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.

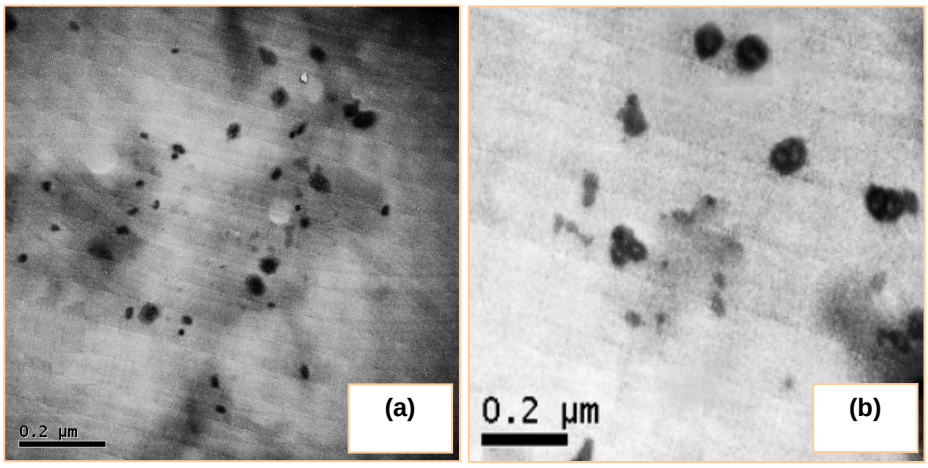


Figure 4.1 Transmission electron micrographs (TEM) of roselle extracts with primary particle size (a) and secondary particle size (b) after nano-grinding

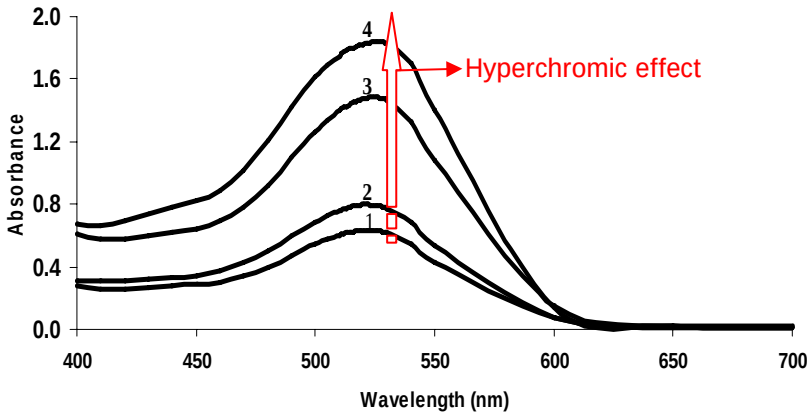


Figure 4.2 The UV-spectrum of roselle extracts in different pH citrate-phosphate buffer solution, 1 = OG in buffer pH 4.0, 2 = OG in buffer pH 3.0, 3 = NG in buffer pH 4.0 and 4 = NG in buffer pH 3.0

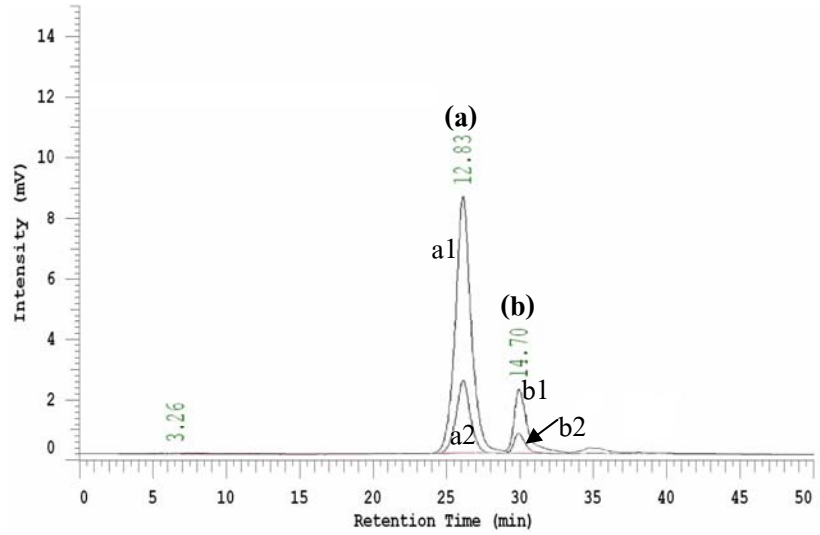


Figure 4.3 HPLC chromatogram of anthocyanins in roselle extracts (a) Delphinidin 3-sambubioside (Dp 3-sam) and (b) Cyaniding 3-sambubioside (Cy 3-sam); (a1) Dp 3-sam of NG roselle, ((b1) Cy 3-sam of NG roselle, (a2) Dp 3-sam of OG roselle and (b2) Cy 3-sam of OG roselle

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.

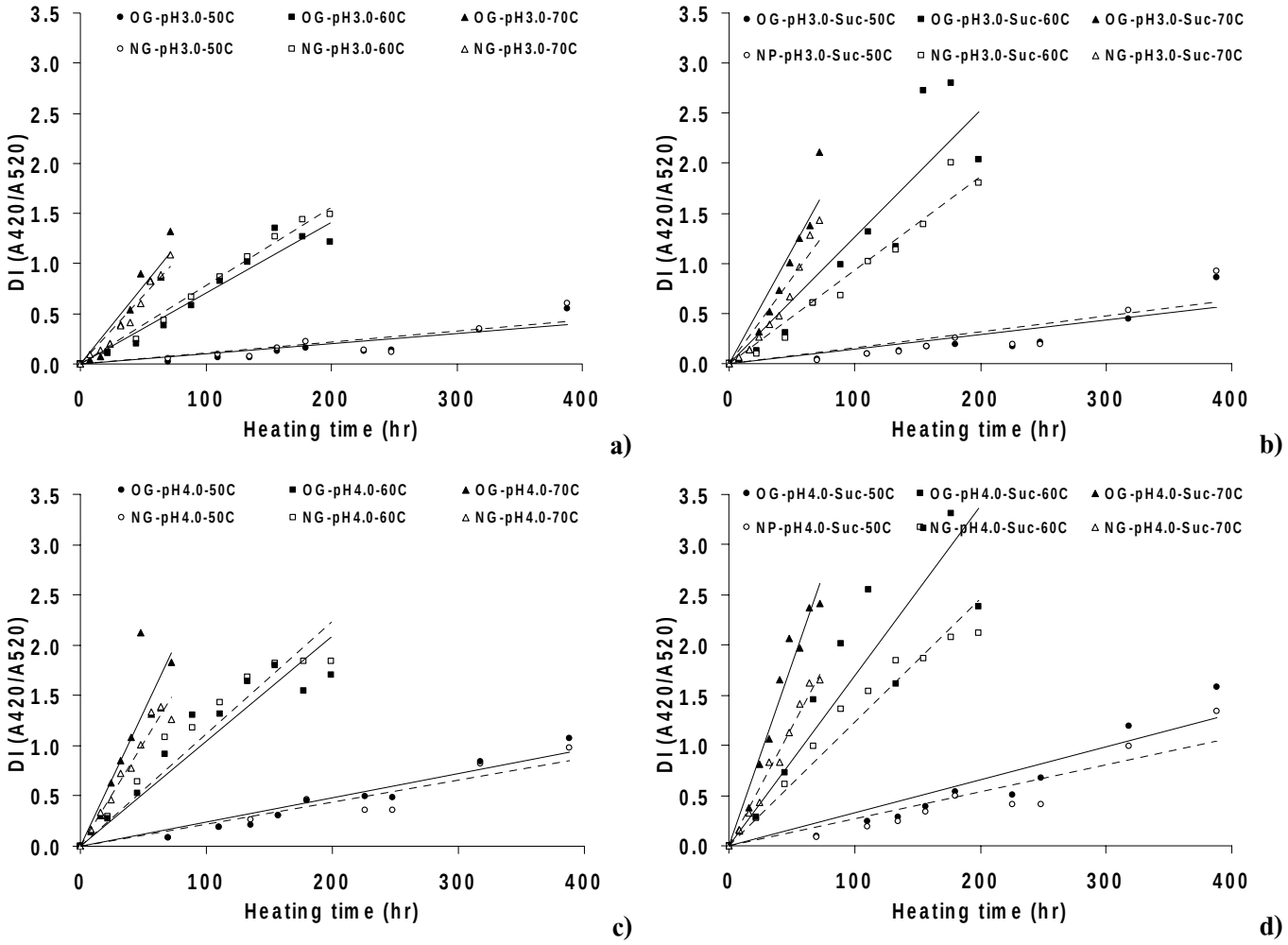


Figure 4.4 Degradation indexes of OG and NG roselle extracts in model solution at pH 3.0 (a) pH 3.0 with 20 % sucrose (b) and OG and NG roselle extracts in model system at pH 4.0 (c) pH 4.0 with 20 % sucrose (d) during heating at 50, 60 and 70 °C

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.

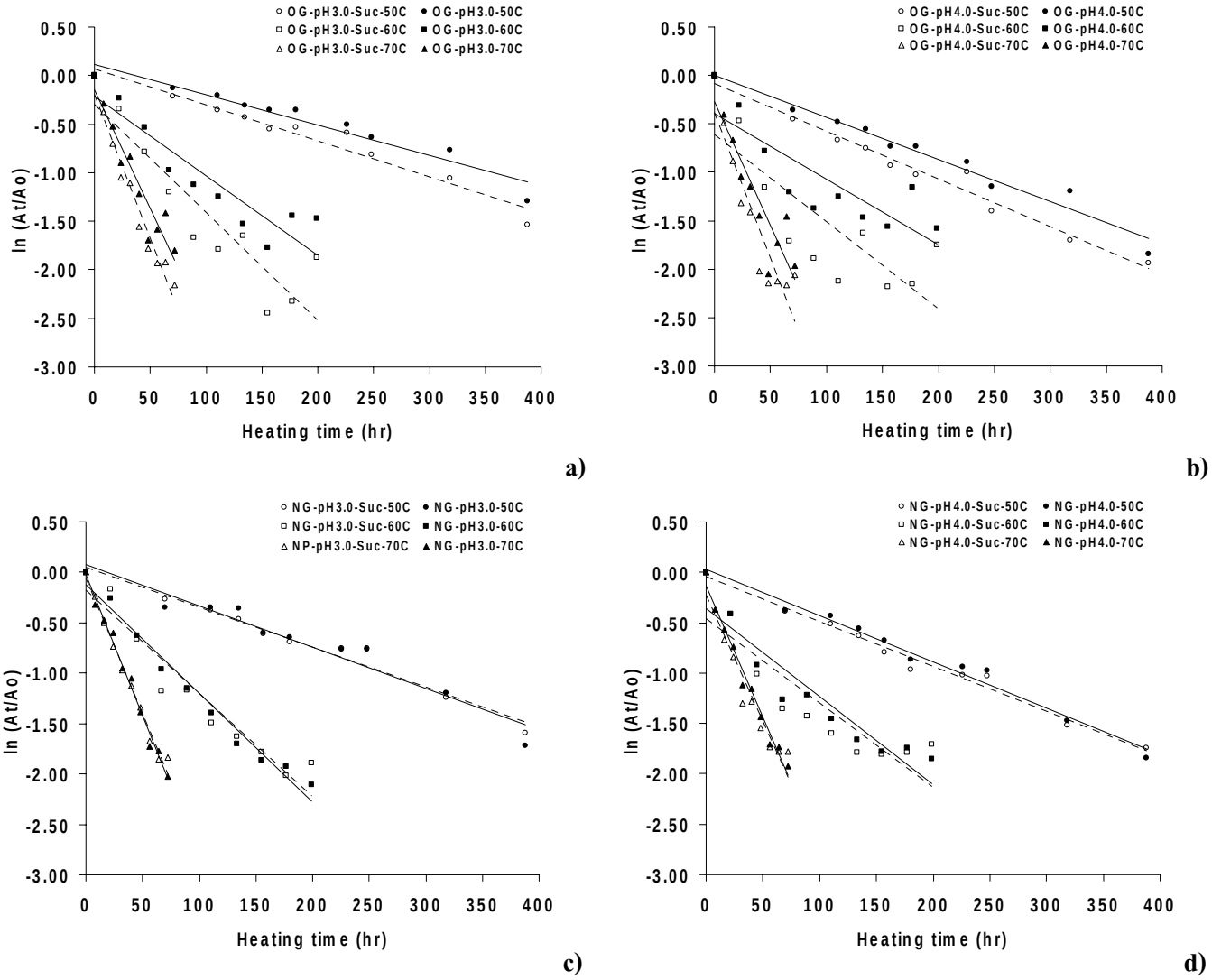


Figure 4.5 Thermal degradation of anthocyanins from OG roselle extract in model solution at pH 3.0 (a) pH 4.0 (b) and from NG roselle extract in model solution at pH 3.0 (c) pH 4.0 (d) during heating at 50 to 70 °C

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)

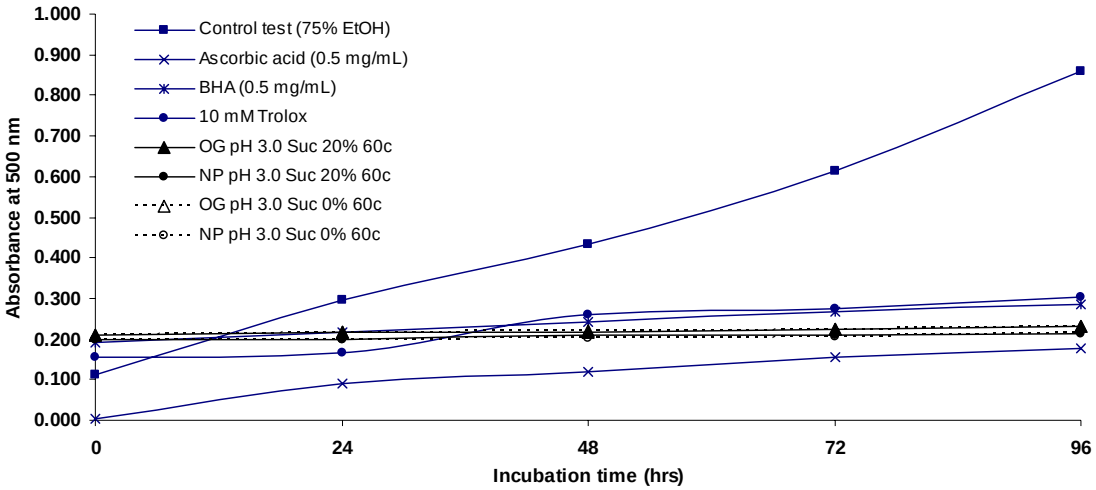


Figure 4.6 Antioxidant properties determined by the ferric thiocyanate method for OG and NG roselle prepared in model solution at pH 3.0 with sucrose after heating at 60 °C, ascorbic acid, BHA and Trolox® was used as positive references

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.

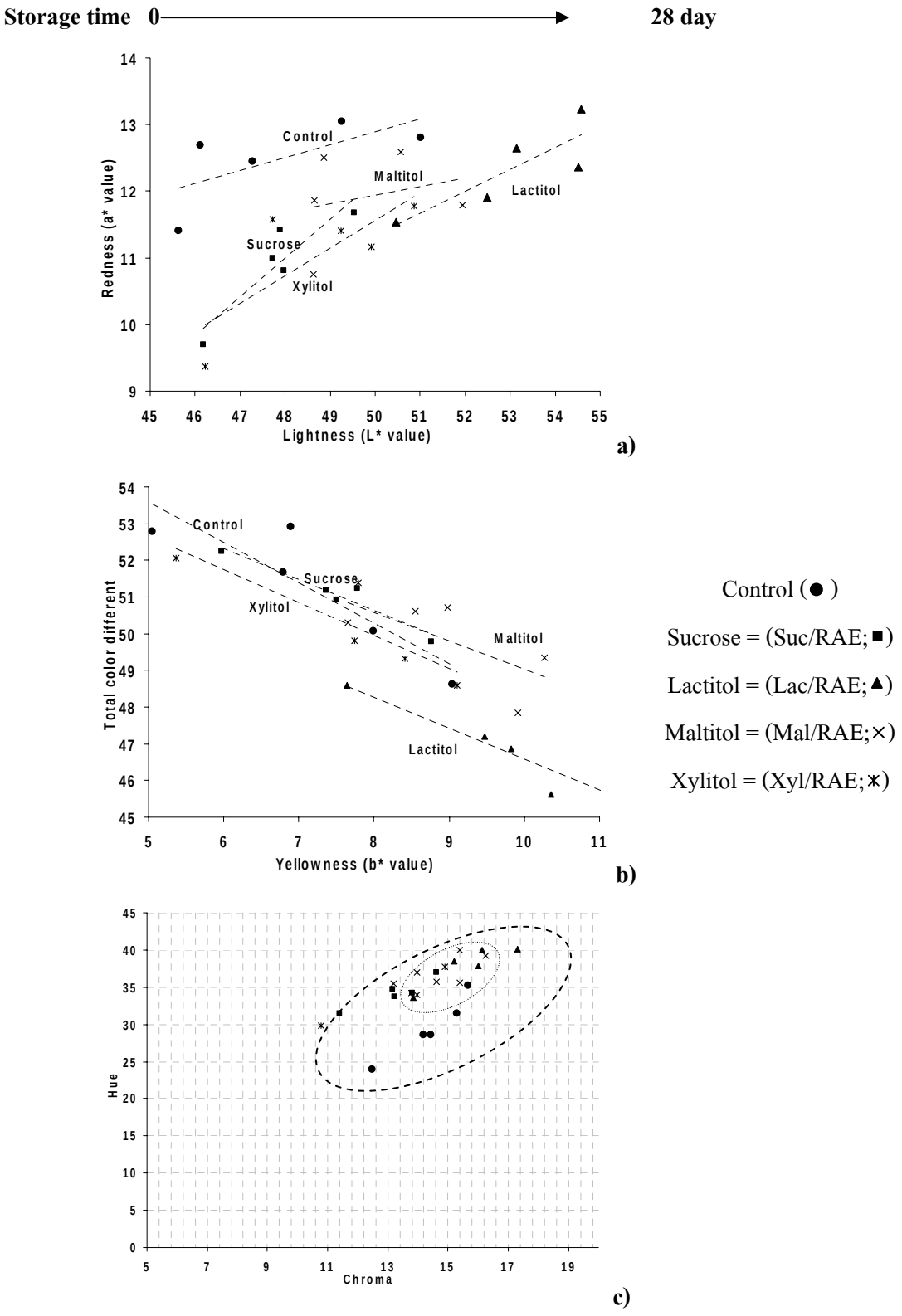


Figure 4.7 Changes of color parameters of Chinese-style sausage as affected by sweeteners during storage at 30±1 °C, a) figure plotted between lightness vs redness , b) yellowness vs total color difference and c) chroma vs hue, n=6. Average coefficient of variation (CV) = 10.25 %

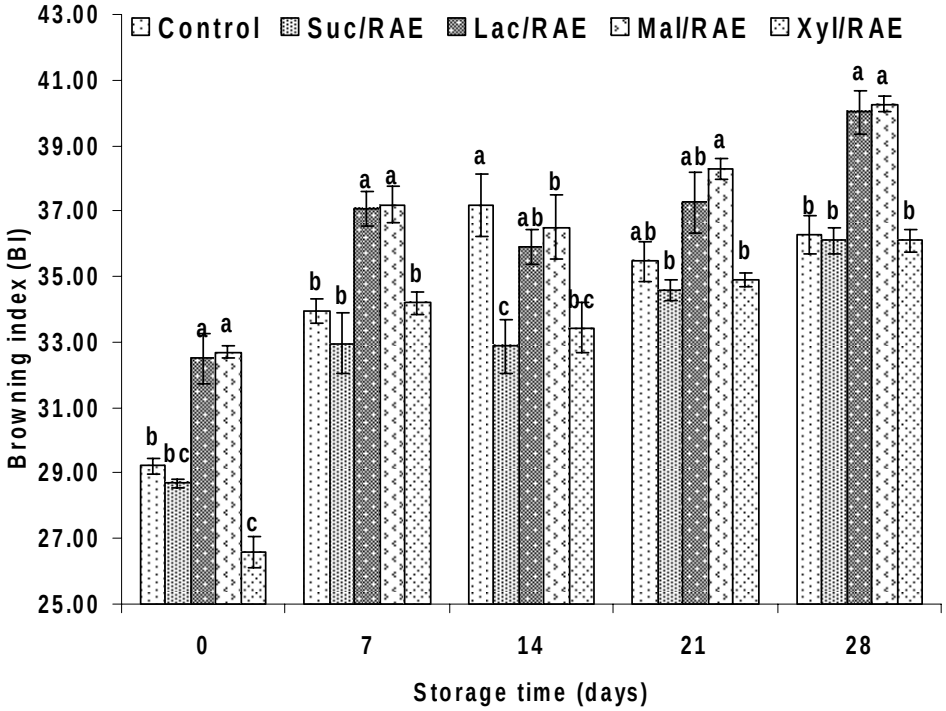


Figure 4.8 Changes of browning index of Chinese-style sausage as affected by sweeteners during storage at 30±1 °C. Bars with different letters are significantly different ($p<0.05$), n=6. Average coefficient of variation (CV) = 10.54 %

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.

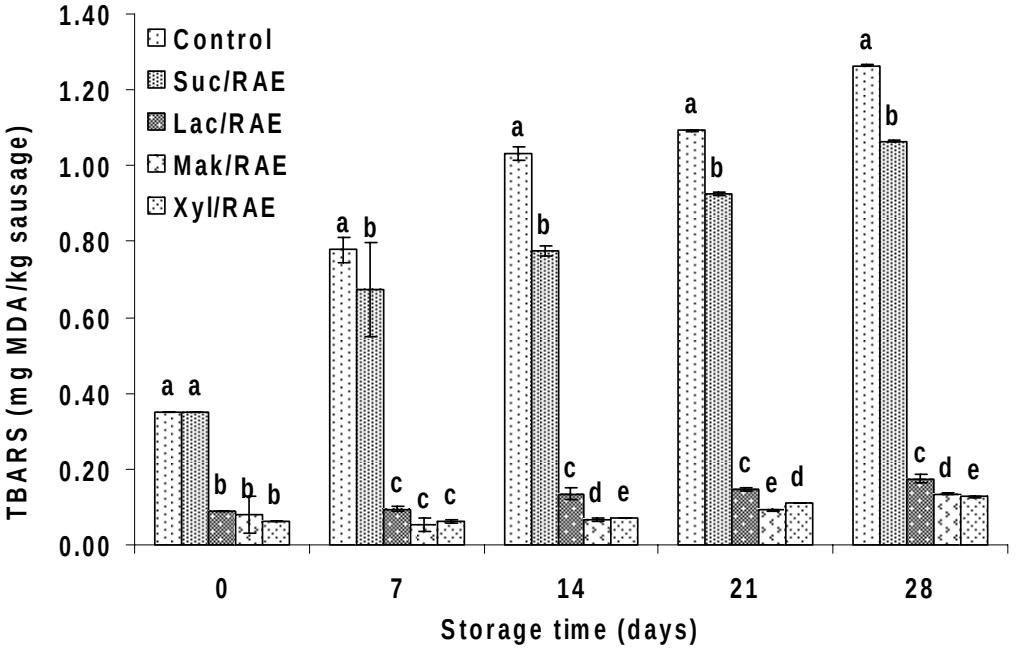


Figure 4.9 Changes of TBARS values of RAE treated Chinese-style sausage with different sweeteners during storage at 30±1 °C. Bars with different letters is significantly different ($p< 0.05$), n=6

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.

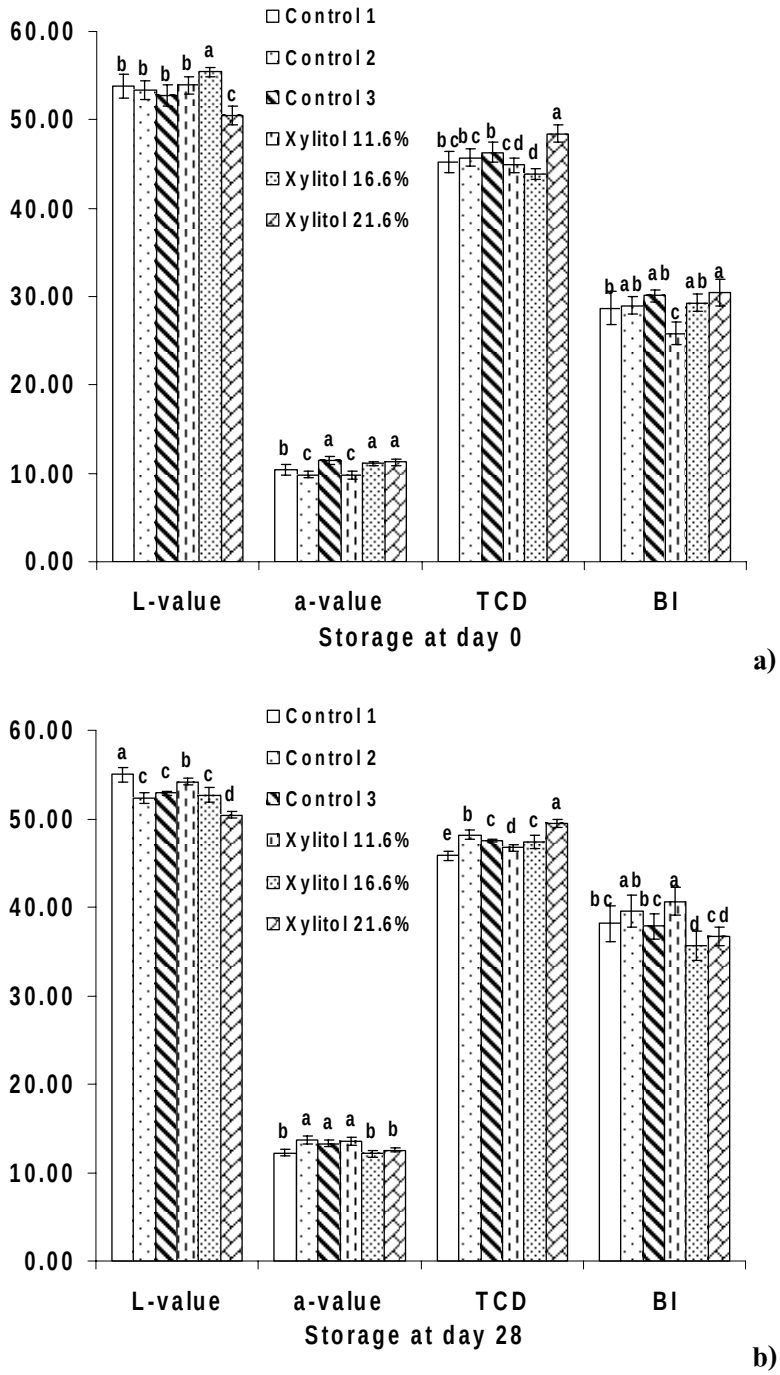


Figure 4.10 Changes of color parameters of RAE treated Chinese-style sausage as affected by different xylitol concentration during storage at 30±1 °C. Bars with different letters are significantly different ($p<0.05$), n=6

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.

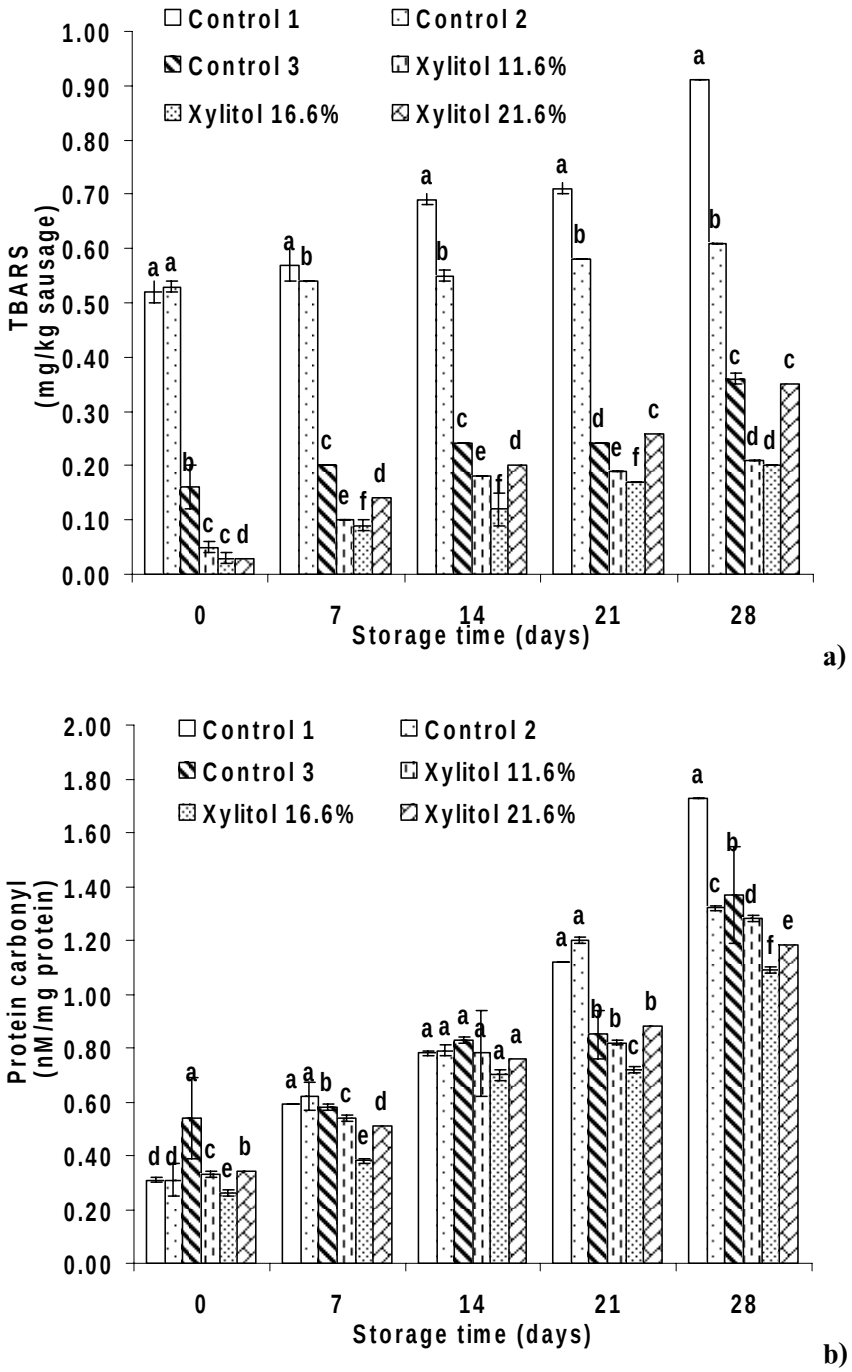


Figure 4.11 Effect of different xylitol concentration on a) lipid oxidation by TBARS values and b) protein carbonyl of Chinese-style sausage during 0-28 day storage at 30±1 °C. Storage time (day) with different letters is significantly different ($p< 0.05$), *n=6*. Average coefficient of variation (CV)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

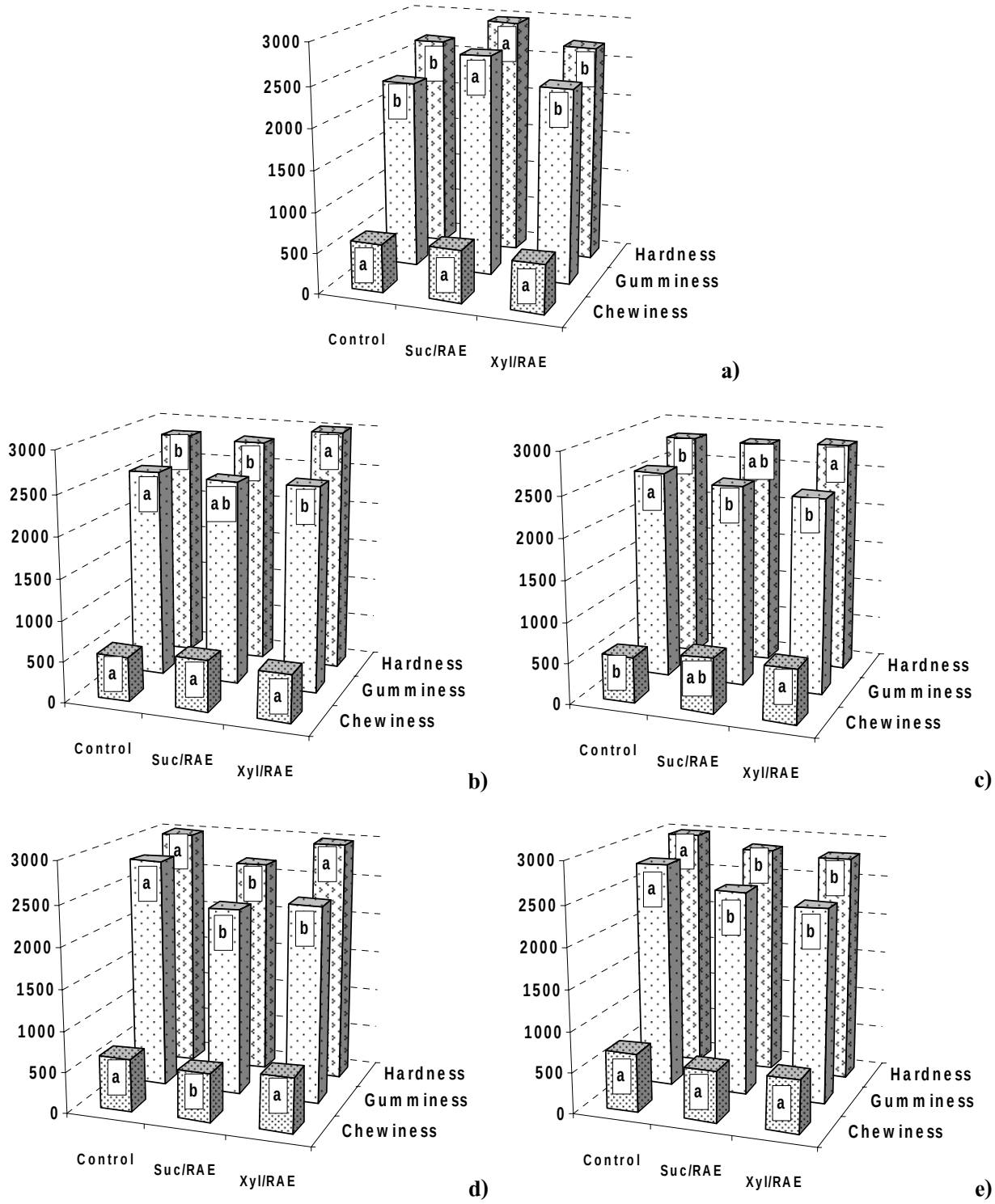


Figure 4.12 Change of hardness (N), gumminess (N) and chewiness (N*mm) of roasted RAE treated Chinese-style sausage with sucrose or xylitol addition during storage for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days at 31±1 °C. Within parameter, bars with different letters are significantly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

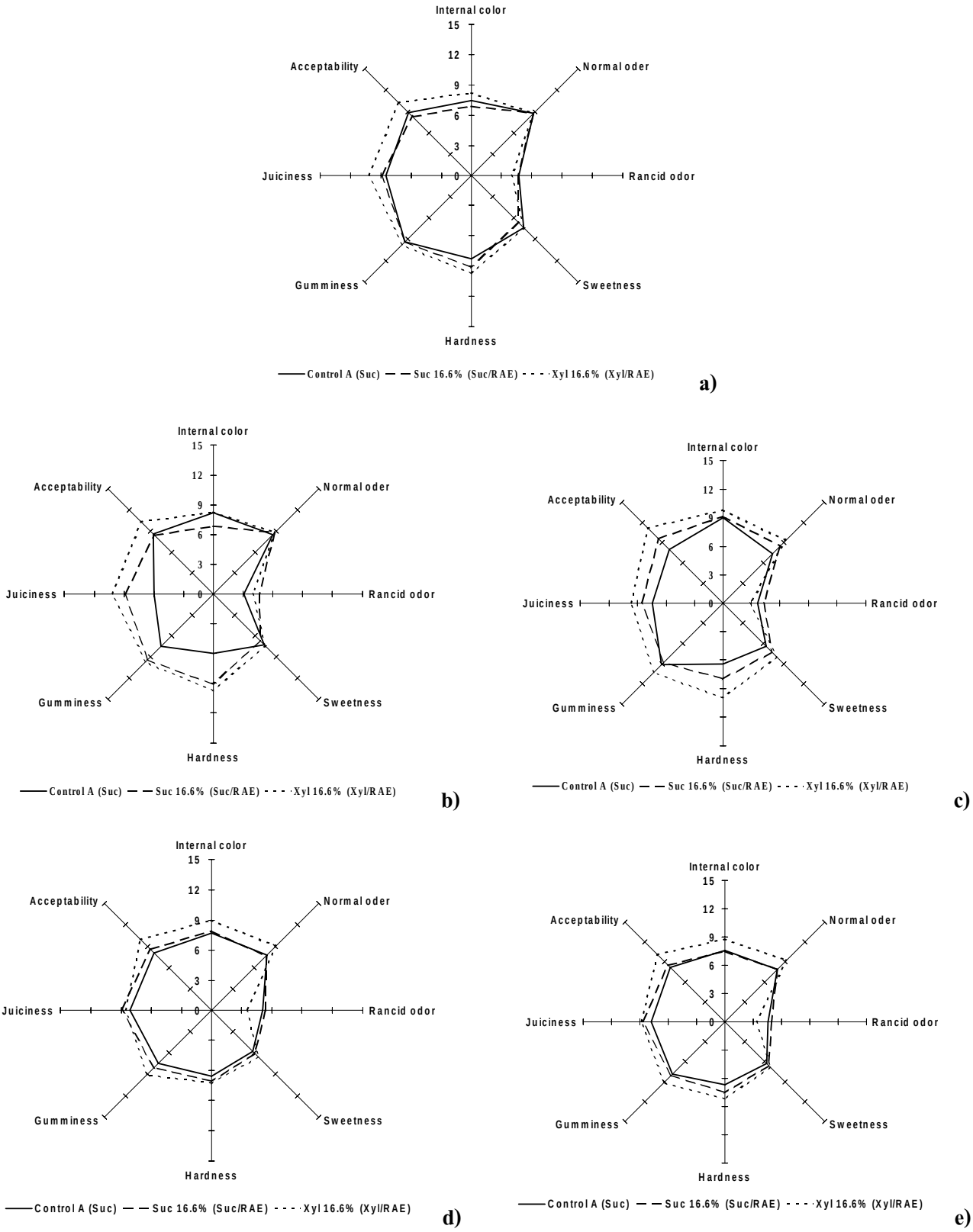


Figure 4.13 Quantitative descriptive analysis of roasted RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days

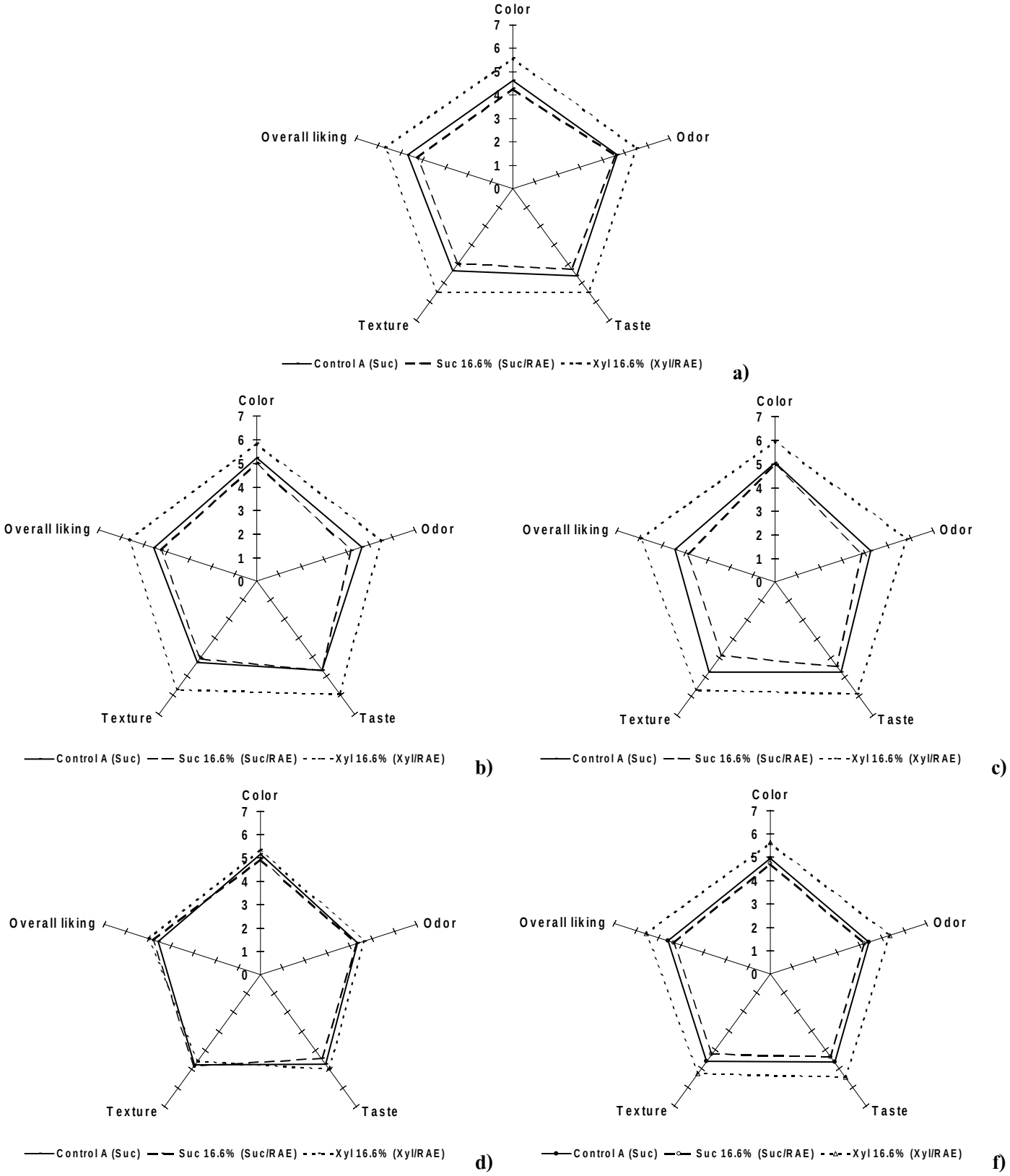


Figure 4.14 Sensory evaluation by 7-point hedonic scale of RAE treated Chinese-style sausage with sucrose or xylitol addition during storage at 31±1 °C for a) 0 day, b) 7 days, c) 14 days, d) 21 days and e) 28 days

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.

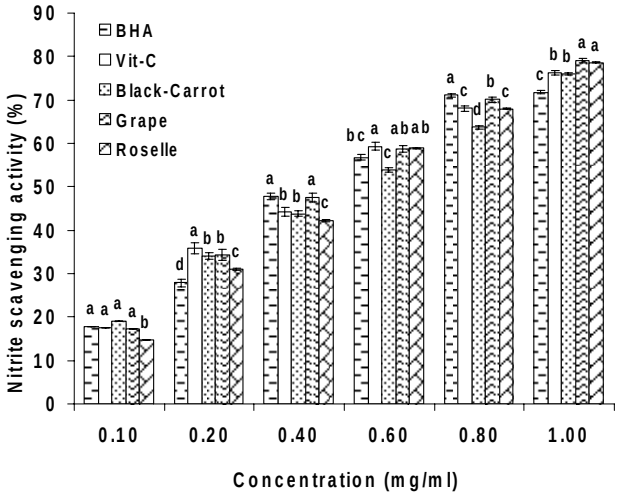
4.3.1.2 Nitric oxide scavenging activity

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)

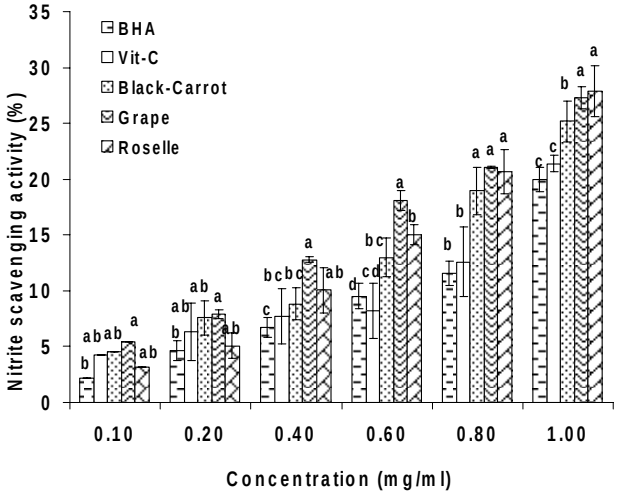
Vitamin C (IC_{50} 0.66±0.01 mg/ml)

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)

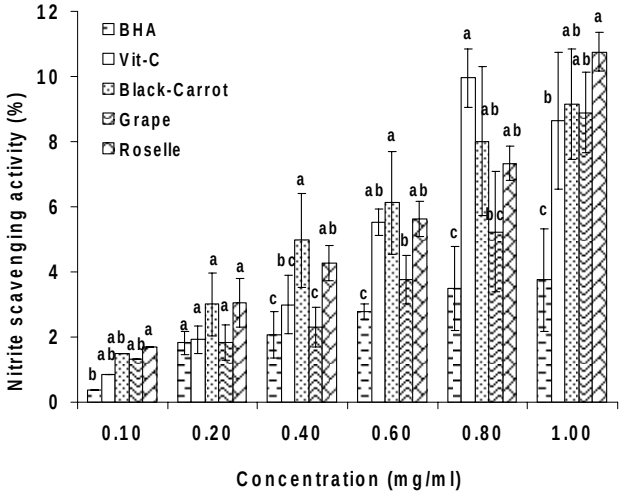
Vitamin C (IC_{50} 2.34±0.07 mg/ml)

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)

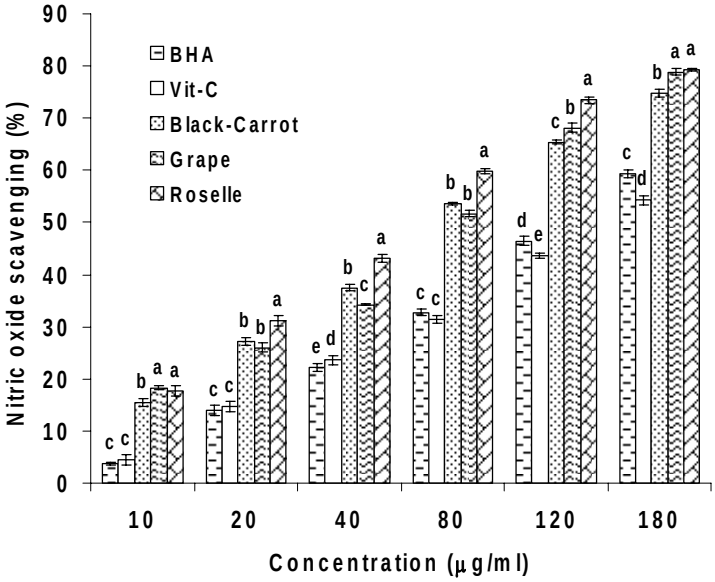
Black carrot (IC_{50} 5.58±0.97 mg/ml)

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)

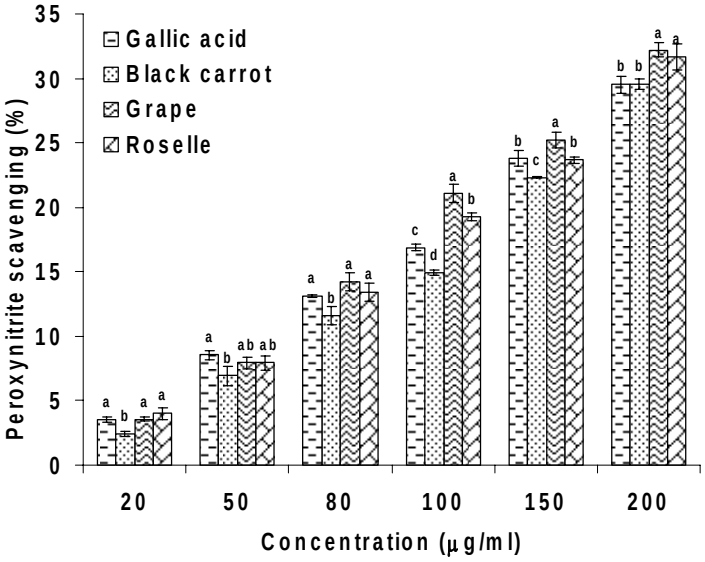
Vitamin C (IC_{50} 165.99±2.64 µl/ml)

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.

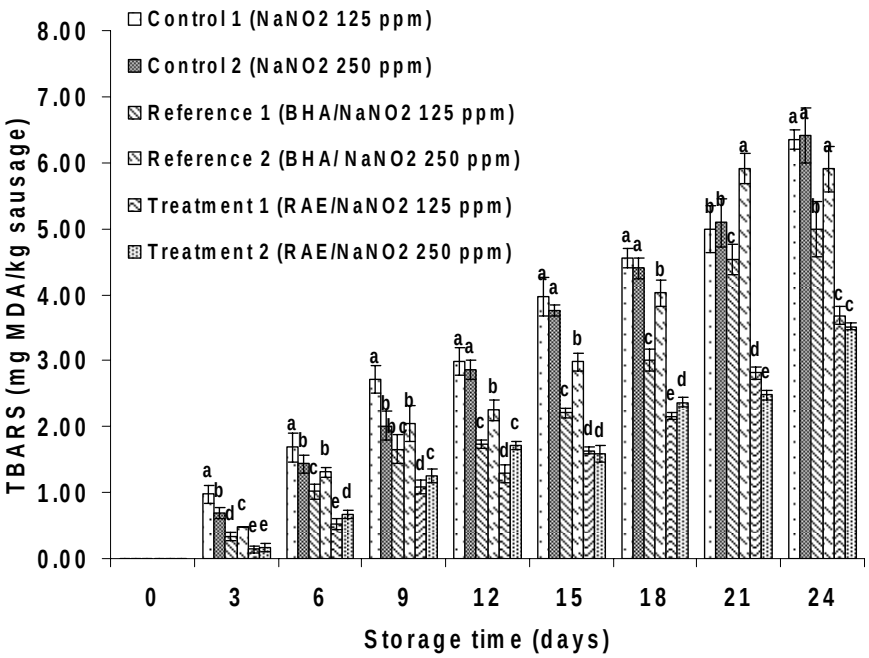


Figure 4.18 TBARS values in Vienna pork sausage during storage at 4±1 °c for 24 days, *n=6*

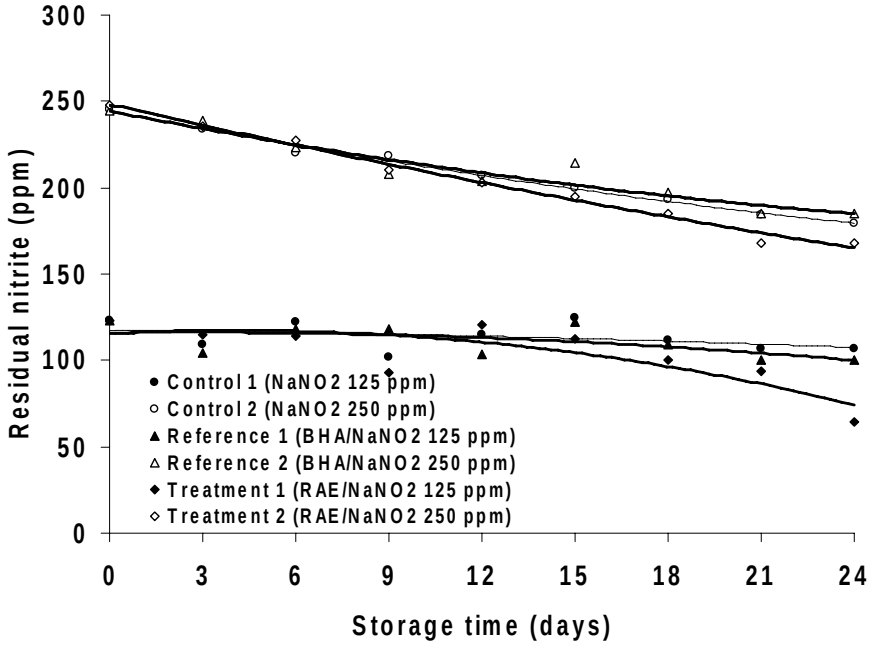


Figure 4.19 Residual nitrite in Vienna pork sausage samples during storage at 4±1 °c for 24 days, *n=6*. Average coefficient of variation (CV) = 4.70 %

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).

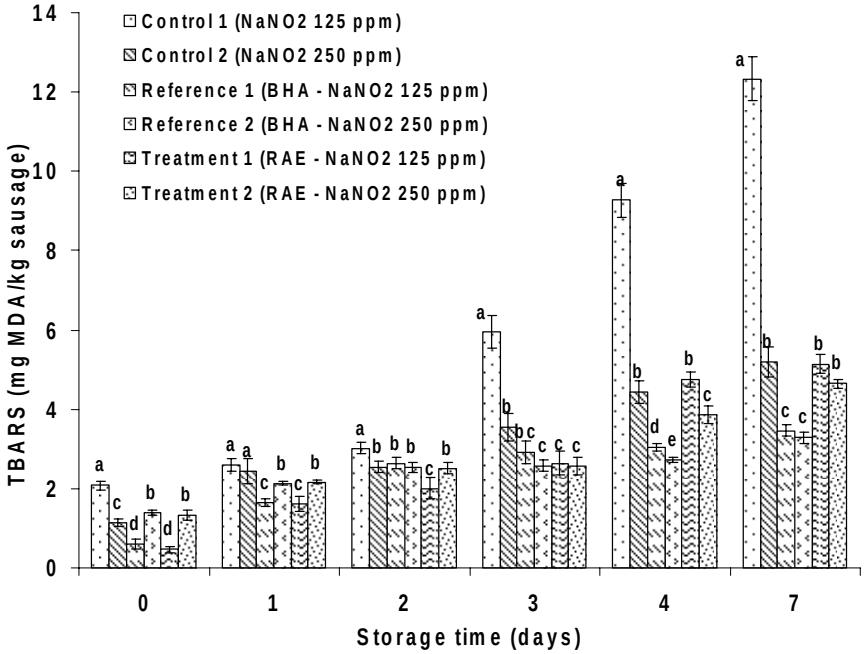


Figure 4.20 TBARS values in traditional Thai fermented pork (Nham) during fermentation at 30±1 °C for 7 days

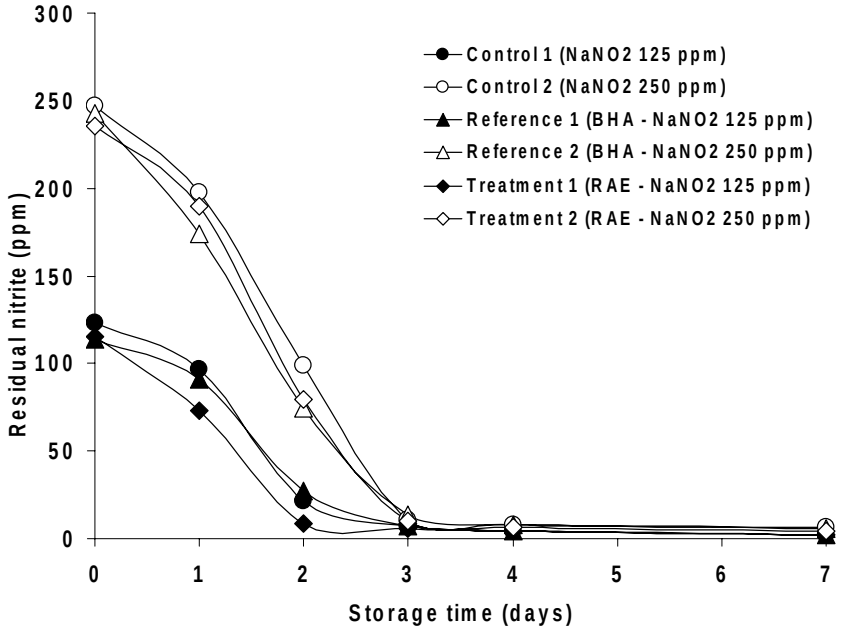


Figure 4.21 Residual nitrite in traditional Thai fermented pork (Nham) during fermentation at 30±1 °C for 7 days, *n=6*. Average coefficient of variation (CV) = 3.18 %

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.

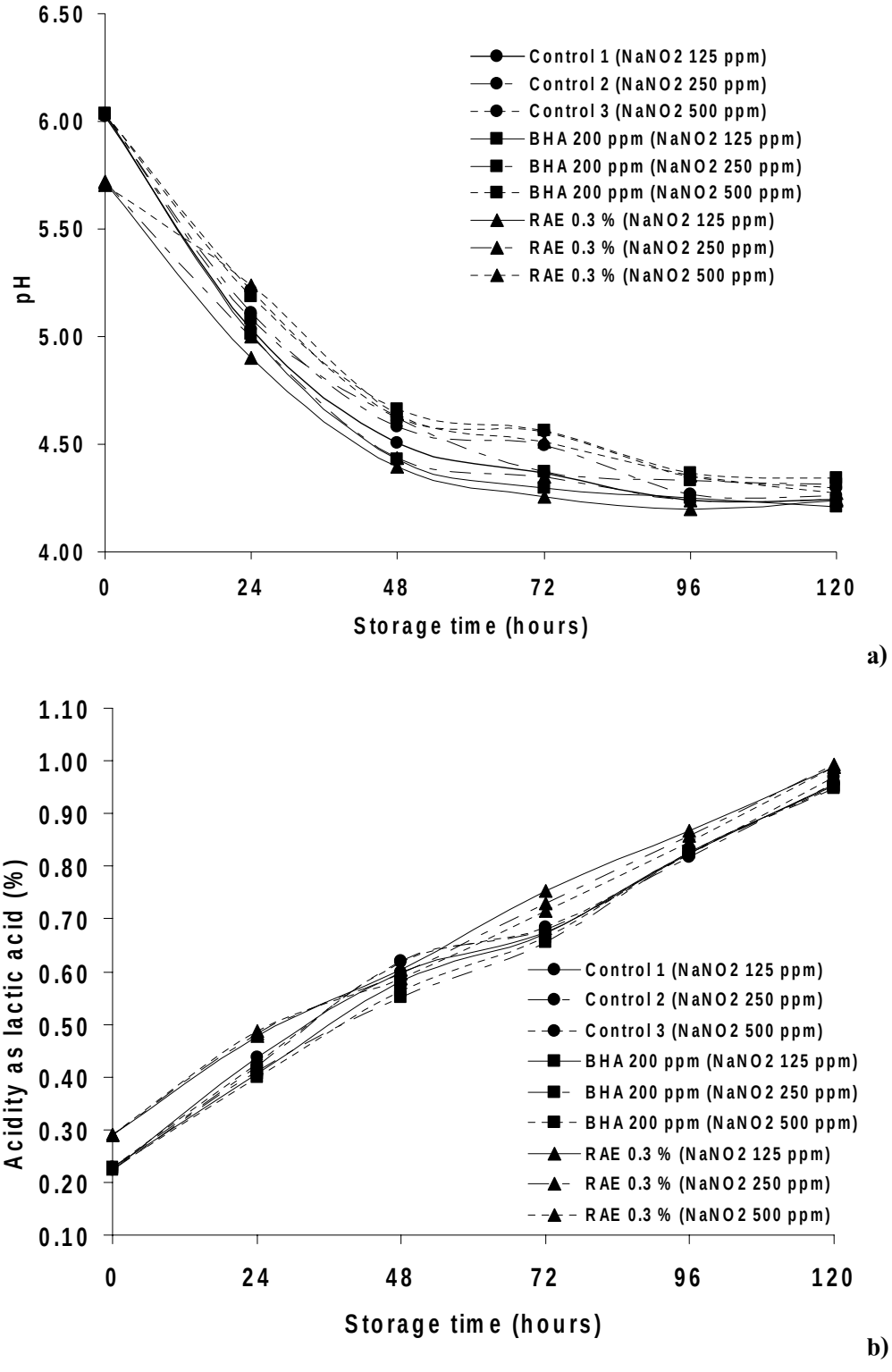


Figure 4.22 Change of a) pH values and b) total acidity as lactic acid in Nham samples with different initial concentration of sodium nitrite during 120 h fermentation time, *n=6*. Average coefficient of variation (CV) = 1.08 and 1.79 % respectively

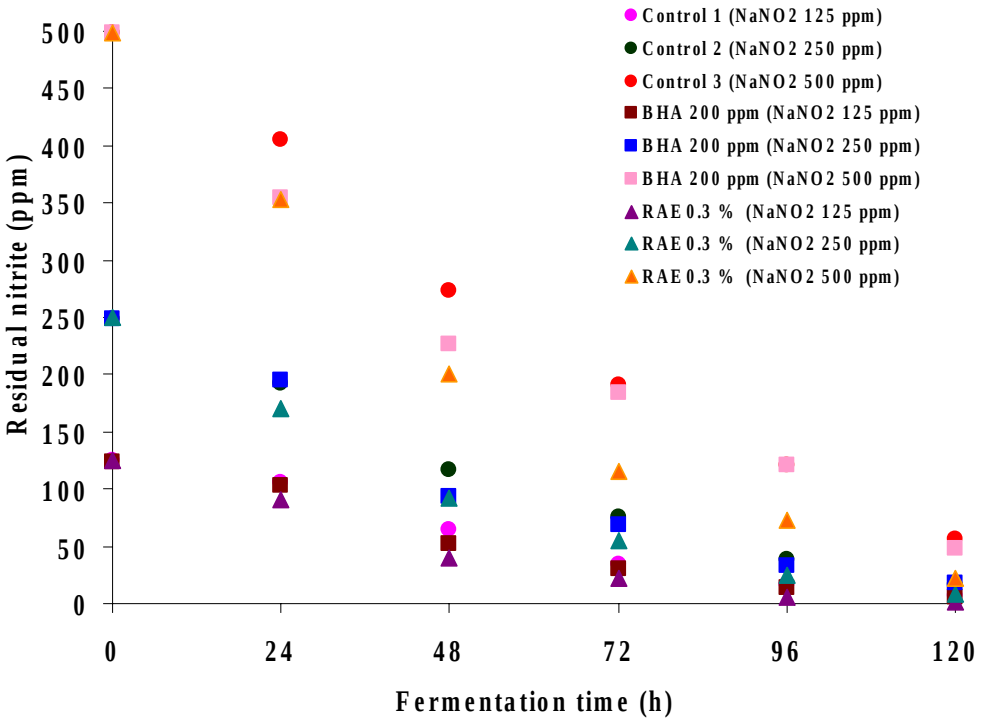


Figure 4.23 Residual nitrite in Nham samples during 120 h fermentation times, *n=6*. Average coefficient of variation (CV) = 1.34 %

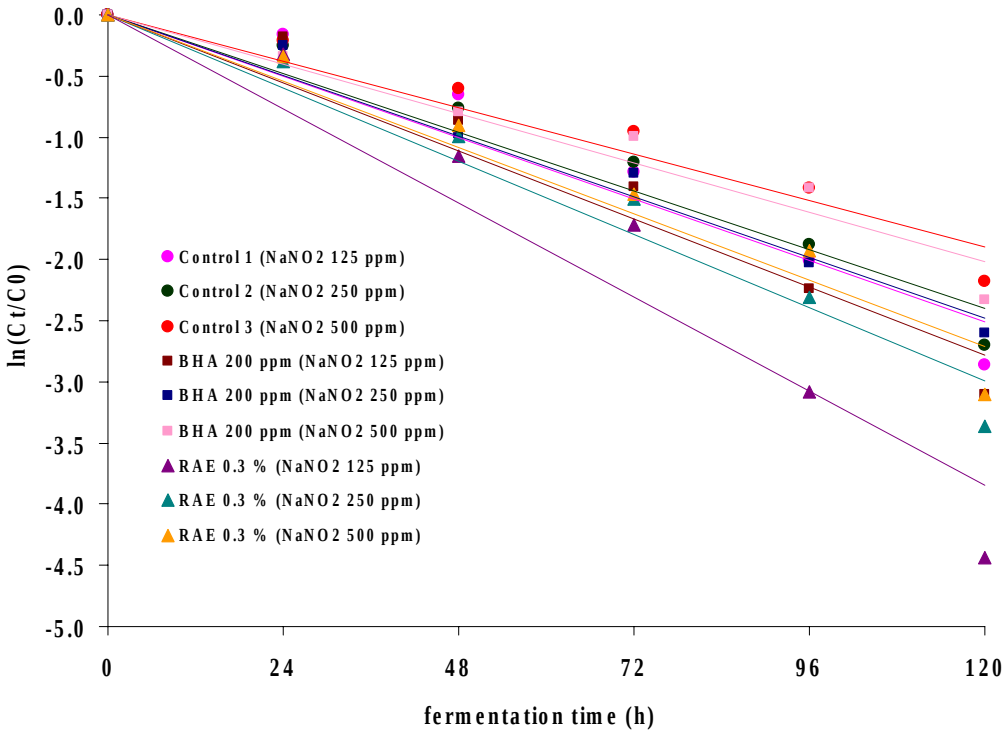


Figure 4.24 Sodium nitrite reductions in Nham samples during 120 h fermentation times.

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.