CHAPTER 2
LITURATURE REVIEWS

2.1 Orange fruits

Production of tangerines in Thailand can be done under a wide range of climatic conditions. The main 3 citrus types that are grown in Thailand and make up to 99% of the total production are tangerine or mandarins *(Citrus reticulata),* pomelo (*Citrus maxima)* and acid lime (*Citrus aurantifolia*). The total production of citrus in Thailand was about 765,000 tones in 2000. Most of this production were consumed by the domestic market, although a small quantity of pomelos was exported (Sethpakdee, 1997). A Khieo Waan (*Citrus reticulata* Blanco) variety of the tangerine occupies the largest growing area in Thailand and is produced as the highest yield citrus. The fruit has a short shelf life under the hot ambient temperature of tropical climates. Mature tangerines are harvested manually and graded mechanically into six size grades. The price of one kilogram of good quality orange is approximately 30-60 baths. Tangerines of large size grade have a greater sugar: acid ratio (Ketsa, 1988). The price of one kilogram of the small size grade fruit is low.

The fresh orange fruits in Thailand can be further manufactured to be jam, confectionary or beverages. The type of beverages will include concentrated orange juice, pasteurised orange juice and canned orange juice. On average, Thai people can consume 3 liters/head/year of fruit juices (Thailand Institute of Scientific and Technological Research, 2004). Beside productions of jam, confectionary or beverages, the by-products of orange fruits can be further utilized to produce specific compounds, such as pectin. The inner layer of orange skins, which is a soft, spongy layer called albedo (Figure 1), is the main source of pectin production. The manufacturing of pectin involves leaching to remove sugar and acid from the fresh peel, an acid extraction, precipitation, purification and standardization. It was reported that liquid pectin is less expensive to manufacture for use in a local market area (FAO, 2003).

A typical structure of a mature orange is shown in Figure 2.1 The most outer part, the flavedo, is composed of cuticle-covered epidermis and oil glands. The oil glands will contain essential oils. Inside the flavedo, there was an albedo, which consists of large and deeply lobed cells with very large intercellular spaces. After the albedo, the orange will have juice sacs, which is fulled of orange juice. When an orange is put under pressure of an extractor, the essential oils in the oil gland is forced out to be mixed with the juices. For the juice sacs, these compounds can be separated, pasteurised, dried and then sold. The juice sacs can be added back later on to orange juice or other beverages to give a more eye appeal for beverages containing low levels of juice solids and to improve mouth feel of the juices (Spiegel-Roy and Goldschmidt, 1996).

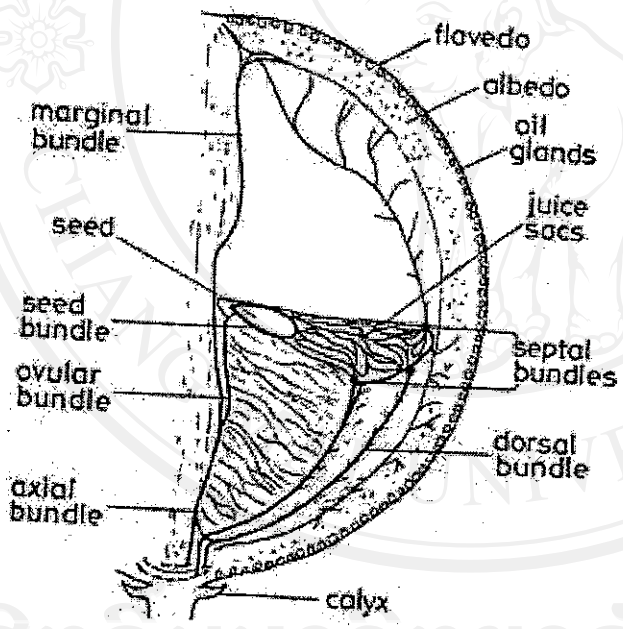

Figure 2.1 Schematic structure of a mature orange
Adapted from Spiegel-Roy and Goldschmidt (1996)

2.2 Chemical composition of orange juice

Table 2.1 An approximate chemical composition of orange juice

Constituents	Proportion per 100g (range)
Protein (g)	0.58- 1.29
Fat (g)	0.00 –0.56
Total soluble solids (g)	8.10 –17.7
Total sugars (g)	6.32-14.3
Total acids (g)	0.58-1.73
Minerals (g)	0.27-0.70
Vitamin C (mg)	26.0–84.0
β-Carotene (mg)	0.23-0.28
Vitamin B_1 (mg)	0.06-0.11
Vitamin B_2 (mg)	0.01-0.06
Vitamin B_6 (mg)	0.02-0.09
Other vitamins (mg)	0.19-0.30

Adapted from Ashurst *et al.* (1999)

Orange juice is rich with vitamin C. Besides that, other nutritional values can be obtained from the presence of protein, carbohydrates, organic acids, a precursor of vitamin A and various types of vitamin B (Table2.1). Since the content of fat is very low in orange juice (0.56% or less), the consumption of the juices will not be a problem for people that are concerned with a healthy-life style. The organic acids of orange juice contain organic acids, mainly citric and malic acids. These acids are the primary acids found in the orange juice. Citric acid accounts for the greatest part of the organic acids. These sugars are sucrose (49-59%), glucose (20-25%) and fructose (20-25%). The ratio of sucrose, glucose and fructose is generally about 2:1:1 (Ashurst *et al.*, 1999). Moreover, the unique flavour of orange juice is a wonderful combination of various component of volatiles as shown in Table2.2.

Table 2.2 Composition of volatile flavour in orange juice

Composition	Sample
Alcohols	Linalool
	α-Terpineol
	Citronellol
	Methanol
	Ethanol
Aldehydes	Acetaldehyde
	Hexanal
	Citronellal
	Geranial
	Neral
Ketones	Carvone
	Acetone
Esters	Ethyl butyrate
	Methyl butyrate
	Ethyl acetate
	Linaly acetate
Hydrocarbons	α-Pinene
	Terpinolene
	Valencene
	Limonene
Acids	Acetic acid
	Butyric acid
Other	Ethyl butyl ether
	Linalool oxides

Adapted from Ashurst *et al.* (1999)

Vitamin C is one of the important nutritional components of orange juice. The content of vitamin C in orange juice was in a range of 26 to 84 mg/100 ml of the juice (Ashurst *et al.*, 1999). Unfortunately, the concentration of this vitamin markedly reduced during storage of the juices (Lee and Coates, 1999; Johnston and Bowling, 2002; Li *et al.*, 1989; Farnworth *et al.*, 2001). For example, vitamin C in fresh squeezed unpasteurised orange juice was declined during a frozen storage in a polyethylene bottle at -23°C (Lee and Coates, 1999). Li *et al.* (1989) also found the concentration of vitamin C in Valencia orange juice was decreased during storage at 5 and 25°C. In addition to these researches, Kabasakalis *et al.* (2000) found that if the juices stored in open containers in a refrigerator for 31 days, the ascorbic acid loss was around 60 to 67%.

A pulsed electric field (PEF) processing that was used to reduce the effect of normal pasteurisation processing by reducing the severity of heat treatment was studied by Ayhan (2000). Since the shelf life, quality and safety of food are also affected by the performance of food packaging, a combination effect of packaging materials, a pulsed electric field processing and storage conditions on the orange juice flavour, colour and vitamin C was investigated in a pilot plant scale using an integrated process between the PEF and storage conditions. The PEF processed orange juice in glass and polyethylene terephthalate (PET) bottles had a shelf life of more than 16 weeks at 4°C storage temperature. The research showed that selecting a compatible packaging material for the orange juice would support the benefits of the PEF processing during the storage period (Ayhan, 2000).

The package that was used for orange juice product in this research is glass bottle. Glass bottle is considered to have the best oxygen barrier properties and it can be washed and pasteurised (Thailand Institute of Scientific and Technological Research, 2004).

The vitamin C content of ready-to-drink orange juice from reconstituted frozen orange juice concentrates that were purchased 4 to 5 weeks before their expired dates was studied in another research. The orange juice was opened, left unsealed and analyzed for the reduced and oxidized vitamin C content. The analysis results showed

that the ready-to-drink orange juice should be purchased 3 to 4 weeks before their expired dates and consumed within one week after being opened. The vitamin contents that were shown on the package was represent the minimum levels that the consumers could expect on the expiration date. Those levels of vitamins were likely to be higher on the day that the consumers purchased the juice. The results of this research also demonstrated that the vitamin C content decreased as the storage time increased (Johnston and Bowling, 2002).

Mexican orange juice were treated in 3 different treatments, which were either bottled without pasteurisation and frozen, or pasteurised with a tetra pak processing system bottled, and frozen or pasteurised and stored at 1°C in plastic bins. The analysis results showed that the pasteurisation treatment reduced the concentration of ascorbic acid in the orange juice from 54.2 to 49.6 mg/100 ml orange juice. The concentrations of acetaldehyde and ethyl acetate were found to be the highest in the unpasteurised juice. Similarly, the concentrations of pinene, myrcene, limonene, terpineol, 1-hexanol, 3-hexen-1-ol and sabinene, which were the flavour compounds of the juices, were also the highest in the unpasteurised juice (Farnworth *et al.*, 2001).

Some methods have been described to determine the amount of total vitamin C in food (ascorbic acid and dehydroascorbic acid), such as a titration method, the UV spectrophotometric method and high-performance liquid chromatography (HPLC). In orange juice, the ascorbic acid levels significantly decreased during a storage period even in unopened containers. Therefore, the presence of dehydroascorbic acid needs to be measured besides the ascorbic acid to get the overall activity of the vitamin C in orange juice (Hoare *et al.*, 1993).

2.3 Spoilage and food-born pathogens in orange juice

Microorganisms are important factor affecting the quality and safety of fruit juices, because they can reduce the shelf life of the juices or produce toxins that can affect the consumer's health. Among the spoilage microflora encountered in orange juice, the more common ones are lactic acid bacteria such as *Lactobacillus* and *Leuconostoc*, and yeast, especially *Saccharomyces cerevisiae*. Both of these microflora groups produce off-flavours due to a production of diacetyl as a metabolic

end product. Yeasts can also cause spoilage due to an ethanol fermentation and a corresponding carbon dioxide production. Other spoilage indicators include a production of hydrogen sulfide and other off-odours (Zook *et al.*, 1999).

The low pH values of some fruit juices greatly limit the number and types of bacteria that can survive or grow. Orange juice have a pH value between 3.4 to 4.0 and in these conditions *Lactobacillus* and *Leuconostoc* can survive and grow. These bacteria can produce undesirable flavours and odours. The growth of lactic acid bacteria in some fruit beverages, like wines, a fermented product from grapes, or cider, a fermented product from apple, can be useful, but in orange juice the lactic acid bacteria considered to be spoilage organisms. *Lactobacilli* play an important role in the spoilage of soft drinks, fruit juices and related products. *Lactobacillus plantarum* and *Lactobacillus brevis* can multiply in orange juice causing the formation of slime, gas, off-flavour, turbidity and changes in acidity (Murdock and Hatcher, 1975).

Microorganisms associated with the spoilage of orange juice included *L. plantarum, Gluconobacter oxydans,* and *S. cerevisiae* (Winniczuk and Parish, 1997). *L. plantarum* and S. cerevisiae are acid-tolerant microorganisms that are able to spoil citrus juices before and after a pasteurisation treatment. The growth of these microorganisms in orange juice with and without pasteurisation treatments was investigated by Alwazeer *et al.* (2002). From the abstract of their paper, they reported that *L. pantarum* had a higher heat resistance than *S. cerevisiae*. The reduction of both microorganisms were higher for at least 2.5 times when they were pasteurised at 60°C for 40 sec rather than at 55°C for 40 sec. However after the pasteurisation processes, *S. cerevisiae* showed a higher growth recovery rate than *L. pantarum* (Parish, 1998).

Bacterial spores are much more heat resistant than their vegetative counterparts. There are a number of non-pathogenic spore-formers including butyric and thermophilic anaerobes that can cause economic losses to food producers. Some unusual spoilage complaints have been reported, for example *Alicyclobacillus acidoterrestris* in apple and orange juice (Brown, 2000).

The number of microorganisms in orange fruits can affect the microbial quality of fresh orange juice. A specific research related to this issue was done to quantify the

transfer rates of microorganisms during an extraction process. It was found that about 1.7–2.6% of total aerobic organisms and 2.3–2.6% of aciduric organisms from the washed oranges were introduced into the fresh juice during the extraction process. Approximately 1.3–9.9% of E. coli and 2.7–8.9% of *L. plantarum* were recovered in the juice extracted from an artificially inoculated fruit. The quality of fresh juice is essentially depending on careful fruit handling and strict processing sanitation (Steven and Davis, 2001). Initial microbial levels of fresh citrus juice could be varied from 1.3 to 5.3 log CFU/ml (Feller, 1988).

Characteristics of some microorganisms that could be found in orange juice are as followed. For *A. acidoterrestris*, the organism is a Gram-positive, spore-forming and thermo-tolerant bacterium. The bacterium is resistant to pasteurisation, capable of growth at a wide range of temperatures, including at 25-44°C and can produce guaiacol and other taint chemicals (Pettipher *et al.*, 1997).

Lactobacillus spp. is spoilage organism of orange juice. The organism is characterized as a Gram-positive rod, non-spore-forming, non-acid-fast and non-motile bacterium. For another spoilage organism, *Leuconostoc* spp., the bacterium is a Gram-positive cocci. This bacterium will show a cell division in one plane and it is a catalase-negative organism (Wiley, 1994; Harrigan, 1998).

S. cerevisiae is a yeast, that has an ellipsoidal shape. The organism is an acid-tolerant microorganism, and usually grows as a unicellular spherical, ovoid or longer cell with rounded end. The vegetative reproduction of the organism is by budding (Harrigan, 1998).

2.4 Processes and hurdle treatments for orange juice

Orange juice is commonly extracted using automatic extractors. These units are designed to separate the juice from the fruit peel, seeds, and large pieces of pulp. Fresh orange juice is a pure juice obtained from mature citrus fruits and has not been further pasteurised, frozen, or concentrated after extraction (Ashurst *et al.*, 1999). A thermal pasteurisation treatment is considered to be the most common method to process and preserve the orange juice. This method extends the shelf life of the product, especially if it is stored at temperatures below 4°C, with a higher assurance

for its safety. However, the processing method may have some effects on the wholesomeness, aroma and flavour of the product. Therefore, alternative preservation technologies that can extend the products shelf-life without sacrificing the quality attributes of the juice were investigated (Hodgins, 2002; Siwaporn, 2002; Marylene, 1998; Parish, 1998; Tajchakavit, 1997; Sadler *et al.*, 1992; Li *et al.*, 1989).

A thermal pasteurisation treatment at 66°C for 10 sec was found to be sufficient to inactivate microorganisms in orange juice. This pasteurisation could make the juices stable for up to 4 weeks storage at 4°C (Sadler *et al.*, 1992).

A study on various temperatures and times for blanching and pasteurising limes and lime juices indicated that the lime juices prepared from the limes that had been blanched in boiling water for 10 sec and pasteurised at 85°C for 30 sec was able to keep for 4 weeks at room temperature without any significant changes in appearance, colour and flavour as compared to that of the fresh lime juices. Total acidity, pH values and total soluble solids of the fresh lime juices were at the ranges of 6.30-6.37, 2.10-2.19 and 7.10-7.70, respectively. No microorganism was found in the pasteurised lime juices (Siwaporn, 2002).

Using a hurdle approach of temperature, acidity, antimicrobial compounds (nisin and lysozyme) and number of pulses was also investigated to maximize a microbial killing. An optimal condition tested resulted in over 6 log reductions in the microbial population when applying 20 pulses at an electric field of 80 kV/cm, a pH solution of 3.5, a heating temperature at 44°C and an addition of 100 IU nisin/100 ml solution. The sensory analysis results of the juice after the hurdle treatment also showed favourable characteristics. A 97.5% retention of vitamin C was found in the treated juice. The juice shelf life based on the microbiological quality was improved and determined to be at least 28 days when stored at 4°C (Hodgins, 2002).

A study on acidification, low storage temperatures and sorbates for storage of orange juice was carried out by Li *et al.* (1989). They found that there was an increase in bacterial population during the first 2 weeks of storage at 5 and 25°C. However, the bacterial populations in the orange juice stored at 5°C were lower than at 25°C. The researchers also found out that the use of 0.03% sorbic acid or potassium sorbate in a combination with acidification at pH 2.5 could store the juices at 10°C for over than 10 weeks.

Valencia oranges were extracted to produce fresh orange juice using commercial extractor conditions. After that, the orange juice were treated by thermal pasteurisation at 75°C for 10 sec (as a minimal heat treatment) or at 98°C for 10 sec (as a traditional heat treatment) and stored at 4 and 8°C for 16 weeks. The results showed that microbial destruction in the pasteurised juices could be achieved from an initial microorganism population of 10^4 CFU/ml to less than 10 CFU/ml. Beside that, processing the orange juice at 98°C for 10 sec would stabilize the cloudiness of the pasteurised orange juice. The pH values and total soluble solids of the juices did not change significantly during the study. The average pH values and total soluble solids were 3.90-3.93 and 12.1-14.7 °Brix, respectively (Parish, 1998).

A pasteurisation processing using a microwave was studied at a heating temperature range of 50 to 90°C. The research was studied to find out the condition to inactivate enzyme and destroy microbial population in apple juices. The rates of enzyme inactivation/ microbial destruction were varied depending on the heating temperatures. For the reduction of microbial population, the worker used *S. cerevisiae* and *L. plantarum* as microbial indicators because these two microorganisms are common to be found in apple juices. The D-values that were found for these microorganisms were varied from 4.75 sec at 52.5°C to 0.378 sec at 60°C (at pH 3.4) for *S. cerevisiae* and 14.1 sec at 57.5°C to 0.327 sec at 65°C (at pH 3.4) for *L. plantarum* (Tajchakavit, 1997).

A study that investigated the quality of orange juice during storage was done by keeping unpasteurised orange juice samples in a frozen condition at -18°C and pasteurised orange juice samples at 1°C in polyethylene bags. The results showed that the optimum quality of the freshly processed orange juice was maintained in the unpasteurised orange juice that was stored in a frozen condition. The juice retained most of its chemical and physical properties and was rated to have a higher sensory score by the sensory panel (Marylene, 1998).

Preservation of orange juice by a combination of mild heat and ginger extract was studied by Linchong (2003). The ginger extract concentrations that were used were 0, 5, 10, 15, 20 and 25 %. The results showed that the addition of 10% ginger extract was the optimum concentration that was accepted by panelists. Subsequently, the orange juice heated at 50°C for 10 min with 10% ginger extract supplementation

to be studied during storage at room temperature. The research found that the processed orange juice had 4 days shelf life at room temperature, whereas fresh orange juice had a shelf life for only 1 day. For the chemical properties of fresh orange juice, it was reported that the orange juice had an average total soluble solids of 12.20, a total acidity expressed as citric acid of 0.72% and a pH value of 4.04 (Linchong, 2003).

2.5 Regulation of fruit juices in Thailand

The Ministry of Public Health of Thailand gave a regulation about fruit juices in Thailand in the name of Food Act number 62. The regulation started that fruit juices must not be toxic for consumers. The juices must not contain pathogenic microorganism, E. coli., yeast and mould. For the coliform bacteria, the microorganism must not be present for more than 2.2 CFU in 100 ml fruit juices when detected by a Most Probable Number (MPN) method. No contaminants should be detected in fruit juices, except for Pb, Cu, Zn, Fe. The presence of these contaminants cannot be exceeded for more than 0.5, 5, 5 and 15 mg /1 kg fruit juices for Pb, Cu, Zn and Fe, respectively.

Fruit juices that are sold in Thailand have to contain specific flavours of the fruits that are used as a raw material. Beside that, the production of fruit juices must use a good quality of water. The final fruit juices must not contain any precipitate except precipitate from natural raw material. Some preservatives that can be used in fruit juice include sulfur dioxide and sorbic or benzoic acid. The maximum level of sulfur dioxide that can be used is 10 mg /1 kg fruit juices, whereas sorbic acid or benzoic acid can be used up to 200 mg / 1 kg fruit juices (Wichailuk, 1993).

2.6 Chilling and Freezing

2.6.1 Chilling

Chilled foods are those foods stored at temperatures near but above their freezing point, typically 0-5°C. This commodity area has shown a massive increase in recent years as traditional chilled products such as orange juice. Three main factors have contributed to this development (Jay, 1992)

(1) The food manufacturer's objective of increasing added value to their products

(2) Consumer demand for fresh foods while at the same time requiring the convenience of only occasional shopping and ease of preparation.

(3) The availability of an efficient cold chain the organization and infrastructure which allows low temperatures to be maintained through the food chain from harvest to consumption.

Chill storage can change both the nature of spoilage and the rate at which it occurs. There may be qualitative changes in spoilage characteristics as low temperatures exert a selective effect preventing the growth of mesophiles and leading to a microflora dominated by psychrotrophs. This can be seen in the case of increased production of carotenoid pigments in some organisms at low temperatures and the stimulation of extracellular polysaccharide production in *Leuconostoc* spp., and some other lactic acid bacteria. In most cases, such changes probably represent a disturbance of metabolism due to the differing thermal coefficients and activation energies of the numerous chemical reactions that comprise microbial metabolism. The ability of organisms to grow at low temperatures appears to be particularly associated with the composition and architecture of the plasma membrane. As the temperature is lowered, the plasma membrane undergoes a phase transition from a liquid crystalline state to a rigid gel in which solute transport is severely limited. The temperature of this transition is lower in psychrotrophs and psychrophiles largely as a result of higher levels of unsaturated and short chain fatty acids in their membrane lipids. If some organisms are allowed to adapt to growth at lower temperatures they increase the proportion of these components in their membranes (Adams *et al.*, 1995).

2.6.2 Freezing

Freezing is the most successful technique for long term preservation of food since nutrient content is largely retained and the product resembles the fresh material more closely than in appertized foods (Adams *et al.*, 1995).

Foods begin to freeze somewhere in the range -0.5 to -3°C, the freezing point being lower than that of pure water due to the solutes present. As water is converted to ice during freezing, the concentration of solutes in the unfrozen water increases, decreasing its freezing point still further so that even at very low temperatures. The temperatures used in frozen storage are generally less than -18°C. At these temperatures no microbial growth is possible, although residual microbial or endogenous enzyme activity such as lipases can persist and eventually spoil a product. This is reduced in the case of fruits and vegetables by blanching before freezing to inactivate endogenous polyphenol oxidases which would otherwise case the product to discolour during storage. Freezer burn is another non-microbiological quality defect that may arise in frozen foods, where surface discolouration occurs due to sublimation of water from the product and its transfer to colder surfaces in the freezer. This can be prevented by wrapping products in a water-impermeable material or by glazing with a layer of ice. Low temperature is not the only inhibitory factor operating in frozen foods; they also have a low water activity produced by removal of water in the form of ice (Jay, 1992).

Microorganisms are affected by each phase of the freezing process. In cooling down to the temperature at which freezing begins, a proportion of the population will be subject to cold shock. At the freezing temperature, further death and injury occur as the cooling curve levels out as latent heat is removed and the product begins to freeze. Initially ice forms mainly extracellular, intracellular ice formation begin favoured by more rapid cooling. This may mechanically damage cells and the high extracellular osmotic pressures generated will dehydrate them. Changes in the ionic strength and pH of the water phase as a result of freezing will also disrupt the structure and function of cell components and macromolecules which depend on these factors for their stability. Cooling down to the storage temperature will prevent any further microbial growth once the temperature has dropped below -10°C. Finally,

during storage there will be an initial decrease in viable numbers followed by slow decline over time. The lower storage temperature, the slower the death rate.

Survival rates after freezing will depend on the precise conditions of freezing, the nature of the food material and the composition of its microflora, but have been variously recorded as between 5 and 70%. Bacterial spores are virtually unaffected by freezing, most vegetative Gram-positive bacteria are relatively resistant and Gram-negatives show the greatest sensitivity. The extent of microbial death is also determined by the rate of cooling Maximum lethality is seen with slow cooling where, although there is little or no cold shock experienced by the organisms, exposure to high solute concentrations is prolonged. Survival is greater with rapid freezing where exposure to these conditions is minimized. Food freezing processes are not designed however to maximize microbial lethality but to minimize loss of product quality. Formation of large ice crystals and prolonged exposure to high osmotic pressure solutions during slow cooling also damage cells of the food material itself causing greater drip loss and textural deterioration on thawing, so fast freezing in which the product is at storage temperature within half and hour is the method of choice commercially. The rate of freezing in domestic freezers is much slower so, although microbial lethality may be greater, so too is product quality loss (Adams *et al.*, 1995).

Thawing of frozen foods is a slower process than freezing. Even with moderate size material the outside of the product will be at the thawing temperature some time before the interior. So with high thawing temperature, mesophiles may be growing on the surface of a product while the interior is still frozen. Slow thawing at lower temperature is generally preferred. It does have some lethal effect as microbial cells experience adverse conditions in the 0 to -10°C range for longer, but it will also allow psychrotrophs to grow. Provided the product is not subject to contamination after thawing, the microflora that develops will differ from that on the fresh material due to the selective lethal effect of freezing. Lactic acid bacterial are often responsible for the spoilage of defrosted vegetables whereas they generally comprise only about 1% of the microflora on fresh chilled produce (Adams *et al.*, 1995).

2.7 Nisin

Nisin, an antimicrobial compound, is produced from a microbial fermentation of Lactococcus lactis subsp. lactis bacteria. In this project, nisin is going to be used to prevent the growth of spoilage bacteria in orange juice. Since nisin is a natural by-product of a fermentation, the uses of the compound were a good alternative in avoiding the uses of chemical preservatives, such as sorbic acid or potassium metabisulfite.

Nisin was discovered in England in 1928 (Table2.3) by Rogers and Whittier during the process of cheese making. After that, in 1947 the first isolation and characterization of the compound was carried out. The compound was then called as "Nisin" because it was produced from strains of lactic streptococci of the serological group N Inhibitory Substance. The suffix "-in" was commonly used for antibiotics at that period. In 1962, the development of nisaplin for commercial used was made in England by Aplin and Barrett, Ltd. Now, the compound has been used in over than 48 countries to prevent spoilage in food products, mainly processed cheese products. In the late 1969, a Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additive recognized nisin as a safe and legal biological food preservative. This bacteriocin was also added in the European food additive list where it was assigned with a number E234 (Thomas *et al.*, 2000; Paul Ross *et al.*, 2002).

2.7.1 General characteristics of nisin

Table2.3 General characteristics of nisin

Characteristic	Description
History	1928 Discovery 1947 first isolation 1951 first use in food 1962-65 development of nisaplin 1969 recognised as a food preservative by FAO/WHO 1971 primary structure determined 1988 granted with a Generally Regarded as Safe (GRAS) status (US. Food and Drugs Administration)
Producer organism	*Lactococcus lactis* subsp. lactis.
Biosynthesis	post- translational enzymatic modification
EU number	E234
Molecular weight	3353 dalton (for the monomer)
Properties	Cationic with a positive charge of 3 (2 for nisin Z) amphiphilic: hydrophobic at N-terminus and hydrophilic at C-terminus.

Table 2.3 (continue)

Characteristic	Description
Stability	Optimum at pH 3.0 (< 5% loss after 115°C for 20 min)
Activity	Bactericidal and bacteriostatic against cells of a wide range of Gram-positive bacteria. Sporostatic against spores of Gram-positive endospore formers (Bacillus and Clostridium).
Methods of application	In solution or as a dry powder, mixed into food, often in a combination with heat treatment. Also for a surface treatment by spray, immersion or in packaging/casing material.
Potential application	Pasteurised fruit juices stored at ambient temperature, meat and meat products, fish products, rehydrated infant formula, vegetarian food.

Adapted from Thomas *et al.* (2000)

2.7.2 Structure and mechanism of nisin

Nisin is a 34- residue long peptide belong to a family, called as lantibiotic. The lantibiotics were originally subdivided into two groups, the elongated type A group and the globular type B group. Nisin is part of the type A group because it has a linear structure rather than a circular structure (group B). It has a pentacyclic structure with one lanthionine residue ring and four β–methyllanthionine residue rings (Breukink and de Kruijff, 1999; Paul Ross *et al.*, 2002). The structure of nisin is illustrated in Figure 2.2

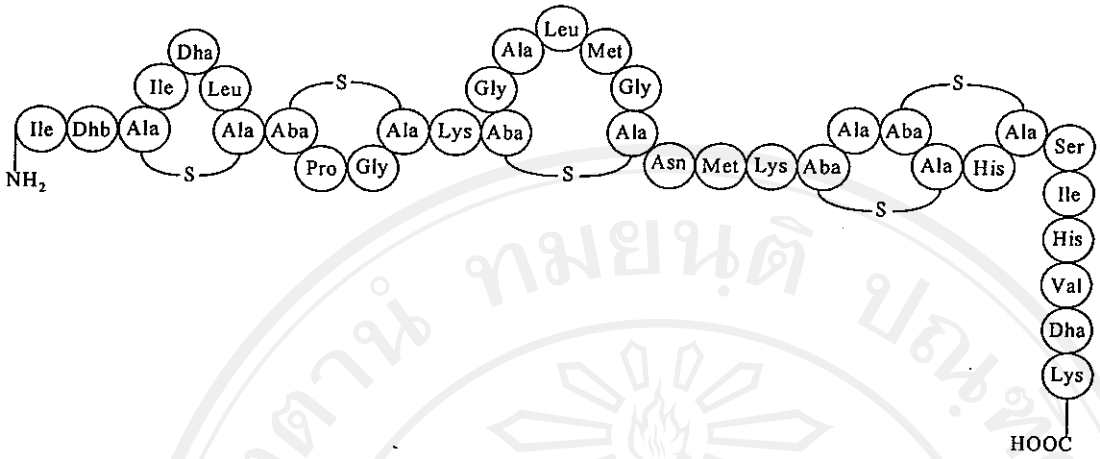

Figure 2.2 Primary structure of nisin A

Adapted from De Vuyst and Vandamme (1994)

Dha: dehydroalanine,Dhb: dehydrobutyrine,Ala-S-Ala:lanthionine,

Aba-S-Ala: β-methyllanthionine.

Nisin is positively charged (+3) in overall and has two rigid ring systems, one is located in N-terminal and the other in C-terminal. The nisin molecule has amphiphathic characteristics, which the half N-terminal of nisin is more hydrophobic than the other half C-terminal (Breukink and de Kruijff, 1999).

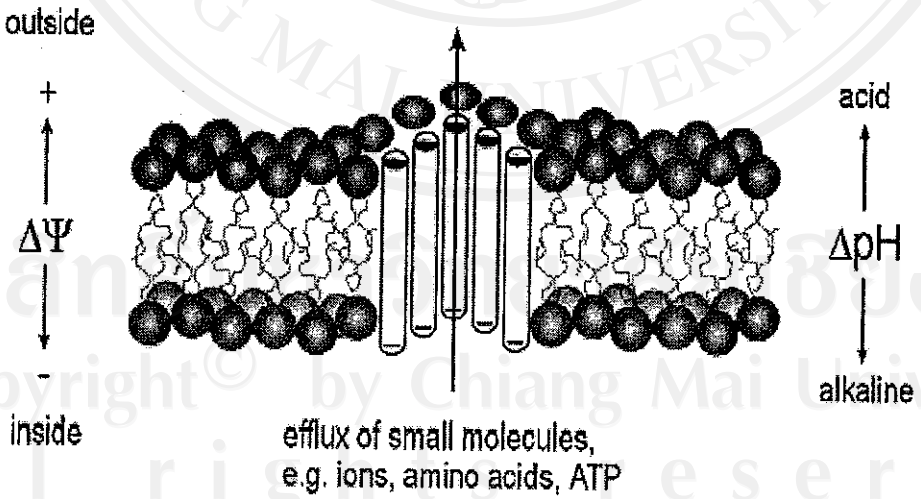

Figure 2.3 Model of barrel-stave pore formation by peptides.

Adapted from De Vuyst and Vandamme (1994)

As a cationic antimicrobial peptide, nisin could kill Gram-positive bacteria by pore formation in the target cytoplasmic membrane cells. The formation of pores by nisin caused a rapid efflux of small cytoplasm compounds, including ions, amino acids, organic acids and adenosine triphosphate (ATP). If the leakage of these compounds was severe, the target cell could die. The detail mechanism of the nisin pore formation is still unclear. The pore formation is generally a cooperative process, which accompanied by aggregation of peptides. The pore formation looked like a barrel-stave could be seen in Figure 2.3. Nisin molecules could join and leave the pore in a dynamic process. Peptides first adapted a transmembrane before they were aggregated to form water filled pore. The pore formation also caused collapse of ion gradients and disperse the proton motive force of the bacteria, which could lead to death of cell (Breukink and de Kruijff, 1999; Paul Ross *et al.*, 2002). Since nisin is positively charged, the compound was found to be selective to interact with negatively charged membranes and bound to membranes containing anionic lipid. Therefore some bacteria were susceptible to nisin, because these bacteria has a high concentration of anionic lipid in their membranes (Breukink and de Kruijff, 1999). In its action against spores, nisin does not have a direct effect on the spore germination. The effectiveness of the compound is more targeted on the post-germination swelling and spore outgrowth (Thomas *et al.*, 2000).

2.7.3 The effectiveness of nisin in food

The effectiveness of nisin in food products are depending on several factors, including:

1) The time and temperature of heat treatments. The length of time and the highness of temperature of a heat treatment have a great influence on the nisin effectiveness. As the time of heat treatment is longer or as the temperature of heat treatment is higher, the availability of nisin in a food product is reduced. Therefore, some losses of nisin should be expected after a heat treatment. To compensate this loss of nisin, the level of nisin added to a food product must be higher than the nisin effective level (Thomas *et al.*, 2000).

2) The food matrix. This factor is important because nisin needs to be mixed properly throughout a food mixture to ensure its effectiveness. Different food matrixes in different food products will influence the distribution of nisin uniformly. A spatial heterogeneity of a food matrix may cause nisin not to be distributed uniformly and lead to a poorer protection of nisin to the product and thus its potential to protect the product (Thomas *et al.*, 2000).

3) pH values of food. Nisin is a cationic molecule due to a combination of three lysine and one or more histidine residues. It is more soluble at acid pH values and becomes less soluble when the pH value is increased. However, the solubility of nisin is not a serious problem in food because generally food products have neutral pH values or lower. It was concluded by Thomas *et al.* (2000) that nisin was effective in a wide range of pH values at 3.5-8.0, although in its application, nisin was more often to be used in acidic foods.

4) The species of bacteria in food products. Nisin can be effective against some Gram-positive bacteria because it is able to perform pore formation, which causes efflux of small cytoplam compounds and lead the cell to death. Gram-positive bacteria have an important role in food products, including orange juice. For the Gram negative bacteria, they are more resistant to nisin because they have outer membrane that prevents the binding and inserting of nisin. However, these bacteria can be more sensitive to nisin if the addition of nisin is combined with other treatments, such as hydrostatic pressure, heat, freezing and thawing. Any of these treatments will damage the outer membranes of the bacteria to allow the bacteriocin having a direct access to the cell membrane (Thomas *et al.*, 2000).

5) The concentration of nisin. The concentration of nisin can be reduced due to food processing, in particular heat treatments. It is important that the level of nisin added to a food product should be adjusted to consider the loss during processing (Thomas *et al.*, 2000). It is desirable to have a good retention level of nisin in the finished products to make sure that nisin is effective in preventing the spore outgrowth and/ or inhibiting the bacteria growth throughout the storage time.

6) The shelf life of food products. The effectiveness of nisin were reduced during storage. This reduction will occur slowly at lower storage temperatures (Delves-Broughton, 1990). Therefore, it is important to maintain the level of nisin

above the minimum level necessary to inhibit food spoilage throughout the storage life of a product.

2.7.4 The safety of nisin

In Thailand, nisin is approved to be used in processed cheese products. This approval can be legalized because there have been enough research results that showed clearly the effectiveness of nisin in the products. In the area of fruit juices, more research is needed before more goverments recognize and can legally approve the use of this natural preservative in fruit juices.

Nisin has been called as a biopreservative, that can be used as a single preservation or in a combination with other preservatives or processing methods to protect food from spoilage and health hazard problems. Nisin has been reported to inhibit both spoilage and pathogenic microorganisms (Paul Ross *et al.*, 2002). Nisin has been consumed for centuries and it is not harmful for human and animal. It is not toxic at the level that is used in food. In fact, the LD_{50} level of nisin is 7 mg/ 1 kg bodyweight (Thomas *et al.*, 2000). This means that a person with a weight of 50 kg can consume 350 mg nisin. This amount of nisin is well-above the necessary level of nisin to be used in fruit juices, which has been reported to be 100 IU/ml nisin or equal to 20 mg nisin for 200 ml fruit juices. Besides that, enzymes can degrade the compound rapidly and nisin does not alter the microflora in the intestinal tract when it is consumed orally.

An international organization, the FAO/WHO Expert Committee had made a conclusion from the available evidence that nisin at a level of 3.3×10^6 unit/kg body weight did not have any advance effect to human. The Committee also permitted an unconditional Acceptable Daily Intake (ADI) at a level of 3.3×10^4units/kg body weight in 1969. In U.S.A., the U.S. Food and Drug Administration has affirmed nisin as a safe compound and it is allowed to be directly used as an ingredient in human food (Thomas et al., 2000). All of these standards are confirming the safety of nisin and its tolerance level of use. Nowadays, more countries have permitted the use of nisin in different products, such as canned tomato juice. In total, it is around 48

countries in the world have allowed the use of the compound legally (Thomas *et al.*, 2000).

2.7.5 Application of nisin in food products

Nisin is an effective preservative in many food products. Types of food products, the permitted level of use and countries in which nisin is approved to be used can be seen in Table 2.4. Nisin is more stable in acid media and heat processing products. Therefore, nisin is generally used as a preservative in heat processed and low pH food products. A research paper in Thailand also reported that researchers could isolate and characterize bacteriocin producing lactic acid bacteria from Nham, a fermented pork sausage which is usually consumed without cooking (Noonpakdee *et al.*, 2003).

Table 2.4 Food products, the permitted levels and countries in which nisin is approved to be used

Products	Permitted level (μg/g food)	Country
Canned tomatoes, canned soups, paste, puree	No limit	Australia
Beer	No limit	Australia, New Zealand
Tomato puree, canned tomato pulp, canned tomato paste, canned tomato juice, pH< 4.5	No limit	Papua New Guinea
Vegetable (raw, peeled, semi-preserved potatoes, green peas)	100	Russia
Dairy products	12.5	China
Pudding (semolina, tapioca, etc.)	3	EU, Czech Republic
Processed (pasteurised) cheese spread (and with vegetables, fruit, meats)	250	USA
Nisin as a permitted preservative	No limit	Peru

Adapted from Thomas *et al.*, (2000)

Nisin is suitable for use in a wide range of food products both liquid and solid, canned or packaged, at chilled or warm ambient temperature storage. The addition of nisin is based on target organisms, such as to prevent spoilage by Gram positive endospore formers (particularly in heat processed foods), to prevent spoilage by lactic acid bacteria and similar organisms and to kill or inhibit Gram positive pathogens. The use of nisin gives many purposes in food. It can extend the shelf life of both chilled and ambient temperature stored foods and/or contribute to a general preservation of a food product, so that a thermal processing can be reduced. The reduction of thermal processing has double advantages, which are improving product quality and reducing manufacturing costs, due to a reduction in both of the time and temperature of the thermal heat treatment. In some cases, distribution costs can also be reduced by the application of nisin. This can happen because the presence of nisin allows the specific food products to be transported at ambient rather than chilled temperatures (Thomas *et al.*, 2000).

Nisin is stable for two years from the date of manufacture provided it is stored dry, away from direct sunlight and between 4 to 25°C. Nisin is best added as an aqueous solution, usually to the liquid portion of a product during processing. It can also be added as a powder, but in all cases, it is essential to ensure uniform dispersal throughout the food matrix. The best time to add nisin is at the last practical stage before heat processing. The addition level of nisin depends on the type of food, its heat process, pH, storage conditions and the required shelf life time. The use level of nisin in fruit juice has been reported to be 5 IU/ml (nisin 1 g= 10^6IU). One of the target organisms in fruit juices *is Alicyclobacillus acidoterrestris* (Komitopoulou *et al.*, 1999).

The effectiveness of nisin to control spoilage bacteria and malolactic fermentation has also been reported. A concentration of 100 IU/ml nisin could prevent the malolactic fermentation and inhibit the spoilage bacteria in the products (Jung, 1992).