CHAPTER 2
LITERATURE REVIEWS

2.1 Angkak

Angkak or red yeast rice is a product resulting from rice fermentation using *Monascus* spp. It has been used extensively in Asia as a natural food colorant in fish, Chinese cheese, red wine and sausages (Went, 1895; Hesseltine, 1965; Blanc *et al.*, 1995; Pinthong and Pattanagul, 2004). In ancient time, angkak production was originated in China and kept as a secret. One report suggested that angkak was used in Philippines for coloring of *bagoong, atsike*-salted fish, and in the preparation of alcoholic beverages such as *anchu* and *somsu*. In addition, Chinese cheese preparation and Chinese beverage known as *anchu* also requires angkak for their production. Later, angkak in the form of cake or ground red powder was exported from China to Eastern Asia. At the present, several countries produce angkak both for internal use and exportation as food additive and dietary supplement (Palo *et al.*, 1960).

Angkak was produced by *Monascus* fungi, an organism which can convert a starch substrate into several metabolites such as enzymes, alcohols, organic acids, vitamins, flavor compounds, fatty acids, antibiotic agents, flocculants, antihypertensives, ketones, and pigments (Yongsmith, 1999). Thus the implementation of *Monascus* pigment as a coloring agent in food provided an additional advantage of specific flavor in the products. Most of angkak products could be used in the powder form or pigment extracts for developing the color of products.

Angkak also has a pharmaceutical characteristic. The ancient Chinese pharmacopoeia, *Ben Cao Gang Mu-Dan Shu Bin Yi*, published during the Ming Dynasty (A.D. 1368-1644), described medicinal function of angkak for the treatment of indigestion and diarrhea, anthrax, bruised muscles, hangovers, colic dyspepsia in children and post-partum problems. Besides, it has been used for improving blood

circulation and promoting the function of the spleen and stomach. Moreover, several books including *Materia Medica for Daily Use*, *Supplements on Developments of Herb Medicine*, and *Compendium of Material Medica* also described the utilization of this pigment as a coloring agent and medicine in the treatment of various diseases (INPR, 2006).

Today, angkak is still used as traditional medicine and food colorant in Asia and in Chinese communities in North America. Considerable interest has been shown in the application of angkak as a nitrite/nitrate substitute for the preservation of meats (Fink-Gremmels *et al.*, 1991). Moreover, applications of some synthetic colorants in food such as azorubin or tartrazin have been limited due to their possible allergeric effects (Multon, 1992). Thus, *Monascus* pigments are used in meat product to replace nitrate or nitrite and improve quality of product. Pinthong and Pattanagul (2004) applied angkak as a red pigment to enhance color of meat sausages. The optimum level of angkak was 1.60%(w/w). The consumers preferred sausages with addition of both *Monascus* pigment and nitrite which was added to improve color stability (Shehata *et al.*, 1998). However, some researchers demonstrated that *Monascus* toxin, known as citrinin, could be synthesized during angkak production process. Therefore, many factors in angkak production must be considered to ensure its safe functional characteristics.

2.2 The Botanical data

Monascus species belong to the group of Ascomycetes and particularly to the family of Monascaceae. The genus *Monascus* is divided into four species: *M. pilosus, M. purpureus, M. ruber* and *M. froridanus*, which account for the majority of strains isolated from traditional oriental food (Sabater-Vilar *et al.*, 1999). The common names of this fungi product are red yeast rice, red rice, angkak, red leaven, beni-koji (Japanese), hung-chu, hong qu, zhitai (Chinese), rotschimmelreis (Europe), red mould (U.S.A.). *M. purpureus* is distinguished by its ascospores which appeared to be spherical shape, of 5 microns in diameter, or slightly ovoid (6×5 microns). The young part of the mycelium is white in the early stage. However, it rapidly changes to a rich pink and subsequently to a distinctive yellow-orange color. The production of yellow orange hyphae reflects the increased acidity of the medium. A deep crimson color is

found at the substrate following the culture ages (Yongsmith, 1999; Erdogrul and Azirak, 2004; INPR, 2006).

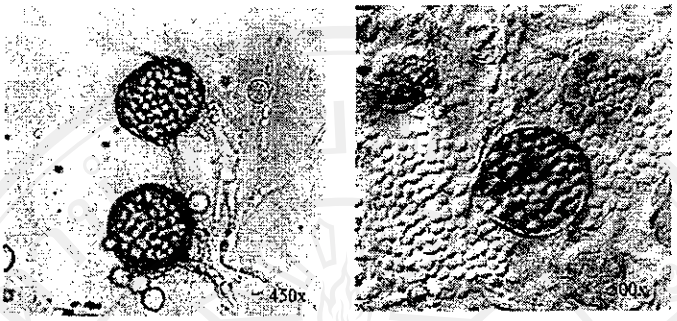

Fig 2.1 Pedicellate ascomata with ascospores of *M. purpureus* Went
Source: Samson *et al.* (2002)

The growth of *Monascus* fungi is a key indicator in the synthesis of pigments and other metabolites. Yongsmith (1999) explained that during the first stage of fermentation period, the fungi utilizes carbon and nitrogen source from substrate for its primary metabolites, bioconversion, energy, carbondioxide and water. On the last stage, fungi use the product produced on the first stage for producing secondary metabolites. Therefore, secondary metabolites such as pigment, citrinin and mevinolin can be detected after the first stage of fungi growth disappear.

2.3 Cultivation conditions of *Monascus* sp.

Monascus sp. can be cultivated on potato-dextrose agar, Sabouraud's agar or Czapek's solution agar for 10 days at 29 to 32°C. The cultures grow rapidly and spread on media. The diameter, color shade and area-texture of the mycelia are all depend on cultural media, strains of fungi and cultivation conditions (Palo *et al.*, 1960).

The procedure for preparing angkak on a laboratory scale is carried out by placing 50 g of polished rice in 8″×12″ autoclavable polypropylene bag with 50 ml water. The shape of polypropylene bag opening was manipulated similarly to Erlenmeyer flask's neck which was later plugged with cotton wool. The bags were then autoclaved at 121°C for 15 min prior cooling down to room temperature. Each bag was inoculated with 1 cm diameter of each *Monascus* strain. The culture was cultivated for 14 days at room temperature. After incubation, the fermented rice was

removed and dried at 55°C for 3 days. After drying, angkak was ground into powder and used in coloring of various foods (Pinthong and Pattanagul, 2004).

For centuries rice have been used as starch substrate for making angkak. All varieties of rice are suitable except the glutinous rice such as Malagkit Sungsung which is considered unsatisfactory because of the gluey texture and the agglomeration of rice grains (Palo *et al.*, 1960). Up to now, several cereal substrates such as oat, wheat, barley and corn had been studied on their possible uses as alternative substrates for *Monascus* sp. cultivation. Each cereal had a different influence on angkak production due to the variation in its composition (Yongsmith, 1999).

Corn was rather used as a substrate for angkak production because it is cheaper than rice and was not consumed as a maindish in China. Palo *et al.* (1960) illustrated that corn, as well as rice, may be used to grow *Monascus* sp. to provide the red color. Ganrong *et al.* (2000) reported the method of preparing corn for angkak production. Firstly, corn was cracked and the outer skin was removed. This step was carried out because the mycelium of *M. anka* could not penetrate the outer skin efficienty. After that, the corn kernels were steeped in water or diluted acetic acid solution for a period of 4 days at room temperature. The soaked corn kernels of 15 g were filled in a 250 ml Erlenmeyer flask with the cotton-plugged before sterilizing at 121°C for 30 min in the autoclave. The sterilized corn kernels were allowed to cool down until its temperature dropped to 32°C prior to inoculation of 2 ml *M. anka* inoculum. The inoculated medium was incubated at the same temperture for 7-10 days, after which the fermented corn kernels were dried and ground.

Adlay is a new substrate for *M. purpureus*. According to the ancient Chinese medical book *Pen-Tsao-Kang-Mu* (Li, 1596; Yang *et al.*, 2004), the seed of adlay was used in China for the treatment of warts, chapped skin, rheumatism and neuralgia and as an anti-inflammatory or antihelmintic agent. The dual benefits to human health may thus be attained because both *Monascus* sp. culture and adlay substrate are effective functional foods.

The preparation of *Monascus* adlay began by inoculation of the fungi to the malt extract agar and incubated at 25°C for 72 h. The pure culture was then re-inoculated into potato dextrose broth and incubated further at 25°C for one week. The

collected culture was subsequently homogenized in a blender and added to the sterilized adlay and incubated again in the same conditions and duration as previously described. The mycelia was air-dried at 40°C before being ground in a mill and sieved at 20 mesh to obtain a coarse powder.

Therefore, several cereals may be used as substrates for angkak production but the high quality product can only be produced at the suitable conditions. Moreover, the addition of carbon/nitrogen source can influence the production of *Monascus* metabolites such as pigment, mevinolin, citrinin and amylase enzyme (Pichyangkura, 1979; Wong *et al.*, 1981; Lin and Demain, 1991; Jůzlová *et al.*, 1994; López *et al.*, 2003).

2.4 Adlay

Adlay or Job's tears (*Coix lacryma-jobi*), an annual crop from Southern Asia, is grown in the United States as an ornament. It is extensively cultivated in Philippine Islands, Indochina, Thailand, Burma, and Sri Lanka. The kernel can be made into a sweet dish by frying and coating/boiling with sugar. Beside that, beers and wines can be made from the grain through a fermentration process. Chinese people use the grain like barley in soups and broths. Adlay is not only used as a food cereal but it also has a medicine functional effect according to an ancient Chinese medical book Pen-Tsao-Kang-Mu (Li, 1596; Duke, 1983; Yang *et al.*, 2004). Recently, there are reports stating that adlay seeds have antiproliferative and chemopreventive effects on lung cancer both in vitro and in vivo (Chang *et al.*, 2003).

Adlay seed (100 g) is reported to contain 380 calories, 11.2 g moisture, 15.4 g protein, 6.2 g fat, 65.3 g total carbohydrate, 0.8 g fiber, 1.9 g ash, 0.5 mg Ca, 435 mg P, 5.0 mg Fe, 0.28 mg thiamine, 0.19 mg riboflavin and 4.3 mg niacin (Duke, 1983). For the nutritional composition of adlay angkak, it was composed of 14.2% of moisture, 37% of carbohydrate, 6.87% of crude ash, 15.4% of crude fat, 15.9% of crude fiber and 24.8% of crude protein. Whereas, using rice as a substrate would produce a rice angkak with a composition of 6.0% moisture, 73.4% carbohydrate, 0.8% fiber, 2.8% fatty acid and 14.7% protein (Ma *et al.*, 2000; Yang *et al.*, 2004).

Numerous reports have indicated that the consumption of adlay seed is beneficial to humans. Chiang *et al.* (2000) studied the effect of adlay as a dietary supplement on culture counts of some important groups of intestinal bacteria and their

metabolism in the gastrointestinal (GI) tract of Sprague-Dawley rats. All animals fed with adlay had normal healthy intestinal walls and no pathogenic signs. The culture counts of fecal lactic acid bacteria were higher in the feces of rats fed with adlay than in the control group. It was concluded that adlay had a significant influence on the growth of intestinal bacteria, which may ultimately affect the physiology and other functions of GI tracts of rats.

Kuo *et al.* (2001) reported methanolic extracts from adlay seed have a moderate antioxidative effect on the peroxidation of linoleic acid. Takahashi *et al.* (1986) reported that coixans A, B, and C isolated from adlay seed had hypoglycemic activities in rats. Park *et al.* (1988) found that lipid components in plasma and feces decreased in rats fed with adlay seed (cited in Kuo *et al.*, 2001).

Yang *et al.* (2004) studied storage stability of monascal adlay by fermentation *Monascus purpureus* on dehulled adlay and polished adlay. It was found that mevinolin content was about 489-497 μg/g for 3 months but it decreased to 397-419 μg/g at the sixth month. Both of monascal adlays (dehulled adlay and polished adlay) were slightly different in proximate composition and comparable in their content of functional components and their fatty acid profiles. Also, these two products were shelf stable at 25°C for three month.

Since both *Monascus*-fermented rice and adlay are used as foods, the newly developed monascal adlay products are undoubtedly consumed as a traditional food. The only concern about their safety for human consumption is the possible existence of citrinin. However, citrinin was not present in these products at the detection limit of 1 μg/g.

From all of these reports, it can be seen that both the *Monascus* cultures and adlay possess functional components effective in maintaining human health. A research that will concentrate on a *Monascus* fungus that is inoculated into cooked adlay may be a new adlay angkak product which can have a synergistic medicinal effect on a human body. Moreover, it seems that the adlay angkak products will become a better nutraceutical grain and a good source of fat, fiber and protein as compared to the conventional rice angkak (Yang *et al.*, 2004).

2.5 The Pigment of *M. purpureus*

The pigments are secondary metabolites in which structures depend on substrate types and other factors during cultivation such as pH, temperature, and moisture content. During growth, *Monascus* spp. will break down starch substrate into several metabolites. The members of the genus *Monascus* produce several azaphilone pigments with a similar polyketide structure. These pigments are produced as a mixture of yellow, orange and red compounds which are commonly used without further separation, although fungi from the genus *Monascus* are traditionally used in oriental countries, originally in China and Thailand, to prepare a fermented red yeast rice with strong red color (Carvalho *et al.*, 2003).

Monascus fungi produces at least six major related pigments which can be categorized into 3 groups based on color as follow (Sweeny *et al.*, 1981).

(1) yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$)

(2) orange pigments: monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_{22}O_5$)

(3) red pigments: monascorubramine ($C_{23}H_{27}NO_4$) and rubropuntamine ($C_{21}H_{23}NO_4$)

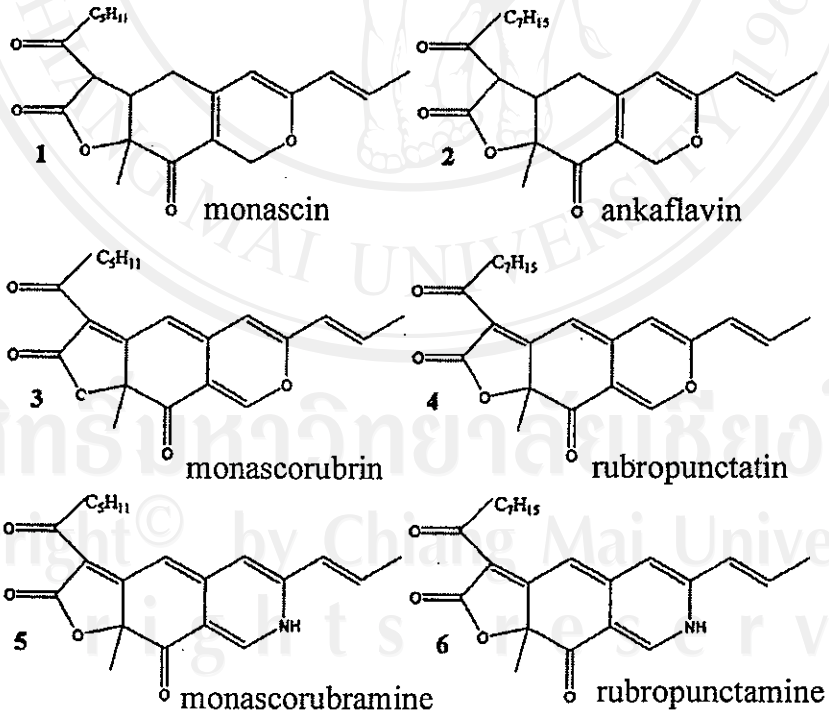

Fig 2.2 Pigments produced by *Monascus* fungi

Source: Jung *et al.* (2003)

For many years it was known that there were six pigments produced by *Monascus*, in the last decade some new colored metabolites have been discovered from the pigments produced by the culture. There are xanthomonascin and yellow II, possibly derived from rubropunctatin (Jůzlová *et al.*, 1996). Recently, two new compounds, monascopyridines A and B, analog in structure to the red pigments but hydrogenated have been described. These compounds have a maximum UV wavelength at 306 nm, and their contributions to *Monascus* color has not yet been investigated (Carvalho *et al.*, 2003).

The orange pigments, monascorubrin and rubropunctatin, are synthesized in the cytosol from acetyl coenzyme A through a multi-enzymatic polyketide synthase complex. These pigments are not hydro-soluble and are unstable in extreme pH (Hajjaj *et al.*, 2000) but present structures with high affinity to compounds containing primary amino groups. Reactions with amino acids lead to formation of water-soluble red pigments, monascorubramine and rubropunctamine. The mechanism of yellow pigment formation is not yet clear; some authors consider that these are products of the alteration of orange pigments, as others believe it to be pigments with their own metabolic pathway (Lin and Demain, 1991; Jůzlová *et al.*, 1996). Among the pigments produced by *Monascus*, the red ones are the most important, since they may be possible substitutes of synthetic colors such as erythrosine (FD & C red no.3) (John and Stuart, 1991). These were considered stable pigments in the range of pH from 2-10 with fair stability to temperature and autoclaving (Lin *et al.*, 1992). The detection of yellow, orange and red pigments of *Monascus* sp. can be analyzed by a spectrophotometer at 400, 470, 500 nm, respectively (Chen and Johns, 1993).

There are many studies about the effect of carbon and nitrogen sources on *Monascus* pigment. Chen and John (1993) summarized that ammonium and peptone were nitrogen sources that gave superior growth and pigment concentrations compared to nitrate when cultured *Monascus purpureus* on a specific medium (glucose 50 g/l, KH_2PO_4 1.0 g/l, $MgSO_4.7H_2O$ 0.5 g/l, trace metals and one of nitrogen sources: $NaNO_3$ 3 g/l, NH_4Cl 2 g/l or bacto-peptone 5 g/l). The maximum absorbances of yellow and red pigments using ammonium as a nitrogen source were 3.6 and 12, respectively. Using peptone as a nitrogen source could make higher

yellow and red pigments, which were equal to 17 and 21, respectively. Both expreriments were based on a medium pH of 6.5.

Lin (1973) studied the effect of carbon and nitrogen sources on the pigment production from *Monascus*. It was found that 1% of galactose, starch, or maltose as a carbon source gave a maximum yield of the pigment in the similar sequence order. On the other hand, monosodium glutamate, $NaNO_3$ or $(NH_4)_2SO_4$ (13.0 mg/ 50 ml) was found to be suitable nitrogen sources for pigment production.

Wong *et al.* (1981) reported that the growth and pigmentation of *Monascus purpureus* were affected by the ratio of glucose and ammonium nitrate concentrations in a synthetic medium. It was found that the total pigmentation was increased with an increase in glucose concentrations. The optimum quantities of glucose at 80 g/l and NH_4NO_3 at 1.0 g/l were the best concentrations to make the highest pigment production.

Chen and Johns (1994) found that the use of 50 g/l maltose improved the production of *Monascus* red pigment (monascorubramine) in a culture media, compared to glucose medium, especially when peptone was used as a nitrogen source.

Júzlova *et al.* (1994) studied ethanol as a substrate for pigment production by the fungus *Monascus purpureus*. Ethanol at a concentration of 2% (v/v) was used as a carbon source in a culture media and the pigment production was higher than that obtained with cultivation on a maltose (3% w/w) media.

Lin and Demain (1991) developed a new chemically-defined medium for higher red pigment production from *Monascus* sp. An initial medium containing 4% glucose, 0.3% NH_4NO_3 (75 mM nitrogen) and inorganic salts became a new chemically defined medium, which was revised in the latter experiment to contain 5% maltose, 1.26% anhydrous monosodiumglutamate, phosphate and $MgSO_4$ at lower concentrations plus other mineral salts. The maximum pigment production in the new medium reached 41 OD_{500} units at 156 hours while this value was only 4.1 in the original medium.

2.6 Mevinolin

Mevinolin ($C_{24}H_{36}O_5$, Lovastatin, Monacolin K) is a potent drug for lowering blood cholesterol. It has a melting point of 157-159°C. It acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG Co-

A reductase) which catalyzes the rate-limiting step of cholesterol biosynthesis. The UV spectrum (methanol) showed maximum absorbances at 229, 237 and 246 nm, respectively. Mevinolin is one of the secondary metabolites produced by the fungi *Penicillium* sp., *Monascus* sp. and *Aspergillus terreus* (Endo, 1979; López *et al.*, 2003).

Mevinolin in *Monascus* cultures at solid state occurs at two different structures: a beta-hydroxy acid form and a lactone form. The ratio of the acid form to the lactone form varies depending on various *Monascus* strains used, the sample pH and other factors, such as the culture media. The functional property of *Monascus* products containing higher ratio of the acid form of Monacolin K should be regarded as a higher quality product (Ganrong *et al.*, 2000). Furthermore, Monacolin J, L, X, M and its derivative forms were found from *M. purpureus* – fermented rice. All of these monacolins were found to be effective as a hypocholesterolemic agent (Li *et al.*, 2004). The structural data of mevinolin in fermented red rice was shown in Fig 2.3

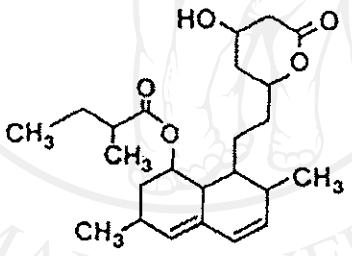

Fig 2.3 Chemical structure of mevinolin
Source: Friedrich *et al.* (1995)

Mevinolin is biosynthetically derived from two polyketide chains joined through an ester linkage. One chain is the diketide 2-methylbutyrate and the other is a nonaketide that includes a distinctive conjugated hexahydronaphthalene ring system.

Over the past several years it has become clear that polyketides are assembled in a variety of mechanistically complex ways. Polyketide synthases are structurally and functionally related to the fatty acid synthases, both catalyzing sequential decarboxylative condensations between ACP-linked acyl-thioesters. Unlike most fatty acid synthases, however, polyketide synthases can omit some or all of the reduction

reaction (β-keto reduction, dehydration and enoyl reduction) that take place after each condensation, thereby yielding products with ketone, alcohol or alkene instead of methylene functional groups at specific positions along the chain.

There are at least three types of polyketide synthase systems: the iterative type II systems of actinomycetes, which generally produce aromatic structures such as actinorhodin or tetracenomycin; the modular type I systems of actinomycetes, which produce macrolides such as erythromycin; and the iterative type I systems typical in fungi, which produce precursors to widely different structures such as patulin, sterigmatocystin or lovastatin.

Hypothetical pathway for lovastatin biosynthesis (Fig 2.4). Independent pathways from the diketide and nonaketide portions of lovastatin, which are catalyzed by lovastatin diketide synthase (LDKS) and lovastatin nonaketide synthase (LNKS), respectively. Both enzymes build their polyketide product from an acetyl starter unit and malonyl extender units, and both add a methyl group from S-adenosyl methionine. The intermediates shown bound to the polyketide synthases are hypothetical pathway (Hendrickson *et al.*, 1999).

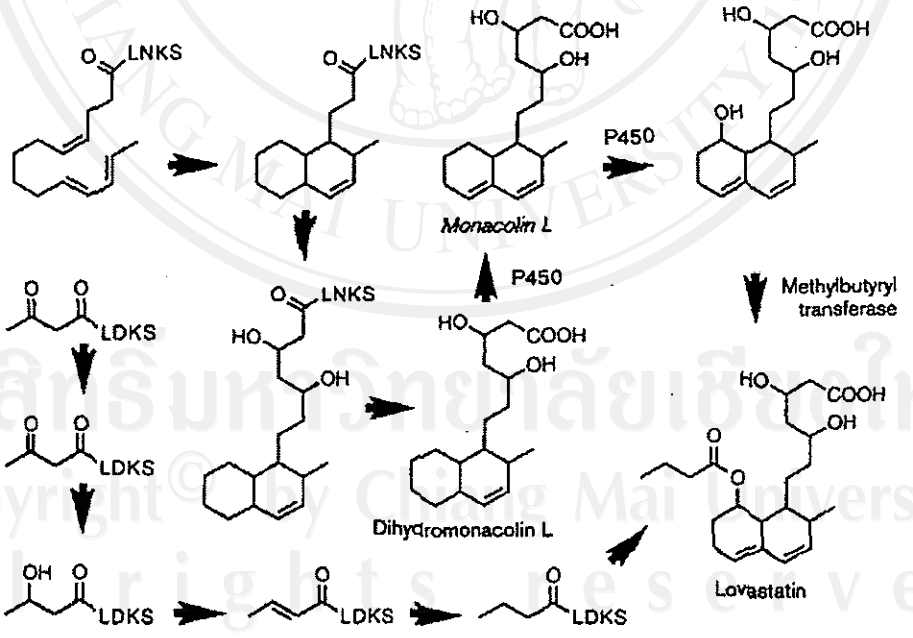

Fig 2.4 The hypothetical pathway of lovastatin biosynthesis, LDKS = lovastatin diketide synthase; LNKS = lovastatin nonaketide synthase; P450 = enzyme

Source: Hendrickson *et al.* (1999)

Chang *et al.* (2002) studied the suitable formula of a complex medium to make the highest lovastatin (mevinolin) production by *Monascus ruber*. It was found that the maximal lovastatin yield was 131 mg/L. This lovastatin production could be achieved using a formula that contained rice powder, peptone, glycerin, and glucose at concentrations around 34.4 g/L, 10.8 g/L, 26.4 ml/L, and 129.2 g/L, respectively. Ganrong *et al.* (2000) found *Monascus* strains that produced a high level of Monacolin K (mevinolin), but did not produce citrinin. They found that *Monascus sp.* 9901 cultured in rice could make the monacolin K content up to 11,000 mg/kg within 20-35 fermentation days. The stationary phase of this strain was 20-35 rice fermentation day which yielded 12,000 mg/kg monacolin K.

López *et al.* (2003) studied a production of lovastatin and the microbial biomass of *Aspergillus terreus* ATCC 20542. The lovastatin production by *A. Terreus* was influenced by the type of carbon sources (lactose, glycerol, and fructose) and the nitrogen sources (yeast extract, corn steep liquor, and soybean meal) in the growth medium. It was found that the maximum value of the lovastatin yield was about 30 mg/g of biomass using the lactose/soybean meal (20 g/l and 3.84 g/l for lactose and soybean meal concentration, respectively) and the lactose/yeast extract (20 g/l and 1.33 g/l for lactose and yeast extract, respectively)

Jyh-Jye *et al.* (2003) produced a higher concentration of monacolin K and reduced the citrinin content from a *Monascus* culture. The influence of different carbon and nitrogen sources on the production of monacolin K and citrinin by *M.purpureus* NTU 601 was studied by these authors. When 0.5% ethanol was added to a culture medium, the production of citrinin decreased from 813 ppb to 561 ppb while the monacolin K production increased from 136 mg/kg to 383 mg/kg. Whereas, when 500 g rice was used as a solid substrate which was added with 120 ml water and 0.3% ethanol and incubated at 30°C, the production of monacolin K content could increase from 136 mg/kg to 530 mg/kg and the citrinin production decreased from 813 ppb to 460 ppb.

2.7 Effects on cholesterol and lipid metabolism

Hypercholesterolemia is a well-known risk factor for coronary artery disease, cerebrovascular disease and peripheral artery disease. Moreover, reducing plasma cholesterol level coincides with a reduced incidence of cardiovascular diseases

complication (stroke, peripheral obstructive arterial disease). The treatment of hypercholesterolemia with a specific drug is costly, while in primary prevention life-style change and dietary habits such as reducing dietary saturated fatty acids, cholesterol, and excess body weight, appear to be more cost-effective than any pharmacological treatment. The inclusion criteria were the following; estimated 10-years cardiovascular disease risk <20%, moderate hypercholesterolemia (TC <300 mg/dL), normal triglyceridemia (TG <250 mg/dL), normal HDL cholesterol (>40 mg/dL) (Cicero *et al.*, 2005).

Several *Monascus* metabolites have been subjected to investigation in order to confirm its pharmacological effects. Monacolin K, called Mevinolin, is the only one of these metabolites which is able to decrease blood cholesterol. It acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG Co-A) which catalyzes the rate-limiting step of cholesterol biosynthesis (Endo, 1979). Monacolin K has two different structures: beta-hydroxy acid and lactone forms. The ratio of the acid form to the lactone form varies depending on the *Monascus* strains being used pH, culture media and other factors. The higher ratio of the acid form was regarded as a higher quality product (Ganrong *et al.*, 2000). Furthermore, Monacolin J, L, X, M and their derivative forms were found from *M. purpureus*-fermented rice. Each of these monacolins was found to be a potent hypocholesterolemic agent (Li *et al.*, 2004).

Heber *et al.* (1999) reported that angkak significantly decreased total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and total triacylglycerol (TG) concentrations in human blood in comparison with the placebo. They evaluated the lipid-lowering effects of angkak dietary supplement in US adults. Eighty-three healthy subjects with hyperlipidemia who were not being treated with lipid-lowering drugs participated in the experiments. Subjects were treated with angkak (2.4 g/day) or placebo and instructed to consume a diet providing 30% of energy from fat with saturated fat of less than 10% and cholesterol (<300 mg). The TC, TG, high-density lipoprotein cholesterol (HDL) and LDL cholesterol were measured at weeks 8, 9, 11 and 12. TC concentration decreased significantly between the baseline and 8 weeks in the angkak-treated group compared with the placebo-treated group. LDL cholesterol and total triacylglycerol were also dropped with the supplement while the level of

HDL cholesterol was not changed significantly. Therefore, angkak provided a novel approach to lowering cholesterol in the general population by applying it in food.

Angkak also affected blood lipids and lipoprotein concentrations in rabbits whose diet was 25% casein to induce endogenous hypercholesterolemia. Within 60 days, its serum cholesterol concentration increased from approximately 1.81 to 7.51 mmol/L. Treatment with angkak for 30 days at dosages of 0.4 and 0.8 g/kg/day had significantly lowered serum TC concentration and TC:HDL-c ratio. Moreover, hypercholesterolemia in rabbits could also be induced exogenously with a diet consisting of 0.5% cholesterol, 15% yolk powder and 5% lard. Rabbits were fed angkak at doses of 0.8 g/kg/day for 40 days which prevented the increase of serum TC, TG concentration and TC:HDL-c ratio. In the hyperlipidemia induced quail with 1% cholesterol, 14% lard, 6% soya-bean oil, angkak was fed orally at doses of 0.1, 0.2 and 0.4 g/kg/day for 2 weeks. It was found that angkak largely prevented the increase of serum TC and TG. This study demonstrated that angkak could be used in the reduction of serum TC and TG in rabbits and quail (Li *et al.*, 1998).

Xuezhikang Jiaonang, is the encapsulated angkak in the capsule form for the treatment of serum TC, TG and LDL cholesterol with the exception for HDL. This drug can be used in the treatment of hyperlipidemia and cardio-cerebro-vascular diseases caused by high blood cholesterol. Furthermore, there are several commercialised names dedicated to blood cholesterol medicines containing mevinolin such as Mevacor, Cholestin, Lovastatin, Zocor, Lipiton, Mevalotin. These are commonly sold in drugstores in China, Japan, United States, Indonesia, Taiwan and Philippines (Erdogrul and Azirak, 2004). It has a relatively mild side effects such as heartburn, wind and dizziness which may occur in some groups of patient taking a drug. Therefore, patients should consult a physician before taking angkak-lowering-cholesterol medicine . It is recommended to take 5-10 mg monacolins per day in a divided dosage for 12 weeks (Heber *et al.*, 1999; Erdogrul and Azirak, 2004).

2.8 Citrinin

Most of *Monascus* strains have a potential capacity to biosynthesize citrinin, a secondary metabolite known to be as mycotoxin and is harmful to consumers. The sole way of avoiding the production of citrinin in angkak is to find a suitable strain

and culture condition for the growth of *Monascus*, including carbon and nitrogen sources as a substrate (Ganrong *et al.*, 2000)

The structural formula of citrinin is shown in Fig 2.5 ($C_{13}H_{14}O_5$, IUPAC (3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid). It has been identified as a secondary fungal metabolite produced by a variety of *Monascus*, *Aspergillus* and *Penicillium* species. Citrinin is an acidic lemon-yellow crystal with maximal UV absorptions at 250 nm and 333 nm (in methanol) with a melting point at 172°C. It is known as hepato-nephrotoxin. Citrinin has been reported to contaminate grains, food and feedstuffs (Sabater-Vilar *et al.*, 1999; Xu *et al.*, 2005). Furthermore, citrinin has an antimicrobial effect on gram-positive bacteria such as *Bacillus subtilis, B. cereus, B. megaterium, Streptococcus lactis* and *Pseudomonas fluorescens*. However, when angkak is used as a food additive or drug, it is necessary to produce angkak without or less amounts of citrinin for the safety of consumers.

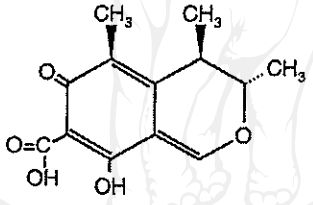

Fig 2.5 Chemical structure of citrinin
Source: Sabater-Vilar *et al.* (1999)

Fig 2.6 showed scheme of the biosynthesis of citrinin by *M. ruber*. The precursor for citrinin formation is a tetraketide arising from the condensation of one acetyl-CoA molecule with three malonyl-CoA molecules instead of a pentaketide (one acetyl-CoA molecule and four malonyl-CoA molecules). Then, an additional acetyl-CoA molecule is added to the tetraketide to form intermediate 1. Futhermore, one can not exclude the possibility that a malonyl-CoA molecule condenses to the tetraketide and that this is accompanied by a decarboxylation. Subsequent reactions include O alkylation and the cleavage of the single bond between C-1 and C-9 (Fig 2.6) in a way similar to that which occurs in the formation of bovilactone or gomphilactone. This

cleavage also agrees with the proximity of C-3 and C-9. In addition, intermediates 2 and 3 were energetically acceptable, with molecular energy levels of 100.6 and 93.8 kcal.mol^{-1}, respectively.

The occurrence of a tetraketide as the precursor for both citrinin and red pigments may account for the differential production of these two polyketides during the growth of *M. ruber*. It will be interesting to further characterize the enzymatic reactions at the tetraketide branch point (Fig 2.7) in order to develop strategies aimed at a selective production of red pigments (Hajjaj *et al.*, 1999).

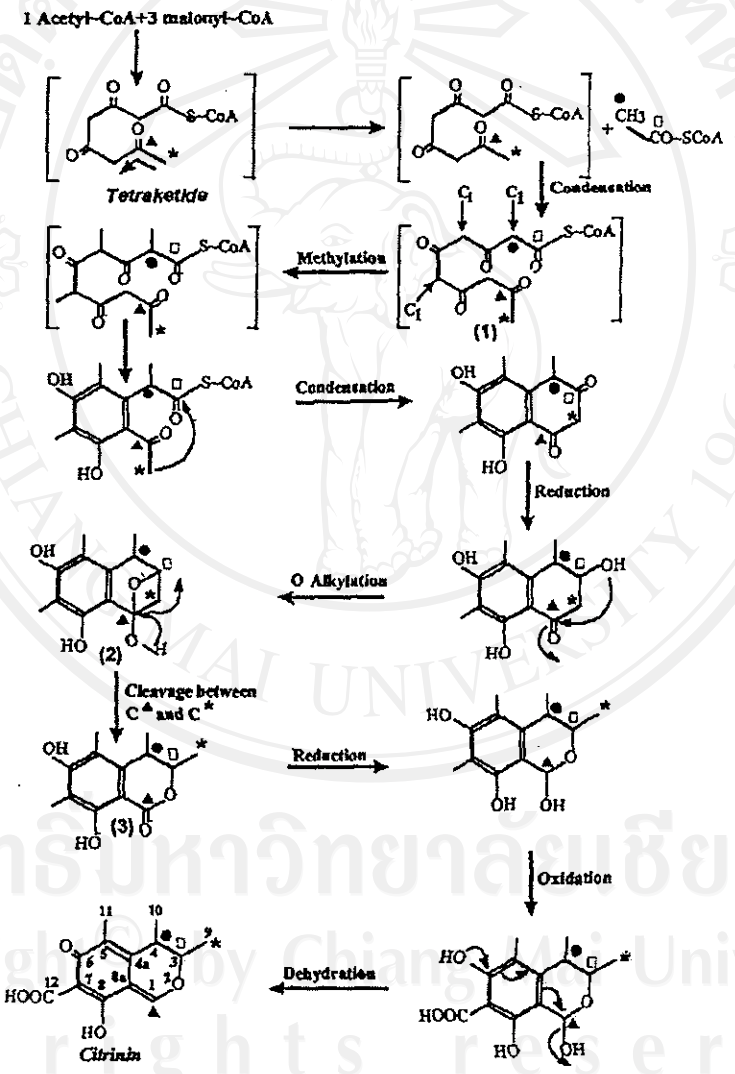

Fig 2.6 Scheme of the biosynthesis of citrinin by *M. ruber*. The start of the condensing reaction is indicated by bent arrow in the upper left panel. Intermediates are numbered. Enrichment of C-1(▲), C-3(□), C-9(*), and C-4(•)

Source: Hajjaj *et al.* (1999)

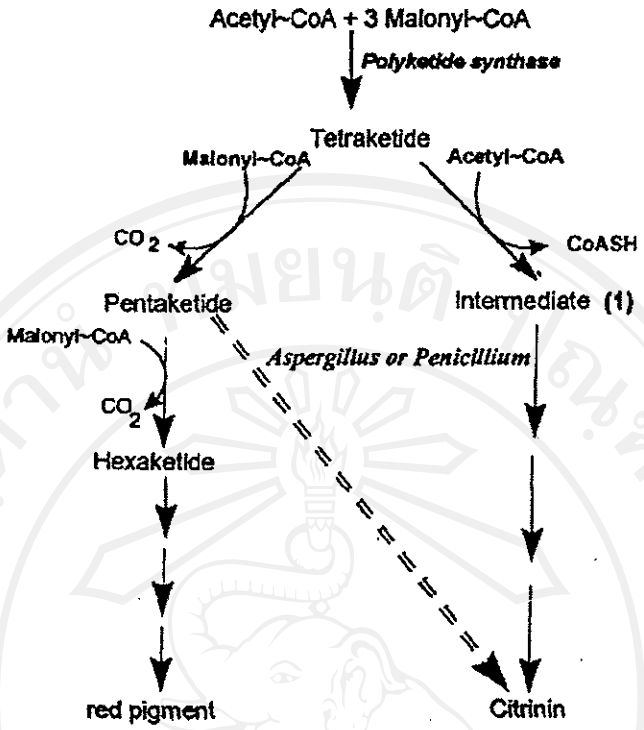

Fig 2.7 Biosynthesis of citrinin and red pigment in *M. ruber*. The toxin pathway in *Aspergillus* and *Penicillium* is indicated by the dashed arrow
Source: Hajjaj *et al.* (1999)

Li *et al.* (2003) studied the production of citrinin by *Monascus* strains used in food industry. The results of thirty-five *Monascus* strains showed that the production of citrinin on rice were at a levels ranging from 0.28 to 2458.80 mg/kg (201.60 mg/kg for the average and 61.99 mg/kg for the medium).

Blanc *et al.* (1995) studied the production of citrinin by various species of *Monascus*. It was found that *M.ruber* cultured on wet rice (50% of water w/w) and incubated at 27°C for 2 weeks could produce a citrinin concentration of 300 mg/kg rice, while *M.purpureus* CBS 109.07 produced 100 mg citrinin/kg rice. Using urea and methionine as nitrogen sources were unfavoured both for citrinin and pigment productions while the growth of microorganisms was not affected.

2.9 Toxicology

Angkak production may be contaminated by citrinin, a potent mycotoxin formerly known as Monascidin A, that could damage kidney and liver. Blanc *et al.* (1995) isolated and identified Monascidin A from various species of *Monascus* sp..

Mass spectroscopy analysis indicated that its structure was identical to citrinin. The antibacterial effect of *Monascus* had been confirmed by Wong and Koehler (1981). Monascidin A, isolated from *M. purpureus* cultures, was able to inhibit *Bacillus* sp., *Streptococcus* sp. and *Pseudomonas* sp. (Wong and Bau, 1977). In all commercial *Monascus* samples, the mycotoxin level of 0.2 to 1.71 ug/g were detected (Sabater-Vilar *et al.*, 1999).

Citrinin could induce a mutagenic response in the *Salmonella*-hepatocyte-assay applying strain TA-98. However, no mutagenicity could be detected in the *Salmonella*-microsome assay. These findings provide further evidence that citrinin requires complex cellular biotransformation to exert mutagenicity (Sabater-Vilar *et al.*, 1999). Wijnands and Leusden (2000) reported that citrinin is able to permeate membranes and hit the mitochondria, giving rise its toxic effect. Citrinin modifies the antioxidant enzymatic defences of cells through the inhibition of glutathione-reductase and transhydrogenase. In this way the toxin increase the generation of reactive oxygen species, stimulating the production of superoxide anion in the respiratory chain, which eventually leads to cell death. Besides effects on the respiratory chain in cells citrinin also affects the transport of calcium ions. Citrinin promotes a dose dependent decrease in the velocity and in the total capacity of Ca^{2+} uptake in mitochondria.

Lethal Dose (LD_{50}) values of citrinin were 35 mg/kg (mouse) and 67 mg/kg (rat) (Wang *et al.*, 2003). However, although *Monascus* extracts and angkak were used as food colorants for centuries without the known case of adverse effects due to the low concentrations applied in food but many country have a legal of citrinin content in angkak. In Japan, the maximum citrinin in angkak could not exceed 200 ng/g. In China and the european Economic community, the maximum allowable citrinin in angkak was still under debate (Chen and Hu, 2005). Therefore, the investigation should focus on the conditions of angkak production that yielded no citrinin or lowest possible concentration before using angkak as food additives or dietary supplement.

2.10 Glucosamine

The advantages of *Monascus* production on solid-state fermentation (SSF) is a high productivity of pigments at a relatively cheap cost. Moreover, the extraction of

pigments from SSF matter seems to be more efficient since the production in solid media is in concentrated form. However, direct biomass estimation in SSF is usually impossible because of the difficulty in efficiently separating biomass from the substrate matrix.

It is difficult to determine growth of *Monascus* by counting mycelia using electron microscope. An easier method could be performed using glucosamine analysis. This compound is a monomer of chitin which is the main component in fungi cell wall (Vignon *et al.*, 1986). The biomass may be indirectly measured by the determination of cell components such as glucosamine (present in chitin, a fungi cell wall component), ergosterol (present in the cell membrane), proteins or nucleic acids (Carvalho *et al.*, 2005).

Most fungi contain chitin, a polymer of *N*-acetylglucosamine, in fungi cell wall. The assay of this compound indicates a mycelial mass contained in the tissues of a higher plant. Moreover, this assay has already been used to estimate the mass of pathogen fungi, saprophilous fungi or endomycorrhizal fungi. This method start with chitin is hydrolysed by alkaline or acid and after that it will be assayed by a colorimetric method or HPLC (Vignon *et al.*, 1986; Shao *et al.*, 2004). Acid hydrolysis enable hydrolysis of chitin into *N*-acetylglucosamine (aldehydes + amino sugars) and then, aldehyde group (C_2H_2O) in *N*-acetylglucosamine is separated out of amino sugar group ($C_6H_{13}NO_5$) called glucosamine. Therefore, fungi growth can be determined using indirect method.

The chemical structure of *N*-acetylglucosamine is $C_8H_{15}NO_6$ and its molecular weight is 221.2. The structure formula was shown in Fig 2.9 (www.sigma-aldrich.com, 2000). Glucosamine was shown the structure formula in Fig.2.8 (Shao *et al.*, 2004). Its molecular weight is 179.0. This substance can be analysed by HPLC conditions following the column used was a 150 mm×4.6 mm, i.d.; 5μm Phenomenex Luna amino column. The mobile phase was 75:25 (v/v) acetonitrile/phosphate buffer (0.02 MKH_2PO_4 adjusted to pH 7.5 with conc. H_3PO_4). The flow rate was 1.5 ml min^{-1}, the wavelength was at 195 nm, the injection volumn was 10 μl, the column temperature was thermostated at 35°C, the run time was 15 min, and quantitation was performed using peak area count (Shao *et al.*, 2004). The other analysis method of glucosamine is colorimetric assay following acid hydrolysis sample (1 ml) was mixed

with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, 6 ml ethanol was added, followed by the addition of 1 ml of Ehrlich reagent and incubated at 65°C for 10 min. After cooling, the optical density was read at 530 nm against the reagent blank. *N*-acetylglucosamine was used as the standard (Babitha *et al.*, 2007)

When glucosamine is assayed to indicate for fungal growth in suitable conditions. The specific growth rate (μ) can be determined for the microbial growth during logarithmic phase by calculating the glucosamine (as biomass) concentration-time profile of batch system. The specific growth rate is defined as shown in Equation 2.1

$$Q_x = \frac{1}{x}\frac{dx}{dt} = \mu \qquad \text{Equation 2.1}$$

In fact, when biomass data is not available, one may use the value of optical density (absorbance) or the number of cells in the estimation of specific growth rate (Leksawasdi, 2007).

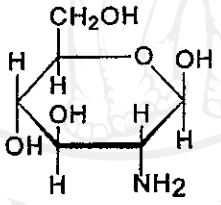

Fig 2.8 Structure formula of glucosamine
Source: Liang *et al.* (1999)

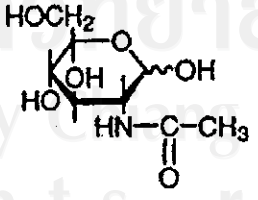

Fig 2.9 Structure formula of *N*-acetylglucosamine
Source: www.sigma-aldrich.com (2005)

2.11 Principles of HPLC/DAD/MSD on angkak metabolites analysis

2.11.1 High Performance Liquid Chromatography (HPLC)

The basic chromatographic process consists of the partition of sample molecules between a mobile fluid and a stationary phase. The stationary phase of liquid chromatography is held in a column and the mobile phase is allowed to flow. The substances distributed preferentially in the mobile phase pass through the chromatographic system faster than those that are distributed preferentially in the stationary phase. As a consequence the substances are eluted from the column in inverse order of their distribution coefficients with respect to the stationary phase.

The instrumentation used in HPLC has changed considerably following the individual components of system in detail. Commonly, HPLC system consists of autosampler, injection valve, solvent reservoir, HPLC pumps, column, temperature control, detector, data system or integrator. The HPLC column is at the heart of any system as here the substrance ischromatographically separated. The injection valve is used to apply the sample mixture to the head of the column. After passing through the column, the eluate passes through the detectors, which detect the separated components of the sample mixture and supply a signal to the chart recorder where the chromatogram is recorded.

It is essential that the chromatogram for the sample to be analysed and those for standards are recorded under identical conditions if comparisons of retention data are to be valid. The importance of certain operating parameters, such as column type and solvent, are self-evident. However, the significance of other factors, for example column temperature, has heen largely ignored, even though this can alter chromatographic data considerably. Further identification of structure thus usually necessitates isolation of the compound from the column eluate, followed by conventional analytical procedures such as infrared spectrophotometry or nuclear magnetic resonance spectrometer.

In the majority of published papers chromatographic similarity is used as the sole criterion for identity, but the limitations of this approach must be realized (Macrae, 1982).

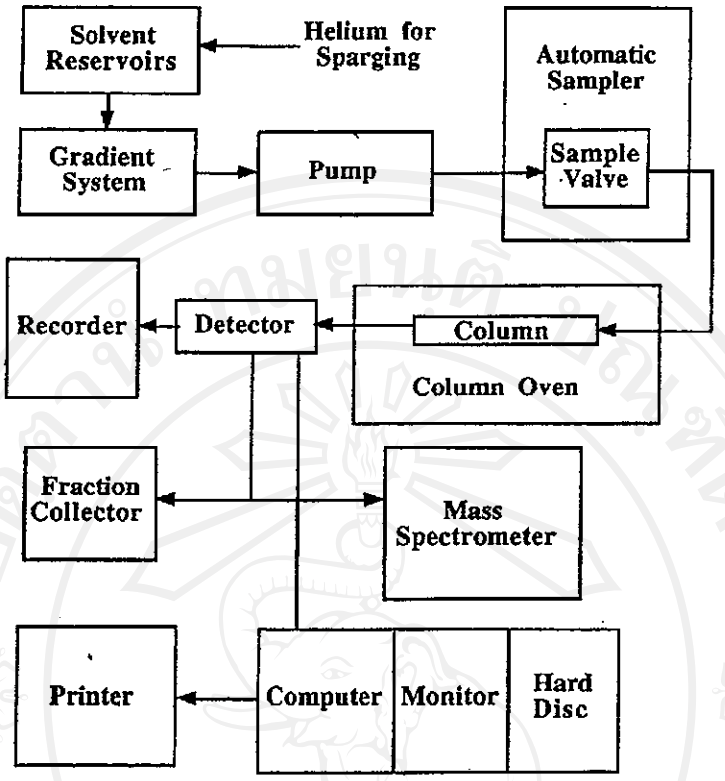

Fig 2.10 High Performance Liquid Chromatography system
Source: Scott, R.P.W. (1995)

2.11.2 The Diode Array Detector (DAD)

The diode array detector utilizes a deuterium or xenon lamp that emits light over the UV spectrum range. Light from the lamp is focused by means of an achromatic lens through the sample cell and onto a holographic grating. The dispersed light from the grating is arranged to fall on a linear diode array. A diagram of a UV diode array detector is shown in Fig 2.11

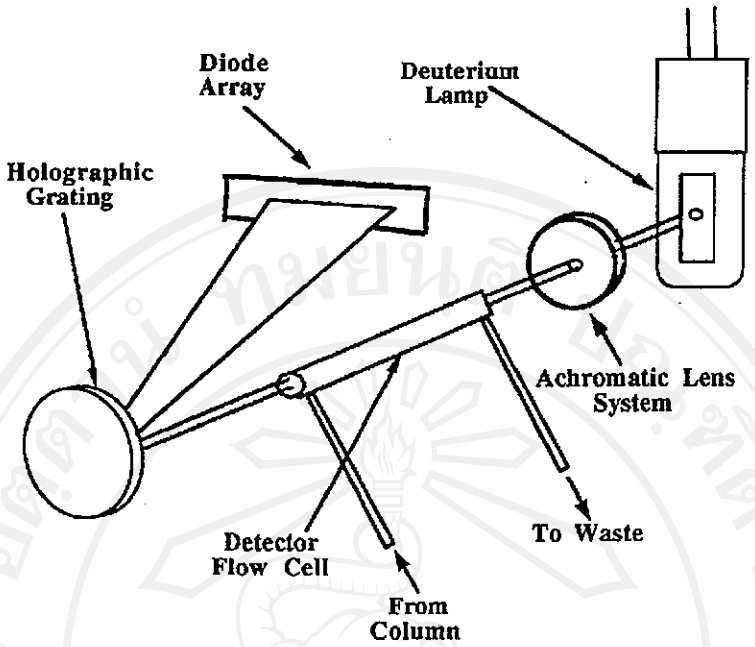

Figure 2.11 The UV Photo Diode Array Detector
Source: Scott, R.P.W. (1995)

The diode array detector takes a UV spectrum of the eluent continuously throughout the complete development of the chromatogram which can be used to a great advantage. It follows that a chromatogram can be reconstructed by monitoring at a specific wavelength and thus depict only those substances that adsorb UV light at the chosen wavelength. Consequently, the chromatogram can be arranged to display only those substances that have unique absorbance characteristics (Scott, R.P.W., 1995).

2.11.3 The Mass Spectrometer Detector (MSD)

For analysis by classical electron ionization (EI) or by chemical ionization (CI) mass spectrometer, is currently the detector for HPLC which can combine sensitivity, versatility, and universality to the highest degree and judging by the outstanding advances made with the help of combined gas. The sample must have a vapor pressure greater than 10^{-2} mm Hg because molecules of the sample must migrate by diffusion from the inlet system into the ionization chamber. Samples may be introduced into the mass spectrometer using a direct probe or batch inlet for pure

solids or volatile liquids. The neutral molecules randomly diffuse throughout the ion source, only a few hundredths of a percent of them are ionized.

The most common ionization process for gas-phase analysis, EI, transfers energy to neutral molecule in the vapor state, giving it sufficient energy to eject one of its own electrons and thereby having a residual positive charge. This process produces a molecular ion with a positive charge and odd electron, as represented by $M^{+\bullet}$ in Fig. 2.12. This $M^{+\bullet}$ may have considerable excess energy that can be dissipated through fragmentation of certain chemical bonds. Cleavage of various chemical bonds leads to the production of fragment ions whose mass is equal to the sum of the atomic masses of the group of atoms retaining the positive charge during the fragmentation process. It is important to realize that not all of the $M^{+\bullet}$ ions decompose into fragment ions. For compounds producing a relatively stable $M^{+\bullet}$, an intense molecular-ion peak will be recorded because the $M^{+\bullet}$ tends to survive or resist fragmentation. Usually, however, most of $M^{+\bullet}$ ions decompose into fragment ions, and in these cases the mass spectrum contains only a small peak for the $M^{+\bullet}$. Various combinations of the above described process are the basis of the chemical fingerprint for a given compound.

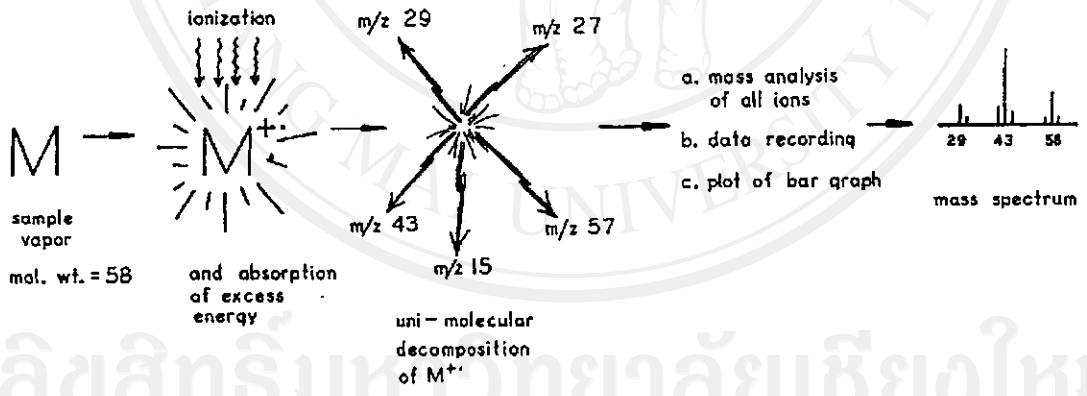

Fig. 2.12 General conceptual scheme for vapor-phase analysis by mass spectrometry
Source: Watson, J.T. (1997)