CHAPTER II

LITERATURE REVIEW

To design capsinoid analogues with inverse position of ester linkage to reduced degradation process *via* *p*-quinonemethide pathway, the basic knowledge of capsinoids, mode of action, stability of capsinoids, degradation process through *p*-quinonemethide pathway, toxicity are necessary explored.

Capsinoids

An analogue of capsinoids, capsiate, was first reported in 1989 by Kobata and co-worker. [1] Capsinoids are a group of non-pungent compounds extracted from a sweet pepper named "CH-19 sweet" and most of their biological activities are similar to capsaicinoids. These substances are consisting of capsiate (**1**), dihydrocapsiate (**2**) and nordihydrocapsiate (**3**) [22] and contain an ester bond instead of amide bond as found in capsaicinoids; however, the acyl residue of capsinoids is identical to capsaicinoids; (Figure 7).

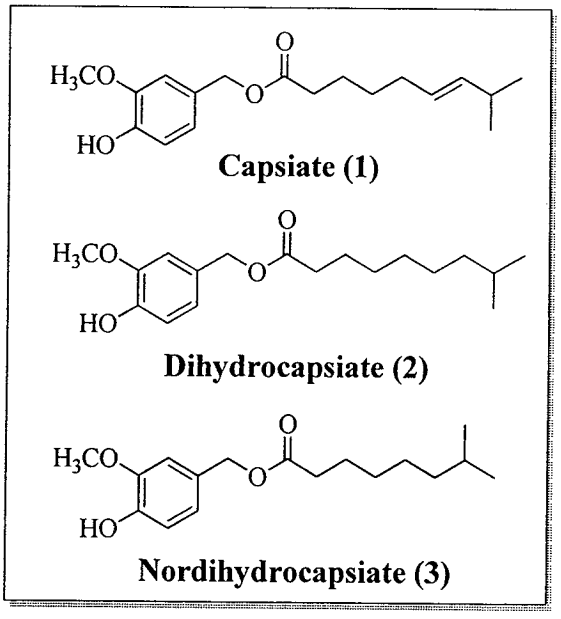


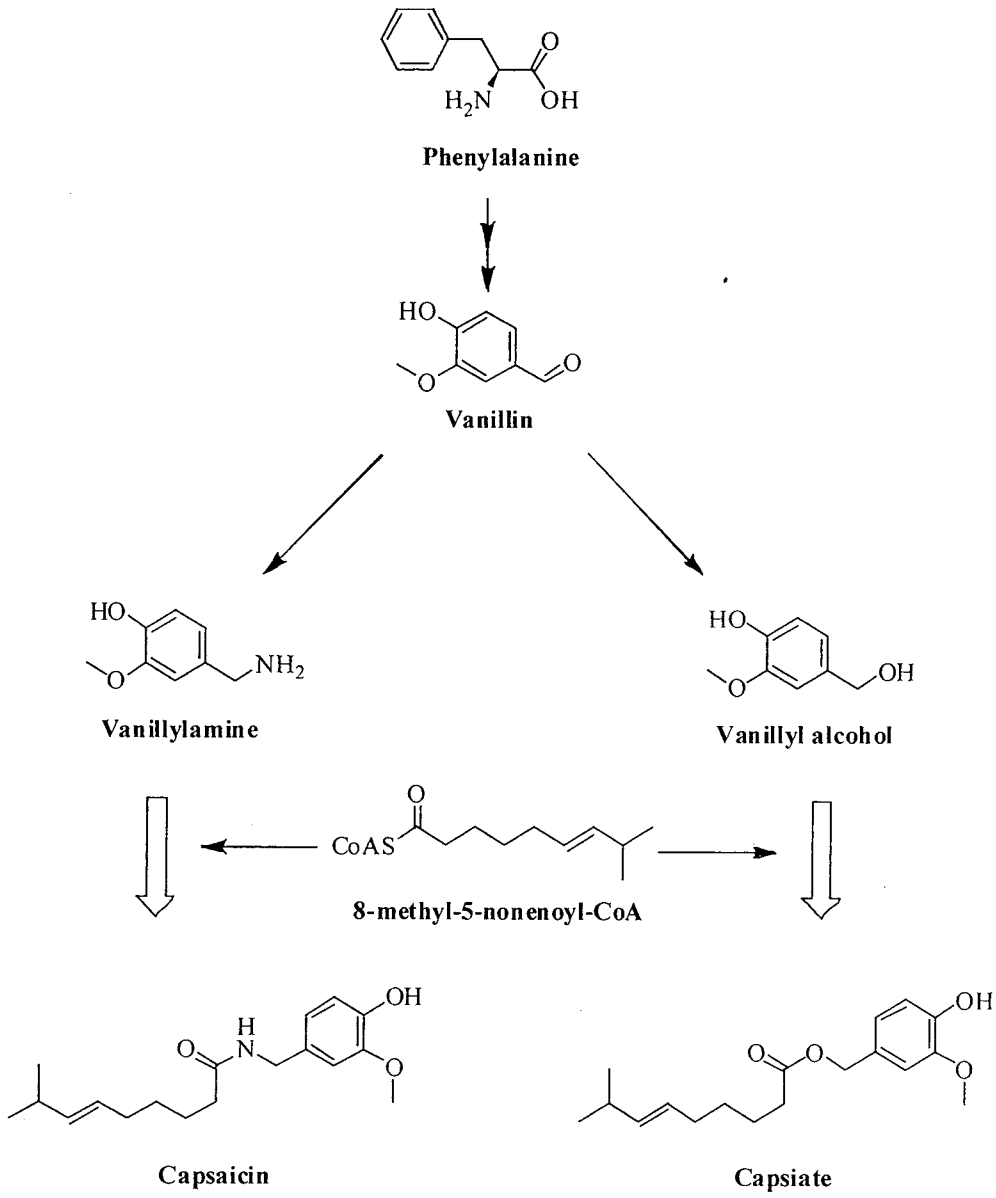
Figure 7 Structures of capsinoids [22]

Large amounts of capsiate can be consumed without irritation. Lacking of pungency while maintaining activity as similar as capsaicin, this make capsiate become a very interesting substance for substitution on capsaicin to provide effective approach for increasing energy expenditure, body temperature and oxygen consumption.

Moreover, the production of capsiate under commercial named "Capsiate Natura" has been approved by the Food and Drug Administration (FDA) in 2007 [23], is a natural product comprising of capsinoids extracted from non-pungent CH-19 sweet pepper plants. Additionally, capsiate have been presented to proliferation post-meal energy expenditure, increase lipid oxidation, reduction of visceral fat, reduce food and fat intake. They have also been displayed to rise fat burning in humans through stimulation of the sympathetic nervous system (SNS) [24]. The advantage of capsiate is enhancement of metabolism without increasing blood pressure or heart rate [25].

Biosynthesis

Biosynthesis pathway of capsinoids is similar to capsaicinoids [26], the aromatic moiety of capsinoids (vanillyl alcohol) is biosynthesized from phenylalanine *via* the phenylpropanoid pathway and the lipophilic chain moiety is generated from branched amino acids such as valine *via* the fatty acid elongation pathway [27]. Generally, putative aminotransferase (pAMT) transforms vanillin to vanillylamine at the end of the phenylpropanoid pathway in capsaicinoid biosynthesis. However, lacking of pAMT in capsinoids; consequently, inhibits capsaicinoid accumulation while induction of capsinoid accumulation in pepper fruits instead. [28, 29] (Scheme 1)



Scheme 1 Biosynthesis pathway of capsaicin and capsiate [26]

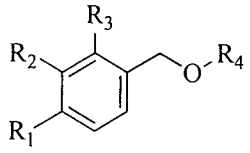
Stability of capsinoids

Even though, capsinoids played an important role of beneficial effects similarly to capsaicinoids without any irritation, their stability is still a problem. It was found that they were highly unstable and easily decomposed when exposed under the light and high temperature over a short periods of time.

Recently, the stability of capsinoids in various solvents was observed by Watanabe and co-worker in 2001 [18]. They found that the quantitative change of

vanillyl nonanoate, a capsinoid analogue, in various solvents was clearly observed by HPLC technique. It was found that vanillyl nonanoate was stable in nonpolar solvents while it was labile in polar solvents such as alcohol and water. In this study, it was shown that the decomposition products from vanillyl nonanoate in methanol and ethanol were methyl and ethyl vanillyl ethers, respectively. To elucidate the decomposition mechanism of capsinoid, six analogues of vanillyl nonanoate were designed and tested (Table 2).

Table 2 Structures of capsiate (1), vanillyl nonanoate, six vanillyl nonanoate analogues and decomposition products, methyl vanillyl ether and ethyl vanillyl ether. [18]



Capsinoid Analogues	R_1	R_2	R_3	R_4
capsiate	OH	OCH_3	H	-C(=O)-$(CH_2)_4CH$=$CHCH(CH_3)_2$
vanillyl nonanoate	OH	OCH_3	H	-C(=O)-$(CH_2)_7CH_3$
4-acetoxy-3-methoxybenzyl nonanoate	OAc	OCH_3	H	-C(=O)-$(CH_2)_7CH_3$
3,4-dimethoxybenzyl nonanoate	OCH_3	OCH_3	H	-C(=O)-$(CH_2)_7CH_3$
o-hydroxybenzyl nonanoate	H	OCH_3	H	-C(=O)-$(CH_2)_7CH_3$
m-hydroxybenzyl nonanoate	H	H	OH	-C(=O)-$(CH_2)_7CH_3$
p-hydroxybenzyl nonanoate	H	OH	H	-C(=O)-$(CH_2)_7CH_3$
p-hydroxybenzyl nonanoate	OH	H	H	-C(=O)-$(CH_2)_7CH_3$
methyl vanillyl ether	OH	OCH_3	H	CH_3
ethyl vanillyl ether	OH	OCH_3	H	C_2H_5

The stability of all capsinoid analogues in organic solvents was observed and it was suggested that the hydroxyl group in the *para*-position of the benzene ring largely contributes to the decomposition of capsinoid analogues. From the table, it was found that capsiate, vanillyl nonanoate and *p*-hydroxybenzyl nonanoate were stable in aprotic polar solvents such as ethyl acetate, chloroform and acetone while it was labile

in protic polar solvents such as methanol, ethanol, DMSO and aqueous surfactant vehicle. (Figure 8)

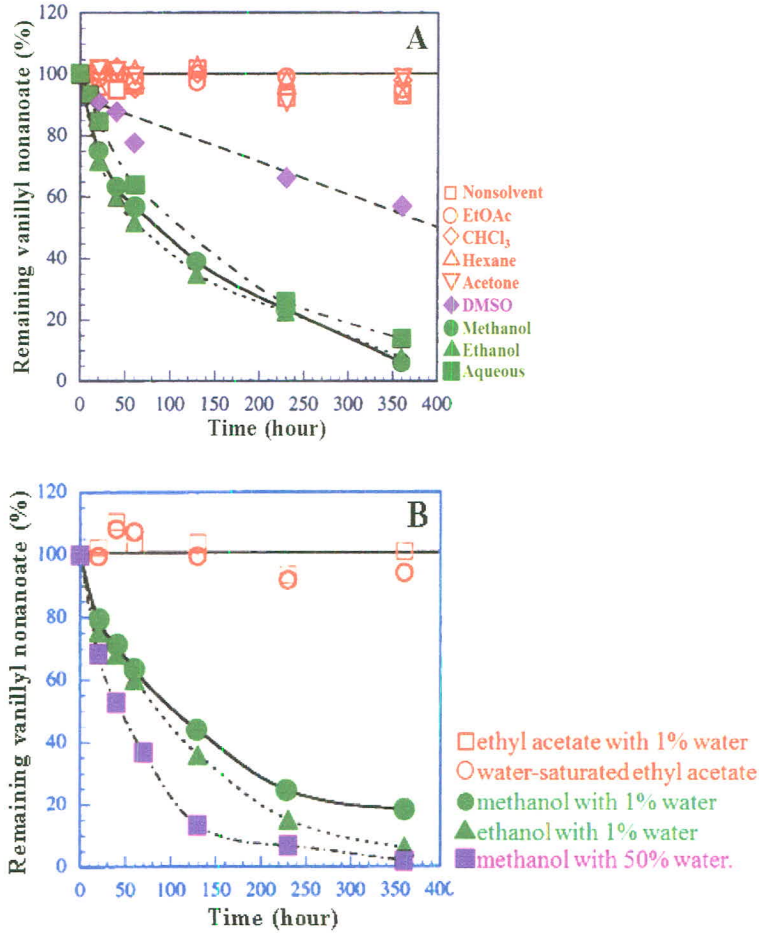
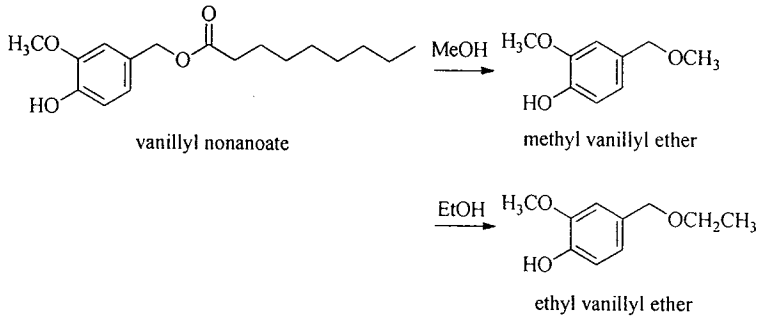


Figure 8 Change in vanillyl nonanoate with time in various solvents (A) and several solvents containing water (B) at 25 °C [18]

The solvolysis of capsinoids by an alcohol gave corresponding alkyl ether and a carboxylic acid. Therefore, the decomposition reaction of capsinoids was quite different from the usual solvolysis of simple esters. They proposed that the decomposition of capsinoid depends on the stability of the benzyl cation formation. In general, the substitution of an electron-donating group such as a hydroxyl group on the benzene ring can delocalize the positive charge at the α-methylene group of the benzyl moiety. The hydroxyl group in a phenol is an ortho/para directing group, therefore the benzyl cations of *o*- and *p*-benzyl phenols are highly stabilized.

Therefore the residue of decomposition depend on the category of solvent, in this case of methanol, methyl vanillyl ether was formed whereas ethyl vanillyl ether was obtained in ethanol (Scheme 2)



Scheme 2 Structures of the decomposition products from vanillyl nonanoate in methanol and ethanol were determined to methyl and ethyl vanillyl ethers [18]

Furthermore, the position of hydroxyl group on the aromatic ring is important role in the decomposition mechanism, the hydroxyl group on *para* and *ortho* position can be stabilized positive charge at the α-methylene leading to the rapid decomposition while the *meta* is highly stable (Figure 9).

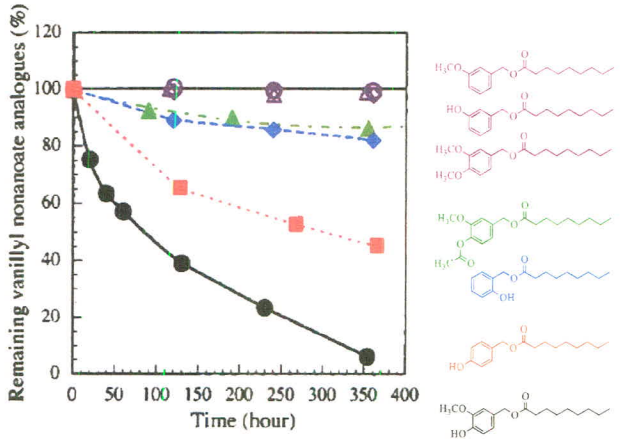
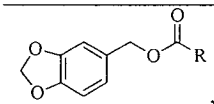
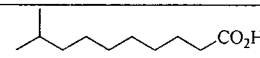
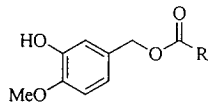
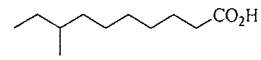
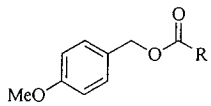
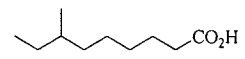
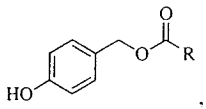
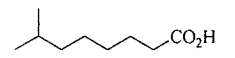
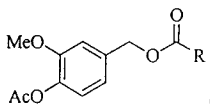
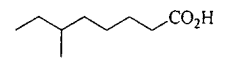
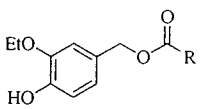
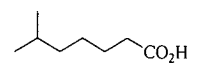
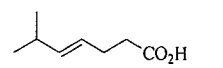
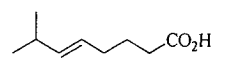
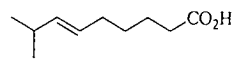
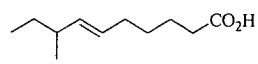
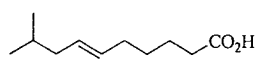
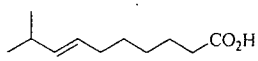
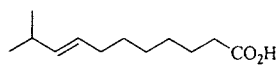
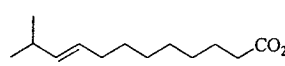


Figure 9 **Change in vanillyl nonanoate analogues with time in methanol at 25 °C: (●) vanillyl nonanoate; (▲) 4-acetoxy-3-methoxybenzyl nonanoate; (Δ) 3,4-dimethoxybenzyl nonanoate; (◊) 3-methoxybenzyl nonanoate; (■) *p*-hydroxybenzyl nonanoate; (○) *m*-hydroxybenzyl nonanoate; (♦) *o*-hydroxybenzyl Nonanoate** [18]

In 2011, the capsinoids stabilization was published in United States Patent by Ajinomoto Co., Inc., Amino and co-worker [30] prepared vanilyl nonanoate, which is a capsinoid, have vanillyl ester bond in molecule that exhibited instability. Moreover, they modified capcinoids structure *via* transforming a substituent on benzene ring which derived from vanillyl alcohol establishing capsinoids derivatives to improve totally sufficient in the stability and achieve stabilization while retaining an ester bond in the structure (Table 3).

Table 3 The substituted benzyl ester derivatives having a branched fatty acid side chain of capsinoids [30]

Substituted benzyl moiety	Carboxylic acid moiety (R-COOH)	Name of carboxylic acid moiety
 ,		9-methyldecanoic acid
 ,		8-methyldecanoic acid
 ,		7-methylnonanoic acid
 ,		7-methyloctanoic acid
 or		6-methyloctanoic acid
		6-methyl-4-heptanoic acid
		(*E*)-6-methyl-4-heptenoic acid
		(*E*)-7-methyl-5-octenoic acid
		(*E*)-8-methyl-6-nonenoic acid
		(*E*)-8-methyl-6-decenoic acid
		(*E*)-9-methyl-6-decenoic acid
		(*E*)-9-methyl-7-decenoic acid
		(*E*)-10-methyl-8-undecenoic acid
		(*E*)-11-methyl-9-dodecenoic acid

In 2007, the US patent publication by Yoko ito and co-worker [19], investigated the stability of capsinoids. They developed a superior stability of the capsinoid compound which can be provided a process of producing a capsinoid-

containing in food and drink. This process involved blending an oil phase which containing capsinoids with an aqueous phase and an emulsifier to prepare emulsion composition, then mixed the emulsion composition with aqueous component to stabilize capsinoids in products

They suggested that capsinoids was unstable due to the ester linkage which formed between a vanilloid region and a fatty acid chain was rapidly hydrolyzed. In this investigation, they suggested that the technique for solubilizing capsinoids in water has been required to add capsinoids into a liquid food. It was successful by emulsifying and emulsion and then solubilizing the capsinoids in water. Moreover, it was found that the variation of concentration of an emulsifier decaglycerin monooleate (DECAGLYNL 1-O) at 0.114%, 0.014% and free from emulsifier may slightly improve the stability during dilution while the presence of an enormous quantity of emulsifier have a negative influenece on the emulsifying process (Figure 10A). The variation of 8 types of emulsifiers were observed which one of emulsifier highly effective that was polyglycerin fatty acid ester, is used an emulsifier (Figure 10B), it was showed the range of usage about 6-10% and capsinoid can be stored at 24 °C for 3 months with improved stability.(Figure 11)

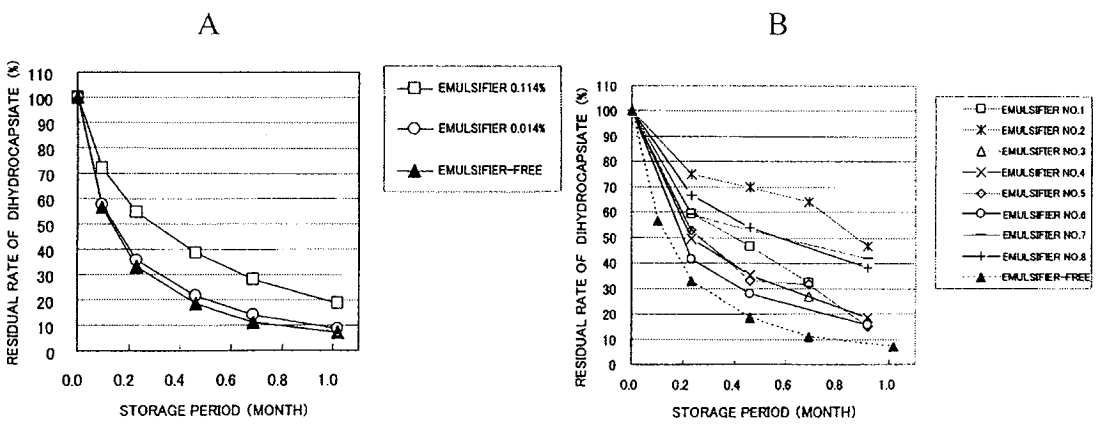


Figure 10 A: the storage stability of dihydrocapsiate in a medium containing decaglycerin monooleate (DECAGLYNL 1-O) B: the storage stability of dihydrocapsiate in a media containing 8 types of emulsifiers [19]

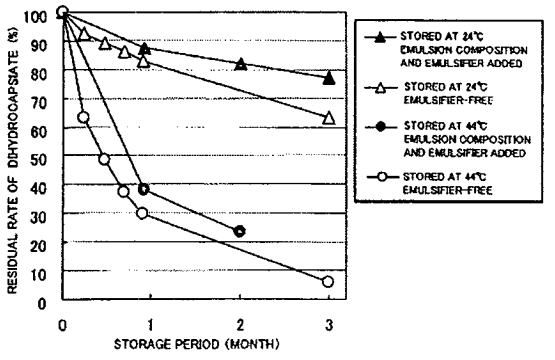


Figure 11 The storage stability of dihydrocapsiate when a capsinoid-containing added a polyglycerin fatty acid ester emulsifier in advance was diluted with an aqueous buffer containing no emulsifier [19]

In 2010, Yusuke and co-worker designed and synthesized capsinoids [31]. They have explored production method of capsinoids *via* dehydrating condensation and stabilizing method of capsinoid and observed degradation of capsinoid composition. They were successfully prepared capsinoids in high yield without using any dehydrating agent at the short period time. It is just only simple esterification reaction between fatty acid and hydroxymethylphenol *via* an immobilized enzyme Novozym 435, which is one kind of lipase enzyme in a non-solvent system or in low-polar solvent which is effective for stabilizing the ester compound of capsinoid.

Moreover, they suggested that the ester compounds can be easily stabilized by adding a small excess amount of fatty acid to ester compound. For example, addition of 9.1% of decanoic acid in acetonitrile to purify vanillyl decanoate separated from decanoic acid, it was found vanillyl decanoate in the range of 97.6 % after storage at 19.5 hours. Therefore, the coexistence of fatty acid in the equilibrium to afford the ester compound led to stabilization of capsinoid.

Toxicity of Capsinoids

Capsinoids exhibit similar physiologicalcal activities as capsaicinoids except pungency. In addition to pungency, toxicity is also one of the major concern for applications and implementation for food and health benefit supplementary. Toxicity of capsinoids was generally studied in both cell and animal as well as in a clinical trial.

In 2007, Dong Huen Shin and co-worker have been studied cytotoxicity of capsinoids in *vitro*, endothelial cells, evaluated through MTT assay at dose of capsinoids in the range of 1, 5, 10, and 25 μM, respectively. It was found no sign of toxicity to cell at high dose [32]. In 2008, a series of toxicity studies has been published dealing with the safety of capsinoids in *vivo* by Kodama and co-worker. These have included the studies of acute toxicity, 26-week chronic toxicity, 2-generation reproductive toxicity, and teratology in rats and rabbits. They continuous published a chronic toxicity study in which rats were administered capsinoids, CH–19 Sweet extract, to rats (containing approximately 7.5% capsinoids). In this experiment, Sprague-Dawley rats received CH–19 Sweet extract by gavage for 26 weeks at dose levels of 0 (vehicle), 1.25, 2.5, and 5.0 ml/kg/day (equivalent to approximately 90, 180, and 360 mg/kg of capsinoids, respectively). There were also shown no sign of toxicological observations within the 4-week recovery period [33].

Moreover, Watanabe and co-worker also investigated the acute oral toxicity of CH-19 Sweet extract containing capsinoids at higher dose. It was examined following a single gavage administration to Sprague-Dawley rats. At dose levels of 0 (vehicle), 5, 10 or 20 mL/kg of body weight of CH-19 Sweet extract, rats received 356.25, 712.5 and 1,425 mg capsinoids/kg, respectively. It was concluded that the toxic dose of CH-19 Sweet extract was greater than 20 mL/kg (1425 mg/kg as capsinoids) for both males and females as no deaths were observed at any dose [34].

For human study, the effective dosage of capsiate was observed, in the recently marketed capsiate supplement contains one milligram per capsule, and the recommended dosage is three capsules every morning (3 mg/day). The supplement comprises the three capsinoids: capsiate, dihydrocapsiate and nordihydrocapsiate. The optimum use dosage of capsiate is unknown. However, one human trail was studied and dosage of 30 milligrams of capsiate was employed and still showed no sign of adverse effect.

Biological Activities of Capsiate

Recently, obesity is a global health problematic and a growing concern for both developed and developing countries [34]. Present anti-obesity medications are associated with undesirable side effects upon chronic usage [35], leaving diet and

physical exercise as the most effective worth for obesity management. Several bioactive dietary molecules have been associated with enhancement of obesity and associated complications [36, 37]. The mechanisms action of anti-obesity can be divided into five groups; boosting fat burning (thermogenesis), inhibiting protein breakdown, suppressing appetite/ boosting satiety (feeling of fullness), blocking fat absorption, and regulating mood (linked to food consumption).

Capsiate, the major capsinoids of red chili pepper and CH-19 sweet, are well known for its potential anti-obesity, enhancing energy expenditure (EE) and body weight regulation in both rodents and animals has been extensively studied. Additionally, in action and pharmacology, capsiate was investigated like an anti-obesity effect. For experimental a two-week study with male mice administered capsiate (10 mg/kg body weight). It was found that increased metabolic rate and promotion of fat oxidation at rest. Moreover, the observation in two-week treatment of capsiate increased the levels of UCP-1 (uncoupling protein 1) and mRNA in brown adipose tissue (BAT), and UCP-2 (uncoupling protein 2) mRNA in white adipose tissue (WAT). These uncoupling proteins that found in the mitochondria of brown adipose tissue used to generate heat and temperature by non-shivering thermogenesis.

Additional study in mice by the same group which found that continuous administration of capsiate suppressed body fat accumulation. Capsiate formation of CH-19 Sweet extract has been also reported to increase body temperature and oxygen consumption in humans. The theoretical model to explain the above results is as follows: Capsinoids and other capsaicinoids activate TRPV1 (transient receptor potential vanilloid 1, or named the capsaicin receptor) found on the digestive tract surface or intestinal system, leading to activation of the sympathetic nervous system followed by activation of UCP-1 in BAT, UCP-2 in WAT, UCP-3 in skeletal muscle, and activation of lipolysis in WAT induced anti-obesity and other functional system to simulate several biological activities such as anti-inflamatory, anti-diabetic and anti-obesity (Figure 12).

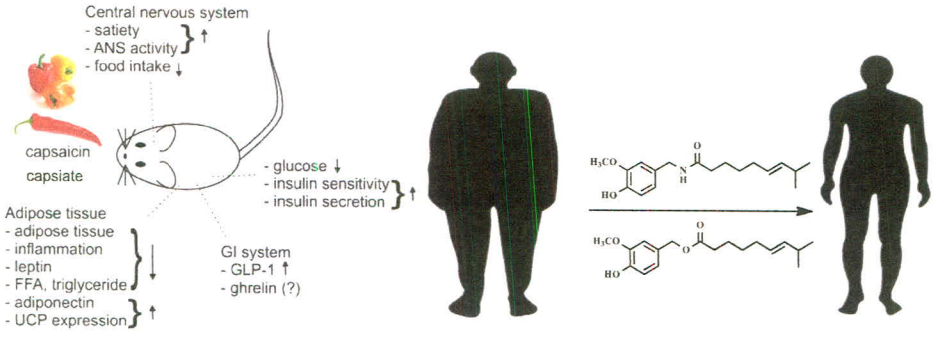


Figure 12 The theoretical model of capsinoids and capsaicinoids activate anti-obesity [38]

In 2006, Fuminori Kawabata and co-worker [39] reported their successful in a study of new non-pungent type of red pepper. The results showed that capsiate activated energy expenditure in similar manner to capsaicin, and the experimental results clearly indicated that the intake of capsiate would promote energy expenditure in both rodents and humans.

A treatment period of two weeks for CH-19 Sweet intake depended on previous results in animal experiments. This short intake period was also selected to minimize the stress on the subjects as much as possible. Nevertheless, they proposed that the highly significant differences would appear within this test period for the following reasons: First, they found that capsiate administration for two weeks clearly decreased the body fat in mice. Second, they also found that the 24 hours oxygen consumption in mice was increased by 25% with capsiate administration (10 mg/kg wt.) for two weeks suggesting that the resting metabolic rate (RMR) may also be increased by repeated capsiate administration. The body weight of rats in the CH-19 Sweet group treatment started to decrease immediately after initiating the treatment period and reached statistical significance compared to the control group on day 3 of the treatment period and clearly loss weight in 14 days (Figure 13).

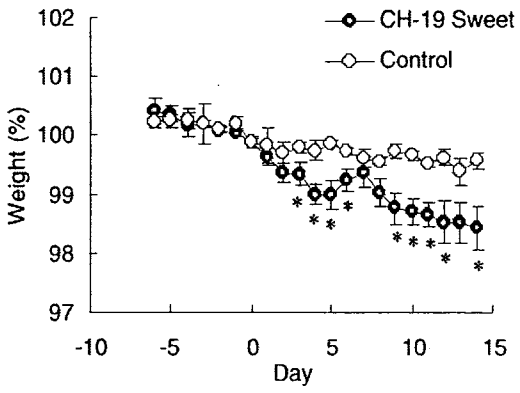


Figure 13 Time-Course Characteristics of Body Weight in the CH-19 Sweet (n = 7) and Control (n = 5) Groups [39]

In Human study, there was no difference in any of the parameters of the body composition and body fat distribution between the CH-19 Sweet group and the control group during both the adjustment period and the treatment period (Table 4). Although the body weight and BMI changes in the control group were minimal after the treatment period, the body fat percentage and fat mass increased, and the fat-free mass decreased (Table 4). On the other hand, in the CH-19 Sweet group, although the body fat percentage was changed very little by the treatment, the body weight, BMI, fat mass and fat-free mass were found to have decreased.

Table 4 Characteristics of Subjects in the CH-19 Sweet (n = 7) and Control (n = 5) Groups When Measurements of Body Fat by BODPOD and CT Scan Were Carried Out during the Adjustment Period for Each Amount of Food Ingested and after 2 Weeks of Treatment [39]

		Adjustment period		Week 2	
		CH-19 Sweet	Control	CH-19 Sweet	Control
body weight	(kg)	69:90±4:29	69:10±5:08	68:13*±4:14	68:65±5:11
BMI	(kg/m^2)	23:77±1:12	22:82±1:83	23:17*±1:07	22:66±1:81
body fat percentage	(%)	20:91±3:46	17:56±3:14	20:66±3:48	18:76*±3:06
fat mass	(kg)	15:10±2:97	12:51±2:89	14:49±2:84	13:28±2:94
fat-free mass	(kg)	54:79±2:74	56:59±2:98	53:64*±2:86	55:38*±2:78

Table 4 (cont.)

		Adjustment period		Week 2	
		CH-19 Sweet	Control	CH-19 Sweet	Control
total fat area	(cm^2)	189:00±43:09	151:18±37:62	173:23*±38:71	149:14±36:82
visceral fat area	(cm^2)	72:44±23:07	36:08±11:48	60:74±15:53	38:36±10:31
subcutaneous fat area	(cm^2)	116:56±26:85	115:10±26:61	112:49±25:27	110:78±26:72

Each value is the mean ± SEM. *P<0:05 vs. baseline by paired t-test.

The changes in each index from the adjustment period are shown in Figure 14 and 15. Significant differences between the two groups were apparent in the change of body fat percentage and percentage change of fat mass (Figure 14A and 14B). The decrease in the total fat area of the umbilicus in the CH-19 Sweet group treatment was greater than that in the control group (Figure 15A). The percentage of visceral fat area also tended to decrease in the CH-19 Sweet treatment group (Figure 15B), but did not change in the control group; no difference was apparent in the change of fat-free mass between groups (Figure 14C).

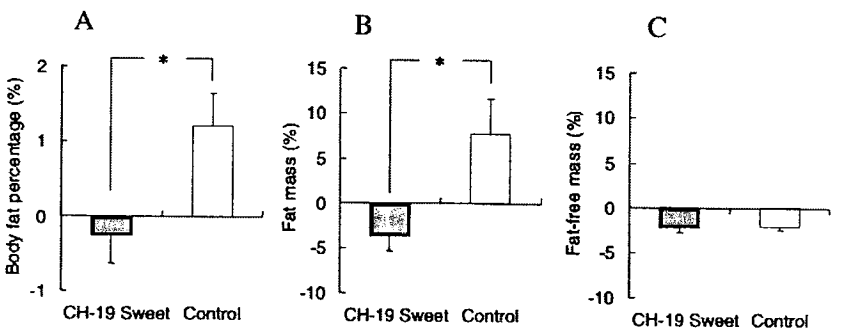


Figure 14 Changes in Body Fat Percentage (A), Fat Mass (B) and Fat-Free Mass (C) Expressed as Relative Values during the Two-Week Treatment Period. [39]

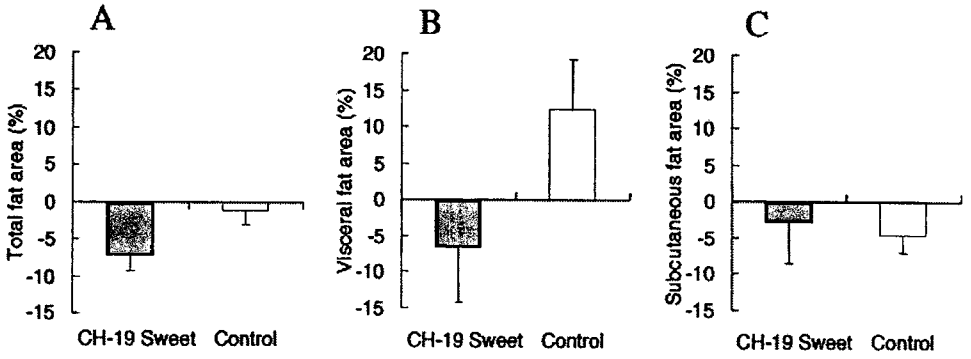
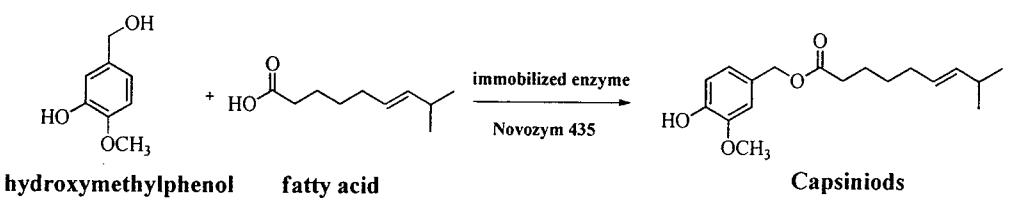


Figure 15 Changes in Total Fat Area (A), Visceral Fat Area (B) and Subcutaneous Fat Area (C) Expressed as Relative Values for the Two-Week Treatment Period. [39]

Modification of Capsiate

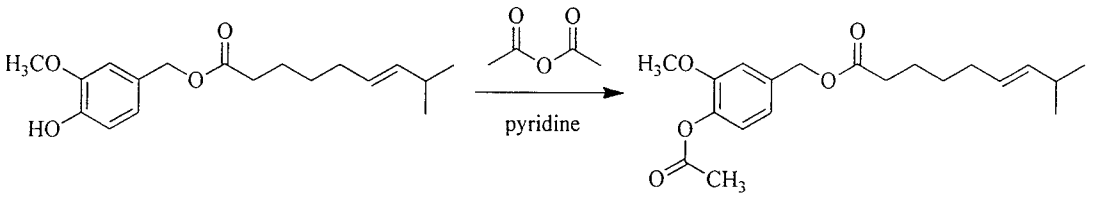
The modification of capcinoids for enhancement of their stability has been interested due to the fact that capsinoids may be usable as food additives or pharmaceutical drugs but it was limited cause of rapid decomposition. However, production of capsinoids from natural sources generally employ of a medium purity grade in a large amount.

Yosuke Amino and co-worker [31] introduced the modification of capsinoids via enzymatic reaction without a solvent or using a low-polarity solvent system. They were successful synthesized the stable capsinoids. However, since the reaction use the enzyme required an equilibrium state resulting from water production during esterification reaction. Accordingly, the reaction utilized a long period of time and afford low yield approximately 60%.



Scheme 3 Enzymatic synthesis of capsinoids with immobilized enzyme, Novozym 435 [31]

Recently in 2013, Parinthorn E. [40] investigated the modification of capsiate in order to stabilize this substance by protecting OH group. Under this study, the OH group on aromatic region was assigned to be non-permanently protected with acetyl group (-$COCH_3$) because the acetyl group can be easily cleaved by acetylcholine in human body. It was found that capsiate analogues exhibited the improved stability in protic solvent system when compare with capsiate.



Scheme 4 Synthesis of capsiate and acetyl-capsiate *via* acetylation [40]