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Optimum Concentration Sample of Herbal Fresh Sausage for Antioxidant Analysis

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The purpose was to investigate the optimum concentration of herbal fresh sausage for antioxidant analysis. Three sausage recipes were applied in this study as following 1) ground pork adding 0.2 g BHT/kg meat (positive lipid peroxidation) 2) herbal fresh sausage recipe 1 (HFS1) and 3) herbal fresh sausage recipe 2 (HFS2). The antioxidant activity of sample was determined using the following assays: 2,2-diphenyl-1-picryhydrazla hydrate (DPPH) radical scavenging activity, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization and reducing power ability. Herbal fresh sausage sample such as 2.5%, 5%, 10%, 15% and 20% (w/v) of meat supernatant were studied. Analysis revealed that the optimum concentration of raw and cooked herbal sausage sample for DPPH activity was 15% (w/v) ranged between 67.26±0.53% and 84.72±0.27%. $ABTS^{+}$ cation decolorization was 5% (w/v) ranged between 58.50±0.80% and 82.49±1.39%. Reducing power ability was 20% - 10% (w/v) ranged between 0.27±0.00 and 0.59±0.01, respectively.

Keywords: optimum concentration, antioxidant, herbal fresh sausage

Introduction

Fresh sausages are the most common processed meat products worldwide and have been a long time favourite in many areas (Da Silveira *et al.*, 2014; Sharma *et al.*, 2017). Herbal fresh sausages are mainly composed of meat and variable amount of fats, which are more or less coarsely minced, mixed with variety of non-meat ingredients such as salt, spices, herbs, colouring agents and preservatives depending on local preparations (Salinas *et al.*, 2014). This products are highly perishable and favorable for microbial growth of spoilage

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and pathogenic agents, since they are made from fresh ground meat. Besides, during the processing and storage of fresh sausages, one of the biggest problems is discoloration and lipid oxidation which cause a decrease in food quality, adversely affecting the color, flavor and texture of the product which lead to a short shelf life (De Silveira *et al.*, 2014; Hugo and Hugo, 2015; Baldin *et al.*, 2016).

Shelf life extension technology in the meat product industry has been developed to include not only synthetic preservatives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), potassium sorbate and nitrite, but also refrigeration technology. The meat industry main challenge is to replace synthetic preservatives and antioxidants with natural ones because synthetic preservatives are known to demonstrate toxicity and cause disease (Jin *et al.*, 2015; Abdulla *et al.*, 2016). Herbs and spices are recognized as preservative agents. Many types of herbs and spices are used mainly as seasonings to improve the flavor of food, as a preservative and potential sources of natural antioxidant (Gholoum, 2013; Yeung *et al.*, 2014). To scavenge these radicals, antioxidant agents are highly needed. Antioxidants are chemical compounds that are capable of donating hydrogen to the free radicals to minimize rancidity and retard lipid peroxidation without any damage to the sensory or nutritional properties of meat products (Lahucky *et al.*, 2010; Kumar *et al.*, 2015). In our body as a result of biological oxidation, free radicals and other reactive oxygen species (ROS) are formed which can lead to cellular and DNA damage as well as homeostatic disruption. The human body has defense this process by producing antioxidants which are either naturally with in the body or externally supplied through food. Therefore, potential dietary antioxidants can be screened with *in vitro* antioxidant. The 2,2-diphenyl-1-picryhydrazla hydrate (DPPH) radical scavenging activity, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization and reducing power ability have been widely used to evaluate the antioxidative activity of plant extracts and foods. (Shah *et al.*, 2014; Poeaim *et al.*, 2016). Jung *et al.*, (2010) and Qwele *et al.*, (2013) found that the concentration range of 5% - 20% (w/v) of meat supernatant was used for antioxidant analysis. However, the suitable concentration of sausage sample to obtain the optimum value of antioxidant analysis is crucial.

Therefore, the objective of this study was to optimize sample concentration of herbal fresh sausage for antioxidant analysis.

Materials and methods

Preparation of herbal fresh sausage samples

Fresh lean pork and back fat were purchased from supermarket in Bangkok, Thailand. After being cut to small pieces, the pork was combined to contain 20% fat (w/w), then ground with meat mincer equipped with 9 mm diameter holes steel plate (Guangdong, China). Three sausage recipe were applied in this study as following: ground pork adding 0.2 g BHT/kg meat; herbal sausage recipe 1 and 2. The experiments were prepared for 3 replications. The sausage samples were packed in polyethylene bag and kept at -20 ℃ for further analysis. Sausage samples were prepared by mixing various ingredients in a mixer (Table 1 and 2). The appearance of both cooked herbal sausage were shown in Figure 1.

Table 1. Ingredients for herbal fresh sausage recipe 1

Ingredients	percentage
1. ground pork	66.33
2. ground fat	16.58
3. chilli powder	1
4. salt	1.16
5. coriander	3.32
6. culantro	1.66
7. sugar	0.83
8. shallots	4.15
9. kaffir lime leaf	0.83
10. apple cider vinegar	4.15

Table 2. Ingredients for herbal fresh sausage recipe 2

Ingredients	percentage
1. ground pork	64.62
2. ground fat	16.16
3. chilli paste	3.23
4. galangal	1.62
5. lemon grass	4.85
6. salt	1.13
7. sugar	0.81
8. kaffir lime leaf	0.81
9. coriander	1.62
10. chilli	1.13
11. apple cider vinegar	4.04

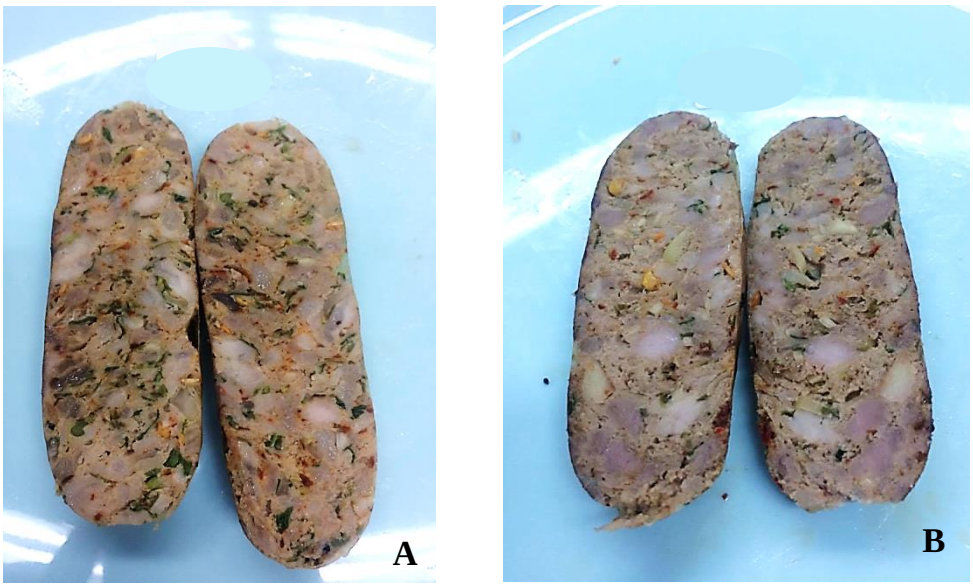

Figure 1. The appearance of both herbal fresh sausage (A) Herbal sausage recipe 1. (B) Herbal sausage recipe 2

Thermal processing

Sausage samples were cooked in an oven at moderate heat (180 ℃) for 15 min, until the skins were golden brown and crispy (inner temperature 72 ℃). Weight loss of sausages after cooking was 29%.

Analytical sample preparation

Sausage sample extractions were prepared by the method described by Jung *et al.*, (2010). Five grammes of each sausage sample was homogenized. A volume of 20% (w/v) homogenate (T-18 Ultra tarrax, Staufen, Germany) was prepared in ethanol. The sausage sample extract was filtered through a Whatman No.1 filter paper. Lipids and the supernatant were separated by centrifuged machine (Avanti JA-20, Beckman Coulter, USA) at 5,160×*g* for 5 min at 4°C. The supernatant such as 2.5%, 5%, 10%, 15% and 20% (w/v) were studied for the estimation of 2,2-diphenyl-1-picryhydrazla hydrate (DPPH) radical scavenging activity, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization and reducing power ability.

Determination of antioxidant activity of herbal fresh sausage

2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH, Sigma, Germany) radical scavenging activity was measured according to the modified of Jung *et al.*, (2010). Briefly, 2 ml of meat supernatant was individually added to 2 ml of 0.2 mM (DPPH) prepared in absolute ethanol. The mixture of ethanol (2 ml) and meat supernatant (2 ml) served as a blank. The control solution was prepared by mixing ethanol (2 ml) and DPPH radical solution (2 ml). The mixtures were incubated for 30 min in the dark at room temperature and the reduction of DPPH radicals was measured at 517 nm using a spectrophotometer (GENESYS 20, Thermo Scientific, USA). The scavenging activity of meat sample against DPPH radical was expressed as percent of control and calculated as:

$$\text{DPPH activity (\%)} = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right] \times 100$$

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization

The antioxidant activity was determined using 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+}$, Sigma, Canada) radical cation decolorization as described by Qwele *et al.*, (2013). The stock $ABTS^{\bullet+}$ solution was generated by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate (Hazardous, New Zealand) in the same ratio and allowed to react in the dark for 12 h at room temperature. This solution was further diluted with ethanol to adjust its absorbance to 0.70 ± 0.02 at 734 nm. Then, 3 ml of diluted $ABTS^{\bullet+}$ solution was added to 0.3 ml of meat supernatant. After 6 mins of incubation at room temperature, the absorbance were recorded at 734 nm using a spectrophotometer (GENESYS 20, Thermo Scientific, USA) against a blank (3 ml of ethanol plus 0.3 ml of meat supernatant), and a control (3 ml of $ABTS^{\bullet+}$ solution plus 0.3 ml of ethanol). The scavenging activity of samples against ABTS radical cation decolorization was expressed as inhibiting percent and calculated as:

$$\text{ABTS activity (\%)} = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right] \times 100$$

Reducing power ability

Reducing power ability was measured by the method described by Vijayalakshmi and Ruckmani, (2016). Briefly, 1 ml of meat supernatant was individually mixed with 2.5 ml of 0.2M phosphate buffer pH 6.6, 2.5 ml of 1% potassium ferricyanide and then incubated at 50 ^{o}C for 30 min. Afterwards, the mixture was added to 2.5 ml of 10% trichloroacetic acid (TCA, Merck, Germany) and centrifuged at 2,200 *g* for 10 min at 25^{o}C. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride (Hazardous, New Zealand). The reaction mixture was allowed to react for 10 min followed by measuring the absorbance at 700 nm using a spectrophotometer (GENESYS 20, Thermo Scientific, USA). Increase absorbance of the reaction mixture indicated higher reducing power of the supplements in the sausage samples.

Statistical analysis

All experimental measurements were carried out in three parallel measurement and expressed as mean ± standard deviation (SD). The data were analyzed by SPSS version 23 software (IBM).

Results and Discussion

2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) radical scavenging activity

Three concentrations 10%, 15% and 20% (w/v) of meat supernatant were used. It was found that different herbal fresh sausage recipe showed different value of % DPPH activity (Table 3). The concentration of 20% revealed the highest for % DPPH activity of herbal sausage recipe in both raw and cooked fresh sausage. The lowest % DPPH activity in both raw and cooked sausage were observed in 10% (w/v). In addition, both herbal sausage recipe expressed higher % DPPH activity than ground pork adding 0.2% BHT and the highest % DPPH activity was found in HFS2. As a result of this, it could be concluded that herbal sausage showed higher antioxidant than ground pork adding 0.2% BHT and could be a healthy food. Therefore, the optimum % DPPH activity was at 15% (w/v) of meat supernatant.

DPPH radical scavenging activity is one of the most used tests for screening the antioxidant activity of compounds. DPPH$^{\bullet}$ shows strong absorption at 517 nm. Antioxidants can react with DPPH$^{\bullet}$ either by electron transfer or via the hydrogen atom which could combine with DPPH$^{\bullet}$ and neutralize its free radical character. The purple color of the DPPH$^{\bullet}$ solution generally fades when an antioxidant is present in the medium. When the

extracts above were tested for DPPH$^{\bullet}$ scavenging ability and ability to inhibit superoxide and hydroxyl radicals. (Luo *et al.,* 2007; Slima *et al.,* 2017). The results implied that herbal fresh sausage could transfer enough hydrogen atoms or electron as well as neutralizing the free radicals. Moreover, our findings are similar to previous works reported by Jung *et al.,* (2010) that the concentration of meat sample at 20% (w/v) of meat supernatant used for DPPH activity. Therefore, the high DPPH scavenging ability of the herbal sausage supplemented with antioxidants source may be attributed to its high hydrogen donating ability.

Table 3. The percentage of DPPH radical scavenging activity from herbal fresh sausage sample

Concentrations of sample		% DPPH radical scavenging activity		
		BHT 0.02%	HFS1	HFS2
Raw	20%	73.25±0.28	90.91±1.21	92.88±0.14
	15%	67.26±0.53	77.67±1.23	81.32±0.24
	10%	49.17±0.30	61.92±0.72	70.31±0.26
Cooked	20%	76.16±0.40	94.24±0.34	95.46±0.38
	15%	69.84±0.84	83.59±0.41	84.72±0.27
	10%	52.86±0.39	75.61±0.17	76.74±0.88

All values were expressed as mean ± standard deviation
BHT - Butylated hydroxytoluene, HFS1 - Herbal fresh sausage recipe 1 and HFS2 - Herbal fresh sausage recipe 2

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization

Three concentrations 2.5%, 5% and 10% (w/v) of meat supernatant were used. The study showed that different herbal fresh sausage recipe displayed different value of % ABTS activity (Table 4). The concentration of 10% revealed the highest of % ABTS activity of herbal sausage recipe in both raw and cooked fresh sausage. The lowest % ABTS activity in both raw and cooked sausage were observed in 2.5% (w/v). The optimum % ABTS activity was at 5% supernatant. Moreover, it was found that both HFS1 and HFS2 showed higher % ABTS activity than ground pork adding 0.2 % BHT Therefore, herbal sausage could be an alternative for healthy food.

The generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that has been applied for the measurement of the total antioxidative activity of food, beverages and meat product. The method for

the screening of antioxidative activity is reported as a decolourization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. A more appropriate format for the assay is the decolourization technique in which the radical is directly generated in a stable form prior to the reaction with putative antioxidants. An improved technique for the generation of $ABTS^{+}$ which is described here involves the direct generation of a blue/green $ABTS^{\bullet+}$ chromophore by the reaction between ABTS and potassium persulfate (Kim and Lee, 2009; Mi *et al.* 2016; Nguyen *et al.,* 2017). Qwele *et al.,* (2013) suggested that the concentration of meat sample at 10% (w/v) of meat supernatant used for ABTS activity. Therefore, sausage samples from all dietary supplementation showed a potential electron donating ability. The fact that sausage supplemented with quantity of antioxidants source had the highest $ABTS^{+}$ reducing activity suggested that these samples had the highest potential to donate electrons for neutralizing free radicals.

Table 4. The percentage of ABTS radical cation decolorization from herbal fresh sausage sample

Concentrations of sample		% ABTS radical cation decolorization		
		BHT 0.02%	HFS1	HFS2
Raw	10%	71.32 ±0.26	93.10 ±0.45	95.57 ±0.60
	5%	58.50 ±0.80	73.74 ±0.64	76.77 ±0.69
	2.5%	30.69 ±0.44	48.06 ±0.76	49.59 ±0.53
Cooked	10%	75.28 ±0.83	96.28 ±0.61	96.46 ±0.81
	5%	59.53 ±1.40	77.70 ±0.95	82.49 ±1.39
	2.5%	33.45 ±0.93	53.36 ±1.37	55.84 ±1.58

All values were expressed as mean ± standard deviation
BHT - Butylated hydroxytoluene, HFS1 - Herbal fresh sausage recipe 1 and HFS2 - Herbal fresh sausage recipe 2

Reducing power ability

Reducing power ability analysis showed that three concentrations 10%, 15% and 20% (w/v) of meat supernatant were studied (Table 5). The result expressed that different herbal fresh sausage recipe showed different value of reducing power ability. The optimum reducing power ability presented in 10 - 20 % meat supernatant. Therefore, both herbal sausage recipe exhibited higher absorbance at 700 nm than ground pork adding 0.2% BHT and high reducing

power might inhibit lipid oxidation by blocking radical chain reaction in the oxidation (Cheng *et al.*, 2017).

The reducing power were generally associated with the presence of reductones which had been reported to exert an antioxidant action by breaking the free radical chains as a result of donating hydrogen atoms. (Sayar *et al.*, 2015) Vijayalakshmi and Ruckmani, (2016) reported that the reducing capacity of the extracts and fractions were performed using Fe^{3+} to Fe^{2+} reduction assay. In this experiment, the yellow color changed to pale green and blue color depending on the concentration of antioxidants in the samples. All the samples showed reducing capacity in a concentration dependant manner. Moreover, our findings are similar to previous works reported by Nguyen *et al.*, (2017) exhibited that the concentration of potential antioxidant and lipid peroxidation inhibition of *Phyllanthus acidus* leaf extract in minced pork at 20% (w/v) of meat supernatant used for reducing power ability.

Table 5. Reducing power ability from herbal fresh sausage sample

Concentrations of sample		Absorbance at 700 nm		
		BHT 0.02%	HFS1	HFS2
Raw	20%	0.42±0.00	0.55±0.00	0.57±0.00
	15%	0.35±0.00	0.47±0.01	0.49±0.00
	10%	0.27±0.00	0.31±0.00	0.35±0.00
Cooked	20%	0.44±0.01	0.57±0.00	0.59±0.01
	15%	0.37±0.01	0.49±0.01	0.52±0.00
	10%	0.30±0.01	0.34±0.01	0.37±0.00

All values were expressed as mean ± standard deviation
BHT - Butylated hydroxytoluene, HFS1 - Herbal fresh sausage recipe 1 and HFS2 - Herbal fresh sausage recipe 2

Conclusion

This study was to investigate the optimum concentration of fresh sausage sample on antioxidant analysis. The antioxidant method were analysed including that 2,2-diphenyl-1-picryhydrazla hydrate (DPPH) radical scavenging activity. 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization and reducing power ability. It was found that 15% and 20% (w/v) of sample were suitable concentration for DPPH and reducing power analysis. In addition 5% (w/v) of sample were observed for optimum concentration for ABTS analysis.

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