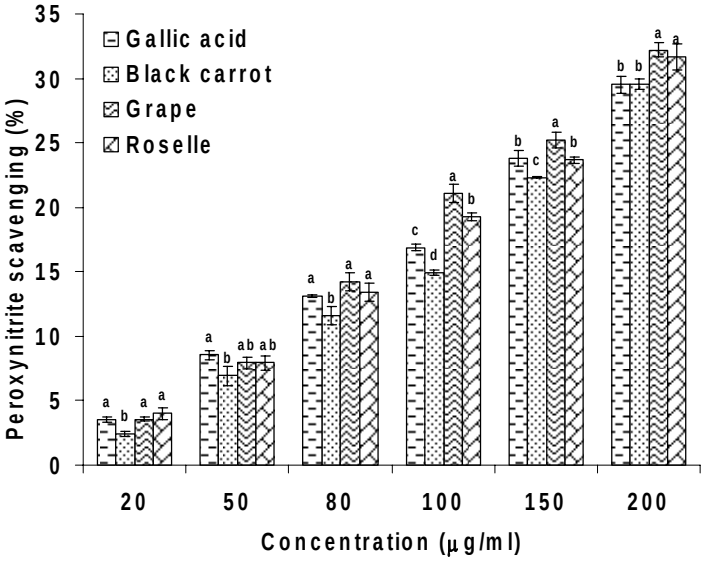
Vitamin C (IC_{50} 165.99±2.64 µl/ml)

Black carrot (IC_{50} 120.31±1.20 µl/ml)

Grape (IC_{50} 114.08±0.81 µl/ml)

Roselle (IC_{50} 113.39±0.40 µl/ml)

Figure 4.16 Nitric oxide scavenging activity of anthocyanins from black carrot, grape and roselle extracts and the standard BHA and ascorbic acid. Bars with different letters are significantly different ($p<0.05$), n=6, The IC_{50} represents the concentration of a sample test that is required for 50% scavenging of nitric oxide in vitro



Gallic acid (IC_{50} 338.72±7.26 µl/ml)

Black carrot (IC_{50} 359.69±5.59 µl/ml)

Grape (IC_{50} 310.56±5.17 µl/ml)

Roselle (IC_{50} 315.95±9.53 µl/ml)

Figure 4.17 The peroxynitrite scavenging activity of anthocyanins from black carrot, grape and roselle extracts and the standard gallic acid. Bars with different letters are significantly different ($p<0.05$), n=6. The IC_{50} represents the concentration of a sample test that is required for 50% scavenging of peroxynitrite in vitro

4.3.2 Effect of RAE on nitrite reduction in meat products

The objective of this study was to evaluate the effect of RAE on residual nitrite reduction in different models of meat products; non-fermented sausage (Vienna pork sausage) and traditional Thai fermented pork (Nham), containing different added sodium nitrite. Resulted are presented below.

4.3.2.1 Vienna pork sausages model

The Vienna sausage sample formulated with 0.3 % roselle extracts (RAE) and sodium nitrite 125 and 250 ppm, then packed in vacuum plastic bag and refrigerated storage at 4±1 °C for 24 days. PH and residual nitrite were evaluated compared to controls (sodium nitrite 125 and 250 ppm without RAE) and positive reference samples (200 ppm BHA added with sodium nitrite 125 and 250 ppm).

1) PH values of the samples during storage

Table 4.9 showed pH values of the Vienna pork sausage samples with different treatments. All sausage samples had pH values in the range of 6.2 to 6.6. As expected from the previous results, samples with RAE addition showed slightly lower pH compared to those without RAE. However, the pH values of all sausage samples were quite stable throughout the storage period.

Table 4.9 Means for pH of Vienna pork sausage with two levels of sodium nitrite during refrigerated storage at 4±1 °C

pH values	Storage time (days)								
	0	3	6	9	12	15	18	21	24
Control 1	6.577^{b}	6.567^{b}	6.565^{b}	6.567^{b}	6.555^{b}	6.547^{a}	6.547^{a}	6.547^{a}	6.547^{a}
Control 2	6.588^{a}	6.575^{a}	6.582^{a}	6.588^{a}	6.577^{a}	6.557^{a}	6.557^{a}	6.557^{a}	6.557^{a}
Reference 1	6.488^{d}	6.477^{d}	6.478^{c}	6.488^{d}	6.475^{d}	6.462^{c}	6.462^{c}	6.462^{c}	6.462^{c}
Reference 2	6.502^{c}	6.490^{c}	6.482^{c}	6.502^{c}	6.487^{c}	6.478^{b}	6.478^{b}	6.478^{b}	6.478^{b}
Treatment 1	6.372^{f}	6.367^{f}	6.370^{d}	6.372^{f}	6.358^{f}	6.358^{d}	6.358^{d}	6.358^{d}	6.358^{d}
Treatment 2	6.383^{e}	6.378^{e}	6.377^{d}	6.383^{e}	6.372^{e}	6.362^{d}	6.362^{d}	6.362^{d}	6.362^{d}

Values represent means±SD, n=6. Means within columns with different letters (a, b, c, d, e) are significantly different ($p<0.05$).
Control 1 = sample with 125 ppm sodium nitrite without RAE, Control 2 = sample with 250 ppm sodium nitrite without RAE, Reference 1 = sample with 200 ppm BHA and 125 ppm sodium nitrite, Reference 2 = sample with 200 ppm BHA and 250 ppm sodium nitrite, Treatment 1 = sample with 0.3 % RAE and 125 ppm sodium nitrite and Treatment 2 = sample with 0.3 % RAE and 250 ppm sodium nitrite.

2) Lipid oxidation of Vienna pork sausage during refrigerated storage

The lipid oxidation in the Vienna pork sausage during storage as measured by TBARS values are shown in Figure 4.18. The TBARS values of all samples increased with the increasing of storage time. However, the RAE treated Vienne pork sausages with 125 and 250 ppm of sodium nitrite obviously showed significantly lower ($p<0.05$) TBARS values compared to the controls (without RAE) and the reference samples (with BHA). In the control samples, there was a tendency of lower TBARS values for the sausage with higher level of sodium nitrite (250 ppm) addition. Fernandez et al. (1997) pointed out that the present of nitrite in the meat products could reduce the TBARS value. However, the concentration effect of sodium nitrite on its antioxidant activity was not clearly observed in both reference samples and samples with RAE addition. On the other hand, sausages with BHA addition tended to had higher TBARS values at higher concentration of sodium nitrite.

Several finding have been reported on the stronger activity of plant extracts in nitrite reduction in meat product models compared to BHA and BHT. For example, Ismail and Yee (2006) showed that the extracts from cocoa shell, roselle seed and a combination of the two extracts could inhibit lipid oxidation in cooked beef more effective than the BHT. The green tea and rosemary extracts were also reported to have stronger antioxidant activity in meat products compared to BHA and BHA/BHT (Sebranek et al., 2005)

3) Residual nitrite in Vienna pork sausage during refrigerated storage

Changes of the residual nitrite levels in Vienna pork sausage samples during refrigerated storage are presented in Figure 4.19. The residual nitrite in all sausage samples tended to decreased with longer storage time and the reduction of nitrite level was ore pronounce in the samples with 250 ppm sodium nitrite. In the control sausages (without RAE), residual nitrite levels reduced from 250 ppm to about 180 ppm after 24 days of refrigerated storage. While the reference samples with BHA addition showed similar results in residual nitrite reduction during storage compared to the controls, sausages with RAE addition clearly proved that RAE was more efficient in reduction of residual nitrite. The nitrite levels reduced 67.73 % and 52.46 % for the RAE treated sausages with 250 and 125 ppm of sodium nitrite, respectively.

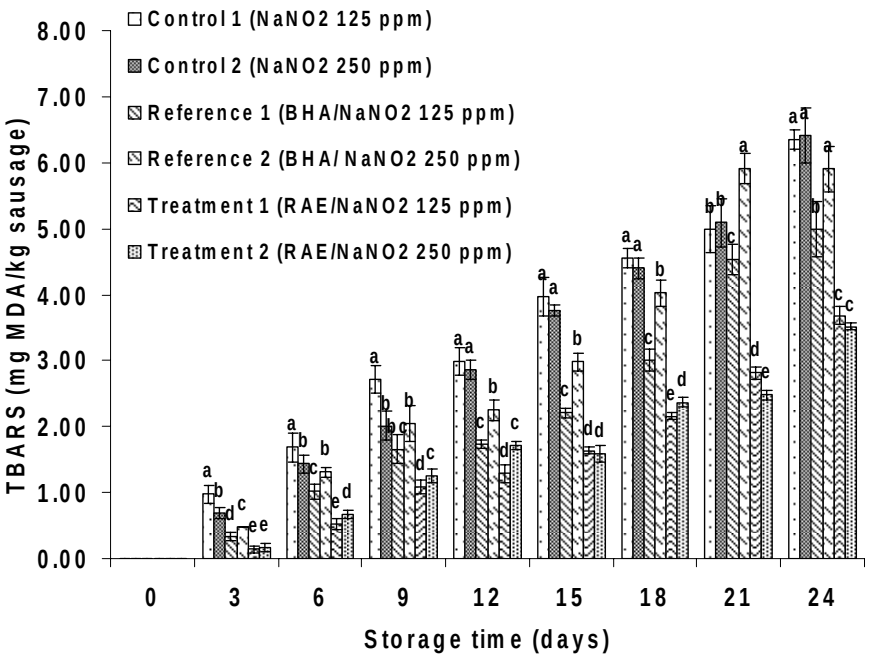


Figure 4.18 TBARS values in Vienna pork sausage during storage at 4±1 °c for 24 days, *n=6*

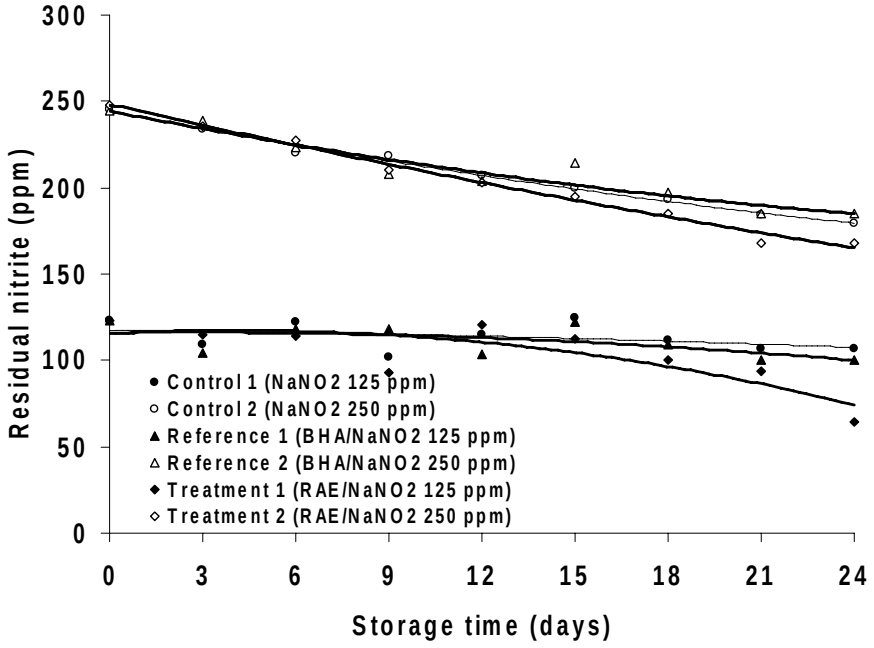


Figure 4.19 Residual nitrite in Vienna pork sausage samples during storage at 4±1 °c for 24 days, *n=6*. Average coefficient of variation (CV) = 4.70 %

4.3.2.2 Traditional Thai fermented pork sausage model

Traditional Thai fermented pork sausage or "Nham" was formulated with addition of 0.3 % roselle anthocyanin extracts (RAE) and sodium nitrite125 and 250 ppm. The 100 g of Nham mixture was stuffed into polyethylene (PE) plastic bag (∅ 1.5 cm; thickness 80 μm) and kept at room temperature (30±1 °C) for fermentation for 7 days. The values of pH, TBARS and residual nitrite were analyzed compared to control samples (sodium nitrite 125 and 250 ppm, without RAE) and the reference samples (sodium nitrite 125 and 250 ppm, BHA 200 ppm).

1) Changes of pH values of Nham samples during fermentation

As seen in Table 4.10, the initial pH of control and reference samples was about 6.12 to 6.13, while Nham with RAE addition showed lower initial pH (5.88 to 5.89) due to the acidic components in the RAE as described earlier. During fermentation, pH values of all Nham samples significantly decreased to 4.3 to 4.4 after 7 days of fermentation. This was due to the production of lactic acid by the natural lactic starter. Similar rate of pH reduction were observed in all samples, indicating that addition of BHA or RAE did not interfere the lactic acid fermentation.

Table 4.10 Changes of pH values of Nham samples during fermentation at 30±1 °C for 7 days

pH value	Storage time (days)					
	0	1	2	3	4	7
Control 1	6.12±0.01^{a}	5.64±0.01^{a}	4.92±0.01^{c}	4.46±0.01^{c}	4.41±0.01^{b}	4.40±0.01^{c}
Control 2	6.13±0.01^{a}	5.63±0.01^{ab}	5.17±0.01^{a}	4.59±0.01^{a}	4.41±0.01^{b}	4.41±0.01^{b}
Reference 1	6.13±0.01^{a}	5.63±0.01^{b}	5.14±0.01^{b}	4.46±0.01^{c}	4.43±0.01^{a}	4.43±0.01^{a}
Reference 2	6.13±0.01^{a}	5.63±0.01^{ab}	4.90±0.01^{d}	4.55±0.01^{b}	4.43±0.01^{a}	4.41±0.01^{bc}
Treatment 1	5.88±0.01^{c}	5.42±0.01^{d}	4.77±0.01^{f}	4.41±0.01^{d}	4.35±0.01^{c}	4.33±0.01^{e}
Treatment 2	5.89±0.01^{b}	5.43±0.01^{c}	4.86±0.02^{e}	4.40±0.01^{d}	4.35±0.01^{c}	4.34±0.01^{d}

Values represent means±SD, n=6. Means within columns and parameter group with different letters (a, b, c, d, e) are significantly different ($p<0.05$).

Control 1 = sample with 125 ppm sodium nitrite without RAE, Control 2 = sample with 250 ppm sodium nitrite without RAE, Reference 1 = sample with 200 ppm BHA and 125 ppm sodium nitrite, Reference 2 = sample with 200 ppm BHA and 250 ppm sodium nitrite, Treatment 1 = sample with 0.3 % RAE and 125 ppm sodium nitrite, Treatment 2 = sample with 0.3 % RAE and 250 ppm sodium nitrite.

2) Lipid oxidation of Nham samples during fermentation

According to the TBARS values shown in Figure 4.20, lipid oxidation continuously increased for all samples of Nham during fermentation. In the control samples, Nham with 125 ppm sodium nitrite had significantly higher TBARS values than Nham with 250 ppm sodium nitrite. This result clearly showed the antioxidant activity of sodium nitrite. However, the effect of nitrite concentration on TBARS values of the reference (with BHA) and RAE treated samples was not clearly observed. Results also showed that RAE could efficiently prevent the lipid oxidation in Nham similar to BHA at the fermentation time within 3 days.

3) Residual nitrite in Nham samples during fermentation

Changes in residual nitrite levels of Nham samples during fermentation are presented in Figure 4.21. With the independent of initial nitrite concentration the residual nitrite in all Nham samples rapidly reduced to lower than 20 ppm after 3 days of fermentation. The pH values of all Nham samples after 3 days of fermentation decreased from 5.9 to about 4.4 (Table 4.12). Comparing to the results observed in the Vienna pork sausage (Table 4.11 and Figure 4.19), the rapid reduction of residual nitrite in Nham was most likely due to the acidic pH caused by lactic acid fermentation. Pegg and Shahidi (2000) suggested that the nitrite depletion rate in meat products in dependent upon products formulation, pH time and temperature relations during processing and storage. In addition, the effect of pH on the nitrite reduction *in vitro* has been reported by Wang et al (2010). They found that the nitrite lost was about 98.5 % at pH 3.0, while only 60-68 % of nitrite depleted at pH between 4.0 to 5.0. As seen in Figure 4.21, the residual nitrite in all Nham samples reduced up to 90 % at pH around 4.5. The over reduction of residual nitrite observed in Nham samples with RAE of BHA addition was also, in part, due to the nitrite scavenging activity of RAE and BHA.

Surprising, the control samples (without RAE and BHA) showed the similar results of residual nitrite depletion. This might be due to the effect of fresh garlic which was used at about 4.3 % as an ingredient in the Nham formula. Sun et al. (2000) have reported that 5 % fresh and 1.2 % garlic powder could reduce residual nitrite in cured Chinese-sausage. Moreover, their extracts exhibited nitrosamine formation in vitro (Choi et al., 2006). The nitrite reduction observed in Nham samples was in agreement with the finding reported by Samelis et al. (1998); who found that residual nitrite in traditional Greek-salami sausages was rapidly decreased from the initial level of 250 ppm to < 10 ppm within 3 days. Furthermore, the residual nitrite found in

Turkish-style sausage was also in the range of 4 to 11 ppm after 3 day ripening (Üren et al., 1997).

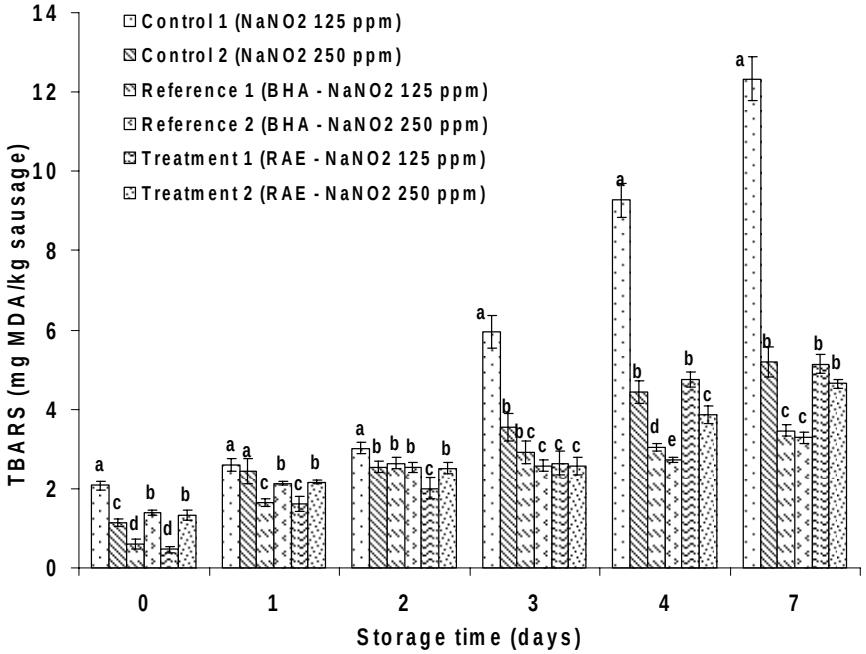


Figure 4.20 TBARS values in traditional Thai fermented pork (Nham) during fermentation at 30±1 °C for 7 days

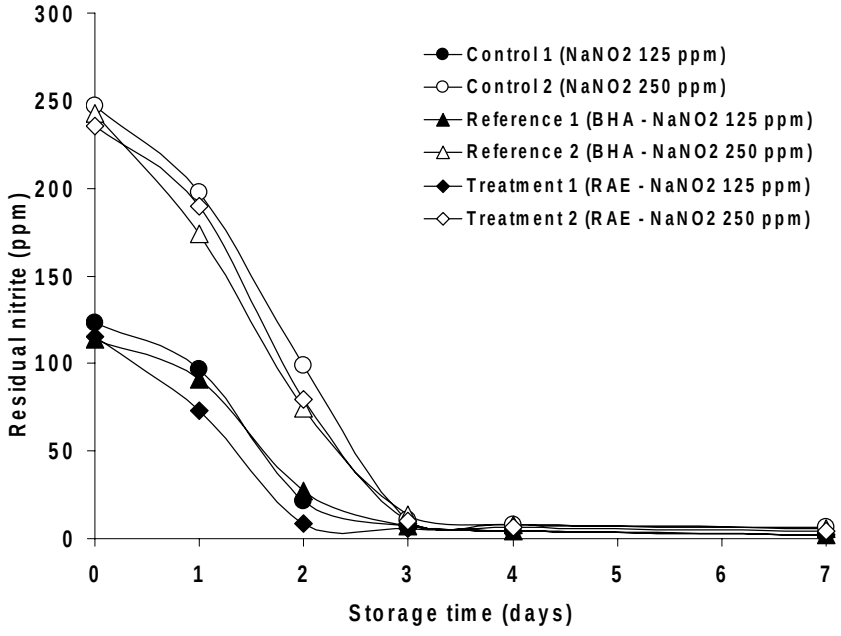


Figure 4.21 Residual nitrite in traditional Thai fermented pork (Nham) during fermentation at 30±1 °C for 7 days, *n=6*. Average coefficient of variation (CV) = 3.18 %

4.3.3 Kinetics study of sodium nitrite degradation in traditional Thai fermented pork model with difference sodium nitrite concentration

The aim of this study was to determine the kinetics of sodium nitrite degradation in Nham model prepared with 0.3 % roselle extract and different levels of sodium nitrite (125, 250 and 500 ppm) during fermentation. Results are discussed below.

4.3.3.1 Changes of pH and total acidity of Nham samples during fermentation

Changes of pH and total acidity (as lactic acid) during fermentation of Nham samples are given in Figure 4.22. The initial pH values of the Nham were 6.15 for control and BHA added samples and 5.70 for RAE treated Nham. During fermentation, pH values of all Nham samples continuously decreased and reached the final values was in accordance to the increasing of total acidity.

As seen in Figure 4.22, the pH values of all samples were almost stable after 72 h of fermentation, while the total acidity still continued to increase up to 0.90 %. Nham is usually consumed when the pH drops to 4.4 to 4.6 (TISI-1219, 2004).

4.3.3.2 Kinetics of sodium nitrite degradation

Results from kinetic analysis for sodium nitrite degradation in Nham samples are shown in Figure 4.23 - 4.24 and Table 4.11. A significant decrease of sodium nitrite content in all Nham samples were observed during 120 h fermentation. As seen in Figure 4.23, the residual nitrite decreased from the initial levels of 125, 250 and 500 ppm to lower than 10 ppm for all samples after 120 h of fermentation.

The rate constant (k_{NaNO2}) and half life ($t_{1/2}$) for the degradation of sodium nitrite as showed in Table 4.11 indicated that the rate of nitrite reduction in Nham models was dependent upon served factors including fermentation time, pH, initial nitrite concentration and nitrite scavenging agent. Considering the effect of fermentation time and pH, it is clear that the increase of fermentation time resulted in pH reduction and increase of k_{NaNO2} with the decrease of $t_{1/2}$, suggesting the greater rate of nitrite degradation at lower pH (or longer fermentation time). Results also revealed that the higher the initial nitrite concentration the lower the rate of nitrite reduction.

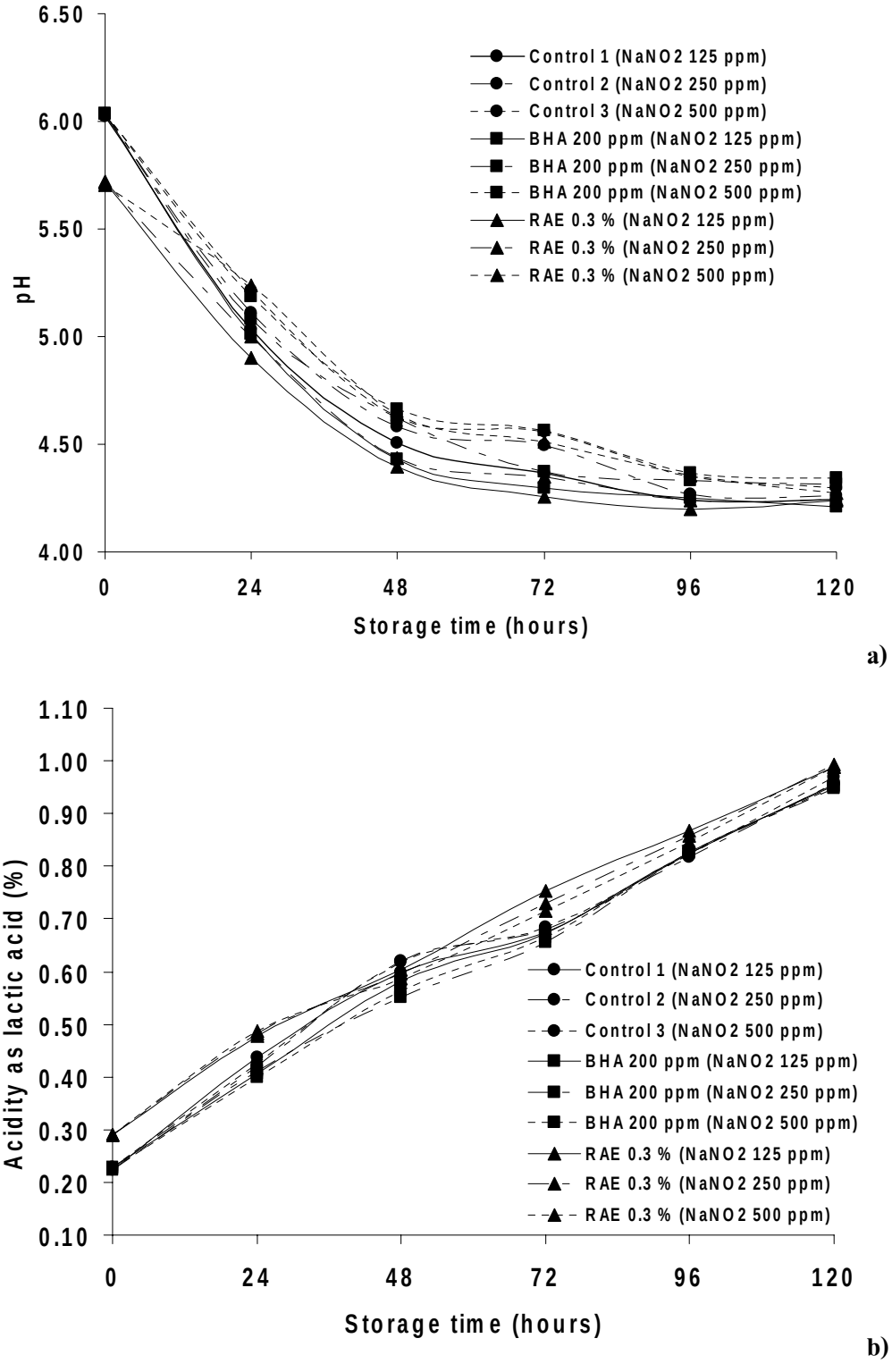


Figure 4.22 Change of a) pH values and b) total acidity as lactic acid in Nham samples with different initial concentration of sodium nitrite during 120 h fermentation time, *n=6*. Average coefficient of variation (CV) = 1.08 and 1.79 % respectively

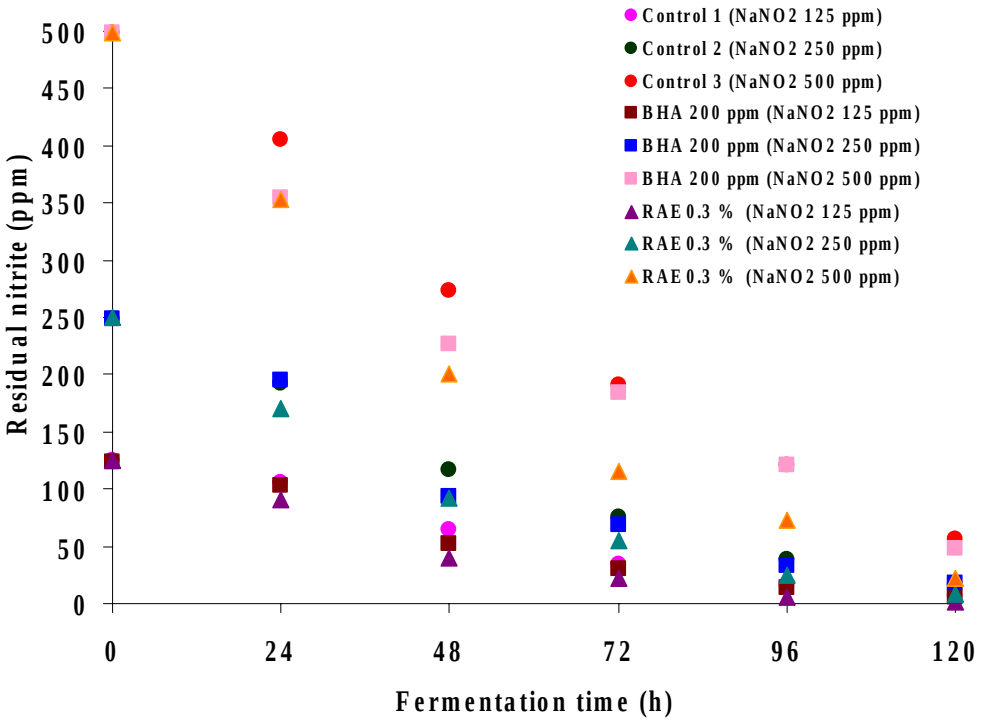


Figure 4.23 Residual nitrite in Nham samples during 120 h fermentation times, *n=6*. Average coefficient of variation (CV) = 1.34 %

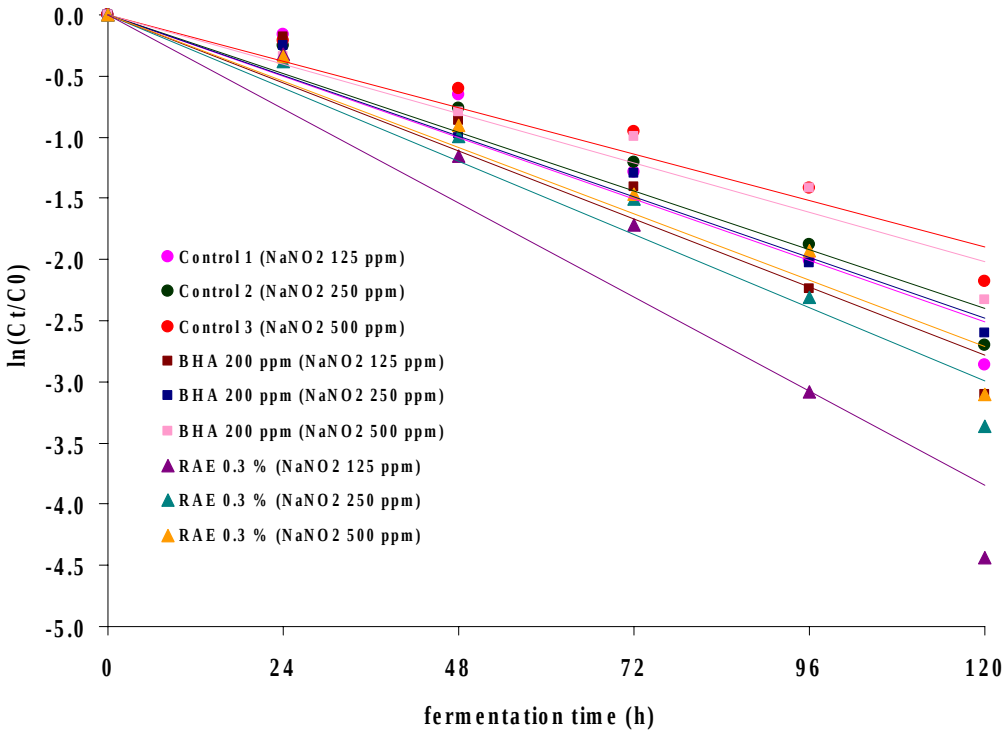


Figure 4.24 Sodium nitrite reductions in Nham samples during 120 h fermentation times.

Table 4.11 PH values and kinetic parameters for the sodium nitrite degradation in Nham with different concentration of sodium nitrite during 120 h fermentation times

Formulation	time (h)	pH	k_{NaNO2} (1/h)	$t_{1/2}$
Control 1 ($NaNO_2$ 125 ppm)	0	6.02	-	-
	24	5.04	0.0067	103.3227
	48	4.51	0.0136	51.0461
	72	4.37	0.0179	38.6898
	96	4.24	0.0209	33.1305
	120	4.24	0.0238	29.0894
Control 2 ($NaNO_2$ 250 ppm)	0	6.03	-	-
	24	5.11	0.0106	65.2408
	48	4.58	0.0158	43.8760
	72	4.50	0.0167	41.5868
	96	4.27	0.0195	35.4940
	120	4.26	0.0225	30.7496
Control 3 ($NaNO_2$ 500 ppm)	0	6.04	-	-
	24	5.21	0.0086	80.3589
	48	4.63	0.0125	55.2245
	72	4.56	0.0133	52.1119
	96	4.35	0.0147	47.0720
	120	4.30	0.0182	38.0882
Reference 1 (BHA 200 ppm and $NaNO_2$ 125 ppm)	0	6.04	-	-
	24	5.01	0.0077	89.6464
	48	4.43	0.0179	38.6901
	72	4.30	0.0195	35.5878
	96	4.25	0.0234	29.6751
	120	4.21	0.0258	26.8464
Reference 2 (BHA 200 ppm and $NaNO_2$ 250 ppm)	0	6.03	-	-
	24	5.08	0.0103	67.2236
	48	4.62	0.0203	34.1151
	72	4.37	0.0180	38.5319
	96	4.33	0.0212	32.7563
	120	4.31	0.0217	31.9376
Reference 3 (BHA 200 ppm and $NaNO_2$ 500 ppm)	0	6.04	-	-
	24	5.19	0.0143	48.4452
	48	4.66	0.0165	41.9982
	72	4.56	0.0138	50.0895
	96	4.37	0.0148	46.8310
	120	4.34	0.0194	35.7393
Treatment 1 (0.3 % RAE and $NaNO_2$ 125 ppm)	0	5.72	-	-
	24	4.90	0.0132	52.3635
	48	4.40	0.0241	28.7872
	72	4.25	0.0238	29.0840
	96	4.20	0.0321	21.6155
	120	4.24	0.0369	18.7591
Treatment 2 (0.3 % RAE and $NaNO_2$ 250 ppm)	0	5.72	-	-
	24	5.00	0.0159	43.5406
	48	4.44	0.0208	33.3947
	72	4.35	0.0209	33.2350
	96	4.24	0.0241	28.7514
	120	4.24	0.0280	24.7463
Treatment 3 (0.3 % RAE and $NaNO_2$ 500 ppm)	0	5.71	-	-
	24	5.24	0.0144	48.1338
	48	4.64	0.0189	36.6167
	72	4.51	0.0204	33.9538
	96	4.35	0.0201	34.4288
	120	4.28	0.0259	26.7617

k_{NaNO2} is the reaction rate constant of sodium nitrite at fermentation time (h), $t_{1/2}$ is the half life (h)

4.3.4 Conclusion

Results from this study supported that roselle anthocyanin extracts and anthocyanins from black carrot and grape exhibited strong activity on the scavenging of reactive nitrogen species (RNS). *In vitro* study revealed the pH-dependent for nitrite scavenging property of RAE, as the higher activity was found at the lower pH. The effect of pH on the nitrite reduction was also confirmed in the models of meat products including Vienna pork sausage and Nham. According to the kinetic parameters of nitrite degradation in Nham model, RAE could be used to enhance the residual nitrite reduction in the meat products.

CHAPTER 5

CONCLUSIONS

The objective of the study was to investigate the physicochemical and antioxidant activities of roselle extracts by different grinding method (OG and NG). The roselle extracts was selected to study under citric acid-Na_2HPO_4 (Mcllvaine) buffer solution (pH 3.0 and 4.0) with and without sucrose (20 %) and heat treatment between 50 to 70 °C. The changes of anthocyanins content, pigment concentration, antioxidant activity and anthocyanins degradation kinetics during heat treatment were determined. The OG roselle extracts was selected for next objective to evaluated the effect of sweeteners on oxidative stability of roselle extracts and determine the physicochemical properties and sensory quality of Chinese-style sausage during storage. The scavenging of reactive nitrogen species (RNS) *in vitro* by roselle extract and anthocyanins from black carrot and grape were determined in next objective. Moreover, nitrite scavenging activity by roselle extracts was monitor in meat model system including Vienna pork sausage and Nham. The results were concluded as follows:

5.1 To study physicochemical and antioxidant activity of roselle extract from difference grinding method were original-grinding (OG) and nano-grinding (NG) method. The NG roselle extracts showed high level of anthocyanins; delphinidin 3-sambubioside and cyanidin 3-sambubioside, and exhibited the higher in color pigment (UV-spectrum) and visual color at similar extracts concentration. Antioxidant activity of roselle extracts tended higher in NG roselle extracts. The temperature and pH were found influenced on physicochemical and antioxidant activity of roselle extracts. Increasing temperature during storage tended to rapidly degradation of roselle extracts in buffer solution, and roselle extract solution was more stable in system pH 3.0 than system pH 4.0. The sugar addition (20 %) into system did not play as the main effect on physicochemical properties of roselle extracts. The observed in each model system with sugar added showed the higher in antioxidant activity. Therefore sugar added could be increase antioxidant of roselle extracts solution during heating. Overall, NG roselle extracts was found better in physicochemical and antioxidant activity. When consideration in degradation rate, the NG roselle extracts exhibited the rapid degradation than OG roselle extracts. Thus, the extraction method should be considered for developing natural antioxidant extract.

5.2 The used of sugar alcohols with roselle extracts did not showed negative effect on physicochemical properties of Chinese-style sausage. However, xylitol added exhibited the similar sausage qualities which are closer the control sausage than other sugar alcohol. Moreover, xylitol added did not effect on antioxidant of roselle extracts in Chinese-style sausage. Thus, xylitol would be a suitable sugar; among the three sugar alcohols studied, to be used as a replacer for sucrose when the RAE is incorporated as a natural antioxidant in Chinese-style sausage. The xylitol concentration was found slightly effective on quality of RAE treated Chinese-style sausage. An equal concentration (16.6 %) between sucrose and xylitol. Xylitol added samples showed similar physicochemical properties compared to control sample (sucrose added), but sucrose added samples showed negative effect on lipid oxidation by TBARS and protein oxidation by total carbonyl contents. However, at high concentration (21.6 %) xylitol play the pro-oxidant activity, which can see the higher in lipid oxidation by TBARS. Thus, 16.6 % xylitol is the suitable level of concentrate was used to replace sucrose in Chinese-style sausage and results showed similar quality compared to original sausage sample. The using xylitol incorporated with roselle extracts in Chinese-style sausage did not showed negative effect on sensory evaluation by quantitative descriptive analysis (QDA) and 7-point hedonic scale. The xylitol added sausage with roselle extracts exhibited a good quality in all sensory parameter including flavor, texture, taste and overall acceptability. This indicated that the xylitol can be used as an alternative sweetener in Chinese-style sausage without unacceptable overall qualities.

5.3 To evaluate the reactive nitrogen species (RNS) scavenging, these species are nitrite scavenging, nitric oxide radical scavenging and peroxynitrite radical scavenging. The roselle extracts can be scavenging these RNS species *in vitro* similar to the anthocyanins from grape but tended more scavenging than anthocyanins from black carrot. Moreover, these anthocyanins were found high activity than BHA, ascorbic acid and gallic acid. However, the nitrite scavenging of roselle extracts in meat model depend on the type of meat products. The roselle extracts exhibited higher reduced nitrite in Nham more than Vienna pork sausage. Nitrite residue in Vienna pork sausage was found slightly decreased when refrigerated storage for 28 day, while nitrite residue in Nham was found decrease after the 3 days fermentation. During storage the RAE treated Vienna pork sausage and Nham were found lower in TBARS value. The effect of roselle extracts on nitrite residue in Nham was monitor with difference nitrite concentration. The residue nitrite in Nham model was found similar trend for nitrite reduction in all Nham model with difference

nitrite concentration when fermentation time increased. However, the result showed that the nitrite degradation was rapid degrade when roselle extracts was presented in the Nham model.

CAHPTER 6

SUGGESTIONS

The following recommendations are made for further study:

6.1 The suitable condition of roselle extracts by original-grinding extraction and nano-grinding extraction, and various factors such as pH, solvent, sucrose, temperature, light and other substances should be study and need to develop to stabilize roselle anthocyanins pigment.

6.2 The content of fat (16 %) in Chinese-style sausage which contains 0.3 % roselle extracts, this fat level might be too low to cause a low significant with TBARS values. Therefore, in the future, an original formula Chinese-style sausage (fat > 30 %) may be analyzed to find out the effect of roselle extracts on lipid oxidation in products.

6.3 Further investigation is also necessary in order to clarify the mechanisms of how roselle extracts affects nitrite reduction in meat products because of activity of roselle extracts are different at different pH as in Vienna pork sausage and traditional Thai fermented pork “Nham”

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APPENDIX

Appendix A: Meat products formula

Appendix B: Buffer solution preparation and analytical methods

Appendix C: Sensory evaluation

Appendix D: HPLC chromatogram trace of roselle anthocyanins

Appendix E: Statistical analysis

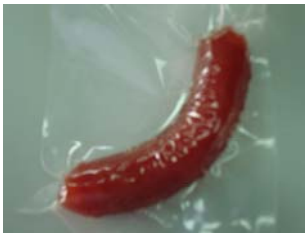
Appendix A

Meat products formula

Appendix A1 Chinese-style sausage formulation by sweeteners treatment (objective 4.2.1)

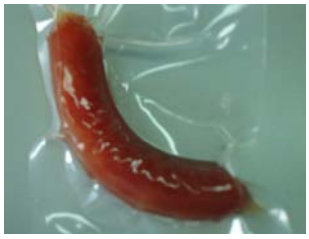
Ingredients	Formulations (Percentage)				
	Control*	**Sucrose**	**Lactitol**	**Maltitol**	**Xylitol**
Lean pork	65	65	65	65	65
Pork lard	16	16	16	16	16
Potassium nitrite	0.2	0.2	0.2	0.2	0.2
Chinese five spices	0.1	0.1	0.1	0.1	0.1
Monosodiumglutamate	0.3	0.3	0.3	0.3	0.3
Salt	1.8	1.8	1.8	1.8	1.8
Sucrose	16.6	16.6	-	-	-
Lactitol	-	-	16.6	-	-
Maltitol	-	-	-	16.6	-
Xylitol	-	-	-	-	16.6
Total (%)	**100**	**100**	**100**	**100**	**100**
Roselle	-	0.3	0.3	0.3	0.3

Control* formula adapted from Pinsirodom (2008)



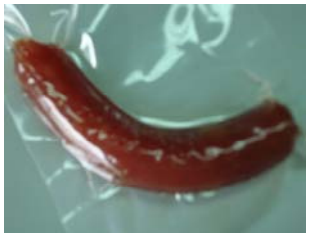
Control sample (Sucrose without RAE)

Sucrose added sample (with 0.3 % RAE)



Lactitol added sample (with 0.3 % RAE)

Maltitol added sample (with 0.3 % RAE)



Xylitol added sample (with 0.3 % RAE)

Figure Appendix A1 Chinese-style sausage formulation by different sweeteners treatment

Appendix A2 Chinese-style sausage formulation by xylitol concentration treatment (objective 4.2.2)

Ingredients	Formulations (Percentage)					
	Control 1	**Control 2**	**Control 3**	**Xylitol 11.6**	**Xylitol 16.6**	**Xylitol 21.6**
Lean pork	77.94	77.94	77.94	77.94	77.94	77.94
Pork lard	19.18	19.18	19.18	19.18	19.18	19.18
Potassium nitrite	0.24	0.24	0.24	0.24	0.24	0.24
Chinese five spices	0.12	0.12	0.12	0.12	0.12	0.12
Monosodiumglutamate	0.36	0.36	0.36	0.36	0.36	0.36
Salt	2.16	2.16	2.16	2.16	2.16	2.16
Total (%)	**100**	**100**	**100**	**100**	**100**	**100**
Sucrose	16.6	16.6	-	-	-	-
Xylitol	-	-	16.6	11.6	16.6	21.6
Roselle	0.3	-	-	0.3	0.3	0.3

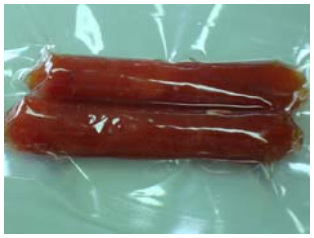
Control = Control samples, Xylitol = Xylitol treatment samples

Control 1 sample

(Sucrose with 0.3 % RAE)

Control 2 sample

(Sucrose without RAE)



Control 3 sample

(Xylitol without RAE)

Xylitol 11.6 %

(11.6 % Xylitol with 0.3 % RAE)

Xylitol 16.6 %

(16.6 % Xylitol with 0.3 % RAE)

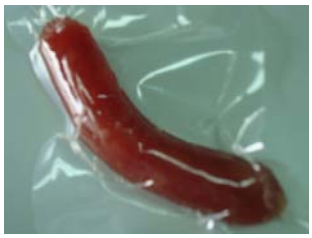
Xylitol 21.6 %

(21.6 % Xylitol with 0.3 % RAE)

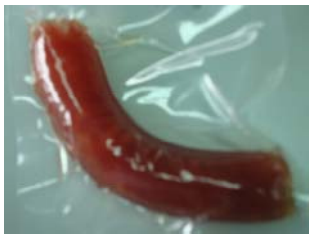
Figure Appendix A2 Chinese-style sausage formulation by xylitol concentration treatment

Appendix A3 Chinese-style sausage formulation by sucrose and xylitol treatment (objective 4.2.3)

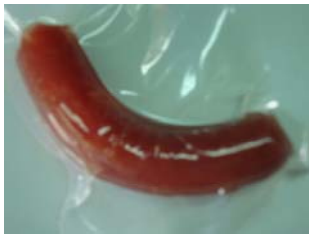
Ingredients (%)	Formulations (Percentage)		
	Control	**Sucrose 16.6**	**Xylitol 16.6**
Lean pork	77.94	77.94	77.94
Pork lard	19.18	19.18	19.18
Potassium nitrite	0.24	0.24	0.24
Chinese five spices	0.12	0.12	0.12
Monosodium glutamate	0.36	0.36	0.36
Salt	2.16	2.16	2.16
Total (%)	**1000**	**1000**	**1000**
Sucrose (%)	16.6	16.6	-
Xylitol (%)	-	-	16.6
Roselle extracts (%)	-	0.3	0.3



Control sample (without RAE)



Sucrose sample (with 0.3 % RAE)



Xylitol sample (with 0.3 % RAE)

Figure Appendix A3 Chinese-style sausage formulation by sucrose and xylitol treatment

Appendix A4 Vienna pork sausage formulation by sodium nitrite treatment (objective 4.3)

Ingredients (%)	Formulations (Percentage)					
	Cont 1*	**Cont 2***	**Ref 1**	**Ref 2**	**Treat 1**	**Treat 2**
Pork meat	54.58	54.58	54.58	54.58	54.58	54.58
Pork lard	21.44	21.44	21.44	21.44	21.44	21.44
Ice	21.44	21.44	21.44	21.44	21.44	21.44
Sodium tripolyphosphate	0.29	0.29	0.29	0.29	0.29	0.29
White pepper	0.29	0.29	0.29	0.29	0.29	0.29
Nutmeg	0.10	0.10	0.10	0.10	0.10	0.10
cardamom	0.05	0.05	0.05	0.05	0.05	0.05
Coriander	0.03	0.03	0.03	0.03	0.03	0.03
Salt	1.00	1.00	1.00	1.00	1.00	1.00
Sugar	0.40	0.40	0.40	0.40	0.40	0.40
Monosodium glutamate	0.19	0.19	0.19	0.19	0.19	0.19
Smoke powder	0.19	0.19	0.19	0.19	0.19	0.19
Total	**100**	**100**	**100**	**100**	**100**	**100**
Sodium nitrite (ppm)	125	250	125	250	125	250
BHA (ppm)	-	-	200	200	-	-
Roselle extracts (%)	-	-	-	-	0.3	0.3

Control formula adapted from Heinz and Hautzinger (2007)

Appendix A5 Thai pork fermented formulation by sodium nitrite treatment (objective 4.3)

Ingredients (%)	Formulations (Percentage)					
	Cont 1*	**Cont 2***	**Ref 1**	**Ref 2**	**Treat 1**	**Treat 2**
Pork	56.86	56.86	56.86	56.86	56.86	56.86
Pork skin	30.62	30.62	30.62	30.62	30.62	30.62
Sticky rice	5.25	5.25	5.25	5.25	5.25	5.25
Garlic	4.37	4.37	4.37	4.37	4.37	4.37
Salt	2.19	2.19	2.19	2.19	2.19	2.19
Sugar	0.44	0.44	0.44	0.44	0.44	0.44
Sodium tripolyphosphate	0.26	0.26	0.26	0.26	0.26	0.26
Total	**100**	**100**	**100**	**100**	**100**	**100**
Sodium nitrite (ppm)	125	250	125	250	125	250
BHA (ppm)	-	-	200	200	-	-
Roselle extracts (%)	-	-	-	-	0.3	0.3

Control formulation adapted from Swetwiwathana et al., (2007)

Appendix A6 Thai pork fermented formulation by sodium nitrite treatment (objective 4.3)

Ingredients	Formulations*									
	%	**C 1**	**C 2**	**C 3**	**R 1**	**R 2**	**R 3**	**T 1**	**T 2**	**T 3**
Pork	56.86	✔	✔	✔	✔	✔	✔	✔	✔	✔
Pork skin	30.62	✔	✔	✔	✔	✔	✔	✔	✔	✔
Sticky rice	5.25	✔	✔	✔	✔	✔	✔	✔	✔	✔
Garlic	4.37	✔	✔	✔	✔	✔	✔	✔	✔	✔
Salt	2.19	✔	✔	✔	✔	✔	✔	✔	✔	✔
Sugar	0.44	✔	✔	✔	✔	✔	✔	✔	✔	✔
Sodium tripolyphosphate	0.26	✔	✔	✔	✔	✔	✔	✔	✔	✔
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Sodium nitrite (ppm)	125	✔	-	-	✔	-	-	✔	-	-
Sodium nitrite (ppm)	250	-	✔	-	-	✔	-	-	✔	
Sodium nitrite (ppm)	500	-	-	✔	-	-	✔	-	-	✔
BHA (ppm)	200	-	-	-	✔	✔	✔	-	-	-
Roselle extracts (%)	0.3	-	-	-	-	-	-	✔	✔	✔

Formulations*; C = Control test, R = Reference test, T = Treatment

Appendix B

Buffer solution preparation and analytical methods

Appendix B1 Mcllvaine's buffer system (pH 2.2-8.0)

Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$, M.W. 210.14); 0.1M-solution contains 21.01 g/l.

Disodium hydrogen phosphate (Na_2HPO_4, M.W. 141.98); 0.2M-solution contains 28.40 g/l, or Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$, M.W. 178.05); 0.2M-solution contains 35.61 g/l.

To prepare 100 ml of the buffer, mix x ml 0.2 M disodium hydrogen phosphate and y ml 0.1 M citric acid as shown below.

pH required	0.2 M Na_2HPO_4 (ml)	0.1 M citric acid (ml)	pH required	0.2 M Na_2HPO_4 (ml)	0.1 M citric acid (ml)
2.2	2.00	98.00	5.2	53.60	46.40
2.4	6.20	93.80	5.4	55.75	44.25
2.6	10.90	89.10	5.6	58.00	42.00
2.8	15.85	84.15	5.8	60.45	39.55
3.0	20.55	79.45	6.0	63.15	36.85
3.2	24.70	75.30	6.2	66.10	33.90
3.4	28.50	71.50	6.4	69.25	30.75
3.6	32.20	67.80	6.6	72.75	27.25
3.8	35.50	64.50	6.8	77.25	22.75
4.0	38.55	61.45	7.0	82.35	17.65
4.2	41.40	58.60	7.2	86.95	13.05
4.4	44.10	55.90	7.4	90.85	9.15
4.6	46.75	53.25	7.6	93.65	6.35
4.8	49.30	50.70	7.8	95.75	4.25
5.0	51.50	48.50	8.0	97.25	2.75

Source: Dawson et al. (2003)

Appendix B2 Sodium carbonate-sodium bicarbonate buffer solutions (pH 8.8-10.6)

Sodium carbonate decahydrate ($Na_2CO_3 \cdot 10H_2O$, M.W. 286.2); 0.1M-solution contains 28.62 g/l.

Sodium carbonate ($NaHCO_3$, M.W. 84.0); 0.1M-solution contains 8.40 g/l.

To prepare 100 ml of the buffer, mix x ml 0.1 M Sodium carbonate and y ml 0.1 M Sodium carbonate as shown below.

pH required	0.1 M Na_2CO_3 (ml)	0.1 M $NaHCO_3$ (ml)
8.8	10	90
9.1	20	80
9.4	30	70
9.5	40	60
9.7	50	50
9.9	60	40
10.1	70	30
10.3	80	20
10.6	90	10

Source: Dawson et al. (2003)

Appendix B3 Ferrous ions chelating ability (FICA)

This method used to determine the ferrous ion chelating activities of roselle anthocyanin. Five-hundred microlitre solution containing extracts was mixed with 1.6 ml of distilled water and then the mixture was reacted with 50 µl of 2 mM $FeCl \cdot 4H_2O$ and 100 µl of 5 mM 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 10 min. The absorbance was read at 562 nm in a UV-Vis spectrophotometer. Five-hundred microlitre of distilled water, instead of roselle extracts solution, was used as control. Lower absorbance of the reaction mixture indicated higher chelating activity. The percentage of chelating activity on inhibition of $ferrozine - Fe^{2+}$ complex formation was calculated by the formula:

$$Ferrous\ ion\ chelating\ capacity\ (\%) = \left[1 - \frac{A_{562nm,sample}}{A_{562nm,control}}\right] x\ 100$$

Appendix B4 Trolox equivalent antioxidant capacity (TEAC) assay

The Trolox equivalent antioxidant capacity assay evaluates the capacity of a crude extract to scavenge $ABTS^{\bullet+}$ radicals. Briefly, a 7 mM solution of ABTS in water was prepared and $ABTS^{\bullet+}$ was formed after the addition of potassium persulfate to the solution at a final concentration of 2.45 mM. After 12–16 hr incubation in darkness at room temperature, the stock solution was diluted with ethanol until an absorbance of 0.7±0.02 at 734 nm was reached. After addition of 4.0 ml of diluted $ABTS^{\bullet+}$ solution to 40 µl of sample (or Trolox standard, 0.5-3 µM), the reaction mixture was incubated for 6 min in cuvett at 37°C. The decrease in absorbance at 734 nm using UV-Vis spectrophotometer (Hitachi U-2001, Japan) was determined at exactly 6 min after initial mixing for all samples. The absorbance of $ABTS^{\bullet+}$ without sample, *i.e.*, the control, also was measured. The TEAC value was calculated using the following formulae:

$$\% \, Inhibition = \left[\frac{Ac - As}{Ac} \right] x \, 100$$

$$TEAC \; value = \frac{\% \, Inhibition}{m}$$

where A_C is the absorbance of the control at t=6 min, A_S is the absorbance of the sample (or Trolox standard) at t=6 min, and *m* is the slope of the standard curve.

Trolox was used as standard and results were calculated based on standard curves such as the one presented here.

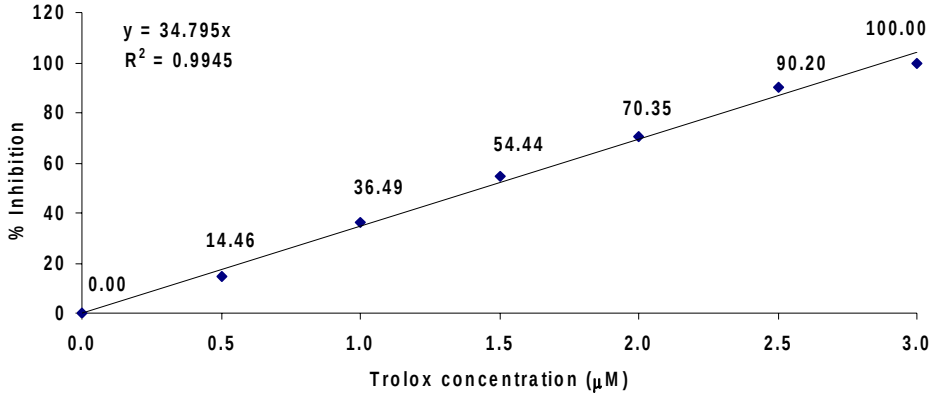


Figure appendix-B1 Trolox equivalent antioxidant capacity (TEAC) standard curve ranging from 0.5-3 micromolar (µM)

Appendix B5 Ferric thiocyanate antioxidant assay (FTC)

The FTC method was used to measure the amount of peroxide at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate. The substance is red, and denser color is indicative of higher absorbance. The TBA method measures free radicals present after peroxide oxidation.

Each sample solution (0.5 ml) was mixed with 0.5 ml of 2.51% linoleic acid in absolute ethanol, 1 ml of 0.05 M phosphate buffer (pH 7), and 0.5 ml of distilled water and placed in a screw capped tube. The reaction mixture was incubated in dark at 40C in an oven. Aliquots of 0.1 ml were taken at every 24 h during incubation and the degree of oxidation was measured by sequentially adding 75% ethanol (9.7 ml), 30% ammonium thiocyanate (0.1 ml) and 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.1 ml). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. The antioxidant activity was calculated as percentage of inhibition relative to the control.

$$OI\ (oxidative\ index) = \frac{Abs_{\ t-96hr}}{Abs_{\ t-0hr}}$$

$$AA\,(Antioxidant\ activity) = \frac{OI_{sample_{t=96hr}}}{OI_{control_{t-96hr}}}\ x\,100$$

$$AA\,(\%) = 100 - \left[\frac{Abs_{sample_{t-96hr}} - Abs_{sample_{t-0hr}}}{Abs_{control_{t-96hr}} - Abs_{control_{t-0hr}}}\right] x\,100$$

Appendix B6 Thiobarbituric acid reactive Ssbstances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined by the modified method of Min et al. (2009). Five-gram samples were weighed into a 50-mL test tube and homogenized with 15 ml of deionized distilled water using the homogenizer (Ultra-Turrax® T25Bbasic, Germany) for 10 s at the highest speed. One milliliter of sample homogenate was transferred to a disposable test tube (13x100 mm), and butylated hydroxyanisole (50 µl, 10%) and TBA/trichloroacetic acid (2 ml) were added. The mixture was vortex and then incubated in a boiling water bath for 15 min to develop color. The sample was cooled in cold water for 10 min, vortex again, and centrifuged for 10 min at 20,000xg. The absorbance of the resulting supernatant solution was determined at 532 nm against a blank containing 1 ml of deionized distilled water and 2 ml of TBA/trichloroacetic acid solution. The amounts of TBARS were expressed as milligrams of malondialdehyde per kilogram of sample. A standard curve was prepared using 1,1,3,3 tetramethoxypropane (TEP).

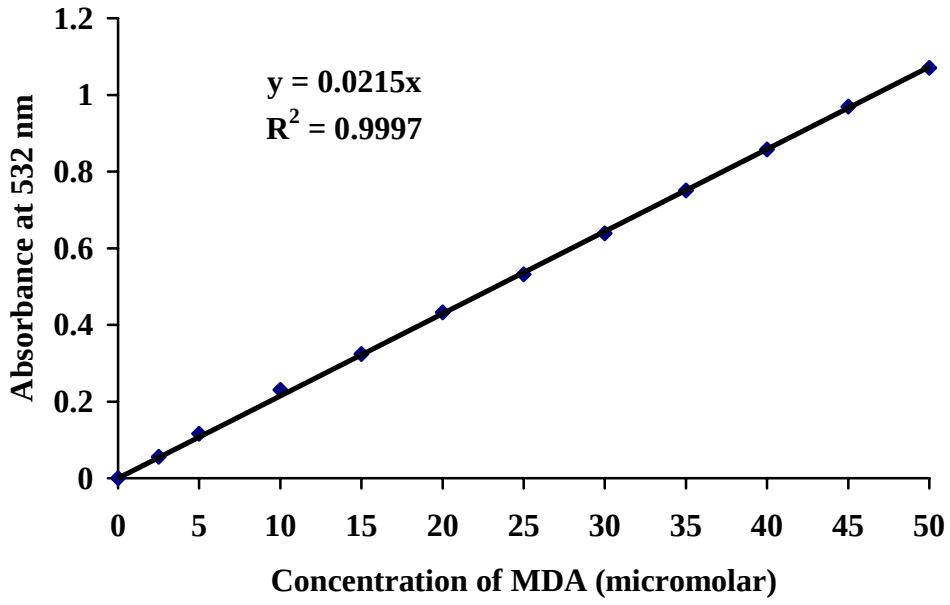


Figure appendix-B2 1,1,3,3 tetramethoxypropane (TEP) standard curve ranging from 0-50 micromolar (µM)

Appendix B7 Protein oxidation (total carbonyls) by 2,4-dinitrophenylhydrazones

Protein oxidation in sausage was followed by measuring the formation of protein carbonyls by converting them to 2,4-dinitrophenylhydrazones (DNPH) and the derivatives were measured spectrophotometrically. Two different measurements were made for protein oxidation: quantification of (a) carbonyls and (b) protein. Meat samples of 1 g were homogenized with 10 ml of 0.15 M potassium chloride (KCl) with the homogenizer (Ultra-Turrax® T25Bbasic, Germany) for 60 s. One ml of homogenate was transferred into a 2 ml Eppendorf vial, where 1 ml of 10% trichloroacetic acid was added. The sample was centrifuged for 5 min at 10,000xg, and the supernatant was removed. For sample (a) 1 ml of 2 M HCl with 0.2% DNPH and for sample (b) 1 ml of 2 M HCl was added. After an incubation of 1 h (shaken every 20 min), 1 ml of 10% trichloroacetic acid (TCA) was added. The sample was vortex and centrifuged for 5 min at 20,000xg. Supernatant was removed carefully without damaging the pellet with a Pasteur pipette. The pellet was washed with 1 ml of ethanol/ethyl acetate (1:1), shaken, and centrifuged for 5 min at 20,000xg; this procedure was repeated two to three times. After this, the pellet was completely dried with nitrogen. The pellet was dissolved in 1.5 ml of 20 mM sodium phosphate buffer with 6 M guanidine hydrochloride, final pH 6.5, shaken, and centrifuged for 2 min at 10,000xg. Carbonyls (sample a) and protein concentration (sample b) were measured at 370 nm and 280 nm, respectively. Concentration of carbonyls was calculated as:

$$Carbonyl\ concentration = \left[\frac{Abs_{370\ nm}}{21.0\ mM^{-1}cm^{-1}}\ x\,1000 \right]$$

Where: $21.0\ mM^{-1}cm^{-1}$ is the molar extinction coefficient of carbonyls. Protein quantification was determined using a standard curve made from BSA.

The inhibitions of roselle extracts against formation of protein carbonyls in Chinese-style sausage was calculated from the equation:

$$\text{Pr}\,otein\ carbonyl\ inhibition\ (\%) = \left(\frac{C_o - C_1}{C_0} \right) x\,100$$

Where: C_0 is the concentration (nM) of protein carbonyls per mg of protein in the control sample and C_1 is the concentration (nM) of protein carbonyls per mg of protein in the tested sample. The inhibitions were expressed as percentages.

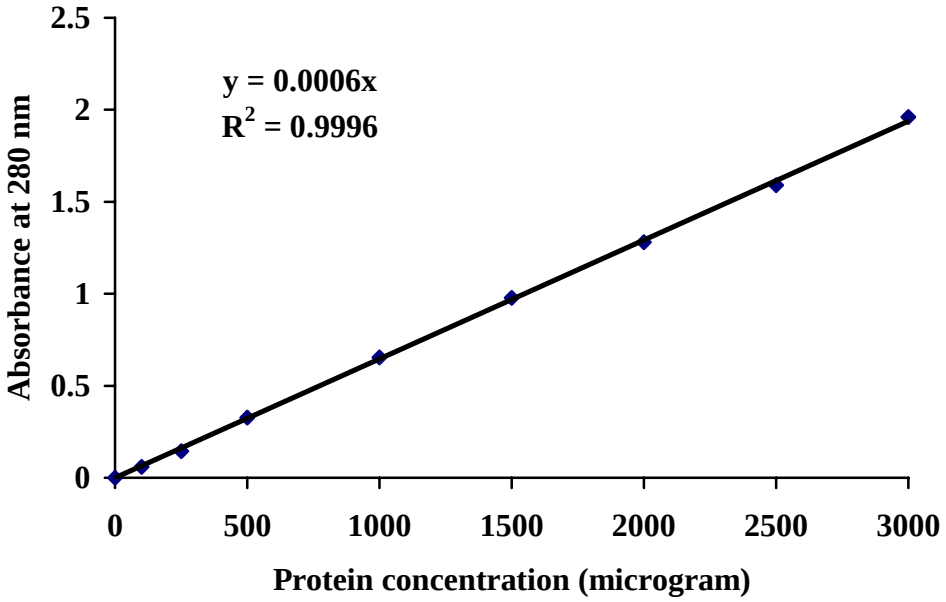


Figure appendix-B3 Bovine serum (BSA) standard curve ranging from 0-3000 microgram

Appendix B8 Nitrite scavenging activity

The anthocyanin extract and/or powder were diluted with the distilled water to a suitable concentration for analysis (up to 200 µg/ml). Three ml of anthocyanin samples were put in the tube (10 ml), then 2 ml of buffer pH 3.0*, 6.0* and 9.0**) and 0.1 ml of 200 µg/ml $NaNO_2$ were added, respectively. Finally, water was added up to 10 ml. The mixture was immediately incubated for 60 min in the water bath at 37°C. Then equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethyline diamine hydrochloride, 2.5% Phosphoric acid) was added to the above mixture. The absorbance was measured, using a Spectrophotometer UV-Vis spectrophotometer (Shimadzu UV-1601, Japan) after 10 min at 538 nm. At the same time the control (without $NaNO_2$) and standard (without $NaNO_2$ and without pigment sample) were also measured. Ascorbic acid and butylated hydroxyanisole (BHA) were used as the positive control compounds. $NaNO_2$ scavenging activity was calculated using the following equation:

$$Sodium\ nitrite\ scavenging\ (\%\ Sa) = \frac{OD_s\left(OD_p - OD_c\right)}{OD_s}\ x\,100$$

Where: Sa is the $NaNO_2$ scavenging rate of tested sample (%), ODs is the OD value of standard, ODp is the OD value in the presence of tested sample and ODc is the OD value of control.

Remark: Buffer pH 3.0 and 6.0 = 0.1 M Citric acid buffer solution pH 3.0 and 6.0, respectively and Buffer pH 9.0 = 0.1 M Sodium carbonate-sodium bicarbonate buffer solutions pH 9.0

Appendix B9 Nitric oxide radical scavenging

Nitric oxide scavenging activity was determined according to Griess Illosvoy reaction. The reaction mixture contained: 10 mM sodium nitroprusside (SNP) in 0.5 M phosphate buffer, pH 7.4, and various doses (0-200 μg/ml) of the test solution (anthocyanin) in a final volume of 3 ml. After incubation for 60 min at 37°C, Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethyline diamine hydrochloride, 2.5% Phosphoric acid) was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with α-napthyl-ethylenediamine was measured, using a Spectrophotometer UV-Vis spectrophotometer (Shimadzu UV-1601, Japan) at 540 nm. Ascorbic acid was used as a positive control. Nitric oxide scavenging ability (%) was calculated by using the formula:

$$Nitric\ oxide\ scavenging\ (\%) = \frac{A_{540\ nm\ of\ control} - A_{540\ nm\ of\ sample}}{A_{540\ nm\ of\ control}}\ x\ 100$$

Appendix B10 Peroxynitrite scavenging activity

Peroxynitrite ($ONOO^-$) was synthesized by the method described by Beckman et al. (1994). An acidic solution 2.11 M H_2O_2 in 1.85 M HNO_3 was mixed with 5 ml 2 M $NaNO_2$ on an ice bath for 1 s and 5 ml of ice-cold 4.2 M NaOH was added. Excess H_2O_2 was removed by treatment with granular manganese dioxide (MnO_2) pre-washed with 1.2 M NaOH and the reaction mixture were left overnight at -20°C. Collect the sample of $ONOO^-$, dilute ~20 times in 0.1 M NaOH and measure the UV/visible absorption spectrum in a quartz cuvette (previously blanked with the NaOH solution alone) between 240 and 400 nm. An absorption band at 302 nm should be evident. Calculate the concentration of $ONOO^-$ was calculated by using the formula:

$$(\varepsilon_{302}\ ONOO^- = 1670\ M^{-1}\ cm^{-1})$$

$$Concentration\ of\ \ peroxynitrite = \frac{\varepsilon_{302nm}}{1670\ M^{-1}cm^{-1}}$$

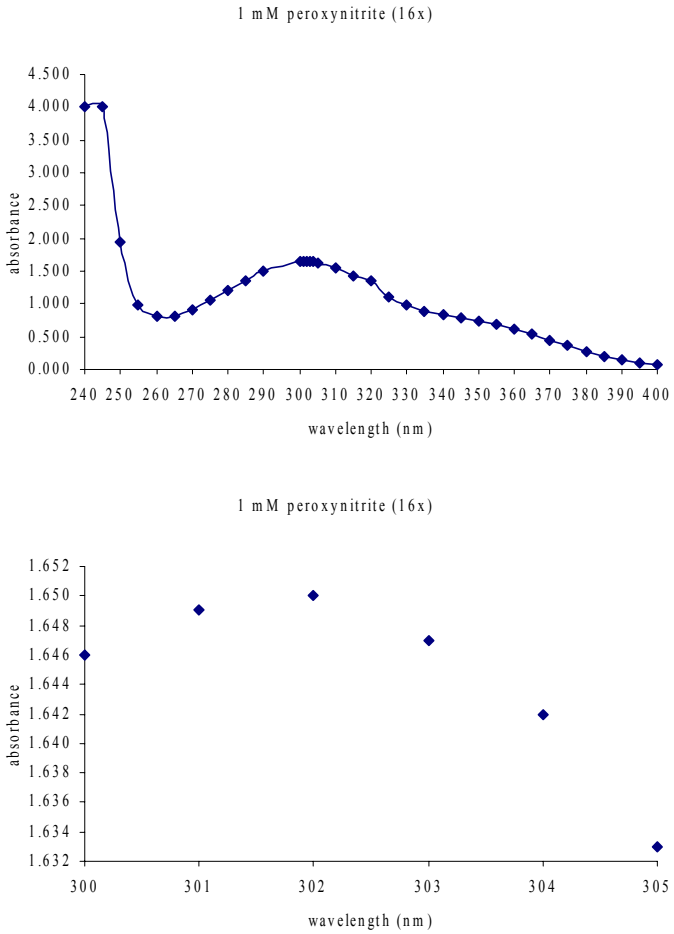


Figure appendix-B4 Absorbance of 1 mM peroxynitrite (16x) [$\lambda_{max\ @\ 301.8} = 1.651$]

An Evans Blue bleaching assay was used to measure peroxynitrite scavenging activity. The assay was performed by a standard method with a slight modification. The reaction mixture contained 50 mM phosphate buffer (pH 7.4) (435 µl), 0.1 mM DTPA (10 µl), 90 mM NaCl (10 µl), 5 mM KCl (10 µl), 12.5 µM Evans Blue (500 µl), various doses of plant extract (0–200 µg/ml) (30 µl) and 1 mM peroxynitrite (5 µl) in a final volume of 1 ml. After incubation at 25°C for 30 min the absorbance was measured, using a Spectrophotometer UV-Vis spectrophotometer (Shimadzu UV-1601, Japan) at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound.

$$Peroxynitrite\ scavenger\ (\%) = \left[\frac{A_0 - A_1}{A_0}\right] x\,100$$

Where: A_0 is the Absorbance of the control, and A_1 is Absorbance in the presence of the sample of extracts and standard.

Appendix B11 Nitrite residue assay

Ground sausage samples (2-5 g) were homogenised with 100 ml of 80°C distilled water in a 250 ml flask, using a homogenizer (Ultra-Turrax® T25Bbasic, Germany) for 60 s at high speed. The homogenate was washed with distilled water to 150 ml totally, sealed with an aluminium foil cap, and heated for 30 min in an 80°C shaking water bath (50 rpm). After cooling with ice water to room temperature, and filtering (Whatman No. 1) immediately, 10 ml of filtrate were transferred into a tube; 2 ml of Griess solution (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethyline diamine hydrochloride, 2.5% Phosphoric acid) were placed in the tube, covered with aluminium-foil, and kept for 30 min. absorbance was measured, using a Spectrophotometer UV-Vis spectrophotometer (Shimadzu UV-1601, Japan) at 540 nm wave-length. The calibration curve was constructed by plotting the absorbance vs. the concentration.

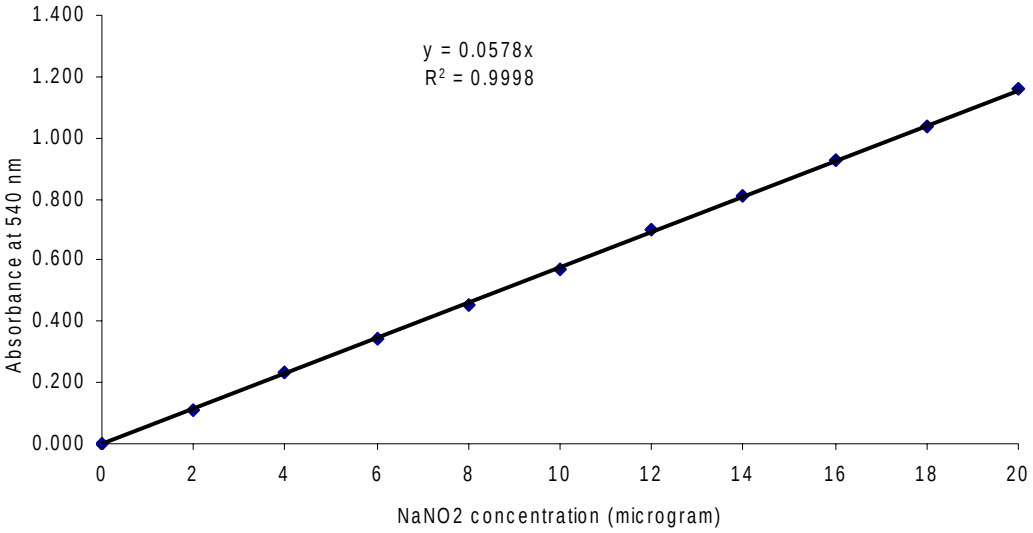


Figure appendix-B5 Sodium nitrite ($NaNO_2$) standard curve ranging from 0-20 microgram

Appendix C

Sensory evaluation

Appendix C1 Questionnaire for sensory evaluation (QDA)

A sensory evaluation test

(Quantitative Descriptive Analysis:QDA)

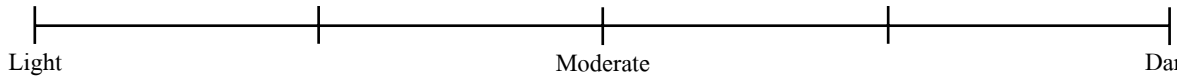
Product: Chinese-style sausage

Name.....Date.....

Instruction: Taste the sausage, one at a time and mark scale of each attribute on the scale line

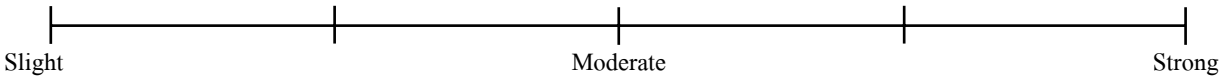
1. Appearance

1.1 Inner color

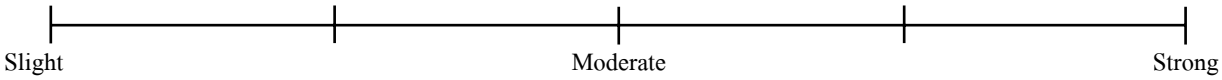


2. Odor

2.1 Normally oder

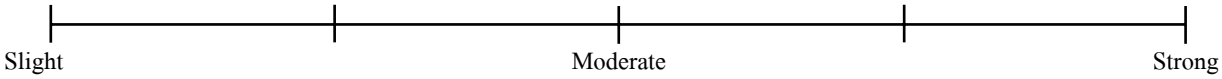


2.2 Rancid oder



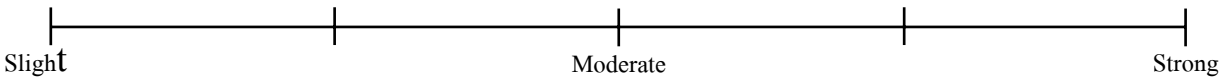
3. Taste

3.1 Sweet

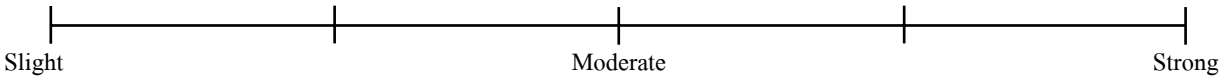


4. Mouthfeel texture

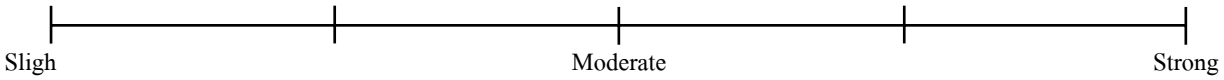
4.1 Hardness



4.2 Gumminess

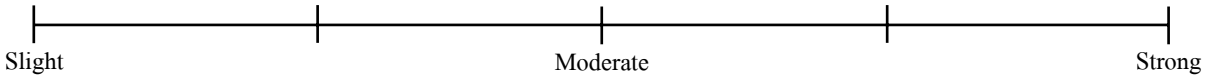


4.3 Chewiness



5. Overall acceptability

5.1 Overall acceptability



Appendix C2 Questionnaire for sensory evaluation (7-point hedonic scale)

A sensory evaluation test

(7-point hedonic scale)

Product: Chinese-style sausage

Name.....Date.....

Instruction: Taste the sample from left to right and put the score

1 = Dislike Extremely

2 = Dislike Moderately

3 = Dislike Slightly

4 = Neither Like nor Dislike

5 = Like Slightly

6 = Like Moderately

7 = Like Extremely

	.....	.....	.....
Color	_____	_____	_____
Odor	_____	_____	_____
Taste	_____	_____	_____
Texture	_____	_____	_____
Overall liking	_____	_____	_____

Suggestion

_____

_____

_____

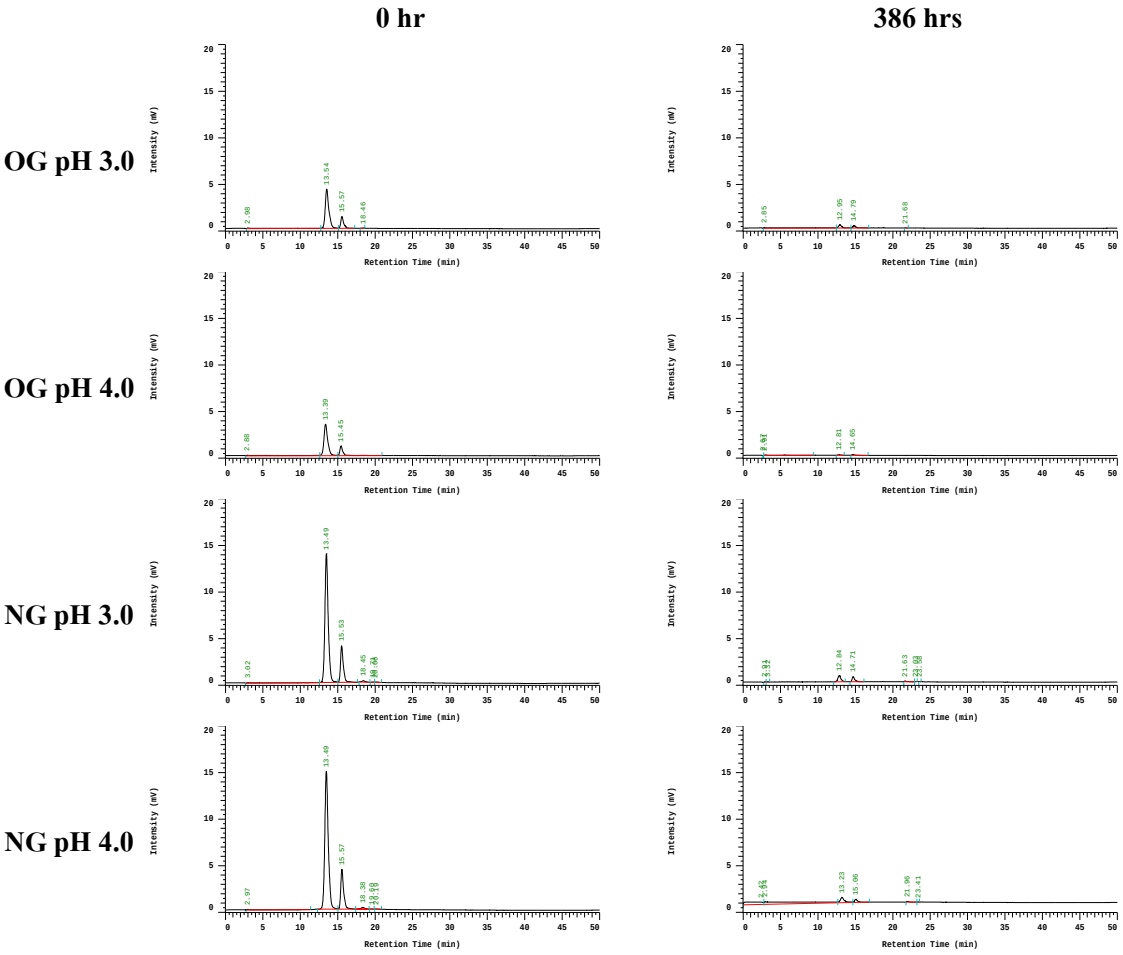
_____

_____

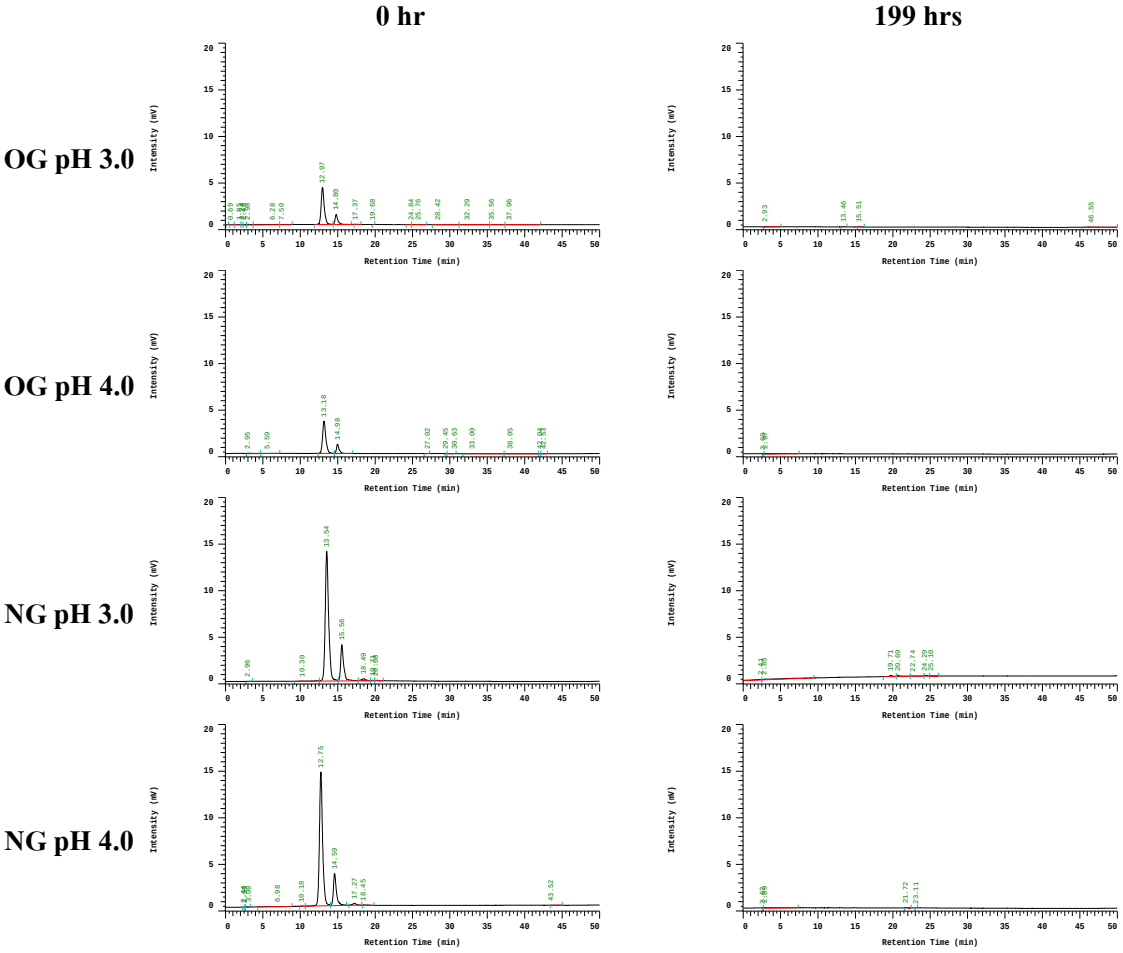
Appendix D

HPLC chromatogram trace of roselle anthocyanins

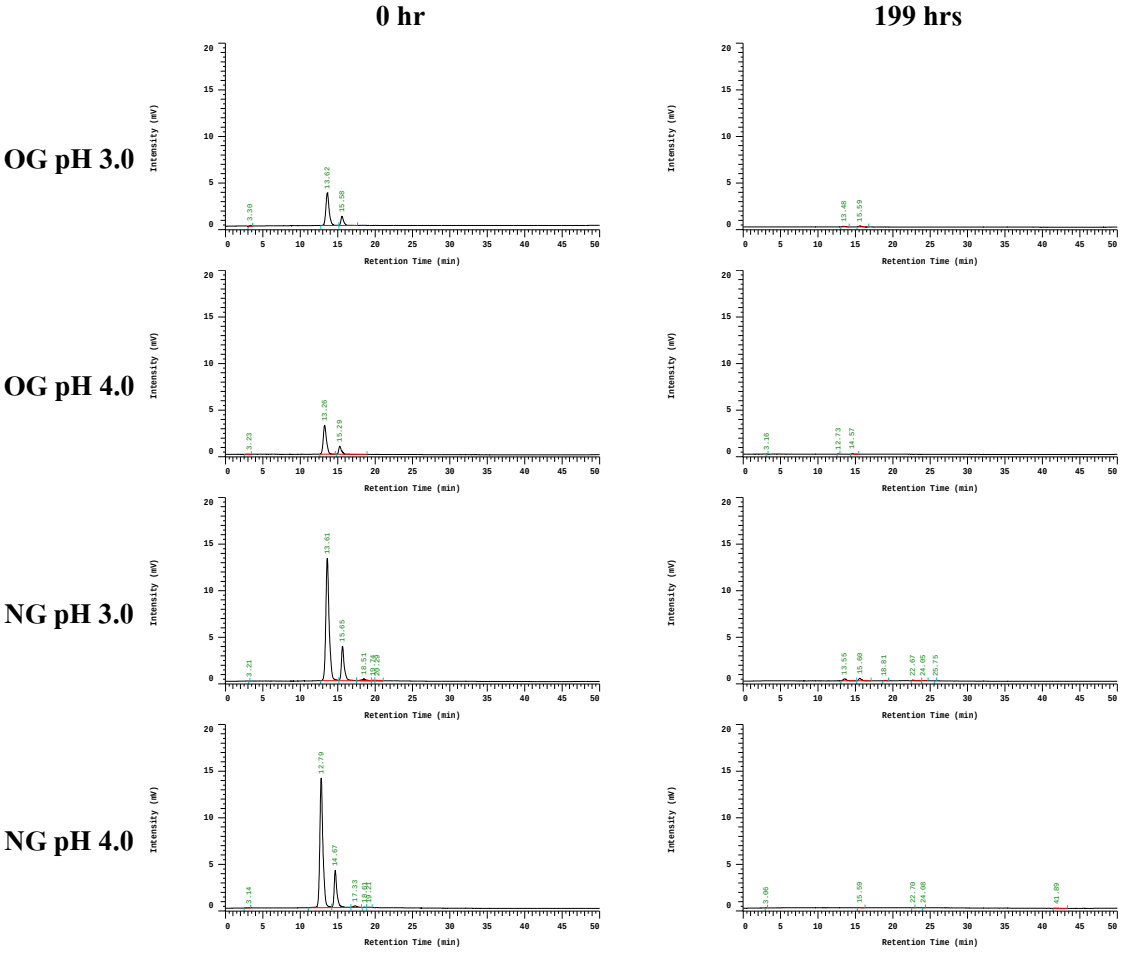
Appendix D1 HPLC chromatogram from roselle anthocyanin prepared in phosphate buffer with sucrose and heat at 50 °C for 0 and 386 hr



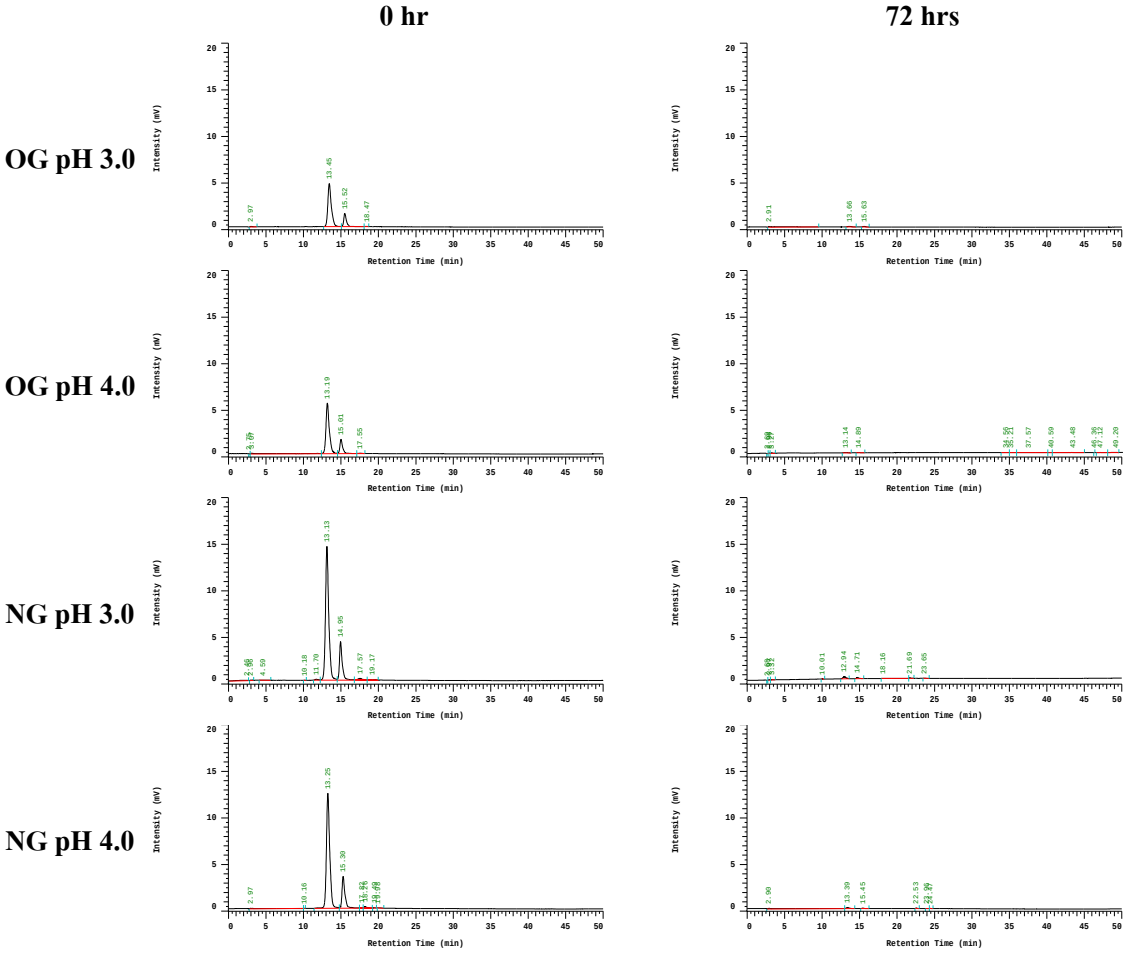
Appendix D2 HPLC chromatogram from roselle anthocyanin prepared in phosphate buffer with sucrose and heat at 60 °C for 0 and 199 hr



Appendix D3 HPLC chromatogram from roselle anthocyanin prepared in phosphate buffer without sucrose and heat at 60 °C for 0 and 199 hr



Appendix D4 HPLC chromatogram from roselle anthocyanin prepared in phosphate buffer with sucrose and heat at 70 °C for 0 and 72 hr



Appendix E

Statistical analysis

Appendix E1 Analysis of variance for Hunter L-value in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	36165.728	207	174.714	713957.594	.000
ACNS	942.373	1	942.373	3850953.080	.000
PH	5139.299	1	5139.299	21001459.451	.000
SUCROSE	1.102	1	1.102	4501.934	.000
TEMP	.000	0	.	.	.
TIME	22160.279	23	963.490	3937249.532	.000
ACNS * PH	9.796	1	9.796	40029.630	.000
ACNS * SUCROSE	10.518	1	10.518	42982.064	.000
PH * SUCROSE	148.092	1	148.092	605171.119	.000
ACNS * PH * SUCROSE	41.205	1	41.205	168382.331	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	424.883	23	18.473	75489.524	.000
PH * TIME	865.331	23	37.623	153744.563	.000
ACNS * PH * TIME	134.128	23	5.832	23830.669	.000
SUCROSE * TIME	454.949	23	19.780	80831.439	.000
ACNS * SUCROSE * TIME	67.157	23	2.920	11931.844	.000
PH * SUCROSE * TIME	220.070	23	9.568	39100.184	.000
ACNS * PH * SUCROSE * TIME	205.415	23	8.931	36496.417	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	.102	416	2.45×10^{-4}		
Corrected Total	36165.830	623			

Appendix E2 Analysis of variance for Hunter a-value in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	144887.793	207	699.941	4918504.522	.000
ACNS	209.892	1	209.892	1474920.157	.000
PH	22416.322	1	22416.322	157520098.850	.000
SUCROSE	3.023	1	3.023	21240.443	.000
TEMP	.000	0	.	.	.
TIME	93321.127	23	4057.440	28511742.587	.000
ACNS * PH	7.840E-02	1	7.840E-02	550.944	.000
ACNS * SUCROSE	29.176	1	29.176	205017.681	.000
PH * SUCROSE	559.859	1	559.859	3934144.637	.000
ACNS * PH * SUCROSE	237.338	1	237.338	1667782.397	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	1532.416	23	66.627	468188.237	.000
PH * TIME	3529.081	23	153.438	1078215.141	.000
ACNS * PH * TIME	412.905	23	17.952	126151.806	.000
SUCROSE * TIME	1146.321	23	49.840	350227.290	.000
ACNS * SUCROSE * TIME	165.649	23	7.202	50609.575	.000
PH * SUCROSE * TIME	702.238	23	30.532	214549.662	.000
ACNS * PH * SUCROSE * TIME	661.282	23	28.751	202036.795	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	5.92×10^{-2}	416	1.42×10^{-4}		
Corrected Total	144887.852	623			

Appendix E3 Analysis of variance for Hunter b-value in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	7540.505	207	36.428	267421.163	.000
ACNS	1933.389	1	1933.389	14193353.250	.000
PH	2.392	1	2.392	17558.391	.000
SUCROSE	10.275	1	10.275	75430.712	.000
TEMP	.000	0	.	.	.
TIME	3316.198	23	144.183	1058469.397	.000
ACNS * PH	.136	1	.136	995.434	.000
ACNS * SUCROSE	4.198	1	4.198	30820.318	.000
PH * SUCROSE	7.986	1	7.986	58629.513	.000
ACNS * PH * SUCROSE	18.900	1	18.900	138745.789	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	161.847	23	7.037	51658.715	.000
PH * TIME	527.872	23	22.951	168487.014	.000
ACNS * PH * TIME	59.750	23	2.598	19071.220	.000
SUCROSE * TIME	85.174	23	3.703	27185.974	.000
ACNS * SUCROSE * TIME	19.492	23	.847	6221.506	.000
PH * SUCROSE * TIME	33.530	23	1.458	10702.184	.000
ACNS * PH * SUCROSE * TIME	31.599	23	1.374	10085.707	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	5.67×10^{-2}	416	1.36×10^{-4}		
Corrected Total	7540.562	623			

Appendix E4 Analysis of variance for chroma in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	91607.438	207	442.548	2209199.667	.000
ACNS	1685.885	1	1685.885	8415938.055	.000
PH	15351.057	1	15351.057	76632477.590	.000
SUCROSE	66.115	1	66.115	330047.311	.000
TEMP	.000	0	.	.	.
TIME	55219.541	23	2400.850	11985041.253	.000
ACNS * PH	30.975	1	30.975	154625.170	.000
ACNS * SUCROSE	39.541	1	39.541	197387.020	.000
PH * SUCROSE	397.541	1	397.541	1984523.174	.000
ACNS * PH * SUCROSE	176.421	1	176.421	880694.386	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	720.794	23	31.339	156443.722	.000
PH * TIME	5217.528	23	226.849	1132430.357	.000
ACNS * PH * TIME	514.536	23	22.371	111676.742	.000
SUCROSE * TIME	889.975	23	38.695	193163.234	.000
ACNS * SUCROSE * TIME	111.973	23	4.868	24302.920	.000
PH * SUCROSE * TIME	496.512	23	21.587	107764.740	.000
ACNS * PH * SUCROSE * TIME	459.526	23	19.979	99737.205	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	8.333E-02	416	2.003E-04		
Corrected Total	91607.521	623			

Appendix E5 Analysis of variance for hue value in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	569293.665	207	2750.211	4394703.269	.000
ACNS	6508.411	1	6508.411	10400123.425	.000
PH	39461.256	1	39461.256	63057167.503	.000
SUCROSE	842.998	1	842.998	1347069.325	.000
TEMP	.000	0	.	.	.
TIME	356345.330	23	15493.275	24757499.998	.000
ACNS * PH	12.404	1	12.404	19820.689	.000
ACNS * SUCROSE	66.108	1	66.108	105636.849	.000
PH * SUCROSE	817.447	1	817.447	1306241.191	.000
ACNS * PH * SUCROSE	128.201	1	128.201	204858.988	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	5652.797	23	245.774	392734.571	.000
PH * TIME	24865.102	23	1081.091	1727531.449	.000
ACNS * PH * TIME	2602.502	23	113.152	180811.842	.000
SUCROSE * TIME	3249.163	23	141.268	225739.303	.000
ACNS * SUCROSE * TIME	1284.371	23	55.842	89233.174	.000
PH * SUCROSE * TIME	2157.934	23	93.823	149924.905	.000
ACNS * PH * SUCROSE * TIME	1713.282	23	74.491	119032.223	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	.260	416	6.26×10^{-4}		
Corrected Total	569293.925	623			

Appendix E6 Analysis of variance for A_{420} in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	1.680	207	8.115E-03	4662.807	.000
ACNS	.935	1	.935	537230.482	.000
PH	.155	1	.155	89045.086	.000
SUCROSE	3.510E-02	1	3.510E-02	20165.171	.000
TEMP	.000	0	.	.	.
TIME	.236	23	1.025E-02	5889.363	.000
ACNS * PH	7.526E-04	1	7.526E-04	432.453	.000
ACNS * SUCROSE	2.957E-03	1	2.957E-03	1698.884	.000
PH * SUCROSE	2.080E-03	1	2.080E-03	1195.151	.000
ACNS * PH * SUCROSE	6.823E-05	1	6.823E-05	39.204	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	2.061E-02	23	8.961E-04	514.879	.000
PH * TIME	.111	23	4.828E-03	2773.842	.000
ACNS * PH * TIME	2.118E-02	23	9.208E-04	529.094	.000
SUCROSE * TIME	4.369E-02	23	1.899E-03	1091.368	.000
ACNS * SUCROSE * TIME	1.070E-02	23	4.653E-04	267.373	.000
PH * SUCROSE * TIME	5.844E-03	23	2.541E-04	146.003	.000
ACNS * PH * SUCROSE * TIME	7.410E-03	23	3.222E-04	185.105	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	7.240E-04	416	1.740E-06		
Corrected Total	1.681	623			

Appendix E7 Analysis of variance for A_{520} in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	28.514	207	.138	125116.587	.000
ACNS	.388	1	.388	352832.620	.000
PH	4.666	1	4.666	4237762.890	.000
SUCROSE	1.295E-03	1	1.295E-03	1175.939	.000
TEMP	.000	0	.	.	.
TIME	17.487	23	.760	690573.309	.000
ACNS * PH	7.202E-03	1	7.202E-03	6541.946	.000
ACNS * SUCROSE	1.600E-02	1	1.600E-02	14530.673	.000
PH * SUCROSE	.116	1	.116	105049.503	.000
ACNS * PH * SUCROSE	3.791E-02	1	3.791E-02	34433.492	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	.436	23	1.898E-02	17237.811	.000
PH * TIME	1.440	23	6.262E-02	56881.154	.000
ACNS * PH * TIME	.232	23	1.009E-02	9161.332	.000
SUCROSE * TIME	.291	23	1.263E-02	11474.604	.000
ACNS * SUCROSE * TIME	3.769E-02	23	1.639E-03	1488.524	.000
PH * SUCROSE * TIME	.151	23	6.571E-03	5968.603	.000
ACNS * PH * SUCROSE * TIME	.147	23	6.385E-03	5799.088	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	4.580E-04	416	1.101E-06		
Corrected Total	28.514	623			

Appendix E8 Analysis of variance for color density in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	38.081	207	.184	7971.938	.000
ACNS	2.378	1	2.378	103035.746	.000
PH	6.507	1	6.507	281974.518	.000
SUCROSE	4.435E-02	1	4.435E-02	1921.872	.000
TEMP	.000	0	.	.	.
TIME	20.983	23	.912	39532.402	.000
ACNS * PH	5.949E-03	1	5.949E-03	257.797	.000
ACNS * SUCROSE	1.473E-02	1	1.473E-02	638.232	.000
PH * SUCROSE	.138	1	.138	5976.544	.000
ACNS * PH * SUCROSE	3.958E-02	1	3.958E-02	1715.303	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	.511	23	2.223E-02	963.153	.000
PH * TIME	2.268	23	9.862E-02	4273.534	.000
ACNS * PH * TIME	.360	23	1.566E-02	678.605	.000
SUCROSE * TIME	.384	23	1.670E-02	723.660	.000
ACNS * SUCROSE * TIME	4.618E-02	23	2.008E-03	87.010	.000
PH * SUCROSE * TIME	.189	23	8.205E-03	355.537	.000
ACNS * PH * SUCROSE * TIME	.181	23	7.870E-03	341.021	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	9.600E-03	416	2.308E-05		
Corrected Total	38.091	623			

Appendix E9 Analysis of variance for degradation index in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	301.589	207	1.457	18366.444	.000
ACNS	4.109	1	4.109	51797.423	.000
PH	24.608	1	24.608	310211.488	.000
SUCROSE	5.059	1	5.059	63768.862	.000
TEMP	.000	0	.	.	.
TIME	189.940	23	8.258	104103.867	.000
ACNS * PH	3.570E-03	1	3.570E-03	45.003	.000
ACNS * SUCROSE	6.765E-03	1	6.765E-03	85.285	.000
PH * SUCROSE	.320	1	.320	4036.039	.000
ACNS * PH * SUCROSE	2.875E-02	1	2.875E-02	362.381	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	2.567	23	.112	1407.169	.000
PH * TIME	6.461	23	.281	3541.021	.000
ACNS * PH * TIME	1.033	23	4.493E-02	566.413	.000
SUCROSE * TIME	6.553	23	.285	3591.516	.000
ACNS * SUCROSE * TIME	.584	23	2.539E-02	320.073	.000
PH * SUCROSE * TIME	1.021	23	4.440E-02	559.757	.000
ACNS * PH * SUCROSE * TIME	.872	23	3.790E-02	477.713	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	3.300E-02	416	7.933E-05		
Corrected Total	301.622	623			

Appendix E10 Analysis of variance for monomeric anthocyanins in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	2219.914	207	10.724	8910.411	.000
ACNS	54.137	1	54.137	44980.436	.000
PH	174.917	1	174.917	145332.840	.000
SUCROSE	4.839	1	4.839	4020.564	.000
TEMP	.000	0	.	.	.
TIME	1451.517	23	63.109	52435.609	.000
ACNS * PH	.416	1	.416	345.400	.000
ACNS * SUCROSE	.384	1	.384	319.125	.000
PH * SUCROSE	2.100	1	2.100	1745.097	.000
ACNS * PH * SUCROSE	1.073	1	1.073	891.468	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	37.238	23	1.619	1345.196	.000
PH * TIME	66.549	23	2.893	2404.074	.000
ACNS * PH * TIME	9.422	23	.410	340.356	.000
SUCROSE * TIME	11.195	23	.487	404.406	.000
ACNS * SUCROSE * TIME	7.362	23	.320	265.950	.000
PH * SUCROSE * TIME	8.675	23	.377	313.380	.000
ACNS * PH * SUCROSE * TIME	7.142	23	.311	258.008	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	.501	416	1.204E-03		
Corrected Total	2220.415	623			

Appendix E11 Analysis of variance for polymeric anthocyanins in roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	2610.047	207	12.609	4785.793	.000
ACNS	53.039	1	53.039	20131.245	.000
PH	195.770	1	195.770	74305.644	.000
SUCROSE	6.926	1	6.926	2628.630	.000
TEMP	.000	0	.	.	.
TIME	1728.251	23	75.141	28520.347	.000
ACNS * PH	.162	1	.162	61.436	.000
ACNS * SUCROSE	.319	1	.319	120.895	.000
PH * SUCROSE	4.390	1	4.390	1666.117	.000
ACNS * PH * SUCROSE	.650	1	.650	246.735	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	35.822	23	1.557	591.154	.000
PH * TIME	74.091	23	3.221	1222.682	.000
ACNS * PH * TIME	11.189	23	.486	184.650	.000
SUCROSE * TIME	14.093	23	.613	232.566	.000
ACNS * SUCROSE * TIME	8.364	23	.364	138.030	.000
PH * SUCROSE * TIME	14.960	23	.650	246.872	.000
ACNS * PH * SUCROSE * TIME	7.671	23	.334	126.593	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	1.096	416	2.635E-03		
Corrected Total	2611.143	623			

Appendix E12 Analysis of variance for ferric ions chelating ability of roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	6791.000	71	95.648	141.622	.000
ACNS	650.694	1	650.694	963.454	.000
PH	201.376	1	201.376	298.169	.000
SUCROSE	4.770	1	4.770	7.063	.009
TEMP	.000	0	.	.	.
TIME	1995.220	6	332.537	492.372	.000
ACNS * PH	1304.195	1	1304.195	1931.063	.000
ACNS * SUCROSE	738.298	1	738.298	1093.165	.000
PH * SUCROSE	429.317	1	429.317	635.670	.000
ACNS * PH * SUCROSE	22.042	1	22.042	32.636	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	165.812	6	27.635	40.918	.000
PH * TIME	36.179	6	6.030	8.928	.000
ACNS * PH * TIME	52.010	6	8.668	12.835	.000
SUCROSE * TIME	95.227	6	15.871	23.500	.000
ACNS * SUCROSE * TIME	263.075	6	43.846	64.921	.000
PH * SUCROSE * TIME	38.505	6	6.418	9.502	.000
ACNS * PH * SUCROSE * TIME	63.889	6	10.648	15.766	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	97.254	144	.675		
Corrected Total	6888.255	215			

Appendix E13 Analysis of variance for Trolox equivalence antioxidant capacity of roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	64.289	71	.905	1287.587	.000
ACNS	52.688	1	52.688	74921.806	.000
PH	.874	1	.874	1242.841	.000
SUCROSE	7.180	1	7.180	10209.246	.000
TEMP	.000	0	.	.	.
TIME	1.440	6	.240	341.366	.000
ACNS * PH	.235	1	.235	333.735	.000
ACNS * SUCROSE	.259	1	.259	368.337	.000
PH * SUCROSE	.714	1	.714	1015.513	.000
ACNS * PH * SUCROSE	.378	1	.378	537.996	.000
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	1.168E-02	6	1.947E-03	2.768	.014
PH * TIME	1.601E-02	6	2.668E-03	3.794	.002
ACNS * PH * TIME	1.706E-02	6	2.843E-03	4.043	.001
SUCROSE * TIME	5.928E-02	6	9.880E-03	14.049	.000
ACNS * SUCROSE * TIME	2.126E-02	6	3.544E-03	5.039	.000
PH * SUCROSE * TIME	1.225E-02	6	2.041E-03	2.903	.011
ACNS * PH * SUCROSE * TIME	2.553E-02	6	4.255E-03	6.051	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	.101	144	7.032E-04		
Corrected Total	64.391	215			

Appendix E14 Analysis of variance for ferrous thiocyanate of roselle extracts prepared in different pH with and without sucrose during heating 50 to 70 °C

Source	SS	df	MS	F	Sig.
Corrected Model	768.523	71	10.824	20.685	.000
ACNS	1.251	1	1.251	2.391	.124
PH	10.560	1	10.560	20.181	.000
SUCROSE	30.150	1	30.150	57.618	.000
TEMP	.000	0	.	.	.
TIME	339.828	6	56.638	108.236	.000
ACNS * PH	.768	1	.768	1.468	.228
ACNS * SUCROSE	.600	1	.600	1.146	.286
PH * SUCROSE	3.481	1	3.481	6.652	.011
ACNS * PH * SUCROSE	2.880	1	2.880	5.503	.020
ACNS * TEMP	.000	0	.	.	.
PH * TEMP	.000	0	.	.	.
ACNS * PH * TEMP	.000	0	.	.	.
SUCROSE * TEMP	.000	0	.	.	.
ACNS * SUCROSE * TEMP	.000	0	.	.	.
PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP	.000	0	.	.	.
ACNS * TIME	9.362	6	1.560	2.982	.009
PH * TIME	6.463	6	1.077	2.059	.062
ACNS * PH * TIME	25.232	6	4.205	8.036	.000
SUCROSE * TIME	27.773	6	4.629	8.846	.000
ACNS * SUCROSE * TIME	11.507	6	1.918	3.665	.002
PH * SUCROSE * TIME	3.050	6	.508	.972	.447
ACNS * PH * SUCROSE * TIME	23.641	6	3.940	7.530	.000
TEMP * TIME	.000	0	.	.	.
ACNS * TEMP * TIME	.000	0	.	.	.
PH * TEMP * TIME	.000	0	.	.	.
ACNS * PH * TEMP * TIME	.000	0	.	.	.
SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * SUCROSE * TEMP * TIME	.000	0	.	.	.
PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
ACNS * PH * SUCROSE * TEMP * TIME	.000	0	.	.	.
Error	75.353	144	.523		
Corrected Total	843.875	215			

Appendix E15 Correlations among physicochemical properties of OG and NG roselle extracts

	L-value	a-value	Chroma	A420nm	A520nm	CD	DI	Mono-ACNs	Poly-ACNs
Roselle extract	-.649**	.430**	.586**	.944**	.521**	.677**	-.125**	-.117*	.080
pH	.163**	-.220**	-.188**	-.050	-.195**	-.164**	.211**	-.224**	.229**
Sucrose	-.155**	.173**	.162**	.032	-.127**	.032	.174**	.118**	-.126**
Temperature	.198**	-.186**	-.177**	-.007	-.039	-.136**	.245**	-.118**	.130**
Time	.399**	-.438**	-.386**	-.054	-.242**	-.307**	.456**	-.420**	.429**
L-value	1	-.952**	-.981**	-.723**	-.857**	-.971**	.708**	-.549**	.573**
a-value		1	.961**	.529**	.861**	.918**	-.779**	.704**	-.719**
Chroma			1	.675**	.882**	.971**	-.676**	.554**	-.574**
A420nm				1	.680**	.802**	-.222**	-.038	.003
A520nm					1	.928**	-.580**	.496**	-.506**
Color density (CD)						1	-.573**	.460**	-.481**
Degradation index (DI)							1	-.827**	.856**
Monomeric-ACNs								1	-.995**

*, ** Correlation is significant at the 0.05 and 0.01 level, respectively.

Appendix E16 Correlation among antioxidative capacities of the OG and NG roselle extracts

	FICA[1]	TEAC[2]	FTC[3]
Roselle extract	.307**	.905**	.039
pH	.171*	.117	-.112
Sucrose	.026	.334**	-.189**
Temperature	-.237**	.002	-.293**
Time	-.382**	-.044	-.424**

FICA[1] = Ferrous Ions Chelating Ability (% inhibition). TEAC[2] = Trolox Equivalence Antioxidant Capacity, FTC[3] = Ferric Thiocyanate Method (Antioxidant activity in linoleic acid emulsion system).

*, ** Correlation is significant at the 0.05 and 0.01 level, respectively.

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