CHAPTER 2

LITERATURE REVIEW

2.1 Pennywort (*Centella asiatica* (Linn.) Urban)

2.1.1 Pennywort description

Pennywort, Buabok, Phak-nok, *Centella asiatica* (Linn.) Urban (Figure 2.1), is a genus of the plant *Apiaceae* (*Umbelliferae* family), which has a mildly bitter taste. It is a prostrate stoloniferous plant, leaves rounded to reinform, the petioles elongated about 4 to 10 cm, flowers umbels with three sessile flower. It is a slender trailing, rooting at the nodes and leaves thin, soft, simple and green, and 1 to 6 in rosette at each node, glabrous and shiny above, paler beneath, 1 to 7 cm in diameter (THP, 2007). It can be found easily in moist habitats and distributes in open or shaded damp area through out many countries.

Figure 2.1 *Centella asiatica* (Linn.) Urban

2.1.2 Pennywort nutrient compositions

The proximate compositions of edible portion (100 g) are 69.0% moisture, 2.0 g protein, 2.0 g fat, 0.7 g fibre, 1.7 g carbohydrate and 23.0 kcal of energy (Sheela *et al*., 2004). In addition, Tee *et al*. (1997) reported the proximate compositions of fresh

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pennywort in Malaysia, which are 87.7% moisture, 2.0 g protein, 0.2 g fat., 1.6 g fibre, 6.7 g carbohydrate, 1.8 g ash and 37 kcal of energy. It contains mineral including potassium, calcium, phosphorus, sodium and ferrous levels that accounted for 391, 171, 32, 21 and 5.6 mg/100 g, respectively (Tee *et al*., 1988). Similarly, it contains vitamin such as carotene, retinol equivalents, ascorbic acid, vitamin B_2, niacin and vitamin B_1 at 2.649, 0.442, 48.5, 0.19, 0.1 and 0.09 mg/ 100 g, respectively (Tee *et al*., 1988). β-Carotene and ascorbic acid are known to have antioxidative activities. They can act as antioxidant and are effective to singlet oxygen or preventing the formation of hydroperoxides (Rajalakshmi and Narasimhan, 1996).

2.1.3 Applications use of pennywort for bioactive natural products

Bioactive natural products have enormous economic importance as specialty chemicals. Bioactive natural products can be used as drugs, biological or pharmacological ingredients, nutraceuticals, and raw materials for the production of drugs (Henkel *et al*., 1999).

Pennywort has been recognized to provide health benefits and used for medicinal purposes to treat a wide range of indications against gastrointestinal diseases, gastric ulcer, indigestion, gastritis and inflammatory diseases of the liver (Brinkhaus, 2000; WHO, 1998). It is listed officially in the Chinese Pharmacopoeia and used as an antipyretic, diuretic, and antidote in the treatment of icterus, heat stoke, diarrhea, ulcerations, eczema and traumatic diseases (Tang and Eisenbrand, 1992). It has been claimed in Thai traditional recipes as a poultice for the wound healing (Farnsworth and Buryyapraphatsara, 1992), mild diuretic (THP, 2007) and mostly used as a vegetable and tonic (Pramongkit, 1995). This plant occupies an important place in indigenous system of Indian medicine as tonic in diseases of skin, nerves (Dutta and Basu, 1962; Rao and Seshadri, 1969), leprosy, bloods (Dutta and Basu, 1962), antiinflammatory, cure in leprosy and syphilis (Pramongkit, 1995). Leaves are used as tonic and for improving memory (Sakina and Dandiya, 1990). Seeds are used for dysentery, fever and headache (Sappakun and Ungwitayatorn, 1982).

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2.1.4 Chemical constituents of pennywort

Over several decades, there has been an increasing interest in various compounds obtained from different sources of pennywort. The different reported compounds may be due to place of origin of the materials or to the differences in variety of the plant (Pramongkit, 1995). The constituents of this plant are classified into main groups, which are triterpenoid saponins (asiaticoside, madecassoside and their aglycones which are asiatic and madecassic acids, respectively) and volatile oil, pectin, trace of alkaloids, etc (THP, 2007). Earlier work on this plant has led to the isolation of more than 70 constituents, such as triterpenoids saponins (Jiang *et al*., 2005; Kuroda *et al*., 2001), polyacetylenes (Schulte *et al*., 1973), flavones (Prum *et al*., 1983), sterols and lipids (Kapoor *et al*., 2003).

The active compound is composed of four related chemical extracts which are asiatic acid (constituting 29-30%), madecassic acid (29-30%), madecassoside (1%) and asiaticoside (40%) (Bosse *et al*., 1979). They are the biologically active constituents in pennywort that have a potential to be promoted as commercial products (Indu Bala and Ng, 1999). In addition, they contain asiaticoside (1-8%) and total phenolics (23,000 mg/100 g) (Brinkhaus *et al*., 2000; Fezah *et al*., 2000). A list of chemical found are summarized as follows:

1) Flavanoid glycosides

Flavonoid components including apigenin, kaempferol, quercetin and rutin have been detected in pennywort. The yield of apigenin was found to be hightest followed by quercetin, kaempferol and rutin (Radzali *et al*., 2001 cited by Kormin, 2005). In addition, Koo and Suhaila (2001) found that the concentrations of quercetin and kaempferol in dried pennywort were 423.5 and 20.5 mg/kg, respectively. Kaempferol-3-glucoside and quercetin-3-glucoside are flavone derivatives isolated from leaves of pennywort (Prum *et al*., 1983).

2) Free amino acids

The amino acids components of pennywort were reported by George and Gnanarethinan (1975). Twenty free amino acids were identified. In leaf, petiole and stolon; glutamate, serine and alanine were presented in larger quantities than any of the other amino acids.

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3) Polyacetylenic compounds

Some polyacetylenic compounds were isolated (Tang and Eisenbrand, 1992). Five of them were identified as pentadeca-2-9-diene-4,6-diyne-1-ol acetate; 3,8-diacetoxypentadeca-1,9-diene-4,6-diyne; 3-hydroxy-8-acetoxy-pentadeca-1,9-diene-4,6-diyne; 3-hydroxy-10-acetoxy-pentadeca-1,8-diene-4,6-diyne and pentadeca-1,8-diene-4,6-diyne-3,10-diol.

4) Terpenoid compounds

Several terpenoids isolated from the whole plant of pennywort are described as follows:

4.1) Monoterpene and sesquiterpene compounds

These compounds are including *α*-pinene, *β*-pinene, myrcene, *γ*-terpinene, bornyl acetate, *α*-copaene, *β*-elemene, *β*-caryophyllene, *trans*-*β*-farnesene, germacrene *D* and bicycloelemene.

4.2) Triterpene compounds

The major compounds isolated from whole plant are triterpene compounds, resulting in the isolation of glycosides and triterpene acids.

Major triterpene compounds namely, asiaticoside, asiatic acid, madecassoside and madecassic acid (Figure 2.2) are of the interest in this research.

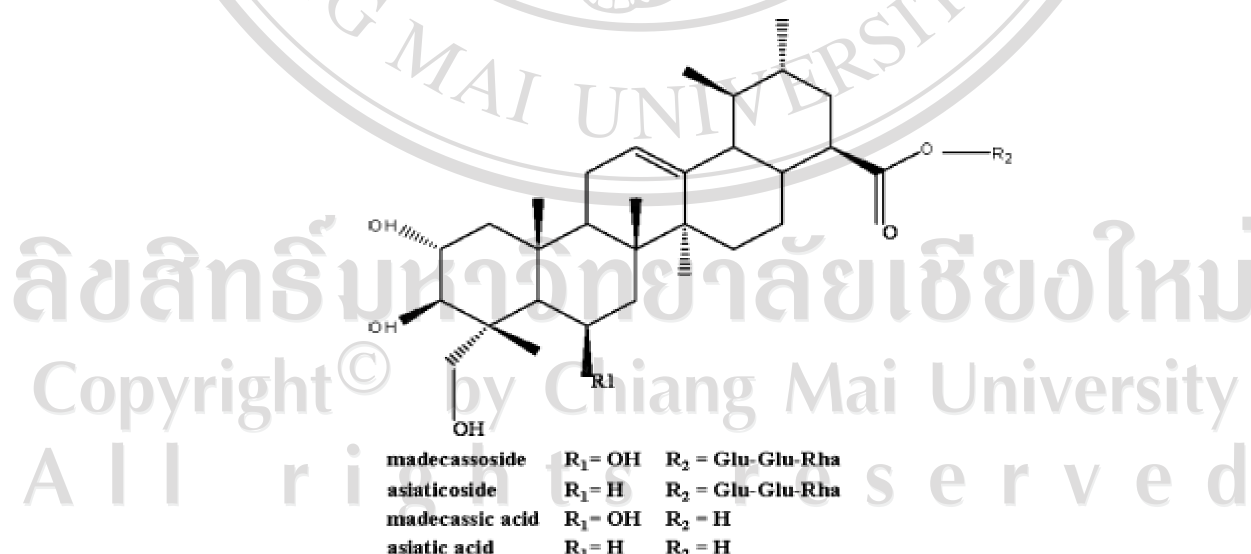

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Figure 2.2 Structure of triterpene compounds of pennywort

(Glu: glucose, Rha: rhamnose)

Source: Rafamantanana *et al*. (2009)

2.1.5 Major bioactive constituents of pennywort

Analysis of bioactive constituents from pennywort extract has been analysed by various methods such as titration, colourimetry, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Rahandrama *et al.*, 1963).

Pramongkit (1995) found that the major triterpene saponin in pennywort was asiaticoside, consisting of sapogenin, asiatic acid and the sugar components which have been identified as one rhamnose and two glucose residues. Asiaticoside was also isolated directly from the plant as well as other sapogenins (6*β*-hydroxy asiatic acid and terminolic acid). The structures of these compounds were elucidated by spectroscopic methods. However, Sribusarakum (1997) investigated spectrophotometric methods for quantification of asiaticoside, by using anthrone as the reagent. This method could not be applied for determination of asiaticoside in pharmaceutical preparations. Analysis of pennywort extract by a reversed phase HPLC showed that it contained asiaticoside, madecassic acid, terminolic acid and asiatic acid. In addition, Yu *et al*. (2006) reported the isolatation of the 95% ethanol extract of pennywort, which is a new urs-type triterpene compound 1. It was a white powder containing ten known compounds, namely asiatic acid, madecassic acid, indocentoic acid, bayogenin, kaempferol, quercetin, euscaphic acid, terminolic acid, 3*β*-6*β*-23-tri-hydroxyolean-12-en-28-oic acid and 3*β*-6*β*-23-tri-hydroxyurs-12-en-28-oic acid (**Figure 2.3**).

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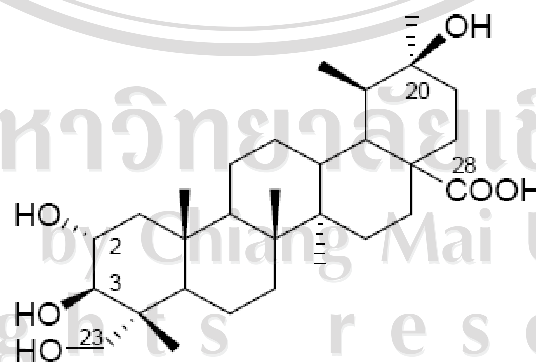

Figure 2.3 Structure of compound 1
Source: Yu *et al*. (2006)

1) Asiaticoside

Asiaticoside, one of the principle terpenoids in pennywort**,** is the marked bioactive ingredient in pennywort since asiaticoside provides most profound antibacterial and fungicidal effects against pathogens and fungi (Hausen, 1993). It is known to stimulate collagen synthesis in fibroblast (Maquart *et al*., 1990; Bonte *et al*., 1995). This compound is clinically used as an agent for wound healing in combination with madecassic and asiatic acids (Hausen, 1993). It is presumed to be converted *in vivo* to asiatic acid by hydrolytic cleavage of the sugar moiety and the metabolic product is responsible for the therapeutic effects (Grimaldi *et al*., 1990; Rush *et al*., 1993).

The structure of asiaticoside is shown in **Figure 2.4**. It was first isolated from the pennywort leaves more than 56 years ago by Polonoski (1951). It was identified as a potent wound healing promoter. However, its isolation using the old method (Bontems, 1941) is too complicated. Its interesting structure and considerable pharmacological activity contribute to more simple ways of the isolation. It is essential to investigate other methods which can isolate those compounds from total triterpene fraction (Bhattacharyya, 1956; Rastogi *et al.,* 1960).

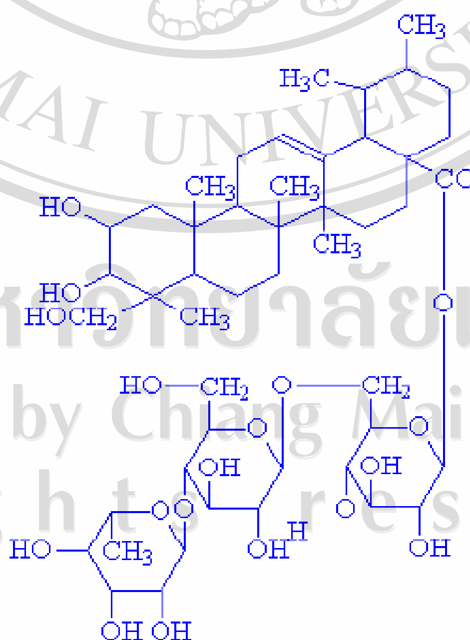

Figure 2.4 Structure of asiaticoside ($C_{48}H_{78}O_{19}$)

Source: Supawantanakul (2003)

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2) Asiatic acid

Asiatic acid (**Figure 2.5**), pentacyclic triterpene compounds, is the aglycone of asiaticoside isolated from the pennywort. It also exhibits bioactive efficacy (Park *et al*., 2007), and is known to control cell division in human hepatoma, melanoma cells and cytotoxic activity on fibroblast cells (Coldren *et al*., 2003). In addition, it has shown various biological affects such as wound healing, protective activities against UV-induced photoaging, induces cell cycle arrest and antiproliferative effects on human (breast, gastric and urine cancer) cells (Park *et al*., 2006).

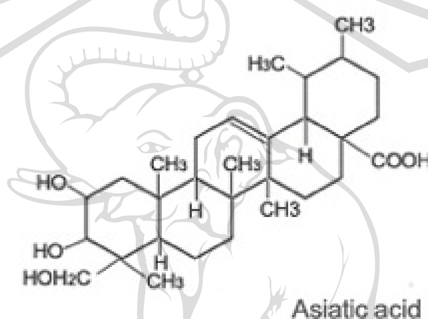

Figure 2.5 Structure of asiatic acid ($C_{30}H_{48}O_5$)
Source: http://jadzuka.files.wordpress.com

3) Madecassoside

Madecassoside (**Figure 2.6**), a glycoside that is a strong antiinflammatory agent (Si-Qi and Huei-Fang, 1981).

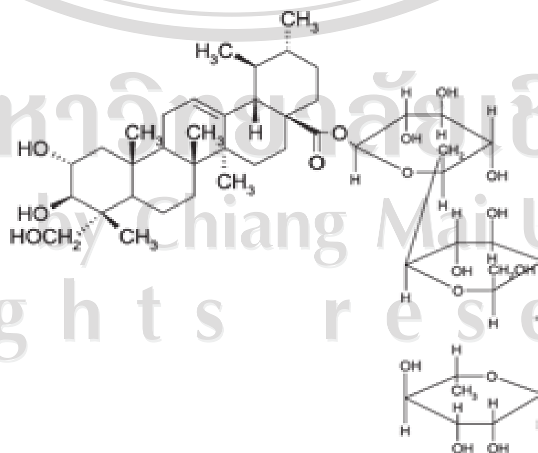

Figure 2.6 Structure of madecassoside ($C_{48}H_{78}O_{20}$)
Source: Si-Qi and Huei-Fang (1981)

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4) Madecassic acid

Madecassic acid (**Figure 2.7**) was isolated from pennywort. Its wound healing property has been attributed to its ability to stimulate collagen synthesis (Si-Qi and Huei-Fang, 1981).

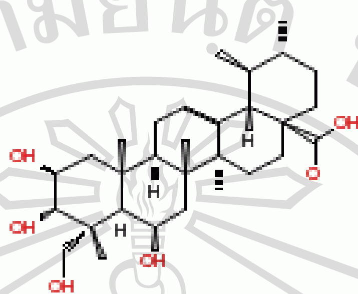

Figure 2.7 Structure of madecassic acid (**$C_{30}H_{48}O_6$**)

Source: Si-Qi and Huei-Fang (1981)

2.2 Pharmacological and health promoting effects of pennywort

Pennywort is valued in indigenous medicine for treatment of leprosy and skin diseases and also improvement memory (Goh *et al.*, 1995). A cold poultice of the fresh herb is used as an external application in rheumatism, elephantiasis and hydrocele. For treating leprosy and other skin diseases it is given as an ointment or dusting powder. Internally it has been valued as a tonic and is used in bronchitis, asthma, gastric catarrh, leucorrhoea, kidney troubles, urethritis (Jaganath and Ng, 1999) and dropsy. A decoction of very young shoots is given for haemorrhoids. It is used for blood conditions, brain and nervous system conditions, cardiovascular conditions, gastrointestinal conditions, glandular conditions, immune system conditions, cough problems, liver conditions, respiratory tract conditions and tissue development (Anonymous, 2004). It is also commonly used as porridge for feeding preschool children in Sri Lanka in combating nutritional deficiencies (Cox *et al.*, 1993).

Umbelliferae are known to have anticancer activities (Yoshinori *et al.*, 1982; Unnikrishnan and Kuttan, 1990). Moreover, some of triterpenes and flavonoids have the anti-tumour activity (Babu *et al.*, 1995) and inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai *et al.*, 1984). The triterpene compounds namely

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asiaticoside, asiatic acid, madecassoside and madecassic acid have influence on biosynthesis of collagen and cured skin problems (Indu Bala and Ng, 1999). Products from pennywort are available as ointments, solutions for injection, powder and tablets in preparations for wound healing (Ramasawamy *et al*., 1970; Bosse *et al*., 1979; Rosen *et al*., 1967; Morisset *et al*., 1987). Flavonoids have potential for treating many diseases (Piskula and Terao, 1998). Faridah (1998) reported that flavonoids from pennywort are used to assist strong, lustrous and healthy hair growth. In addition, they exhibit a wide range of biological effects including anti inflammatory, anti bacterial, anti viral and anti allergic (Cook and Samman, 1996).

Foods and beverages, which are rich in phenolics content, contribute to a reduced incidence of heart disease (Muhammad Idris *et al*., 1999). Pharmaceutical preparations from pennywort have been widely used in Europe. The extract is commonly composed of asiaticoside, asiatic acid and madecassic acid (Sribusarakum, 1997).

The pharmacological actions of asiaticoside was the principal active ingredient for successful treatment of leprosy (Boiteau *et al*., 1949), which stimulated many investigators to study its effect as a healing agent (Anonymous, 1945). Asiaticoside is reported to have effect to treat leprosy (Boiteau and Ratsimamanga, 1956) and it is also used as an antiinflammatory (Newall *et al*., 1996), antimicrobial activity (WHO, 1998) and antioxidant (Shukla *et al*., 1999). Madecassol, a formula based on pennywort extract, when applied locally on wounds in rats prompted the proliferation of granulation and increases tensile (Vogel *et al*., 1990). It decreased the wound area of the skin necrosis induced by burn (Manuel and Eduardo, 1976).

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2.3 Antioxidant properties of plant

In traditional applications, herbs, fruits and vegetables have been widely used as major sources of antioxidants. Most tropical herbs are rich with antioxidant activities. There is wide range of components identified as antioxidant compounds in herbs. Many studies have reported concerning the relationships between antioxidant activity and quercetin content in *Polygonum hydropiper* (Haraguchi *et al*., 1992), vitamin E in green leaf vegetables (Mallet *et al*., 1994) and total polyphenols in

Chrysanthemum morifolium and *Hordeum vulgare* (Duh and Yen, 1997). Polyphenol compounds appear to be major contributors to the antioxidant potential of green tea beverage (Wang *et al*., 2000) and non citrus juice (apple juice) (Miller *et al*., 1995). In addition, Shukla *et al*. (1999) reported that asiaticoside (0.2%) improved healing of surface wounds in rats and significantly increased the level of enzymatic and non-enzymatic antioxidants. In tomato products, ascorbic acid and polyphenols (flavonoids and hydroxycinnamic acids) are reported to be the major antioxidant hydrophilic components, including vitamin E and carotenoids mainly constitute the hydrophobic fraction (Takeoka *et al*., 2001; Martinez-Valverde *et al*., 2002).

Abdul Hamid *et al*. (2002) reported that pennywort have a high antioxidant activity. It has been established that the presence of polyphenols in the extract contribute its antioxidative activity (Zainol *et al*., 2003). Vimala *et al*. (2003) also reported that pennywort leaves were found to have high antioxidant activity in different pathways including superoxide free radical scavenging activity (86.4%), inhibition of linoleic acid peroxidation (98.2%) and radical scavenging activity (98.2%). Pennywort was useful to protect the cells from oxidative damage, to destroy excess free radicals and it helped to keep the oxidative stress state in balance. Asiaticoside from this plant has been attributed to increase the antioxidant levels at an initial stage of healing (Shukla *et al*., 1999). Yusuf *et al*. (2000) observed the antioxidative activities of carotenoid and ascorbate, which inhibited peroxidase (POD) in pennywort. It exhibited optimum antioxidant activity at neutral pH and the activity remained stable up to 50˚C and increased when concentration was increased from 1,000 to 5,000 ppm (Abdul Hamid *et al*., 2002).

Several methods have been developed to quantify natural antioxidants. FRAP is a method for assessing antioxidant power through their reduction of ferric to ferrous at low pH. The combination of ferrous-tripyridyltriazine is caused to formation of blue colour and detected at wavelength of 593 nm (Benzie and Strain, 1996). The antioxidant activity has been detected on fresh plasma. This method also applied on beverages (Tsai *et al*., 2002) and vegetables (Hunter and Fletcher, 2002).

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2.3.1 Phenolic compounds

The biologically active phenolic compounds containing one or more aromatic rings are found naturally in plant foods, which provide flavour, colour and texture. Phenolic compounds are primary antioxidants that are major contributors to the antioxidative activities of pennywort. Phenolic composition is also observed to be responsible in taste characteristics, including bitterness and astringency (Lea and Arnold, 1978). Pennywort leaves show the highest total polyphenol (0.23 µg/mg dried methanol extract) (Fezah *et al*., 2000). Zainol *et al*. (2003) also found total polyphenol in pennywort to be varied from 3.23 to 11.7 g/100 g (dry basis). These compounds can prevent hydroperoxide formation, stop the reactions and provide a longer shelf life for foods. They can react with peroxyl radicals and unsaturated lipid molecules and then converted them to more stable products. The mechanism for the reaction of phenolic compounds is associated with their ability to donate hydrogen atoms to free radicals.

Phenolic antioxidants, particularly flavonoids, are widely-occurring groups of secondary metabolites in plants. They function as primary antioxidants, chelators and superoxide anion scavengers (Rajalakshmi and Narasimhan, 1996). They also have much stronger antioxidant activities against peroxy radicals than vitamin E, ascorbic acid and glutathione (Cao *et al*., 1996). Quercetin was found as the main antioxidant in medicinal herbs (Haraguchi *et al*., 1992) and onions (Makris and Rossiter, 2001). It has been effective in inhibiting copper-catalyzed oxidation.

2.3.2 Ascorbic acid

Ascorbic acid (vitamin C or *L*-dehydroascorbic acid), secondary antioxidant, is an oxygen scavenger compound. It is present in high concentrations in citrus fruits, and well known as an antioxidant nutrient, which provides health protection due to its action against the presence of free radicals and consequently participates in the prevention of many degenerative diseases including coronary diseases and also cancers (Diplock, 1994). Kaack and Austed (1998) reported that ascorbic acid protects some flavonoids against oxidative degradation during juice processing and storage. Its present in processed foods is considered as an indicator for the product quality due to its relative instability to heat, oxygen and light (Biech *et al*., 1974). It is

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able to scavenge oxygen and prevent food oxidation, regenerate phenolic or fat-soluble antioxidants, maintain sulfhydryl groups and act synergistically with chelating agents (Madhavi *et al*., 1996). Ascorbic acid is usually added to food drinks with headspace of air. It increases the foods acidity and prevent the growth of aerobic bacteria, and also fortified as an antioxidant or nutrient supplement in many food products (Kormin, 2005). This compound is sensitive to various modes of degradation such as temperature, salt, sugar, pH, oxygen, enzymes and metal catalyst (Tannenbaum *et al.*, 1985). It can be degraded by active oxygen and reaction initiated by transition metals, and also easily destroyed through oxidation at high temperature. The amount of ascorbic acid decreases during processing and storage (Kormin, 2005).

2.3.3 Carotenoids

Carotenoids are preventive or synergistic activities which retard the rate of chain initiation and also reduce the rate of lipid autooxidation. These compounds may act as electron or hydrogen donors to primary antioxidant redicals. Carotenoids are secondary antioxidant (Lindley, 1999 cited by Kormin, 2005). Lycopene, major carotenoid and antioxidant, in tomato was lost by 35% when temperature was increased from 90 to 150˚C. Heat treatment below 100˚C caused small change or no effect on the lycopene degradation (Shi and Le Maguer, 1999). Takeoka *et al*. (2001) reported that lycopene concentration of tomato paste was decreased from 9 to 28% due to longer thermal processing time. However, caroteniods and β-carotene are very heat stable after prolonged thermal processing (Elkin, 1979; Miki and Akatsu, 1971).

2.4 Chlorophyll

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Chlorophyll is vital for photosynthesis, and is one of the most complex and abundant organic metal compounds of low molecular weight. The colour change in leaves from green to yellow or red is the most conspicuous in the loss of chlorophyll (Takamiya *et al.*, 2000). However, it is not chlorophyll degradation products that turn green leaves to yellow or red but mainly carotenoids and anthocyanins. Chlorophyll degradation occurs on cell death caused by external factors including injuries sustained by low or high temperature and pathogen attack (Hendry *et al*., 1987).

Rapid degradation of free chlorophyll or its coloured derivatives is necessary to avoid cell damage by their photodynamic action. Degradation intermediates have been identified, especially those concerning the cleavage of the tetrapyrrole macrocycle and of successive reactions (Matile *et al*., 1999; Hörtensteiner, 1999). However, the mechanism responsible for the degradation of chlorophyll in some reactions was unclear (Takamiya *et al.*, 2000).

Chlorophyll degradation

Takamiya *et al*. (2000) reviewed that the generally accepted pathway of chlorophyll degradation (**Figure 2.8**) comprises of 2 stages, before (early stage) and after (late stage) the cleavage of the tetrapyrrole macrocyclic ring. The products of the early stage are greenish, whereas those of the late stage are essentially colorless.

The early stage includes modification of the side chain of the tetrapyrrole macrocycle; hydrolysis of a phytol residue in ring IV (dephytylation), release of Mg^{2+} from the tetrapyrrole macrocycle by displacement with $2H^+$ (dechelation) and some modifications of the macrocycle that are probably specific for the plant species.

The chlase, enzyme catalyzing the dephytylation, chlorophyllase (chlorophyll–chlorophyllido hydrolase, chlase) reaction, is the first step in chlorophyll degradation and therefore the location of chlase is a factor in determining the site of chlorophyll degradation. There is more than one compartment for chlase localization, which implies the presence of multiple pathways for chlorophyll degradation. The chlase isozyme is located in plastids.

The late stage includes the cleavage of the tetrapyrrole macrocycle by an oxygenase and subsequent reactions, such as reduction to yield colorless fluorescent and further non fluorescent catabolites. The second stage is thus essential in the degreening of the chlorophyll molecule and therefore it determines chlorophyll degradation in leaf senescence and fruit ripening. In most cases of leaf senescence and fruit ripening, degradation intermediates do not accumulate to an appreciable extent, suggesting that there is a series of degradation reactions.

Late stage, this stage is composed of three steps: (1) oxygenolytic cleavage of the macrocyclic ring of tetrapyrrole (2) conversion of the cleavage product to colorless fluorescent compounds (3) conversion of the colourless fluorescent compounds to non fluorescent compounds.

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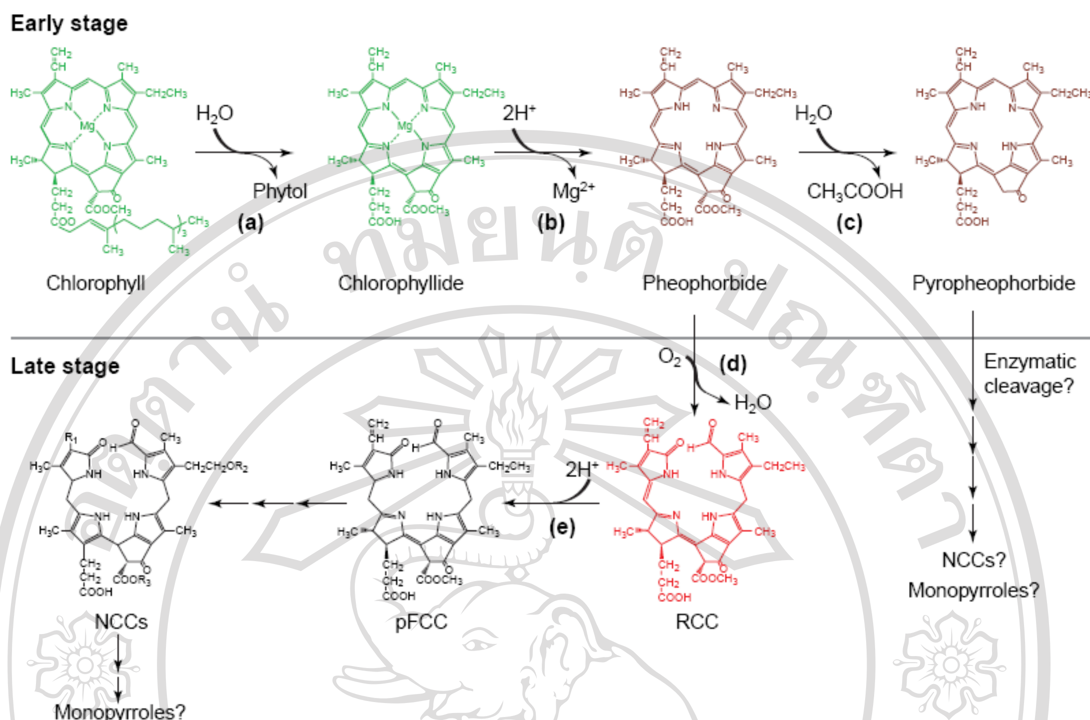

Figure 2.8 Chlorophyll degradation pathways in higher plants. The pathway is composed of two stages, an early stage before the cleavage reaction of the tetrapyrrole macrocyclic ring and a late stage that includes the cleavage reaction and steps after the reaction. The products in the early stage are green, whereas those in the late stage are colorless. (a) Chlorophyllase. (b) Magnesium dechelatase (c) Pheophorbidase (d) Pheophorbide *a* oxygenase. (e) Red chlorophyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite.

Source: Takamiya *et al*. (2000)

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Oxygenolytic cleavage of pheophorbide *a*

Pheophorbide *a* oxygenase (**Figures 2.8-2.9**) is considered to catalyse this reaction to yield 'red chlorophyll catabolite' in barley, rape and other plants (Hortensteiner *et al*., 1998). Only the formyl oxygen is derived from O_2 (Brandis *et al*., 1996), the lactam oxygen at C-4 being derived from water, indicating that this enzyme is a mono-oxygenase (Hortensteiner *et al*., 1998; Curty *et al*., 1995). The activity is found in the envelope membrane. The expression of oxygenase activity requires reduced ferredoxin and NADPH, both of which are supplied in gerontoplasts

(Hortensteiner *et al*., 1998). This activity can be detected in the senescent leaves of several plant species as well as in ripening fruits (Hortensteiner *et al*., 1995; Moser and Matile, 1997). Furthermore, pheophorbide *a* is the only substrate for the oxygenase, which is consistent with the exclusive occurrence of final degradation products of chlorophyll *a* (Hortensteiner *et al*., 1995).

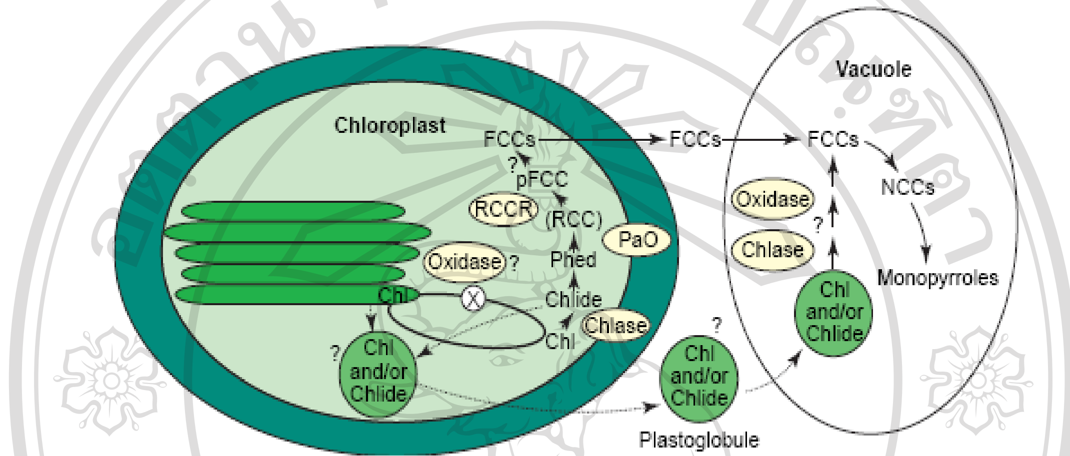

Figure 2.9 Hypothetical compartmentation of the chlorophyll (Chl) degradation pathway. Solid lines indicate the chlorophyll degradation pathway and broken lines indicate additional hypothetical pathways depending on plant species. Abbreviations: Chlase, chlorophyllase; Chlide, chlorophyllide; FCCs, fluorescent chlorophyll catabolites; NCCs, non-fluorescent chlorophyll catabolites; PaO, pheophorbide *a* oxygenase; pFCC, primary fluorescent chlorophyll catabolite; Phed, pheophorbide; RCC, red chlorophyll catabolite; RCCR, RCC reductase; X, a hypothetical chlorophyll carrier. Enzymes are represented by ellipses

Source: Takamiya *et al*. (2000)

Reduction of the red chlorophyll catabolite

Closely associated with the oxygenase reaction is a reduction of the δ-methine bridge of the red chlorophyll catabolite by a stromal enzyme to yield a colorless fluorescent product, primary fluorescent chlorophyll catabolite (**Figures 2.8-2.9**) (Rodoni *et al*., 1997). The primary fluorescent chlorophyll catabolite and all fluorescent chlorophyll catabolites have the same absorption spectrum. The reduction requires reduced ferredoxin and anaerobiosis. This means that the reactions of the

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oxygenase and the reduction of the red chlorophyll catabolite are closely coupled so that the anaerobic microenvironment is maintained for the reductase, whereas the oxygenase reaction requires molecular oxygen (Takamiya *et al*., 2000).

Chlorophyll *b* degradation

It has been suggested that chlorophyll *b* enters the degradation pathway after it is converted to chlorophyllide *b* by chlase. Chlorophyllide *b* is then converted to chlorophyllide *a* by 'chlorophyll *b* reductase'. This might be the reason for why there is no final degradation product of chlorophyll *b* in higher plants (Takamiya *et al*., 2000).

Oxidative chlorophyll bleaching

There are several reports on chlorophyll degradation of chloroplast lysate or leaf extracts induced by intrinsic (per)oxidation with phenolic compounds and hydrogen peroxide, and by lipoxygenation with linolenic acid ('oxidative chlorophyll bleaching') (Janave, 1997; Johnson-Flanagan and Spencer, 1996; Adachi *et al*., 1999). In some plants, the rise in peroxidase activity or oxidase activity paralleled the degreening of seeds or cotyledons (Johnson-Flanagan and Spencer, 1996; Adachi *et al*., 1999). However, only a few of the reaction intermediates formed by cleavage of the macrocycle ring have been firmly identified (Adachi *et al*., 1999).

Although these oxidative enzymes are certainly in the chloroplast, they are also present in the vacuole. Thus, oxidative (and/or oxygenolytic) cleavage of the macrocycle, whether it is catalyzed by a pheophorbide *a*-oxygenase-like enzyme and/or (per)oxidase, might occur outside the chloroplast. The cleavage reaction has never been examined using a fraction completely lacking the extracts derived from chloroplasts. However, the participation of POD in chlorophyll degradation *in vivo* has been argued in the case of cucumber (Abeles and Dunn, 1989).

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2.5 Effects of sugar contents on food quality

Sugar is normally added in processed foods to increase the product stability via lowering the water activity (a_w). Thus, sugar addition also increased the products concentration. Wrolstad *et al*. (1990) reported that a sugar concentration of more than 20% prevented the loss of anthocyanins, and the stability of anthocyanins increased

with the decreased water activity (a_w). Jackman and Smith (1996) also found that increasing amounts of antioxidant compounds are degraded at lower sugar levels. The loss of antioxidant properties in tomato, including lycopene content, is increased by 25-30% (Takeoka *et al*., 2001). The enzymatic and chemical oxidation rates of phenolic compounds are associated with some intrinsic food variables such as a_w and processing conditions (Nicoli *et al*., 1999).

2.6 Effects of pre-thermal processing on food quality

Most food processing procedures significantly reduced phenolic concentration. For example, quercetin in onions was reduced from 41 to 25 mg/100 g after blanching and to 22 mg/100 g after boiling for 3 min but it has been reported to be heat stable and its loss may occur before heat processing including peeling, chopping and trimming (Ewald *et al*., 1999). Chopping in asparagus significantly reduce rutin by 18.5% (Makris and Rossiter, 2001). Blanching treatment prevented enzymatic oxidation, which causes loss of natural components in raw materials (Nicoli *et al*., 1999). After blanching of peas (97˚C for 85 s) and blanching of spinach (97˚C for 90 s), the antioxidant activity level was reduced by 50 and 20%, respectively, but it remained constant and stable at frozen storage after blanching (Hunter and Flatcher, 2002).

2.7 Thermal processing for fruit and vegetable products

Fruit and vegetable products are often subjected to heat treatments for longer shelf life or preserve their quality and also prevent the microbial growth. The pasteurization processes often heat at slower heating up rates, using considerably higher temperatures. This method is based on time and temperature relationship (Moyer and Aitken, 1971). Most of the batch pasteurization of acidified beverages applied at below 93˚C in order to maintain the sensory quality and to reduce the nutrient loss. The pasteurization for highly acid juice ($pH<4.2$) would normally be processed at 71.1 to 100˚C (Pederson, 1980). Moreover, Chuah (1984) reported that pasteurization usually consists of heating food to 60-90˚C and acidic beverage products are heated at 60˚C for 10-20 min. Potter (1986) also reported that acid juices

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were pasteurized at 63˚C for 30 min or 77-82.2˚C for 20-30 min (Pederson *et al*., 1980).

The beverage quality depend on the degree of heat processing due to over processing may lead to undesirable changes in flavour, texture and nutritive value. However, under processing may not destroy the spoilage organisms, and is a potential health-hazard (Kormin, 2005). Flash pasteurization, high temperature short time treatment, is effectively retained the flavours and nutritional value of juice but the short holding time required a special equipment. Milk flash pasteurization at 71.1˚C for 15 s is equivalent in bacterial destruction to batch pasteurization at 62.8˚C for 30 min (Potter, 1986). Suitable heat processing can be obtained from a_w, pH and thermal conductivity of the product (Desrosier and Desrosier, 1977). Time and temperature relationship of pasteurization process requires the knowledge D and Z value for the destruction of the target organisms. The D value, a measure of heat resistance of microorganism, is the time in minutes at a given temperature required to destroy 1 log cycle (90%) of the target microorganism. The Z value reflects the temperature dependence of the reaction, is defined as the temperature change required changing the D value by a factor of 10 (Potter, 1986; Desrosier and Desrosier, 1977). However, time and temperature are chosen according to heat sensitivities of the foods and the effects on microorganism survival (Potter, 1986 cited by Kormin, 2005).

The heat resistance of the microorganisms and spores is affected by the factors including age of organisms or spores, composition of medium in which the organisms or spores are grown, pH and a_w of heating medium, heating temperature and initial concentration of organisms or spores (Chuah, 1984). The important factor determining the minimum thermal process is the pH of the product (Noraini, 1984 cited by Kormin, 2005). Desrosier and Desrosier (1977) reported the effect of pH on heating temperature and the time required to destroy the heat resistance of spores. Thermal processing for low acid foods is designed to inactivate the spore of *C. botulinum*. Low acid foods usually processed in steam under pressure at high temperature (116, 121 or 140˚C) (Noraini, 1984 cited by Kormin, 2005).

Beverage pasteurization processes depend on type of juice. For example, the pineapple juice applied temperature and time at 85-90˚C for 1-5 min (Che Rahani, 1998), orange juice was treated at 80˚C for 6 min (Scalzo, 2004), guava and carrot

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juice were recommended at 82˚C for 5 min (Bao and Chang, 1994) and mango juice was pasteurized at 87.8˚C for 1 min (Muhammed *et al*., 1965 cited by Kormin, 2005).

The sterilization of fruit and vegetable beverage is depended on the microbial level of raw materials, acidity of products, the size of can and the thermal conductivity of the product. For example, canned mango puree was heated in boiling water for 20 min (Godoy and Rodriguez-Amaya, 1987); canned guava juice was boiled for 30 min (Padula and Rodriguez-Amaya, 1987). The sterilization process causes losses of sensorial and nutritional qualities but it is still used, and it is optimized to maintain product quality.

Previously pasteurized and sterilized acid juices are hot filled at 78-93˚C for 1-3 min before cooling (Noraini, 1984). Pasteurized pineapple juice is transferred into cans at 90˚C and held for 1-3 min or the juice is pasteurized at 60˚C then transferred into cans and boiled (Mehrlich and Felton, 1971). Some juice is pasteurized at 80-87˚C for 1-10 min then filled and boiled for 10-30 min (Godoy and Rodriguez-Amaya, 1987; Padular and Rodriguez-Amaya, 1987; Che Rahani, 1998).

Effects of thermal processing on food qualities

1) Phytochemicals

Pasteurization is designed to achieve a target shelf life for the refrigerated product. Conventional thermal treatment of fruit juices has been widely and efficiently used, but the thermal process has a negative effect on the sensory and the nutritional characteristics of the juices (Arena *et al*., 2001; Manso *et al*., 2001; Yeom *et al*., 2000).

Some heated food often result in loss of nutritional quality and losses of their resistance against lipid oxidation. The phytochemical retention of products during processing and storage depends on the nature of raw materials such as their content and oxidative state (Anese and Nicoli, 2001).

Heat processing is the major cause of detectable changes on nutritional value and phytochemical contents (Pokorny, 2001). Most of the phytochemical amounts are reduced after thermal processing (Bender, 1987). However, the bioavailability and the human uptake are found to be higher in thermally processed food. Gartner, *et al.* (1997) reported the concentration of lycopene serum is higher in heat processed sample rather than fresh tomatoes after consuming. Hussein and EI-Tohamy (1990)

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suggested that cooking practices is able to increase bioavailability by physically disrupting or softening plant cell walls. The loss of phytochemicals depends on many parameters during food processing and storage. Heat, cold, light, oxygen, moisture, dryness, natural enzymes and microorganisms can affect the food quality (Potter, 1986). Low storage temperature, low oxygen contents and protection of the product from light in storage are also suggested to increase the compounds retention (Shi *et al*., 2002).

The pasteurizing of pineapple and asam jawa drink at 85- 90˚C for 1-5 min still maintained the sensorial quality of products (Che Rahani, 1998). The carrot juice heated at 82˚C for 5 min retained 57% of *α*-carotene (Bao and Chang, 1994). However, caroteniods content are very heat stable after prolonged thermal processing (Elkin, 1979).

Komin (2005) investigated the effect of heat processing at 65˚C for 15 min, 80˚C for 5 min, and pasteurization at 80˚C for 5 min followed by canning and boiling at 100˚C for 10 min on phytochemicals and compared to fresh drink pennywort. The results revealed that the fresh sample exhibited higher ($p \leq 0.5$) antioxidant activity than the heat treated samples. The FRAP values was 860 µmol/L for fresh sample and in the range of 404-740 µmol/L for heat-treated samples. The concentration of ascorbic acid and total polyphenol after heat treatment were 0.7-1.76 mg/100 ml and 730.27-903.23 mg/100 ml, respectively. Heat processing resulted in several fold decrease of total triterpene glycosides, the amount of fresh sample was 10.8-17.3% higher than heat-treated samples. However, the stock solution of asiaticoside was found to be stable under refrigeration, and remained at 99.2% after storage 90 days (Qi *et al*., 2000).

2) Antioxidants

Processing of food significantly changes the natural antioxidants due to most of these chemical constituents being unstable (Erdman, 1979; Hurt, 1979). The antioxidant activity during heat processing can be lost, remain stable or unchanged and even enhanced due to the presence of heat stable natural antioxidants, polyphenols and formation of new compounds having pro-oxidant and antioxidant properties (Nicoli *et al*., 1999). Several oxidation-reduction reactions occur during fruit and vegetable processing, in which an electron is removed from molecules and

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they are oxidized. This reaction leads to colour, flavour, odour, texture and nutritional changes during processing and storage of foods at high temperature. Antioxidant activity depends on many factors, including lipid composition, antioxidant concentration, temperature, oxygen, other antioxidants, and food components (Pokorny *et al*., 2001). The oxidation level is influenced by temperature, light, oxygen, physico-chemical changes and the presence of a catalyst (Frankel and Meyer, 2000). Heat and oxygen cause an acceleration of the chain initiation and propagation of the oxidation process and a decrease in the oxidation stability or the antioxidant activity (Yanishlieva-Maslarove, 2001).

The equivalent antioxidant activity of orange juice increased from 126.8 (fresh) to 135.2 mg/100 ml after pasteurization at 75˚C and decreased from 150.1 to 143.7 mg/100 ml after pasteurization at 95˚C for 30 s (Gil-Izquierdo *et al*., 2002). According to Scalzo (2004), thermal treatment of blood orange juice at 80˚C for 6 min reduced the inhibition of DPPH from 49.1 to 43.2%. Conversely, after pasteurization at 80˚C for 1 min, the concentration of anthocyanins of this juice was higher than that of a non-thermal treatment (Cabrita *et al*., 2000).

3) Nutrients

Oxidative and degradation of lipid and protein lead to the development of undesirable changes including off-flavour, rancidity, softening, loss of solubility and loss of nutritive value (Cheftel *et al*., 1985), and vitamins and minerals also degrade.

The effects of processing and storage on vitamins and minerals are well known. For example, ascorbic acid degradation in canned peas at 110-132˚C increased with temperature was used with the Arrhenius activation energy for the reaction of 41 kcal/mol (Lathop and Leung, 1980). The ascorbic acid level in food products depends on the raw material composition and processing method employed (Martin *et al*., 2002). Fresh apple contain ascorbic acid at 100 ppm but the vitamin is rapidly lost during juice processing (Lea, 1992). The loss of ascorbic acid in medicine plants dried at 50˚C for 9 hr (75.60%) was found to be higher than in freeze dried samples (21.13%) (Mahanom *et al*., 1999). *L*-Ascorbic acid in orange juice increased from 126.8 to 135.3 mg/100 ml after pasteurization at 75 ˚C but decreased from 150.1 to 143.7 mg/100 ml after pasteurization at 95˚C for 30 s (Gil-Izquierdo *et al*., 2002).

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Potassium, sodium, and phosphorus in spinach were reduced by 56, 43 and 36%, respectively after blanching (Bengtsson, 1969). Meiners *et al.* (1976) reported that processing of navy beans caused a decrease in the minerals including iron, zinc, magnesium and phosphorus in the range of 50-65%.

Carbohydrates can be lost by hydrolysis to an extent that depends on the conditions including pH, temperature and anomeric configuration of the material.

2.8 High pressure processing of foods

2.8.1 Evolution of high pressure processing

High pressure processing (HPP) has advanced further than the other alternative physical technologies applied to food. To some extent, this is because of the efficacy of HPP. If the applied pressure is high enough, all vegetative and spore forms of microorganisms can be inactivated. In addition, the engineering aspects of HPP have advanced to such an extent that commercially economic process has become viable within the last decade or so, at least for high-value products. As with so many new technologies, the original observations on which HPP was built a long time ago. The first reports that microorganisms could be inactivated by high pressure were made more than 100 years ago (Hite, 1899).

HPP has been developed as an alternative to conventional heat treatments for the preservation of foods. This technique offers several advantages such as the possibility of obtaining conserved products with characteristics very similar to those present before processing; homogeneity of treatment since the pressure is always the same at each point of the product; significant energy saving in comparison with thermal stabilization techniques (Rovere *et al*., 1996); products which are new from physical, functional and nutritional points of view (Balny, 1995; Tauscher, 1995); microbial reduction (Patterson *et al*., 1996), with better food quality parameters including nutrients, flavour and sensorial preservation (Sancho *et al*., 1999).

HPP could preserve nutritional value (Oey *et al*., 2007) and the delicate sensory properties of fruits and vegetables due to its limited effect on the covalent bonds of low molecular mass compounds including colour and flavour compounds (Oey *et al*., 2008). However, food is a complex system and the compounds

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responsible for sensory properties coexist with enzymes, metal ions, etc (Oey *et al*., 2008). During HPP at 100-1,000 MPa at -20 to 60˚C, various changes including (i) cell wall and membrane disruption (Michel and Autio, 2001; Prestamo and Arroyo, 1998; Van Buggenhout *et al*., 2005); (ii) enzyme catalysed conversion processes (Ludikhuyze *et al*., 2000; Verlent *et al*., 2004; 2005); (iii) chemical reactions (Oey *et al*., 2004a; 2004b; 2005; Nguyen *et al*., 2003; 2006; Oey *et al*., 2006) and (iv) modification of biopolymers including enzyme inactivation, protein denaturation and gel formation (Balny *et al*., 2002; Kunugi and Tanaka, 2002; Ludikhuyze *et al*., 2003; Randolph *et al*., 2002; Van der Plancken *et al*., 2005) can occur at the same time (Oey *et al*., 2008).

2.8.2 Mode of action of high pressure processing on microorganisms

HPP can be expected that the mode of action of pressure on whole organisms is not necessary the same, but depend on the pressure level. Elevated pressure between 30 and 50 MPa can influence gene expression and protein synthesis. High pressure can induce tetraploidy in *Saccharomyces cerevisiae*, indicating that HPP can interfere with replication of DNA. At pressure of approximately 100 MPa the nuclear membrane of yeasts is affected, and at more than 400-600 MPa further alteration occurs in the mitochondria and the cytoplasm. In particular, metal ions are released at pressure over 300 MPa. Pressure-inducible proteins have been found in *Methanococcus thermolyticus*, *Rhodothorula rubra* and *E. coli*, a pressure of 53 MPa can induce protein similar to those found at elevated temperature. Although it is not yet known whether pressure can indeed enhance resistance to physical treatment, cells subjected to stress other than temperature (e.g. by sub-lethal heat) become more resistant to pressure. The mechanism might be stabilization of the structures of membrane-bound enzymes. A perturbation of the bacterial membrane is almost always involved during pressure treatment (Smelt, 1998).

Cell membranes which are held together by hydrogen bonds and separate the intracellular constituents from their environment, are comprised of a twin layer of phospholipids consisting of hydrophobic moieties (fatty acids) and relatively hydrophilic moieties (glycerol), with a protein layer between the two phospholipids layers. Cell membranes, which play an important role in cell transport, permeability

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and respiration, are the first point of attack of high pressure, which breaks up phospholipids molecules, denatures proteins and alters permeability (Chong and Cossius, 1993). Fatty acids of barophilic microorganisms become more polyunsaturated with increase in growth pressure (De Long and Yayanos, 1985). A food spoilage organism, *Lactobacillus plantarum*, in the exponential phase is more resistant to pressure when the cells are grown at sub-optimum temperature (Smelt *et al*., 1994). Under these conditions, fatty acids are more unsaturated than in cells grown at optimum temperatures. When cholesterol is included, the fluidity of cell membranes of prokaryotes decreases and the cells become more sensitive to pressure. The protective effect of different carbohydrates on the membrane during freezing is in the order of glycerol < fructose < glucose < sucrose < trehalose and the same order was found for the protective effect of these carbohydrates against pressure (Smelt, 1998). Propidium iodide and ethidium bromide bind to nucleic acids, but they can only penetrate into the cell when the membrane is damaged. By contrast with untreated cells, pressurized bacteria can be stained with propidium iodide and ethidium bromide (Mackey *et al*., 1995; Smelt *et al*., 1994). Pressure-inactivation is also accompanied by an increase of extra cellular ATP, showing leakage of the membrane. Integral and peripheral membrane proteins become more detached from the plasma membrane when the membrane bilayers are sufficiently perturbed by pressure. HPP can also induce enzyme denaturation. There is an optimum temperature at which enzymes are most resistant to pressure (Hawley, 1978). As this is similar to that found for microorganisms and bacteriophages, there is some circumstantial evidence that some microbial enzymes constitute the main target of pressure inactivation (Smelt, 1998). Enzymes of extreme thermophiles are not only more heat resistant but also more pressure resistant than mesophilic microorganisms stabilized by pressure (Jaenicke, 1991). High pressure can presumably directly affect enzymes and carriers of transport systems. A decrease intracellular pH after pressure treatment has also been found.

The observations on membrane damage, protein denature, decrease intracellular pH and the observations on yeasts suggest that membrane-bound enzymes associated with efflux of protons may be at least one of the major targets in HPP inactivation. Elevated pressure can influence gene and protein expression in both

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0.1 MPa adapted and HPP adapted microorganisms. A further important effect of pressure on membranes would be on ion movements mediated by ATPase enzymes. *Streptococcus faecalis* could be adapted to grow at a pressure as high as 20 MPa. This stain had a regulatory defect and it produced large amounts of ammonia. Under pressure, non-adapted of *S. faecalis* is hypersensitive to acid and the ammonia acted to neutralize metabolic acids. DNA and RNA are very resistant to pressure. However, an extreme condensation of the nuclear material was observed in *Listeria monocytogenes* and *Salmonella typhimurium* (Smelt, 1998).

The structure of spores is much more complex than of vegetative cells. In addition to peptidoglycan, spores content, dipicolinic acid as well as calcium ions. This complexity affords spores higher resistance to heat, pressure, drying, radiation, acids and chemical disinfectants. The mode of action on bacterial spores is still a matter of speculation. Bacterial spores are killed directly by pressure higher than 1,000 MPa and more rapidly at low pH values. Taki *et al*. (1991) used a pressure of 600 MPa at 60˚C for 20-60 min to inactivate spores of *Bacillus licheniformis*, while pressure of 800 MPa at 50˚C for 20 min was used to reduce the number of spores of *B. cereus*, (Fonberg-Broczek *et al*., 1995). Spores of *C. sporogenes* were completely inactivated by pressure of 800 MPa at 80-90˚C or 1,500 MPa at 60˚C (Tawari *et al*., 1999). However, spores are sensitive to pressures between 50 and 300 MPa (Sale *et al*., 1970; Timson and Short, 1965; Wuytack *et al*., 1997). It is generally agreed that at such pressure, spores germinate, followed by death of the germinated spore. Sale *et al*. (1970) suggest a two step process to inactivate spores involving a low pressure-temperature regime to initiate germination and then a more severe regime to inactivate the germinated forms. This method could inactivate 4 x 10^5 endospores/ml of *B. cereus* at room temperature by treatment at 200 MPa for 1 min followed by treatment at 900 MPa for 1 min (Tawari *et al*., 1999). Wuytack *et al.* (1997) and Hölters *et al*. (1997) used pressures of 60-200 MPa to induce germination of dormant spores of *B. subtilis* and *B. stearothermophilus* and pressures greater than 300 MPa to inactivate the spores.

Under different pressures, holding times, solvents and concentrations, aids etc., the denaturation of protein and the destruction of membranes are different.

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2.8.3 Effects of high pressure processing on fruit and vegetable products

HPP has been proposed as an alternative method for the stabilization of freshly squeezed orange juice and the extension of its shelf life (Nienaber and Shellhammer, 2001; Parish, 1998a). Compared to conventional processes, HPP treatment has been found to be less detrimental to low molecular weight food compounds, due to the stability of covalent bonds to high pressure. Therefore, vitamins, pigments, flavouring agents and other compounds associated with sensory, nutritional and health related qualities of the product, are not greatly affected by HPP (Hoover, 1993; Knorr, 1993). The effect of HPP is variable, and dependent on HPP operating conditions and food composition (Hendrickx *et al*., 1998; Matser *et al*., 2004). The effect of HPP can vary depending on processing conditions including pressure, time, pH and temperature and form (whole, puree and juice). The food matrix can be altered by these variables consequently impacting on the effectiveness of HPP. The type of plant material (such as species) is also important but rather less is known about the impact of cultivar within species on food quality following HPP (Wolbang *et al*., 2008).

Oey *et al*. (2008) described in a review that HPP is a unique technology compared to other food processing technologies as pressure can result in enhancement or retardation of chemical and biochemical reactions as well as in both desired and undesired modification of biopolymers (including enzyme activation or inactivation and gel formation). Elucidation of the HPP effects on sensory properties of fruit-based and vegetable-based food products, including colour, flavour and texture, are not straightforward due to the presence of various enzymatic and chemical reactions both during processing and storage. HPP at moderate temperatures must be applied with precautions. At elevated temperatures, the effects of pressure-enhanced chemical reactions on sensory properties could give additional contributions to the effects of pressure-induced enzymatic reactions and enzymatic inactivation.

The preparation of fresh fruit products is of great interest for the food industry because of consumer's renewed interest in natural and healthy foods. Conventional thermal processing yields products that loose their typical attributes of freshness. In recent years, the use of optimized thermal treatments and the introduction of new ingredients have improved the organoleptic properties of conventional products. This has resulted in products that differ from fresh ones. The short shelf life and low safety

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level of fresh, unprocessed fruits are significant drawbacks. Crispiness, sharp cutting surfaces, color and taste are attributes that any fresh product must keep. HPP can help foods achieve these qualities. The high rate of microbial inactivation in acid products may correspond to microbial stability. On the other hand, the low enzyme inactivation limits the shelf life of plant products. Nevertheless, measures to control the residual enzyme activity (such as refrigerated distribution or suitable packaging materials) are in line with the high-quality standards required for fresh, minimally processed foods (Oey *et al*., 2008).

The remarkable increase in firmness of fruit tissue during storage is most likely due to the selective inactivation of pectic enzymes that cause the complexation of calcium ions with demethylated pectin chains. HPP enhances the rate of this long-term reaction as a consequence of the pressure-induced cell disruption and the loss of compartmentalization. However, some times the process may affect the taste and flavour of the product. These changes are the result of the biochemical oxidation of fatty acid traces, a mechanism that involves a sequence of enzymes that are not usually sensitive to pressure. Different ways of solving this problem have been suggested. The most sample measures are in agreement with good manufacturing practices: fast material flow on the processing time, minimal dead times, maximum deaeration and refrigeration of the raw material before processing. In fact, a low starting temperature and a fast treatment slow down or suppress the lipase activity before the concentration of precursors (free fatty acids) is sufficiently high to initiate the formation of off-flavours. Thus, with proper techniques, natural colour, taste and typical attributes can be retained in sensitive fruits such as kiwi, strawberries or raspberries and related products over a comparably long period of time. The proper combination of techniques allows food manufacturers to prepare high-quality conventional foods and to develop completely new products (Rovere, 2001).

Moreover, consumers are more likely to accept these products when the visual freshness is accompanied by a natural behavior. The fact that a fruit salad has an oxidative stability up to several hours after opening the package is important to confirm its natural characteristics and to limit the life of a product formulated without any added preservative. The use of ascorbic acid can help increase the oxidative

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stability of the product and reduce the technical hurdles to be overcome before packaging.

Fruit juices have been recognized as vehicles of food borne outbreaks since many year ago (Paris, 1998), but the inclement of consumption of non-pasteurized has caused a greater number of incidents associated with fruit juice (Cook *et al.*, 1998; Paris, 1998). Although *S. aureus* is not known to have caused outbreaks through consumption of fruit juice, it is able to resist acidic conditions and low pH in orange juice (Mothershaw and Jaffer, 2001). Some foods pasteurized by this process are reported to have better flavour, texture, nutritional retention and colour, compared to thermally processed foods. The products in both pre-packaged and post-aseptically packaged forms can be treated under elevated pressure up to 880 MPa (Shouqin *et al.*, 2004). The HPP is capable to preserve the nutritional substances in juices. Houlka *et al*. (2006) reported that the HPP (500 MPa for 10 min.) of broccoli juices was comparable in substance, sulforaphane content and antimutagenic activity with frozen products. It was found that freezing of broccoli before juicing decreased sulforaphane formation and the content of ascorbic acid depending on the holding time of the pressurization, but it is independent of pressure level. The effects of HPP on the qualities of fruit and vegetable products as follows;

1) The effects of HPP on colour

Oey *et al*. (2008) reviewed that HPP at low and moderate temperatures has a limited effect on pigments (including chlorophyll, carotenoids, anthocyanins, etc) responsible for the colour of fruits and vegetables. The colour compounds of HPP fruits and vegetables change during storage due to incomplete inactivation of enzymes and microorganisms, which can result in undesired chemical reactions (both enzymatic and non-enzymatic) in the food matrix.

Chlorophyll is a green compound found in the leaves and green stems of plants. Chlorophylls *a* and *b* have different stabilities towards pressure and temperature. At room temperature, chlorophylls *a* and *b* exhibit extreme pressure stability but at temperatures higher than 50˚C, HPP affects their stability for example; a significant reduction in the chlorophyll content of broccoli juice was found (Butz *et al*., 2002; Van Loey *et al*., 1998). The temperature dependency of the degradation rate constant of chlorophyll *a* is higher than that of chlorophyll *b*. At a constant pressure

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level, the values of the degradation rate constants of the chlorophyll pigments increase with increasing temperature (Van Loey *et al*., 1998) whereas at constant elevated temperatures, pressure increase accelerates the degradation of chlorophyll *a* and *b*. The pressure dependency of the degradation rate constant of chlorophyll *b* at 70˚C is higher than that of chlorophyll *a*. For example, elevating pressure from 200-800 MPa accelerates the degradation of chlorophyll *a* and *b* of broccoli by 19.4% and 68.4%, respectively (Van Loey *et al*., 1998). Matser *et al*. (2004) also reported that chlorophyll degradation of green beans and spinach due to HPP occurred at elevated temperatures, even for a short exposure time (two pulses of 700 MPa for 1 min at 90˚C).

HPP at ambient and moderate temperatures results in limited colour changes of green vegetables. In many cases, the green colour of vegetables becomes even more intense (decrease in *L**, *a** and *b** values) for example the colour of green beans intensified after HPP of 500 MPa for 1 min at ambient temperature (Krebbers *et al*., 2002a). This might be caused by cell disruption during HPP resulting in the leakage of chlorophyll in to the intercellular space yielding a more intense bright green colour on the vegetable surface. However, at elevated temperatures, the green colour shifted visibly to olive-green with a concomitant increase in the *a** value for example, green beans after HPP at elevated temperature (two pulses of 1,000 MPa for 80 s at 75˚C) (Krebbers *et al*., 2002a) or basils after HPP of 860 MPa for 80 s at 75˚C or 700 MPa for 80 s at 85˚C (Krebbers *et al*., 2002b).

Carotenoids are important for the orange yellow and red appearance of fruits and vegetables. Carotenoids are rather pressure stable. HPP increases the extraction yields of carotenes from the plant matrix (De Ancos *et al*., 2000; Fernandez Garcıa *et al*., 2001a, 2001b; Tauscher, 1998). Pressure-induced isomerization of all-*trans* lycopene in hexane was observed during HPP at 500 and 600 MPa (room temperature for 12 min). This phenomenon was not observed in food matrices such as in tomato puree (Qiu *et al*., 2006). The colour of tomato puree remained unchanged after HPP up to 700 MPa for 1 hr at 65˚C (Rodrigo *et al*., 2007a).

Anthocyanins are water-soluble vacuolar flavonoid pigments responsible for the red to blue colour of fruits and vegetables. Anthocyanins are stable during HPP at moderate temperatures, for example, pelargonidin-3-glucoside and pelargonidin-3-

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rutinoside in red raspberry (*Rubus idaeus*) and strawberry (*Fragaria xananassa*) during HPP at 800 MPa (for 15 min at 18-22˚C) (Garcia-Palazon *et al*., 2004). Anthocyanins in pressure-treated vegetables and fruits are not stable during storage. A shelf life study (for 7 days at 5, 20 and 30˚C) of pressurized (200, 400, 600 and 800 MPa for 15 min at 20-22.5˚C) blackcurrants (*Ribes nigrum*) during a week-long storage at selected temperatures showed that cyanidin-3-rutinoside and delphinidin-3-rutinoside had different stabilities. Anthocyanins in pressurized blackcurrants remained unchanged during storage at 4˚C (Kouniaki *et al*., 2004).

There are various hypotheses on the degradation mechanism of anthocyanins in pressurized fruits during storage. The first hypothesis of anthocyanin degradation is a reaction caused by incomplete enzyme inactivation. A link between enzyme inactivation (β-glucosidase, POD and polyphenoloxidase (PPO)) and anthocyanin stability has been found in several fruits (Garcia-Palazon *et al*., 2004; Suthanthangjai *et al*., 2005; Zabetakis *et al*., 2000a). For example, PPO inactivation was linked to the stability of pelargonidin-3-glucoside and pelargonidin-3-rutinoside in pressurized (800 MPa for 15 min at 18-22˚C) red raspberry and strawberry. Since HPP at 800 MPa (for 15 min at 18-22˚C) caused complete PPO inactivation, the stability of these two pelargonidins was maintained during storage (Garcia-Palazon *et al*., 2004). This finding is also supported by Suthanthangjai *et al*. (2005). Besides β-glucosidase, POD and PPO also play important roles in anthocyanin degradation during storage. Suthanthangjai *et al*. (2005) showed that cyanidin-3-glucoside and cyanidin-3-sophorosides (the major pigments in raspberry) had the highest stability during 9 days of storage at 4˚C after pressurization at 200 or 800 MPa (for 15 min at 18-22˚C) compared with HPP at 400 or 600 MPa. A high loss of both pigments after HPP at 400 and 600 MPa is probably due to a lower degree of inactivation of β-glucosidase, POD and PPO.

The second hypothesis of anthocyanin degradation deals with the substrate specificity of β-glucosidase acting on anthocyanins (Gimenez *et al*., 2001; Zabetakis *et al*., 2000a). Zabetakis *et al*. (2000a) found different levels of anthocyanin (such as pelargonidin-3-glucoside and pelargonidin-3-rutinoside) losses in strawberry after HPP of 200, 400, 600 and 800 MPa (for 15 min at 18-22˚C) and a higher residual activity of β-glucosidase after HPP at 400 MPa than at 200, 600 and 800 MPa. They

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also found a higher loss of pelargonidin-3-glucoside, compared to pelargonidin-3-rutinoside at the same level of residual enzyme activity probably because *β*-glucosidase, an enzyme naturally present in many plants, has higher substrate specificity for pelargonidin-3-glucoside than for pelargonidin-3-rutinoside. A similar finding was also observed in strawberry jam (Gimenez *et al*., 2001).

The third hypothesis concerns the effect of ascorbic acid on the stability of anthocyanins. Ascorbic acid apart from being an antioxidant also tends to accelerate the degradation of anthocyanins (Kouniaki *et al*., 2004). Anthocyanin losses can be reduced by storing HPP treated products at low temperature.

Besides the instability of colour pigments, browning plays an important role in the discoloration of HPP food products. In fruit-based food products, no visual colour differences (based on *L**, *a** and *b** values) are observed immediately after HPP, for example in white grape juice after HPP at 400 MPa (at 2˚C), 500 MPa (at 2˚C) and 400 MPa (at 40˚C) for 10 min (Daoudi *et al*., 2002) or in mango pulps after HPP at 100-400 MPa for 15 or 30 min at 20˚C (Ahmed *et al*., 2005). Ahmed *et al*. (2005) observed that colour parameters such as (*a**/*b**), Chroma and hue angle values of mango pulps remained constant after HPP indicating pigment stability, while increasing pressure intensity decreased the value of ΔE. Oey *et al*. (2008) reported that the structure and pigmentation of food interact with each other to affect both colour and translucency or opacity. Texture modification may result in changes in the nature and extent of internally scattered light and the distribution of surface reflectance, which in turn may produce, changes in colour appearance rather than the changes in pigment concentration (Mac Dougall, 2002). Colour changes in HPP treated fruits and vegetables can be related to changes in textural properties. This phenomenon was observed in tomato based products. HPP (400 MPa for 15 min at 25˚C) resulted in an increase in the *L** value of tomato puree indicating a lightening of the puree surface colour. The CIELAB parameters were significantly higher both in the untreated and in the HPP treated tomato puree compared to the thermally treated purees (Sanchez-Moreno *et al*., 2006). The reason could be the formation of a jelly-like translucent structure of tomato puree as observed by Verlent *et al*. (2006) when pressures were below 400 MPa.

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2) The effects of HPP on texture

Oey *et al*. (2008) reviewed the origin of texture changes in fruits and vegetables, and reported that they can be related to transformations in cell wall polymers due to enzymatic and non-enzymatic reactions (Sila *et al*., 2007). Due to cell disruption, HPP facilitates the occurrence of enzymatic and non-enzymatic reactions. Substrates, ions and enzymes which are located in different compartments in the cells can be liberated and interact with each other during HPP. At the same time, pressure can enhance the action of pectinmethylesterase (PME), lower the polygalacturonase (PG) activity (occurring mostly at moderate temperature), and avoid β-elimination, a reaction where loss of two substituents from adjacent atoms including carbon, nitrogen and oxygen results in the formation of new unsaturated bonds, which can possibly occur at elevated temperatures. Pectinases, including orange PME (Van den Broeck *et al*., 2000), strawberry, carrot and banana PME (Ly Nguyen *et al*., 2002, 2003a, 2003b), tomato PG (Fachin *et al*., 2003), pepper PME (Castro *et al*., 2006) and plum PME (Nunes *et al*., 2006) showed differences in their pressure and temperature stability. As a consequence, different pressure and temperature combinations can be used to activate or inactivate some specific pectinases during processing to create textures, which cannot be formed by thermal processing. Moreover, the use of HPP can be combined with pre-treatments such as infusion of exogenous pectinases (Duvetter *et al*., 2005) and/or soaking in calcium chloride solutions (Sila *et al*., 2004; 2006), which can result in increased firmness of the processed fruits and vegetables.

Oey *et al*. (2008) also reported that HPP can disturb the cell permeability of fruits and vegetables, which enables movement of water and metabolites in the cell. The degree of cell disruption is not only dependent on the applied pressure level but also on the type of plant cell. Disruption of cell integrity was observed in HPP (400 MPa for 30 min at 5˚C) vegetables including spinach and cauliflower by microscopic examination (Prestamo and Arroyo, 1998). HPP affects the organization of the parenchyma cells. The plant cells disintegrate and the intercellular spaces are no longer filled with gas. After HPP, cavity formation occurs and a firm texture and a soaked appearance are noticed after HPP.

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2.1) Hardness and firmness

In studies of HPP effects on texture, the solidity of fruits and vegetables is importance, and hardness and firmness are mostly used as quality parameters. Basak and Ramaswamy (1998) studied the effect of HPP (100-400 MPa for 5-60 min at room temperature) on the firmness of different fruits and vegetables including apple, pear, orange, pineapple, carrot, celery, green pepper and red pepper. They found a rapid loss of firmness during compression. During the pressure holding period (for 30-60 min), the firmness either decreased further or recovered gradually, which was observed for pear, orange, pineapple, carrot, celery, green pepper and red pepper treated at 100 and 200 MPa. PME activity was suggested to be the major reason for the observed increase in firmness. Upon HPP, PME is liberated and contacts its substrate, the highly methylated pectin, leading to demethylation. The deesterified pectin (low methoxy-pectin) is capable of forming a gel-network with divalent ions resulting in increased hardness. After HPP was carried out at elevated temperatures, pronounced texture preservation was found in contrast to thermal treatment at atmospheric pressure, as shown for HPP (two pulses of 1,000 MPa for 80 s at 75˚C) of green beans (Krebbers *et al*., 2002a) and HPP (600 MPa for 90 min at 80˚C) of carrot disks (De Roeck *et al*., 2007). Both the action of pectinases (for example, PME) and reduced chemical reactions (for example, β-elimination) probably contribute to texture preservation during HPP at elevated temperatures. However, the exact mechanisms behind texture preservation at elevated temperature and pressure are not known (Oey *et al*., 2008).

2.2) Softening

Fruits and vegetables, including apple, pear, orange, pineapple, carrot, celery, green pepper and red pepper, were experienced softening at pressures more than 200 MPa (for 5-60 min at room temperature) (Basak and Ramaswamy, 1998). At HPP 100 MPa, pear was the most pressure sensitive fruit followed by apple, pineapple and orange, respectively. While at 200 MPa, apple was more sensitive than pear. Softening under pressure was also observed for cherry tomatoes (Tangwongchai *et al*., 2000). HPP at 200-400 MPa (for 20 min at 20˚C) resulted in increased texture damage while pressures greater than 400 MPa (500 and 600 MPa for 20 min at 20˚C) led to less apparent damage. The softening of cherry tomatoes HPP at 200-400 MPa

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may be a result of simultaneous activity of PME and PG, since PG is able to depolymerize pectin that has been demethylated by PME.

2.3) Viscosity

Oey *et al*. (2008) reported that HPP can affect the rheological properties of food products including crushed fruits and vegetables, puree, pulp and juice. The observed effects are dependent on the conditions of the HPP and the type of fruit and vegetable. Ahmed *et al*. (2005) reported that the viscosity of mango pulp increased after HPP at 100 and 200 MPa (for 15 and 30 min at 20˚C), while a reduction in viscosity was observed after HPP at 300 and 400 MPa (for 15 and 30 min at 20˚C). The viscosity of tomato homogenate decreased considerably at pressures lower than 400 MPa but increased at higher pressure levels, such as 500 MPa combined with temperatures up to 60˚C (Plaza *et al*., 2003; Sanchez-Moreno *et al*., 2006; Verlent *et al*., 2006). However, in the presence of NaCl (0.8%), the effect of pressure was the opposite, the viscosity increased with increasing pressure up to 400 MPa (Plaza *et al*., 2003). For some fruit juices, cloud stability is an important quality aspect. Polydera *et al*. (2005) studied the effects of HPP treatment on the shelf life of navel orange juice and showed that (i) pressure treatment (600 MPa for 4 min at 40˚C) resulted in a higher viscosity than thermal treatment (for 60 s at 80˚C) and (ii) a limited cloud loss and a small decrease in the viscosity of HPP juice were observed during storage (for 64 days at 0, 5, 10, 15 and 30˚C) even at an elevated storage temperature (30˚C). It is suggested that residual PME activity is responsible for the quality loss of orange juice during storage.

3) The effects of HPP on flavour

Flavour is the sensory impression of a food that is determined mainly by the chemical senses of taste and smell. The human tongue can distinguish only 5 distinct qualities of taste, of which sourness; sweetness and bitterness are the most important ones regarding the flavour of fruits and vegetables. On the other hand, the human nose can distinguish effects of a vast number of volatile compounds, even in minute quantities. Any changes in the compounds responsible for the sourness, sweetness, bitterness or odour of fruits and vegetables may result in changes in their flavour (Oey *et al*., 2008).

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The fresh flavour of fruits and vegetables is not altered by HPP since the structure of low molecular weight flavour compounds is not directly affected by HPP (Oey *et al*., 2008). This has been observed by chemical and sensory analysis of strawberry puree (Lambert *et al*., 1999), mandarin juice (Takahashi *et al*., 1993), orange-lemon-carrot juice (Fernandez Garcia *et al*., 2001a), white grape juice (Daoudi *et al*., 2002) and guava juice (Yen and Lin, 1999), which have been treated at pressures of 200-600 MPa combined with ambient temperature. As HPP can enhance and retard enzymatic and chemical reactions, it could indirectly alter the content of some flavour compounds and disturb the whole balance of flavour composition in fruits and vegetables. As a consequence, HPP could result in undesired changes in flavour (Oey *et al*., 2008).

Hexanal is a volatile compound associated with the smell of foliage and grass. Gas chromatographic (GC) studies showed changes in the hexanal content of fruits and vegetables as a result of HPP (Oey *et al*., 2008). Navarro *et al*. (2002) observed that HPP at 400 MPa (for 20 min at ambient temperature) more than doubled the hexanal content of strawberry puree. On the other hand, Lambert *et al*. (1999) observed less pronounced effects of pressure on the hexanal content of strawberry puree but pressurization at 800 MPa (for 20 min at ambient temperature) resulted in a slight decrease in the hexanal content. For HPP (300 MPa for 30 min at 25˚C) treated onions, an increase of 40% in the concentration of hexanal and the generation of a braised or fried odour probably due to increased contents of propyl *trans*-propenyl disulfide and 3,4-dimethylthiopene have been found (Butz *et al*., 1994). Porretta *et al*. (1995) reported that HPP at 500, 700 and 900 MPa for 3, 6 and 9 min at room temperature of fresh tomato juice resulted in the generation of such a strong rancid taste, that the juice was unsuitable for sensory analysis. *n*-Hexanal was suggested to be responsible for the rancid taste, because the *n*-hexanal content in all pressure-treated tomato juices was much higher (6.4 mg/kg) than in the fresh juice (0.3 mg/kg). At concentrations lower than about 1.2 mg/kg, *n*-hexanal contributes to the typical fresh flavour of tomatoes. Higher concentrations impart a rancid flavour. The increased concentration of *n*-hexanal was considered to be a result of HPP-induced oxidation of free fatty acids, including linoleic and linolenic acids. Lipoxygenase and hydroperoxide lyase, which are naturally present in tomato, are

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partly responsible for the development of the rancid taste as they catalyze the oxidation of polyunsaturated fatty acids. Lipoxygenase and hydroxyperoxide lyase endogenously present in tomato juice have different pressure stabilities at 20˚C. When HPP is applied at pressures lower than 500 MPa at 20˚C, tomato hydroperoxide lyase is more labile than tomato lipoxygenase, while their stabilities are reversed at pressure 500 MPa (Rodrigo *et al*., 2007b). In diced tomatoes, lipoxygenase activity was reduced by almost 50% as a result of HPP treatment at 400 MPa (for 1-5 min at 25 and 45˚C) and it was very low after treatment at 800 MPa (for 1 min at 25 and 45˚C) (Shook *et al*., 2001).

HPP at 800 MPa (for 20 min at 20˚C) modified the flavour profile of strawberry puree (Lambert *et al*., 1999). Some new compounds were formed, such as *γ*-lactone, which correlates with the flavour of peach. The concentration of many volatile compounds contributing to fresh strawberry flavour, including nerolidol, furaneol, linalool and some ester compounds was significantly lower in strawberry puree processed at 800 MPa (for 20 min at 20˚C) than in the fresh puree. After cold storage (1 day, 4˚C), the concentrations of acids including butanoic acid, 2-methyl-butanoic acid and hexanoic acid, and the ketone compound 2,4,6-heptanetrione of HP treated (200, 400, 600 and 800 MPa for 15 min at 18-22˚C) strawberries were lower than in the untreated strawberries (Zabetakis *et al*., 2000b). The best acid retention was observed for strawberries HPP treated at 400 MPa. The concentration of the alcohol 1,6,10-dodecatrien-3-ol increased in strawberries treated at 800 MPa. The level of 2,5-dimethyl-4-hydroxy-2H-furan-3-one (DMHF), one of the most important flavour compounds in fresh strawberry, was not much altered after HPP and during storage.

Ester compounds are amongst the most important flavour compounds in strawberries but the stability of ester compounds during HPP is still under discussion (Oey *et al*., 2008). Lambadarios and Zabetakis (2002) observed only a small decrease in ester concentration when model systems containing fruit esters in buffer solution were subjected to HPP (400 and 800 MPa for 15 min at 18-22˚C) at various pH values (pH 4, 6 and 8). Lambert *et al*. (1999) also reported the presence of many esters in HPP (200, 500 and 800 MPa for 20 min at 20˚C) strawberry puree. On the other hand, Zabetakis *et al*. (2000b) found no ester compounds in HPP (200, 400,

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600 and 800 MPa for 15 min at 18-22˚C) treated strawberries. It is possible that the ester compounds in the study of Zabetakis *et al*. (2000b) were lost during sample extraction (Oey *et al.*, 2008).

Gimenez *et al*. (2001) reported that strawberry jam HPP (400 and 800 MPa for 5 min at 22˚C) smelled more chemical, rancid and less fruity than traditionally processed jam. However, none of the flavour compounds generated by sterilization (for 20 min at 120˚C) was found in HPP treated (200-800 MPa for 20 min at ambient temperature) strawberry puree (Lambert *et al*., 1999). The findings of better flavour retention of HPP (600 MPa for 5 min at ambient temperature) strawberry puree in comparison with heat-treated (for 5 min at 80˚C) puree has been supported by a study where an electronic nose detector was used to analyze the volatiles of the treated purees. HPP strawberry puree differed from heat treated and fresh strawberry puree. Cross validation of the electronic nose data showed that heat treatment changed volatile compounds more than HPP. Corresponding results were reported for similarly processed raspberry and black currant purees (Dalmadi *et al*., 2007).

According to sensory evaluation, the flavour of HPP (500 MPa for 90 s or 5 min, 700 MPa for 60 s and 800 MPa for 5 min at room temperature) orange juice was not as fresh as the flavour of untreated orange juice (Fernandez Garcia *et al*., 2001a; 2001b; Parish, 1998). Fernandez Garcia *et al*. (2001a) reported that the carrot aroma was more intense in HPP (800 MPa for 5 min at room temperature) than in fresh orange-lemon-carrot juice. The taste of HPP orange juice was judged better than that of traditional pasteurized juice (Parish, 1998; Polydera *et al*., 2003; 2005) and the typical off-flavour of heat treated mandarin juice was not detected in HPP treated (400 MPa for 10 min at ambient temperature) juice (Takahashi *et al*., 1993). Baxter *et al*. (2005) observed no differences in the concentration of volatile flavour compounds between freshly frozen, heat treated (85˚C for 25 s) or HPP treated (600 MPa for 60 s at 18-20˚C) orange juice. The results of the chemical analysis were supported by the results of a trained sensory panel and a consumer panel, which did not notice any differences in odour or flavour between the differently treated orange juices.

Oey *et al*. (2008) reported that HPP at room temperature does not necessarily result in inactivation of pressure resistant bacterial spores and enzymes,

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which may spoil the HPP treated product during storage. Therefore, cold storage is needed to preserve the high quality of the treated product. Navarro *et al*. (2002) reported that for HPP treated (400 MPa for 20 min at ambient temperature) strawberry puree, stored for 30 days at 4˚C, increases in the contents of methyl butyrate, mesifurane, 2-methyl-butyric acid, hexanoic acid, ethyl butyrate, ethyl hexanoate, 1-hexanol and linalool were observed. During storage, the content of 1-hexanol in HPP treated strawberry puree increased probably due to residual lipoxygenase activity. In this case, POD was not considered responsible for flavour changes during storage, as the activity of POD was very low after HPP (Oey *et al*., 2008). Daoudi *et al.* (2002) showed that the sweetness and acidity of HPP (500 MPa for 10 min at 2˚C) treated grape juice were maintained for 60 days during storage at 4˚C but the fresh fruit and grass aroma were slightly reduced during storage (Daoudi *et al*., 2002). Similar results were observed for HPP treated guava juices. The volatile flavour compounds in HPP treated (600 MPa for 15 min at 25˚C) guava juice remained stable during 30 days of storage at 4˚C, but changes in the concentrations of volatiles were observed after 60 days of storage. The concentrations of methanol and ethanol increased and the concentrations of many ester and aldehyde compounds decreased probably due to residual enzyme activity (Yen and Lin, 1999).

The flavour of HPP treated basil (two pulses of 860 MPa at 75˚C and two pulses of 700 MPa at 85˚C) was more intense than the aroma of heat sterilized, frozen or dried basil. The contents of methylchavicol and linalool, two essential oils important for the characteristic fresh basil flavour, were not changed by HPP. In conventionally heat sterilized basil the concentrations of methylchavicol and linalool were reduced by more than 80%. After 2 months storage at 20˚C, the characteristic basil aroma was still observed in HPP treated basil (Krebbers *et al*., 2002b).

A significant concentration of important flavour compounds accumulates in many fruits as non-volatile and flavourless glycoconjugates, which are known as glycosidic aroma precursors. These glycosides can be hydrolyzed to volatile aglycones by the action of *β*-glucosidases (Pogorzelski and Wilkowska, 2007). Linalool, nerol, geraniol and citronellol are some examples of glycosidically-bound aroma compounds typically present in fruits (Pogorzelski and Wilkowska, 2007). HPP may have potential in releasing flavour compounds from plant-based foods since the

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activity of for example strawberry β-glucosidase is enhanced after HPP at 200 and 400 MPa (at 18-22˚C for 15 min) (Zabetakis *et al*., 2000b). The increased activity of β-glucosidase did not result in decreased levels of 2,5-dimethyl-4- hydroxy-2H-furan-3-one-glucoside (DMHF-glucoside), which is a precursor of 2,5-dimethyl-4-hydroxy-2H-furan-3-one (DMHF), one of the most important volatile flavours in fresh strawberries. The increased levels of benzaldehyde in HPP treated (400 MPa for 10 min at 20˚C) peaches was attributed to β-glucosidase activity (Sumitani *et al*., 1994). In this context, it may be possible to boost the release of bound aroma compounds by adding commercial enzyme preparations containing β-glucosidase activity to fruit products. For example, Gueguen *et al*. (1996) reported a 705% increase in volatile flavour compounds in strawberry juice treated with *Candida molischiana* β-glucosidase. The enzyme treatment resulted in an increase in the concentrations of linalool, benzyl alcohol and 2-phenylethanol. It would be interesting to find out whether the release of flavour compounds could be further enhanced by performing the β-glucosidase treatment under pressure. However, this approach must be applied with caution for anthocyanin-containing fruits since β-glucosidase can also hydrolyze glucosylated anthocyanins leading to colour loss (Oey *et al*., 2008).

Oey *et al*. (2008) reported in their review that HPP is a promising preservation method for fruits and vegetables, even though the original fresh sensory properties are not always fully retained. The sensory properties of many HPP fruit and vegetable products are still superior to those of products preserved in the traditional way by heat treatment. Regarding flavour, it is difficult to evaluate how HPP induced changes in volatile compounds affect the overall flavour of fruits and vegetables. In strawberries, for example, more than 350 volatile compounds have been identified (Zabetakis and Holden, 1997). A complex mixture of furanone, esters, aldehydes, alcohols and sulphur compounds are considered responsible for the strawberry flavour. Some compounds have a greater impact on overall flavour than others due to differences in the odour thresholds of the compounds. Due to interactions between individual flavour compounds, even a small change in the concentration of one compound may have major effects on the overall flavour. Therefore it is obvious that sensory analysis is needed in addition to pure chemical and mechanical analysis to

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gain a better understanding of the HPP effects on the overall sensory properties of plant-based foods (Oey *et al*., 2008).

2.9 Comparison of high pressure processing, pasteurization and sterilization on qualities of juices during long-term storage

Oey *et al*. (2008) reported that during HPP, different pressure and temperature combinations can be used to achieve desired effects on texture, colour and flavour of foods. The quality of HPP fruits and vegetables can change during storage due to coexisting chemical reactions (including oxidation) and biochemical reactions when endogenous enzymes or microorganisms are incompletely inactivated. In a safe food the spoilage and pathogen microorganisms are eliminated by a pasteurizing technique. If harmful spores are present in the product, their elimination requires normally a sterilization treatment to obtain a product with long and steady shelf life (Parton *et al*., 2006). Heat treatment is an efficient and economical process for microbial inactivation in perishable liquid foods, but it can not be used to treat heat-labile compounds. (Wuytack and Michels, 2002; Vachon *et al*., 2002). In recent years, considerable research efforts have been directed towards the development of new non-thermal processes for food preservation, such as the use of high hydrostatic pressure, pulsed electric field, ultraviolet light, ionizing radiation, pulsed light and more recently, ultra-high pressure homogenization (UHPH) which is also called dynamic high pressure (Gervilla *et al*., 2000).

Pehrsson (1996) described experiments on HPP of microbial stable citrus juice, where they processed juices for 60-90 s at refrigeration or freezing temperature. The product was stored and distributed under refrigeration. The pressure-treated juice was stable for 6 months at 4˚C without loosing any freshness (as compared to juices that were thermally treated at 98˚C for 10 s). Kloczko and Radomski (1996) studied preservation of fresh fruits, vegetables, fruit-vegetable juices by subjecting them to pressures and subsequently storing them at 6˚C. They reported that HPP had no beneficial effect on keeping quality of fruits and vegetables, whereas immediately after pressurization, and after 55 days of refrigerated storage, pressure-treated juices had better aroma, flavour and microbiological quality than untreated controls, and

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ascorbic acid content remained the same or declined slightly. Donsi *et al.* (1996) studied the high pressure stabilities of orange juice by evaluating microbial activity and the chemical composition of orange juice treated at different pressure levels for various operating times. They obtained a 2 months shelf life for pressure-treated orange juice (at 350 MPa for 1 min at 30˚C) stored under refrigeration. Butz *et al.* (1997) studied the effects of HPP on antimutagenic activities of fruits and vegetables juice. The antimutagenic activity was compared with raw and heated samples (100˚C or 50˚C for 10 min). They reported that antimutagenicity of strawberry and grapefruit juices was not affected by heat and pressure. Also, vegetable juices exhibited moderate to strong antimutagenicity, whereas, the anti-mutagenic activity of carrot, leek, spinach, kohlrabi and cauliflower juices was sensitive to heat treatment but remained unaffected by pressure treatment. In addition, Triska *et al*. (2007) studied the influence of preservation technology (HPP, freezing and pasteurization) on total isothiocyanates content in vegetable juices. It was found that the total isothiocyanates content was lowest in white cabbage and the highest in Brussels sprouts. With exception, broccoli juice is decreased with pasteurization and freezing is the most gentle preservation method for these substances.

During storage, the green colour of vegetables treated by HPP at room temperature turned into a pale yellow colour (decrease in *a** value) probably due to chemical reactions including oxidation. By comparison, vegetables pressurized at elevated temperatures, which results in inactivation of some enzymes, showed no further colour change during storage. The colour of pressure-treated green beans and basil was still acceptable after storage times of 1 and 2 months, respectively (Krebbers *et al*., 2002a; 2002b).

Discoloration of pressurized food products was reported during storage (3˚C) due to enzymatic browning. Guerrero-Beltran *et al*. (2005) observed enzymatic browning in HPP (379-586 MPa for 0.033, 5, 10, 15 or 20 min at room temperature) mango puree. Additions of ascorbic acid and cysteine inhibited the PPO activity resulting in less browning. This inhibition was enhanced by HPP. Polydera *et al*. (2003) found discoloration (based on *L**, *a** and *b** values) of HPP (500 MPa for 5 min at 35˚C) reconstituted orange juice during storage (at 0, 10 and 15˚C for 120 days) and the degradation trend was not significantly different between pressure and

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thermally treated juices. Similar results were observed by Polydera *et al*. (2005) in pressurized (600 MPa for 4 min at 40˚C) navel orange juice. The colour change (based on *L**, *a** and *b** values) had a linear correlation with the ascorbic acid loss during storage (0, 5, 10, 15 and 30˚C for 64 days) but it was not dependent on the type of processing (comparison between temperature pasteurization and HPP). An increase in storage temperature resulted in higher rates of browning of orange juice. The activation energy for colour degradation of HPP juice due to browning was higher than that of temperature pasteurized juice.

A shelf life study on navel orange juice (Polydera *et al*., 2005) showed that HPP 600 MPa for 4 min at 40˚C resulted in a higher viscosity than thermal treatment at 80˚C for 60 s. A limited cloud loss and a small decrease in the viscosity of HPP juice were observed during storage for 64 days at 0, 5, 10, 15 and 30˚C even at an elevated storage temperature (30˚C). It is suggested that residual PME activity is responsible for the quality loss of orange juice during storage.

2.10 Flavour profile by solid-phase micro-extraction technique

Solid-phase micro-extraction (SPME) can have a significant impact on the analysis of raw materials and finished products. SPME will not provide a complete chemical profile for every sample, but as a rapid isolation technique it can alert the chemist to the types of compounds occurring in the sample during the time it takes to prepare an extract using a more typical isolation scheme (Harmon, 1997). Head space-SPME is a technique based on the chemical equilibrium among three phases in the system (sample matrix, head space and fibre coating). Although the aqueous matrix and the coating of the fibre compete to trap analytes, the affinity of the fibre for each analyte is crucial to the study of volatile components by SPME (Zhang *et al*., 1994).

2.10.1 Flavour profile of fresh foods

The natural chemicals comprising the aroma of fresh fruits are usually complex mixtures of alcohols, aldehydes, esters and terpenoids that may transform markedly during the ripening cycle. These chemicals are generally recovered from the fruit pulp or their juices by vacuum or steam distillation before separation and

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analysis using capillary GC or GC-MS. Such isolation techniques require relatively large amounts of fruit, sometimes on the order of several kilograms, to obtain a suitable analytical sample, which then is diluted and contaminated with the organic solvents used during the isolation. Additionally, traditional isolation methods require from 4-24 hr before the identification can begin. Purge- and trap-techniques will reduce both the amount of sample required and the time needed to prepare suitable isolate, but the equipment is expensive and requires time to establish the operating interface to GC or GC-MS systems. Head space SPME can be utilized with small samples of fruit, the extract can be prepared in a few minutes with little sample preparation, and it is readily transported to any number of GC systems (Harmon, 1997).

Fresh fruits have been sampled by simply removing 3 or 4 small cores of fruit pulp using the blunt end of a disposable Pasteur pipette and depositing the pieces into a 15 ml head space vial. After fitting the vial with a Teflon-lined seal, the fruit is immediately extracted using headspace SPME at room temperature for 10 min before injection and analysis by GC-MS (Harmon, 1997).

Cantaloupe is an orange, delicately flavoured fruit of *Cucumis melo* L., which becomes progressively stronger in flavour and aroma with increasing ripeness. It has been reported that isobutyl acetate, butyl acetate, and ethyl butyrate are present in cantaloupe at a level of 0.1 ppm and that hexyl acetate is present at 0.04 ppm (Maarse and Visscher, 1992). Obviously these amounts will vary from sample to sample, but these levels are indicative of the concentrations in the melon, and the headspace SPME technique is able to detect very low levels of non-polar volatile chemicals.

A ripe banana (*Musa sapientum* L.) was also examined in the same manner. After transferring about 2 g of banana cores to a vial, the headspace SPME extract provided the GC-MS chromatogram. This chromatogram is considerably more complex than a typical banana flavour, which usually is highly concentrated in isoamyl acetate. Quantitative values have been reported (Maarse and Visscher, 1992) for several of the compounds from banana extracts, among them isoamyl alcohol (2-12 ppm), isobutyl acetate (47 ppm), isoamyl acetate (12-75 ppm), isoamyl isobutyrate (0.7 ppm), isoamyl butyrate (6 ppm), isoamyl caproate (0.07 ppm), eugenol (1.2 ppm) and elemicin (7.5 ppm). The relative peak areas shown in a chromatogram suggest a

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different quantitative profile, but this might have been affected by differences in the ripeness of the sample and the relative extraction efficiency of the SPME fibre. 3-Hexenyl caproate was found in the SPME extract but was not listed among the banana flavour compounds in the reference (Harmon, 1997). Bartlett pear aroma contains very low levels of butyl acetate (0.16 ppm), hexyl acetate (0.09 ppm), methyl *trans*-2-*cis*-4-decadienoate (0.5 ppm), ethyl *trans* -2-*cis*-4-decadienoate (0.04 ppm) and *α*-farnesene (0.04 ppm) (Maarse and Visscher, 1992).

SPME can be a powerful tool for the rapid isolation of volatile chemicals from fresh fruit and fruit juice products. It should be possible to establish a method to determine the degree of ripeness of different fruits by matching the chemical profiles with sensory or other established parameters. The changes occurring during ripening can be observed within a few minutes, and the analysis can be repeated using the same piece of fruit if shallow core samples are taken and proper storage conditions are maintained between analyses. A different treatment of the samples using buffers, salt solution, homogenization, different SPME fibre types, separation with chiral capillary columns, etc. might provide a completely different insight into the chemical profile and biochemistry of these plants (Harmon, 1997).

2.10.2 Flavour profile of juices

Different analytical methods have been developed to determine the concentration of flavour components in fruit juices. SPME is a technique widely applied in food chemistry research. The direct liquid immersion SPME of a sample of fruit juice beverage was comparable or higher in sensitivity to a conventional solvent extraction using dichloromethane for most of the recovered flavour chemicals. Although the sensitivity of headspace SPME was not compared in the same study, it would have provided a similar result (Yang and Peppard, 1994).

Dimick and Hoskin (1983) published a comprehensive review of apple flavour. Almost 200 compounds have been listed in 22 references as flavour components of apple juices (Nijssen *et al*., 1996). It is known that processing (such as pasteurization and filtration) can influence the flavour of apple juice (Su and Wiley, 1998).

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Pfannhauser *et al*. (1987) reported the changes in light-exposed orange lemonades in which *α*-pinene, *β*-pinene, mycrene, linalool and *γ*-terpinene reduced in concentration and *α*-terpineol, carvone and *trans*-carveol increased. The component *α*-terpineol is formed by oxidative degradation of limonene and is well known for its contribution to the off flavour of orange juice when its concentration exceeds 2 mg/kg (Tatum *et al*., 1975). However, Jordan *et al.* (2001) indicated that a decrease in the pulp content from 10-15 to 3% of insoluble solid content, results in a reduction in the concentration of *α*-terpineol and aldehydes. Presumably, reducing the pulp content would be beneficial in maintaining the quality of the flavour and aroma profile of stored orange juice. Ahmed *et al*. (1978) showed that limonene has an important role in the deterioration of orange juice aroma when it is present in concentrations greater than 190 ppm. Qiao *et al*. (2008) determined the aroma composition and aroma active compounds of Jinchen sweet orange juice and peel oil. Totals of 49 and 32 compounds were identified in juice and peel oil. Limonene, followed by linalool, terpinen-4-ol, *β*-myrcene, *α*-terpineol, octanal and *γ*-terpinene were the components found in greatest proportions in orange juice but limonene was the one found in greatest concentrations, representing 90.85% of all volatiles in peel orange oil. Hashizume (2007) studied the light-induced off-flavour in apple juice from concentrates stored in glass bottles under fluorescent light. A strong metallic off-flavour was formed by photooxidation. A major contributor to the off-flavour was identified as 1-octen-3-one. In addition, pentanal, 2-methyl-1-penten-3-one, hexanal, (*E*)-2-heptanal, 6-methyl-5-hepten-2-one and (*E*)-2-octanal were increased significantly after light-exposure and could contribute to the off-flavour.

Umekawa (2000) also found metallic off-flavours present in grapefruit juices stored in a polyethylene terephthalate (PET) bottle and detected 1-octen-3-one, 3-methoxy-1-butanol, anis-aldehyde and *α*-cardinal as volatile components.

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2.10.3 Flavour profile of processed foods

For many spices and herbs, aroma and flavour will vary depending upon the country of origin, processing conditions, the age of the sample, the type of packaging, the ratio of essential oil volatiles to heat-producing principles, and many other factors. Black pepper is one of the most widely consumed spices in the world. It might be of

benefit to be able to evaluate the chemical composition of the volatile oil of single peppercorns to correlate with sensory attributes. A chromatogram was reported as the result of a 5 min room temperature head space SPME of a single black peppercorn (42 mg) that had been crushed with pliers and rapidly transferred to a 4 ml vial. The composition was somewhat atypical of a normal chromatogram obtained by steam distillation, but the general appearance was readily identified. Considering that the whole sample could have provided no more than 1.5 mg of volatile oil, the sensitivity of the extraction is remarkable (Harmon, 1997).

Most herbs have their own characteristic smell due to the presence of volatile compounds. More than 65 different volatiles have been reported to be present in the volatiles of *Eurycoma longifolia* extracts (Guo *et al*., 2004) but a freeze dried extract contained a maximum of 83 volatiles whereas a spray dried sample contained a maximum of 28 volatile compounds (Shafiqul Islam *et al*., 2006).

Since the spice was known to contain a unique aroma chemical, it was an easy matter to transfer a small amount of the curry to a vial, perform a headspace extraction, and determine whether the spice had been added. The complete analysis required less than one hour from the time the sample was received (Harmon, 1997).

2.10.4 Flavour profile of pennywort

Chou (2005) identified 19 compounds in the essential oil from pennywort in Taiwan as linalool, *p*-meth-1-en-8-ol, copaene, elemene, sesquiphellandrene, caryophyllene, thjosene, cadinene, acoradien, humulene, alloaromadendrene, farnesene, selinene, 1,5,5,8-tetramethyl-1,2-oxabicyclo [9,1,0] dodeca-3,7-diene, eudesmene, cuparene, caryophyllene oxide, phytol and hexahydrofarnesylacetone. Ali (2008) reported 23 compounds in this plant from Malaysia including *α*-pinene (1.2%), germacrene *D* (1.6%), (+)-cyclosativene (2.3%), *β*-farnesene (4.8%), *β*-cubebene (17%), *α*-caryophyllene (17%), *α*-humulene (22%), *γ*-murolene (22%) and various components with concentrations less than 1% including *β*-pinene, *m*-cymene, *D*-limonene, *β*-*trans*-ocimene, *γ*-terpinene, 1-bromoallene, *β*-linalool, *trans*-3-nonen-2-one, terpinen-4-ol, *o*-menth-8-ene, 4-isopropylidene-1-vinyl, *α*-cubebene, *n*-decyl acetate, *β*-cedrene, *δ*-cadinene and caryophyllene oxide. In addition, Oyedeji and Afolayan (2005) also reported that the essential oil extracted from pennywort grown

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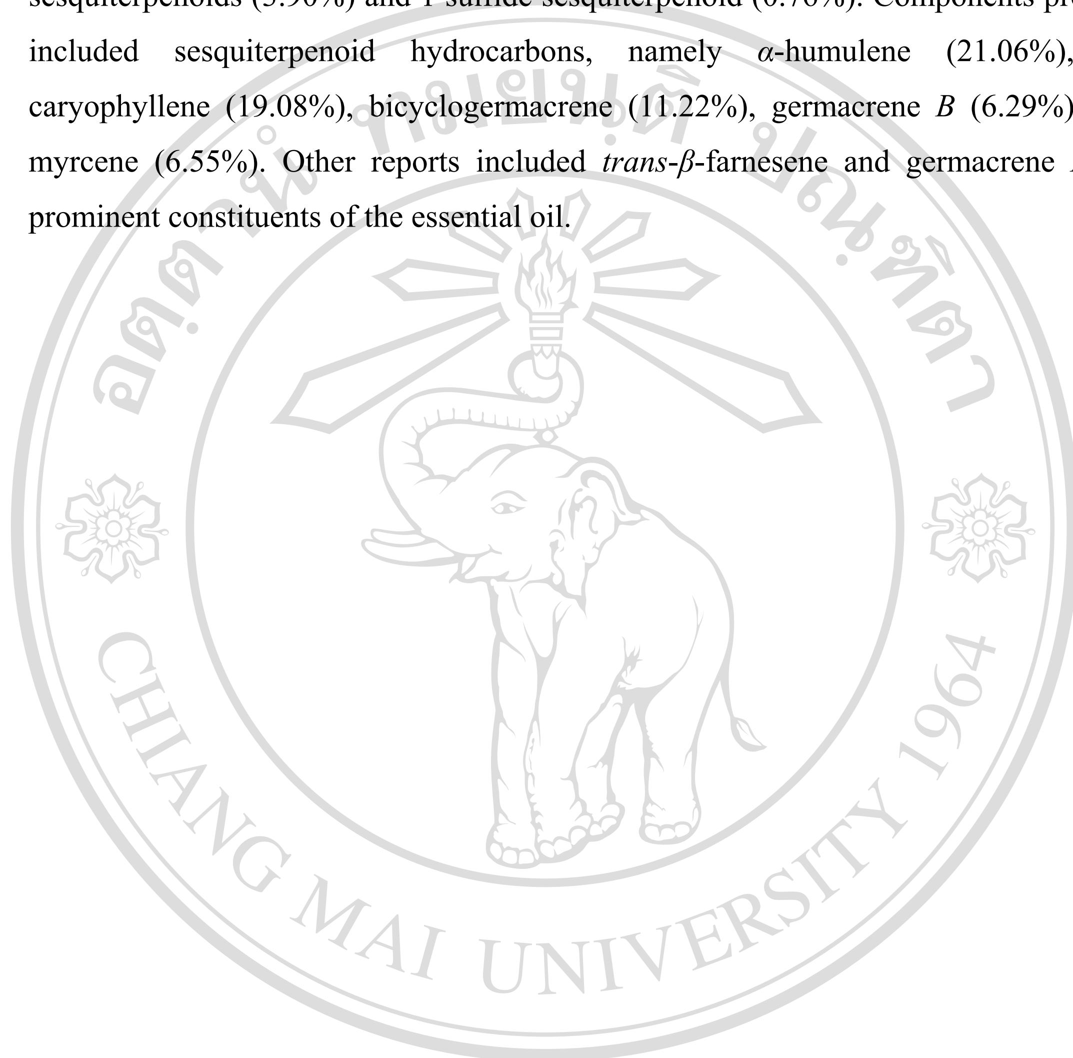
in South Africa contained 11 monoterpenoid hydrocarbons (20.20%), 9 oxygenated monoterpenoids (5.46%), 14 sesquiterpenoid hydrocarbons (68.89%), 5 oxygenated sesquiterpenoids (3.90%) and 1 sulfide sesquiterpenoid (0.76%). Components present included sesquiterpenoid hydrocarbons, namely *α*-humulene (21.06%), *β*-caryophyllene (19.08%), bicyclogermacrene (11.22%), germacrene *B* (6.29%) and myrcene (6.55%). Other reports included *trans*-*β*-farnesene and germacrene *D* as prominent constituents of the essential oil.



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