



## **The Effect of Antioxidant Capacity of the Capsaicinoids Extracts from Hot Chilli Pepper on the Autoxidation of Oxymyoglobin**

**Saksit Chanthai<sup>\*1</sup>, Jureerat Juangsamoot<sup>1</sup>, Chalerm Ruangviriyachai<sup>1</sup> and Suchila Techawongstien<sup>2</sup>**

<sup>1</sup>Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

<sup>2</sup>Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University 40002, Thailand

E-mail: [sakcha2@kku.ac.th](mailto:sakcha2@kku.ac.th)

---

### **ABSTRACT**

*An antioxidant capacity of the capsaicinoids extracts from hot chilli pepper based on the measurement of the absorbance decreases as a result of the radical scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH<sup>•</sup>) radical was conducted by spectrophotometric method. The extracts of total capsaicinoids were comparatively obtained by using both magnetic stirring extraction (MSE) and accelerated solvent extraction (ASE) methods. The average percentages of DPPH radical scavenging of the methanolic extracts using MSE and ASE were ranged from 68.1-82.6% and 70.0-85.8%, respectively. In application, the retardation of an autoxidation of oxymyoglobin (MbO<sub>2</sub>) in the presence of the capsaicinoids as expressed by the observed rate constant ( $k_{obs}$ ) of the kinetics reaction was measured under the optimum conditions. The average percentages of the retardation effect on the autoxidation reaction when monitoring at the absorbance of 543 nm ( $A_{543}$ ) and 581 nm ( $A_{581}$ ) were found in the ranges of 34.0-74.5% and 39.5-79.2% for MSE and in the ranges of 38.9-75.8% and 44.7-80.5% for ASE, respectively. Among ten varieties of the chilli pepper samples used, trends in the retardation effect according to their antioxidant activity on the autoxidation of MbO<sub>2</sub> were found higher than 70%. From these results, it was, therefore, shown that the antioxidant capacity of the capsaicinoids in the extracts could enhance stabilizing the MbO<sub>2</sub> by acting as the radical scavenging capacity.*

**Keywords:** Capsaicinoids, chilli pepper, antioxidant activity, autooxidation, myoglobin, accelerated solvent extraction, magnetic stirring extraction.

## INTRODUCTION

Hot chilli peppers are the dried ripen fruits of the species of genus capsicum. They are also called red peppers or capsicums constituting an importance, well known commercial crop used both as condiment or culinary supplement and as vegetable. Thai cuisine is inextricably linked with hot chilli peppers. Together with a few other spices and herbs, hot chilli pepper is carefully blend, combine to give Thai food its unparallel range of delicately nuance flavors. Capsaicinoids are an important group of organic compounds closely related to the family of alkaloids and they are responsible for the hot flavor, of which capsaicin is the principal component. The capsaicinoids are known to be biosynthesized and accumulated in the placentas of *Capsicum* fruits [1,2].

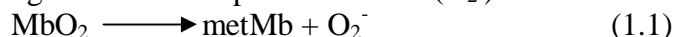
The study of capsaicinoids is, thus, of great importance in hot chilli peppers. These compounds play an important role in the flavor, color, pungent and quality of food and act as natural antioxidants. Furthermore, biological activities have been found in association with human health. Two major capsaicinoids present in most varieties of the chilli are capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide). Other minor capsaicinoids are also found including nordihydrocapsaicin, norcapsaicin, homocapsaicin, nornorcapsaicin, nornornorcapsaicin and nonivamide [3]. The determination of capsaicinoids in hot peppers, topical cream [4], self-defense weapon [5] and aerosol defense spray [6] has, therefore, been of increasing interest for many reasons. Various extraction methods of capsaicinoids from hot peppers have been reported, such as maceration by magnetic stirring extraction [7], Soxhlet extraction [8], supercritical fluid extraction [9], enzymatic extraction [10]. Accelerated solvent extraction (ASE) is recently applicable method utilizing organic solvents at high temperature and pressure to extract analytes. It is rapid, small volume of organic solvent and efficient removal of the analytes from various matrices. After extraction of the capsaicinoids using suitable solvents, analysis could be performed by several chromatographic techniques, including gas chromatography (GC) [6,11], supercritical fluid chromatography, and high performance liquid chromatography (HPLC) [12-14]. Practically, RP-HPLC-PDA still provides an advantage as routine separation method over other techniques, except a sophisticated instrument like LC-MS, including high efficiency, short analysis time and low sample consumption.

Besides the extraction and analytical methods, in neurological research capsaicin has generally been used to stimulate sensory nerves and also to treat bladder inflammation. Blending in topical ointments can be used for arthritis and neuralgia [4]. It exerts its effect on the sensory nerves by interacting with the vanilloid receptor, promoting the release of substance P as well as other cytokines. The release of cytokines from the peripheral sensory neurons causes a sensation of intense burning and pain [15]. In recent years, capsaicinoids have attracted the interest of researchers because they show promise of being powerful antioxidants, protecting the human body from free radicals, the formation of which is associated with antiradical activity of flavonoids and phenolics. The antiradical activity is principally based on the redox properties of their hydroxyl group and the structural relationships between different parts of their chemical structures [16]. Various methods have been used to determine and compare the antioxidant activity of foods. Numerous methods have been published for their determinations also involving electron spin resonance (ESR) [17]. These analytical methods are used to measure the radical-scavenging activity of antioxidants against free radicals like 1, 1-diphenyl-2-picrylhydrazyl (DPPH<sup>•</sup>) radical, superoxide anion radical (O<sub>2</sub><sup>•-</sup>), hydroxyl radical (OH<sup>•</sup>), or peroxy radical (ROO<sup>•</sup>) [18-25]. The ABTS [2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical

cation has been used to screen the relative radical-scavenging abilities of capsaicinoids and flavonoids through their properties as electron or H-donating agents [24]. The procedure of oxygen radical absorbance capacity (ORAC) has been used to determine antioxidant capacities of fruits and vegetables [22,26].

A rapid, simple and cheap method to measure antioxidant capacity of *Capsicum* plants involves using of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of *Capsicum* plants [16,17]. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm with a purple color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces when the odd electron of DPPH radical becomes paired with hydrogen to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to a number of electrons captured. Alternative methods have been used to retard an autoxidation of oxymyoglobin (MbO<sub>2</sub>) to be met-form of myoglobin (Mb) from useful natural antioxidants. Addition of antioxidants to meat products is known affecting on the metMb formation and lipid oxidation. The application of vitamin E [27], rutin [28], ascorbate acid [29], rosemary [30] to meat products is well documented. Although, studies on total antioxidant activity of capsaicinoids with DPPH have been reported in a wide variety of food, their effects of antioxidant activity on biological systems such as Mb is still limited.

In muscle, Mb is known as a small, single subunit intracellular oxygen binding protein. It is one of the most important and plentiful proteins of most vertebrates. The richest sources of myoglobin are the muscle of aquatic diving vertebrates such as whales, seals or porpoises [31]. Moreover, it is also found in aerobic muscle and heart vertical of mammals, reptiles, amphibians, teleosts, chondrichthid fish [32] and lower animal species [33]. Since it is generally known that fish meat gradually darkens during frozen storage as well as subsequent thawing, this darkening is mostly due to the autoxidation of MbO<sub>2</sub>. Under air-saturated conditions, MbO<sub>2</sub> is oxidized easily to metMb with generation of superoxide anion (O<sub>2</sub><sup>-</sup>) as



$$k_{\text{obs}} = -d [\text{MbO}_2] / dt \quad (1.2)$$

Where  $k_{\text{obs}}$  is the observed rate constant of the kinetics reaction of autoxidation. Some chemical and physical properties of certain fish Mb have previously been reported in line with this phenomenon, indicating that the autoxidation reaction is mainly dependent on temperature [34]. Thus, an investigation of the autoxidation reaction of MbO<sub>2</sub> in the presence of capsaicinoids is very interesting.

Therefore, the present study was aimed to determine the effect of antioxidant capacity of the capsaicinoids in the extract of hot chilli pepper samples obtained from both MSE and ASE methods on the retardation of the autoxidation of MbO<sub>2</sub> solution under the optimum conditions. The observed rate constants of the kinetics reaction of MbO<sub>2</sub> were then obtained, indicating the sensitivity of the capsaicinoids contents in association with their antioxidant capacity.

## MATERIALS AND METHODS

**Chemicals:** All reagents used were at least analytical reagent (AR) grade. Methanol was of HPLC grade (Lab Scan (Thailand)). Myoglobin from horse skeletal muscle (>90% purity) and 1,1'-Diphenyl-2-picrylhydrazyl radical (DPPH) were from Fluka (Switzerland). Capsaicin, dihydrocapsaicin and  $\alpha$ -tocopherol with the highest purity (99.5%) and 2-Amino-2-(hydroxymethyl) propane-1,3-diol were from Sigma (USA). Sodium dithionite was from Riedel-

de Haën (Germany). Aqueous solutions were prepared with deionized water obtained from RiOs™ type 1 simplicity 185 (Millipore Waters, USA) throughout the experiments. All solutions of both standard compounds were kept cool at 4°C.

**Instruments :** The experiments were carried out on a Waters liquid chromatograph (Waters, USA). It consists of a Waters 600E multi-solvent delivery system, a Waters in-line degasser AF, a Rheodyne injector with sample loop of 20 µL, a Waters 2996 photodiode array detector, and a Waters temperature control system. Empower software was used for data acquisition. Hypersil® ODSC<sub>18</sub> column (4.6 mm i.d. × 10 cm, 3 µm) was used. Accelerated solvent extractor, ASE-200, was from Dionex (USA). It consists of oven, sample extraction cells, the collection vial volume (30 mL), needle, solvent reservoir and control panel. The C<sub>18</sub> cartridge (500 mg/4 mL) was obtained from Alltech (USA).

**Plant materials :** Ten varieties of hot chilli pepper samples were collected from cultivating sites and seed products Co Ltd. Most of the samples were *Capsicum annum L.* (S01, Munpama; S02, Super hot; S05, Yodson, Nonethai-Nakhon Ratchasima; S07, Jinda, Chumsang- Nakhon Sawan; S08, Jinda, Muang-Phetchaboon; S09, Yodson, Theparak-Nakhon Ratchasima; S10, small Jinda), *Capsicum chinense L.* (S03, Pag-puan; S06, South Africa), and *Capsicum frutescens L.* (S04, Doi-ded-gan) obtained from Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University. The dried samples were ground using a kitchen grinder, kept in plastic bag and stored in desiccator before use.

**Magnetic stirring extraction (MSE) :** Two grams of ground chilli sample were macerated using magnetic bar stirring with 20 mL methanol at 60°C for 2 h. The extract solution was subjected to centrifuge to remove the plant material and then filtered through a Whatman No. 42 filter paper. The solvent in the extract was evaporated to dryness using a rotary evaporator and the residue was dissolved with the solvent to the final volume of 5.0 mL. The extract solution was then cleaned up using C<sub>18</sub> SPE tube and subsequently filtered through 0.45 µm nylon filter membrane prior to HPLC analysis.

**Accelerated solvent extraction (ASE) :** Practically, two pieces of Whatman No.42 filter paper (19.8 mm, i.d.) were placed onto cellulose disk (19.8 mm, i.d.) at the outlet end of the extraction cell. The ground chilli sample (2.0 g) and silica gel (6.0 g) were loaded into the cell and then another filter paper was place again on the top of the sample. The ASE conditions were carried out by using methanol as the extraction solvent at 200°C for 5 min extraction time and 3 extracting cycles. The extract obtained was filtered through the Whatman No. 42 filter paper, evaporated the solvent, and diluted to the final volume with 5.0 mL methanol. An aliquot of the extract was also cleaned up in the same manner as in the MSE method prior to HPLC analysis.

**Determination of radical scavenging capacity :** Standard solutions of both capsaicinoids were diluted with methanol from their stock solutions (2,640 µg/mL capsaicin and 2,140 µg/mL dihydrocapsaicin). For standard solution of α-tocopherol, 11.0 mM of α-tocopherol solution was prepared by dissolving 50 µL α-tocopherol in 10.0 mL methanol. The α-tocopherol solution was transferred to a covered aluminium foil vial and kept cool at 4°C prior to use. A DPPH solution (40.1 µM) was prepared by dissolving of 0.0080 g DPPH in 500 mL methanol. The DPPH solution was transferred to a dark bottle and also kept cool at 4°C before use. Tris-HCl buffer

solution of 0.1 M pH 7.8 was prepared by dissolving 2-amino-2-(hydroxymethyl) propane-1,3-diol 6.057 g in 400 mL of distilled water, next adjusted with 6.0 M HCl to pH 7.8 and diluted with deionized water to a final volume of 500 mL.

For myoglobin (Mb) solution, 0.5 g of Mb was dissolved in 40.0 mL Tris-HCl buffer solution (pH 7.8), filtered through Whatman No.1 paper and transferred to a bottle and kept cool at 4°C before use. Oxymyoglobin (MbO<sub>2</sub>) was prepared by using 12.0 mL of the Mb solution (12.5 mg/mL) mixed with 180.0 mL of 10 mM Tris-HCl buffer (pH 7.8) and then a solid crystal of sodium dithionite (48.0 mg) was added. The Mb solution was mixed thoroughly with the reducing agent by shaking the flask up and down, deoxymyoglobin (deoxyMb) was first occurred. Later, the deoxyMb solution was left in the air and then oxygenated by an excessive bubbling (about 3 min) until the reddish pink color of MbO<sub>2</sub> had appeared.

**Effect of incubation time :** Scavenging potentials of free radicals were tested in the methanolic solution of DPPH. The absorption spectra of DPPH were recorded over the wavelength range 200-800 nm using UV-visible spectrophotometer (Agilent, USA). The absorbance at the maximum wavelength 517 nm (A<sub>517</sub>) was measured. Standard solutions of capsaicin and dihydrocapsaicin (20.0 µM) in methanol were prepared. Then 1.0 mL of each solution was added into 2.0 mL DPPH solution (40.1 µM). The A<sub>517</sub> was measured every 10 min intervals, starting from the moment of the solution mixing. A reference sample, α-tocopherol (20.0 µM), was prepared in 1.0 mL methanol. The antiradical activity was calculated as percentage of DPPH decolorization compared with the control solution within the time when all standards reached plateau (80 min).

**Effect of concentration of capsaicinoids :** An aliquot (2.0 mL) of 40.1 µM DPPH solution was mixed with various concentrations (0.0, 20.0, 40.0, 60.0, 80.0 and 100.0 µM) of capsaicin and dihydrocapsaicin and then diluted to a final volume of 3.0 mL methanol. To determine the linearity of the method, 5 levels of standard solutions with concentration ranging from 0.0-100.0 µM were prepared and analyzed. The A<sub>517</sub> was measured after 40 min.

Radical scavenging capacity of the chilli extract: Briefly, 40.1 µM DPPH in methanol was prepared and 2.0 mL of this solution was added into 1.0 mL of the chilli extract (this is the same sample used for quantitative analysis by HPLC to fix the exact concentration of both capsaicinoids). The mixture of each chilli extract and DPPH solution was placed at ambient temperature for 40 min and measured the A<sub>517</sub>.

#### **Determination of retardation effect of the autoxidation reaction of MbO<sub>2</sub>**

Effect of temperature on the autoxidation rate of MbO<sub>2</sub> : For each set of the autoxidation measurements, 3.0 mL of 0.78 mg/mL MbO<sub>2</sub> (freshly prepared) was added into sample quartz cell (1.0 cm path length). An equal volume of 10 mM Tris-HCl buffer solution (pH 7.8) was used in a reference quartz cell. The experiments were run at 45, 50 and 55°C with a time course of 115 min. The absorbance changed at 581 (A<sub>581</sub>) and 543 (A<sub>543</sub>) nm was recorded. Immediately, the visible spectrum (450-700 nm) was scanned on the same chart at 5 min time intervals.

Effect of capsaicinoids on the autoxidation rate of MbO<sub>2</sub> at various temperatures : An aliquot (2.5 mL) of 0.78 mg/mL MbO<sub>2</sub> (freshly prepared) was mixed with each mixture of 0.5 mL of 10.0 mg/L and or 30.0 mg/L capsaicinoids. The experiments were run at 45, 50 and 55°C with a time course of 115 min. The absorbance changed at the two wavelengths was recorded in the same manner.

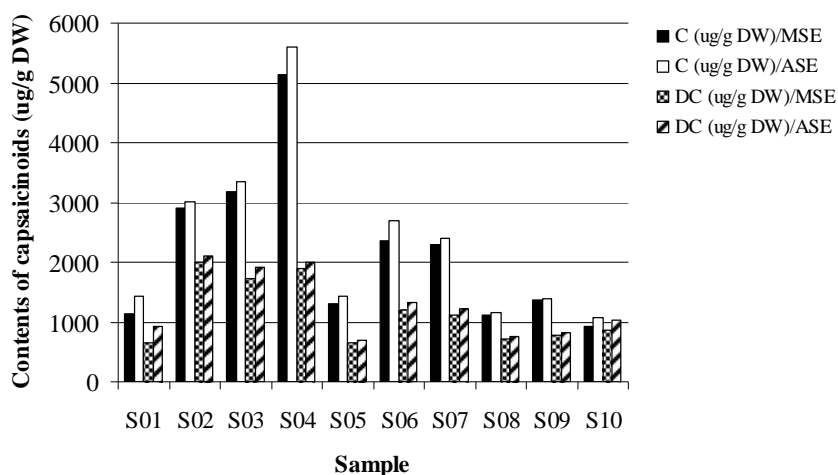
Effect of concentration of capsaicinoids on the autoxidation rate of MbO<sub>2</sub> : The absorption spectra of MbO<sub>2</sub> were recorded in the in the range of 200-800 nm. An aliquot (2.5 mL) of 0.78 mg/mL MbO<sub>2</sub> solution was mixed with 1.0 mL of various concentrations (5.0-80.0 mg/L) of mixture of capsaicin and dihydrocapsaicin under the optimum conditions. Both A<sub>581</sub> and A<sub>543</sub> nm were then measured with respect to blank solution.

Effect of various volumes of the sample extract on the autoxidation rate of MbO<sub>2</sub> : Ten kinds of the hot chilli pepper extracts (0.1-0.5 mL) were added into 2.5 mL of 0.78 mg/mL MbO<sub>2</sub> (freshly prepared) and adjusted to final volume 3.0 mL. The solution was incubated at 50°C in a water bath for 35 min. The absorbance at both wavelengths was measured.

Effect of the capsaicinoid extract on the retardation of autoxidation of MbO<sub>2</sub>: An aliquot (2.5 mL) of 0.78 mg/mL MbO<sub>2</sub> (freshly prepared) was added into the chilli pepper extract 0.5 mL. Then, after incubated at 50°C in a water bath for 35 min the absorbance at A<sub>581</sub> and A<sub>543</sub> was measured.

## RESULTS AND DISCUSSION

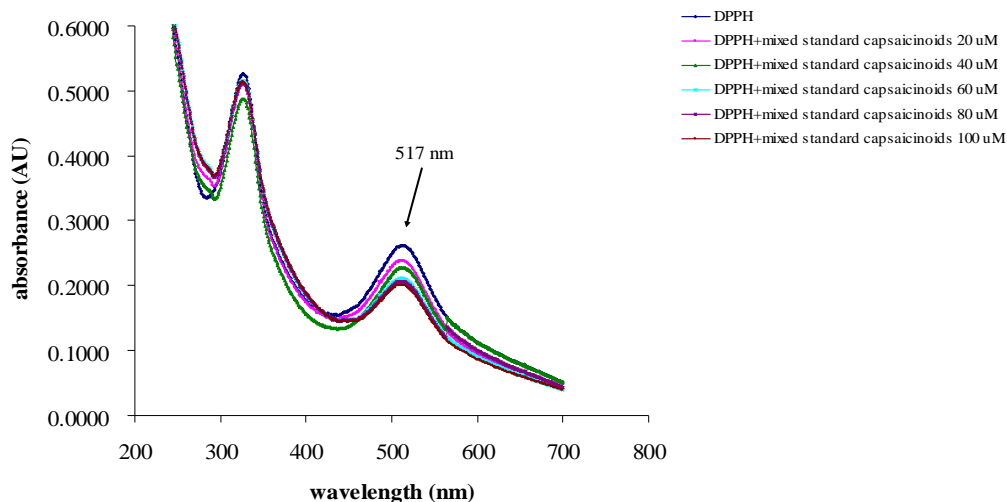
**Antioxidant capacity of capsaicinoids :** Since the capsaicinoids in the chilli pepper samples had been determined by reversed phase HPLC-PDA detector, the contents of capsaicin and dihydrocapsaicin in ten varieties of the samples after extraction by both MSE and ASE methods were found to be 910.5-4981.4 µg/g DW and 692.6-2087.5 µg/g DW, respectively, as shown in Figure 1. The contents of both capsaicin and dihydrocapsaicin obtained from using both of the extraction methods were comparable without statistically significant difference ( $p < 0.05$ ). Among these chilli samples, sample S04 gave the highest contents of capsaicin. Some samples contained quite low amounts of the capsaicinoids in average (S01, S05, S08, S09 and S10). Those of the others were in average not higher than 2,000 µg/g DW.



**FIGURE 1: The contents of capsaicin and dihydrocapsaicin in hot chilli pepper samples extracted by using both MSE and ASE methods and determined by RP-HPLC-PDA**

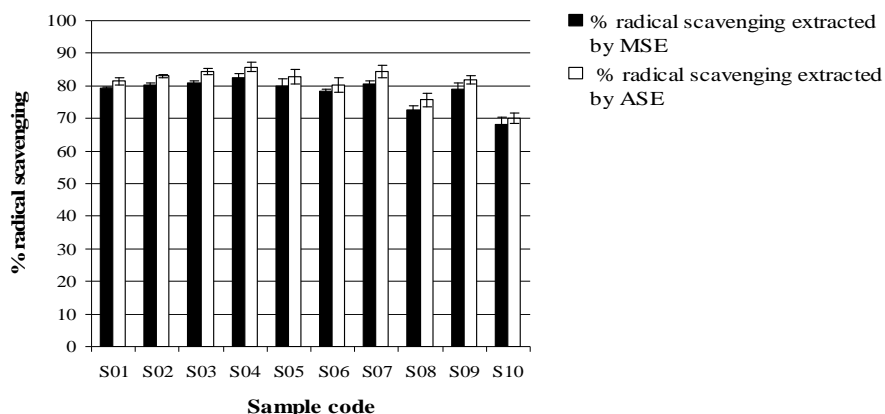
Concerning on antioxidant capacity, the absorbance decreases as a result of a color change from purple to yellow as the DPPH<sup>•</sup> is scavenged by hydrogen donating antioxidant due to the formation of non-radical form (DPPH-H). The remaining DPPH<sup>•</sup>, measured after a certain time, corresponds inversely to the radical scavenging activity of the sample. So, the most rapidly the

absorbance decreases, the most potent the antioxidant activity of the compound in terms of hydrogen donating ability [16,17]. Furthermore, the different reaction kinetics of antioxidant with DPPH depends on the nature of antioxidants. Figure 2 shows the spectra of  $4.0 \times 10^{-5}$  M DPPH<sup>•</sup> solution with various concentrations of the mixture of capsaicinoids standard, giving its maximum wavelengths at 327 nm and 517 nm. But the reaction capability of DPPH<sup>•</sup> was determined by the decrease in its absorbance at 517 nm ( $A_{517}$ ) as induced by the antioxidants. The absorbance of DPPH<sup>•</sup> decreased with an increasing of the concentrations of the capsaicinoids. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The effect of capsaicin, dihydrocapsaicin and  $\alpha$ -tocopherol used as a control at 20.0  $\mu$ M on the radical DPPH in methanolic solution was also investigated (data not shown). It was found that the percentage of its radical scavenging of  $\alpha$ -Tocopherol instantly increased and attained a maximum within 10 min, since its capability for H<sup>+</sup> donor is very quickly. In contrast, both capsaicin and dihydrocapsaicin exhibited the radical scavenging activity slowly and found constantly within 40 min. However, after incubated at ambient temperature for 40 min, the free radical scavenging activity of both solutions of capsaicin and dihydrocapsaicin standard and  $\alpha$ -tocopherol solution also increased with an increasing of their concentrations. Thus, both capsaicinoids exhibited their concentration depending of the radical scavenging power. However,  $\alpha$ -tocopherol of 100.0  $\mu$ M still showed constantly (saturation) for its radical scavenging activity.



**FIGURE 2: UV-visible spectra of DPPH<sup>•</sup> as a control and DPPH<sup>•</sup> with various concentrations of the mixture of the capsaicinoids standard**

The total antioxidant activity of capsaicinoids was then related with the radical scavenging of DPPH. In this study, the capsaicinoids were methanolic extracted using two extraction methods, MSE and ASE, from hot chilli pepper samples. The average percentages of antiradical scavenging of these extracts were ranged from 68.1-82.6% for MSE and 70.0-85.8% for ASE technique as shown in Figure 3. It suggests that both extraction techniques gave comparatively a high percentage of the radical scavenging according to the amounts of the obtained capsaicinoids. However, the power of radical scavenging of the crude extract using ASE was considerably higher than that of MSE.

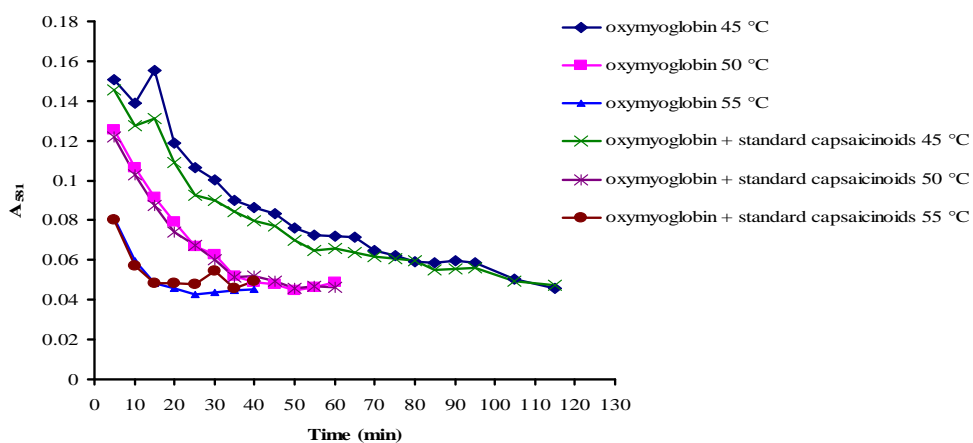


**FIGURE 3: Percentage of radical scavenging of capsaicinoids obtained from hot chili pepper extracts using both MSE and ASE methods.**

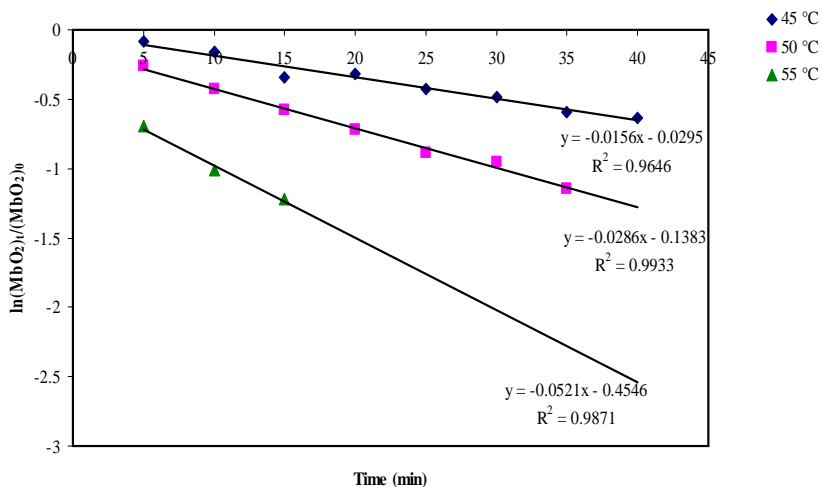
**The retarding of the autoxidation of oxymyoglobin by capsaicinoids:** An alternative method to retard the autoxidation of MbO<sub>2</sub> to be metMb has been concerning on natural antioxidants. Addition of antioxidants to meat products is known affecting on metMb formation and lipid oxidation. The aim of the study was to investigate the ability of antioxidants present in chili pepper extract to provide protection against autoxidation of MbO<sub>2</sub>.

The MbO<sub>2</sub> of horse skeletal muscle was prepared by monitoring the Soret peak at 413 nm after being saturated with O<sub>2</sub> and then slightly blue-shifted to 411 nm after it settled down for a while at room temperature and or in a cool ice-box container. It gave the visible bands at 581 ( $\alpha$  peak) and 543 ( $\beta$  peak) nm (data not shown). The effect of temperature (45, 50 and 55°C) was investigated in detail to activate the autoxidation rate of MbO<sub>2</sub> to be metMb both in the presence and in the absence of capsaicinoids. The visible bands at A<sub>581</sub> and A<sub>543</sub> within a time interval of 115 min were monitored in order to fix suitable time for the autoxidation reaction (Figure 4). It was found that an increasing of temperature affected directly the autoxidation of MbO<sub>2</sub> done very quickly. Therefore, in fact, the thermal effect influences the reaction kinetics of the autoxidation of MbO<sub>2</sub>. The observed first-order rate constants ( $k_{obs}$ ) for the autoxidation of MbO<sub>2</sub> at 45, 50 and 55°C were drawn from their slope of the linear equation (Figure 5). From the slopes of the straight lines, the  $k_{obs}$  at those temperatures were obtained. The  $k_{obs}$  values obtained from the MbO<sub>2</sub> increased along with an increasing of temperature. Then, the effect of the capsaicinoids on the retarding of the autoxidation reaction of MbO<sub>2</sub> could be studied under the optimum conditions. The results showed that the capsaicinoids standard could retard the autoxidation of MbO<sub>2</sub> of which was different in the  $k_{obs}$  values between MbO<sub>2</sub> as control and MbO<sub>2</sub> in the presence of capsaicinoids standard as shown in Figure 6. For instance, at a typical concentration of 30 mg/L capsaicinoids and at 50°C incubation the obtained  $k_{obs}$  decreased from  $2.86 \times 10^{-2}$  to  $2.72 \times 10^{-2}$ .

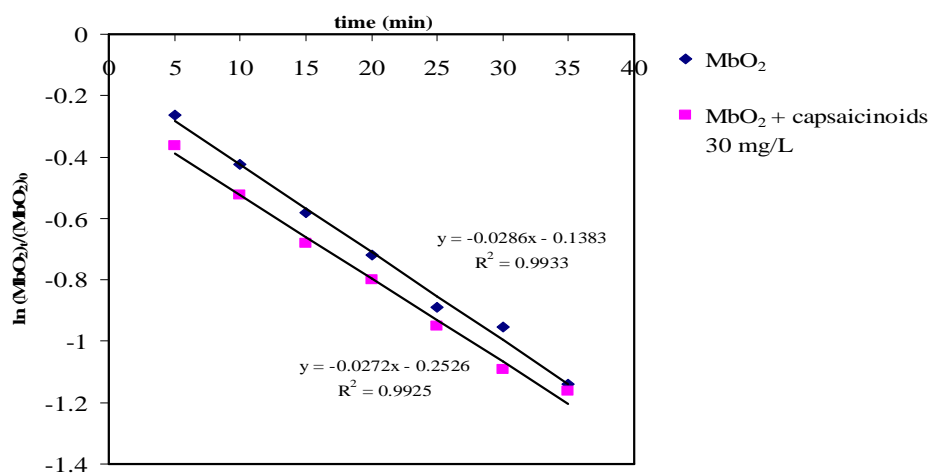




**FIGURE 4:** Effect of temperatures (45, 50 and 55 °C) and capsaicinoids (the mixture of standard solution of both capsaicin and dihydrocapsaicin containing of 5  $\mu\text{g}$ ) on  $\text{MbO}_2$  measured at  $A_{581}$ .



**FIGURE 5:** First-order plots for the autoxidation of  $\text{MbO}_2$  solution as control at 45, 50 and 55 °C. Myoglobin concentration was 0.78 mg/mL.



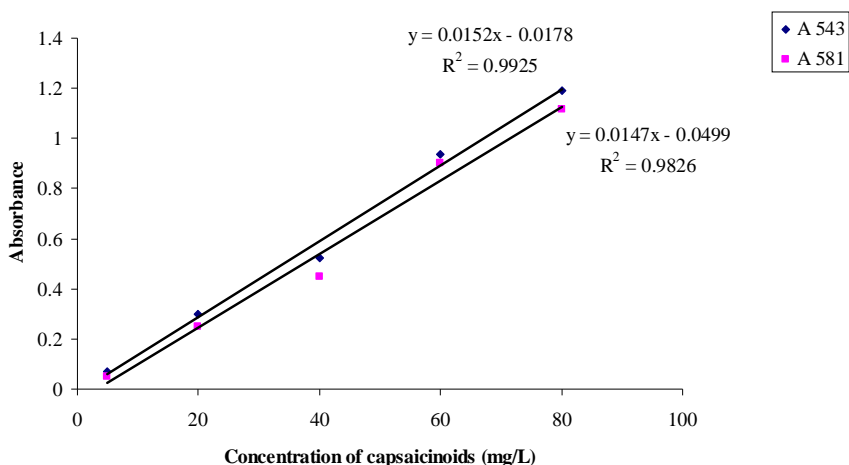
**FIGURE 6: First-order plots for the autoxidation of MbO<sub>2</sub> solution at 50°C in the present of capsaicinoids (30 mg/L). Myoglobin concentration was 0.78 mg/mL.**

Table 1 shows the  $k_{\text{obs}}$  values of the autoxidation of MbO<sub>2</sub> at various temperatures and both in the presence and absence of capsaicinoids (10 mg/L). Although it seemed to be not much different, but the retarding effect of the autoxidation of the oxygen binding protein is considerably confined at higher temperatures. So, the content of capsaicinoids could be somewhat attributed to the first-order rate constant. Thus, the effect of concentration of the capsaicinoids on the autoxidation of MbO<sub>2</sub> was further investigated, resulting that an increasing of the capsaicinoid concentrations was linearly related to stabilize the A<sub>543</sub> and A<sub>581</sub> (Figure 7). It can be experimentally confirmed that the antioxidant capacity of capsaicinoids could retard the autoxidation reaction of MbO<sub>2</sub> (to stabilize Fe<sup>2+</sup> ion bound strongly with O<sub>2</sub>) at 50°C for 35 min as for the studied conditions.

**TABLE 1: The observed first-order rate constants ( $k_{\text{obs}}$ ) obtained from a temperature-dependent and the effect of capsaicinoids on the autoxidation of MbO<sub>2</sub>.**

Temperature (°C)	$k_{\text{obs}}$ of MbO <sub>2</sub> solution	$k_{\text{obs}}$ of MbO <sub>2</sub> in the present of capsaicinoids (10 mg/L)
45	$1.56 \times 10^{-2}$	$1.55 \times 10^{-2}$
50	$2.86 \times 10^{-2}$	$2.81 \times 10^{-2}$
55	$5.21 \times 10^{-2}$	$5.10 \times 10^{-2}$

In this study, the effect of sample volume (0.1-0.5 mL) of the sample extract of capsaicinoids adding into MbO<sub>2</sub> solution was also investigated under the optimum conditions (data not shown), resulted in the linear increasing of the retardation of the autoxidation of MbO<sub>2</sub>. The sensitivity of the capsaicinoids expressed as the slopes obtained from ten varieties of chilli samples were rather different as shown in Table 2. These results suggest that different varieties of hot chilli peppers have different levels of the retardation activity of the autoxidation of MbO<sub>2</sub>. While their antioxidant capacities were comparable (Figure 2). The capsaicinoids from sample S09 gave the most positive effect against the autoxidation compared among other samples, whereas sample S03 gave less retardation effect.

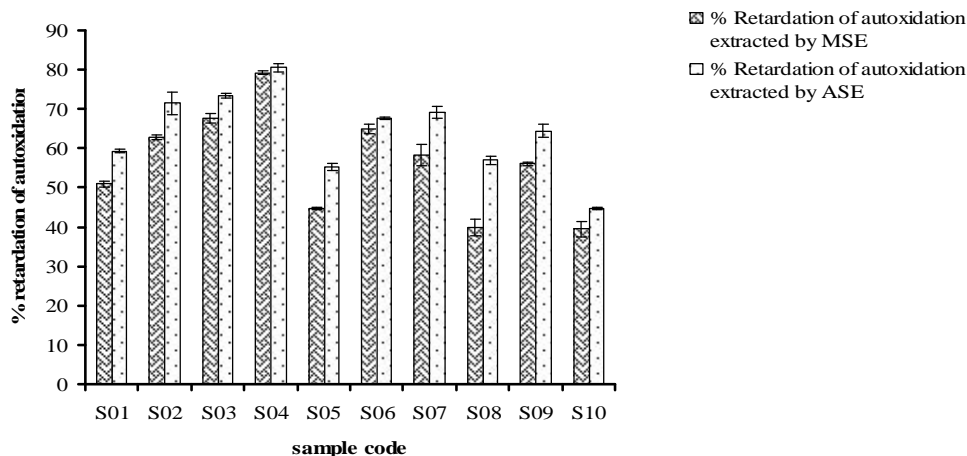


**FIGURE 7: The effect of concentration of capsaicinoids; the mixture of standard solution of capsaicin and dihydrocapsaicin (5.0-80.0 mg/L) on the autoxidation of MbO<sub>2</sub> solution.**

As the capsaicinoids could retard the autoxidation of MbO<sub>2</sub> after incubated at 50 °C for 35 min, the average percentages of the extracts for the retardation of autoxidation reaction were found to be 34.0-74.5% (at A<sub>543</sub>) and 39.5-79.2% (at A<sub>581</sub>) for the capsaicinoids extracted by MSE and 38.9-75.8% (at A<sub>543</sub>) and 44.7-80.5 (at A<sub>581</sub>) for the capsaicinoids extracted by ASE as shown in Table 3 and Figure 8. It can be noted that measuring the absorbance due in course of MbO<sub>2</sub> transformation is slightly different in the percentage ranges. However, mostly the slopes obtained from A<sub>543</sub> were slightly higher than those of A<sub>581</sub>. In addition, the contents of capsaicinoids obtained from the ASE method also exhibit higher percentages of the retarding effect of the autoxidation reaction. Moreover, this trend is shown somewhat differences in the corresponding power of the radical scavenging capacity and total capsaicinoids found in those samples. It is suggested that it is not only antioxidant property of the capsaicinoids being affected on the retarding of the autoxidation of MbO<sub>2</sub>. Other natural products in the sample extracts might play additionally concerning the oxidation reaction of the heme group in the protein.

**TABLE 2: Regression equations and correlation coefficients of the sample extracts of capsaicinoids on the autoxidation of MbO<sub>2</sub> measured at A<sub>543</sub> and A<sub>581</sub>.**

Sample	Measured at A <sub>543</sub>		Measured at A <sub>581</sub>	
	Equation	r <sup>2</sup>	Equation	r <sup>2</sup>
S01	y=1.1166x+0.1678	0.9154	y=0.9217x+0.1213	0.9154
S02	y=0.8650x+0.1793	0.9124	y=0.6997x+0.1333	0.8466
S03	y=0.4390x+0.2149	0.5863	y=0.7320x+0.1678	0.8600
S04	y=0.8125x+0.2281	0.9765	y=0.8125x+0.2281	0.9765
S05	y=0.6771x+0.1899	0.8903	y=0.6771x+0.1899	0.8903
S06	y=0.8510x+0.2395	0.8273	y=0.9190x+0.1805	0.6891
S07	y=0.5557x+0.2023	0.9049	y=0.4469x+0.1579	0.8286
S08	y=0.9577x+0.1744	0.9008	y=0.7997x+0.1298	0.8504
S09	y=1.5316x+0.1878	0.9814	y=1.3006x+0.1368	0.9637
S10	y=0.6627x+0.2059	0.8990	y=0.5304x+0.1548	0.8336



**FIGURE 8: The retardation effect of autoxidation of MbO<sub>2</sub> in the presence of the extract of hot chilli peppers using both MSE and ASE methods and determined at A<sub>581</sub> nm.**

The selectivity of some organic compounds present in the crude extract of the hot chilli pepper would be taken into accounts. Typically, sample S04 which contains the highest contents of both capsaicinoids exhibit the highest retardation capacity of the autoxidation of MbO<sub>2</sub>. In other meaning, the antioxidant activity of the capsaicinoids can stabilize the heme group of the protein to prolong its oxygen molecule, slow down the kinetics phenomenon of meat darkening, in the muscle. These results are, therefore, implied for meat preservation by some natural products.

**TABLE 3: The retardation of autoxidation of MbO<sub>2</sub> solution against the crude extracts obtained from ten varieties of hot chilli pepper samples (mean  $\pm$  SD,  $n = 3$ ).**

Sample	MSE		ASE	
	% Retardation of autoxidation at A <sub>543</sub>	% Retardation of autoxidation at A <sub>581</sub>	% Retardation of autoxidation at A <sub>543</sub>	% Retardation of autoxidation at A <sub>581</sub>
S01	44.9 $\pm$ 0.6	50.9 $\pm$ 0.7	53.4 $\pm$ 0.5	59.3 $\pm$ 0.5
S02	56.8 $\pm$ 0.7	62.8 $\pm$ 0.6	66.3 $\pm$ 2.9	71.5 $\pm$ 2.8
S03	61.7 $\pm$ 1.4	67.6 $\pm$ 1.3	68.0 $\pm$ 0.7	73.5 $\pm$ 0.6
S04	74.5 $\pm$ 0.5	79.2 $\pm$ 0.4	75.8 $\pm$ 1.1	80.5 $\pm$ 1.0
S05	38.9 $\pm$ 0.7	44.7 $\pm$ 0.2	49.4 $\pm$ 0.9	55.4 $\pm$ 0.9
S06	59.0 $\pm$ 1.3	64.9 $\pm$ 1.3	62.1 $\pm$ 0.3	67.7 $\pm$ 0.3
S07	51.0 $\pm$ 2.6	58.2 $\pm$ 2.7	63.8 $\pm$ 0.7	69.1 $\pm$ 1.5
S08	34.7 $\pm$ 1.9	39.9 $\pm$ 2.1	50.8 $\pm$ 0.9	57.0 $\pm$ 1.0
S09	49.7 $\pm$ 0.8	56.1 $\pm$ 0.4	58.4 $\pm$ 1.5	64.4 $\pm$ 1.6
S10	34.0 $\pm$ 1.8	39.5 $\pm$ 2.0	38.9 $\pm$ 0.3	44.7 $\pm$ 0.2

## APPLICATIONS

The spicy taste as often the so-called capsaicin in chilli pepper plays an important role as an antioxidant capacity of food ingredients and or food products. The antioxidant activity of the capsaicinoids can stabilize the heme group of the protein to prolong its oxygen molecule, slow down the kinetics phenomenon of meat darkening, in the muscle. These results are, therefore, implied for meat preservation by some natural products. Different varieties of hot chilli peppers have different levels of the retardation activity of the autoxidation of MbO<sub>2</sub>

## CONCLUSION

The present study reported the crude extract of capsaicinoids mostly consisting of two major components as capsaicin and dihydrocapsaicin which had been extracted using MSE (methanol, 60 °C, 2 h) and ASE (methanol, 5 min static time, 3 cycles) and determined by RP-HPLC-PDA. The total contents of both capsaicinoids were found in the range of 2,110.9-8,766.9 µg/g DW. Concerning these data, an antioxidant capacity of the capsaicinoids based on the measurement of the absorbance decrease as a result of the antioxidant scavenging of DPPH<sup>•</sup> was determined within the reaction time of 40 min to obtain the steady state of this measurement. The average percentages of the radical scavenging of the extracts were found to be 68.1-82.6% and 70.0-85.8% for MSE and ASE techniques, respectively. In application, the retardation of autoxidation of MbO<sub>2</sub> in the presence of capsaicinoids was measured under the optimum conditions (35 min incubation at 50°C and at the maximum wavelength of both A<sub>543</sub> and A<sub>581</sub>). The average percentages of the retarding of autoxidation rate reaction were ranged from 34.0-74.5 % using A<sub>543</sub> and 39.5-79.2% using A<sub>581</sub> for MSE, and 38.9-75.8% using A<sub>543</sub> and 44.7-80.5% using A<sub>581</sub> for ASE, respectively. The DPPH radical scavenging capacity of the sample extracts were found higher than 70% in average corresponding with the trend in the retardation of MbO<sub>2</sub> to be metMb by the capsaicinoids. From these results, it is implied that the spicy taste as often the so-called capsaicin in chilli pepper plays an important role as an antioxidant capacity of food ingredients and or food products.

## ACKNOWLEDGEMENTS

The authors are grateful for financial support from the Center for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand, and the Hitachi Scholarship Foundation, Tokyo, Japan.

## REFERENCES

- [1] J. C. Tapia, R. Garcia, M. Eleazar, G. Calva, and J. A. Rocha, "Capsaicin recovery from a cell culture broth," *Indian Engineering and Chemistry Research*, vol. 32, pp. 2242-2246, **1993**.
- [2] K. Sato, S. S. Sasaki, Y. Goda, T. Yamada, O. Nunomura, K. Ishikawa, and T. Maitani, "Direct connection of supercritical fluid extraction and supercritical fluid chromatography as a rapid quantitative method for capsaicinoids in placentas of *Capsicum*," *Journal of Agricultural and Food Chemistry*, vol. 47, pp. 4665-4668, **1999**.
- [3] G. F. Barbero, M. Palma, and C. G. Barroso, "Pressurized liquid extraction of capsaicinoids from peppers," *Journal of Agricultural and Food Chemistry*, vol. 54, pp. 3231-3236, **2006**.
- [4] E. Kaale, A. V. Schepdael, E. Roets, and J. Hoogmartens, "Determination of capsaicinoids in topical cream by liquid-liquid extraction and liquid chromatography," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 30, pp.1331-1337, **2002**.
- [5] C. Reilly, D. Crouch, G. Yost, and A. A. Fatah, "Determination of capsaicin, dihydrocapsaicin, and nonivamide in self-defense weapons by liquid chromatography-

- mass spectrometry and liquid chromatography-tandem mass spectrometry,” *Journal of Chromatography A*, vol. 912, pp.259-267, **2001**.
- [6] O. J. Spicer, and J. R. Almirall, “Extraction of capsaicin in aerosol defense sprays from fabrics,” *Talanta*, vol. 67, pp. 377-382, **2005**.
- [7] P. K. Titze, E. M. Seitz, and M. Petz, “Pungency in paprika (*Capsicum annuum* L.). 2. Heterogeneity of capsaicinoid content in individual fruits from one plant,” *Journal of Agricultural and Food Chemistry*, vol. 50, pp. 1264-1266, **2002**.
- [8] F. Korel, N. Bagdatlioglu, M. O. Balaban, and Y. Hisil, Y. “Ground red peppers: capsaicinoids content, scoville scores, and discrimination by an electronic nose,” *Journal of Agricultural and Food Chemistry*, vol. 50, pp. 3257-3261, **2002**.
- [9] A. P. Uzunalic, M. Skerget, B. Weinreich, and Z. Knes, “Extraction of chilli pepper (var. Byedige) with supercritical CO<sub>2</sub>: effect of pressure and temperature on capsaicinoid and color extraction efficiency,” *Food Chemistry*, vol. 87, pp. 51-58, **2004**.
- [10] R. I. Santamaria, M. D. Reyes-Duarte, E. Barzana, E. Fernando, D. Gama, M. Moto, and A. Lopez-Munguim, “Selective enzyme-mediated extraction of capsaicinoids and carotenoids from chili guajillo puya (*Capsicum annuum* L.) using ethanol as solvent,” *Journal of Agricultural and Food Chemistry*, vol. 48, pp. 3063-3067, **2000**.
- [11] B. V. Thomas, A. A. Schreiber, and C. P. Weisskop, “Simple method for quantitation of capsaicinoids in peppers using capillary gas chromatography,” *Journal of Agricultural and Food Chemistry*, vol. 46, pp. 2655-2663, **1998**.
- [12] G. F. Barbero, M. Palma, and C. G. Barroso, “Determination of capsaicinoids in peppers by microwave-assisted extraction-high performance liquid chromatography with fluorescence detection,” *Analytica Chimica Acta*, vol. 578, pp. 227-233, **2006**.
- [13] F. Higashiguchi, H. Nakamura, H. Hayashi, and T. Kometani, “Purification and structure determination of glucosides of capsaicin and dihydrocapsaicin from various *Capsicum* fruits,” *Journal of Agricultural and Food Chemistry*, vol. 54, pp. 5948-5953, **2006**.
- [14] U. Schweiggert, R. Carle, and A. Schieber, “Characterization of major and minor capsaicinoids and related compounds in chili pods (*Capsicum frutescens* L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry,” *Analytica Chimica Acta*, vol. 557, pp. 236-244, **2006**.
- [15] Y. J. Surh, and S. S. Lee, “Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential,” *Life Sciences*, vol.56, pp. 1845-1855, **1995**.
- [16] M. Materska, and I. Perucka, “Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L.),” *Journal of Agricultural and Food Chemistry*, vol. 53, pp. 1750-1756, **2005**.
- [17] K. Kogure, S. Goto, M. Nishimura, M. Yasumoto, K. Abe, C. Ohiwa, H. Sassa, T. Kusumi, and H. Terada, “Mechanism of potent antiperoxidative effect of capsaicin,” *Biochimica Biophysica Acta*, vol. 1573, pp. 81-92, **2002**.
- [18] D. E. Henderson, and A. M. Slickman, “Quantitative HPLC determination of the antioxidant activity of capsaicin on the formation of lipid hydroperoxide of linoleic acid: a comparative study against BHT and melatonin,” *Journal of Agricultural and Food Chemistry*, vol. 47, pp. 2563-2570, **1999**.
- [19] A. Rosa, M. Deiana, V. Casu, S. Paccagnini, G. Appendino, M. Ballero, and M. A. Dessi, “Antioxidant activity of capsinoids,” *Journal of Agricultural and Food Chemistry*, vol. 50, pp. 7396-7401, **2002**.

- [20] T. Ochi, Y. Takaishi, K. Kogure, and I. Yamauti, "Antioxidant activity of a new capsaicin derivative from *Capsicum annuum*," *Journal of Natural Products*, vol. 66, pp. 1094-1096, **2003**.
- [21] D. Zhang, and Y. Hamauza, "Phenolic compounds, ascorbic acid, carotenoids and antioxidant properties of green, red and yellow bell peppers," *Food and Agricultural Environment*, vol. 1, no. 2, pp. 22-27, **2003**.
- [22] D. Huang, B. Ou, and R. L. Prior, "The chemistry behind antioxidant capacity assays," *Journal of Agricultural and Food Chemistry*, vol. 53, pp. 1841-1856, **2005**.
- [23] I. Gülçin, I. "Antioxidant and antiradical activities of L-carnitine," *Life Sciences*, vol. 78, pp. 803-811, **2006**.
- [24] S. Chatterjee, Z. Niaz, S. Gautam, S. Adhikari, P. S. Variyar, and A. Sharma, "Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (*Myristica fragrans*)," *Food Chemistry*, vol. 101, pp. 515-523, **2007**.
- [25] X. Lui, S. Ardo, M. Bunning, J. Parry, K. Zhou, C. Stushnoff, F. Stoniker, L. Yu, and P. Kendall, "Total phenolic content and DPPH\* radical scavenging activity of lettuce (*Lactuca sativa* L.) grown in Colorado," *LWT-Food Science and Technology*, vol. 40, pp. 552-557, **2007**.
- [26] T. H. Tsai, P. J. Tsai, and S. C. Ho, "Antioxidant and anti-inflammatory activities of several commonly used spices," *Journal of Food Science*, vol. 70, pp. 93-97, **2005**.
- [27] T. L. Mc Carthy, J. P. Kerry, J.F. Kerry, P.B. Lynch, and D. J. Buckley, D.J. "Assessment of the antioxidant potential of natural food and plant extracts in fresh and previously frozen pork patties," *Meat Sciences*, vol. 57, pp. 177-184, **2001**.
- [28] A. E. D. Bekhit, G. H. Geesink, M.A. Ilian, J.D. Morton, and R. Bickerstaffe, "The effects of natural antioxidants on oxidative processes and metmyoglobin reducing activity in beef patties," *Food Chemistry*, vol. 81, pp. 175-187, **2003**.
- [29] A. Sánchez-Escalante, D. Djenane, G. Torrecano, B. Giménez, J. A. Beltrán, and P. Roncalés, "Evaluation of the antioxidant ability of hydrazine-purified and untreated commercial carnosine in beef patties," *Meat Sciences*, vol. 64, pp. 59-67, **2003**.
- [30] J. G. Sebranek, V. J. H. Sewalt, K. L. Robbins, and T. A. Houser, "Comparison of a natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork sausage," *Meat Sciences*, vol. 69, pp. 289-296, **2005**.
- [31] R. E. Dickerson, and I. Gels, "The structure and action of proteins," *Molecular Carriers*, Benjamin/Cummings Publishing Company, California, USA, pp. 44-66, **1969**.
- [32] W. M. Peter, J. B. Michael, E. V. Michael, and E. C. Robert, "Structural and kinetic characterization of myoglobins from eurythermal and stenothermal fish species," *Comparative Biochemistry and Physiology B*, vol. 137, no.3, pp. 341-350, **2004**.
- [33] Y. Tsubamoto, A. Matsuoka, K. Yusa, and K. Shimaka, "Protozoan myoglobins from *Paramecium caudatum*: Its autooxidation reaction and hemichrome formation," *European Journal of Biochemistry*, vol. 193, pp. 55-59, **1990**.
- [34] S. Chanthai, H. Neida, M. Ogawa, T. Tamiya, and T. Tsuchiya, "Studies on thermal denaturation of fish myoglobins using differential scanning calorimetry, circular dichroism and tryptophan fluorescence," *Fisheries Sciences*, vol. 64, no. 3, pp. 923- 927, **1996**.