CHAPTER 1

INTRODUCTION

The consumption of foods from vegetable origin has been associated with reducing risk of a range of chronic diseases [1, 2]. Owing to their antioxidative properties, the components such as phenolic compounds may play a role in the aetiology of chronic disease through oxidative damage to body cells and molecules. As several types of seaweeds have been consumed by people with a belief that seaweeds can reduce risk of chronic diseases, it is worthwhile to investigate for phenolic compounds exhibiting antioxidative properties in seaweeds.

1.1 Phenolic Compounds

Phenolic compounds are important components of many fruits, vegetables and beverages to which they contribute to flavor, color and sensory properties such as astringency. There are a wide range of compounds that possess an aromatic ring bearing a hydroxyl substituent, including their functional derivatives such as esters, methyl esters and glycosides [3].

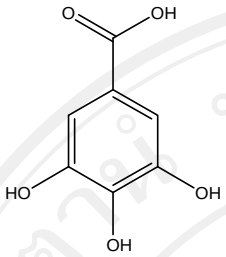
Plants and foods contain a large variety of phenolic derivatives, including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans and lignins. These rather varied substances are essential for the growth and reproduction of plants and also act as antifeedants and antipathogents [4]. The contribution of phenolics to the pigmentation of plant foods is also well recognized. In addition, phenolics have been established to possess powerful antibiotic, antioxidant and anticarcinogenic properties.

Many properties of plant products are associated with the presence, type and content of their phenolic compounds. The astringency of foods, the beneficial health effects of certain phenolics or their potential antinutritional properties when present in large quantities are significant to producers and consumers of foods.

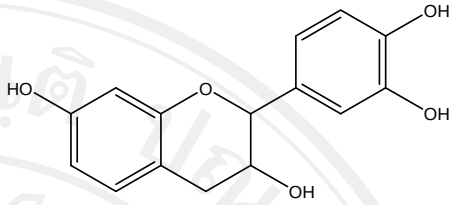
A large number of biological activities have been associated with phenolic compounds, including antiproliferative, antioxidant and antiviral activities. Also, there is epidemiological evidence of an inverse correlation between phenolic compounds intake and mortality from coronary heart disease and stroke.

Gallic acid, catechin, epicetechin, caffeic acid, rutin and quercetin as shown in **Fig. 1.1** are common dietary phenolic compounds and are of particularly interest as antioxidant.

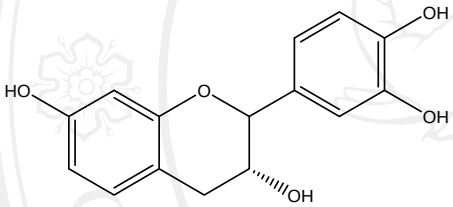
The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Crude extracts of fruits, herb, vegetables, cereals, nuts and other plant materials rich in phenolics are increasingly of interest in the food industry. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer has also raised an interest in this matter. This promted Sang *et al*. to investigate the chemical composition of almond skins and isolated five flavonol glycosides, catechin, protocatechuic acid, vanillic acid, and *p*-hydroxybenzoic acid [5].



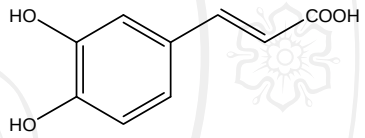
Gallic acid (MW = 170.12)



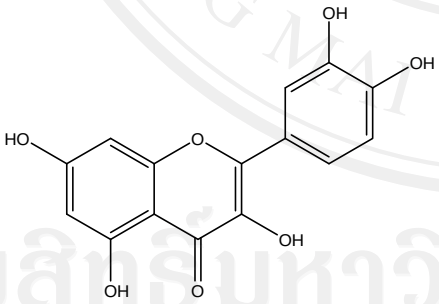
Catechin (MW = 290.27)



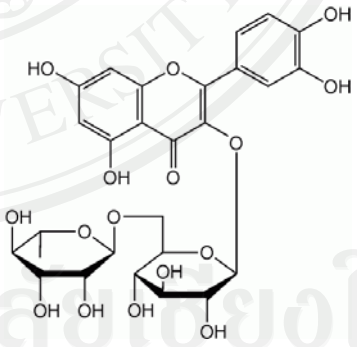
Epicatechin (MW = 290.27)



Caffeic acid (MW = 180.16)



Quercetin (MW = 302.24)



Rutin (MW = 610.53)

Fig. 1.1 Chemical structures of common phenolic compounds [6]

Phenolic compounds are considered as nonnutrient biologically active compounds. The functionality of these compounds is expressed through their action as an inhibitor or an activator for a large variety of mammalian enzyme systems, and as metal chelators and scavenger of free oxygen radicals. Oxygen free radicals are involved in many pathological conditions such as atherosclerosis, cancer and chronic inflammation. Phenolics interfere with the pathways that regulate cell division and proliferation, platelet aggregation, detoxification, inflammatory and immune response. Among these phenolic substance, flavonoids and in particular anthocyanins, are of interest because of their high occurrence in foods, especially in fruits, vegetables and green leafy vegetables including green tea.

There have been many reports that phenolic compounds in tea [7, 8], wine [9, 10], cacao [11], fruits and vegetables [12] had beneficial effects for our health, especially as an antioxidant. However, there was a few information on the presence of phenolic compounds in various seaweeds.

1.2 Seaweeds [13, 14]

Seaweeds are algae that live in the sea or in brackish water. Scientists often call them "benthic marine algae", which just means "attached algae that live in the sea". Seaweeds come in three basic colors, as shown in **Fig.1.2**: red, green and brown: dulse is the red seaweed; sea lettuce is amongst the green algae; and the brown is a young wrack. Red and brown algae are almost exclusively marine, whilst green algae are also common in freshwaters and in terrestrial situations. Many of these algae are very ancient organisms and although lumped together as "algae" are not really closely related, having representatives in 4 of the 5 kingdoms of organisms. There are about 10,500 species of seaweeds, of which 6,500 alone are red algae (Rhodophyta).

Brown seaweeds

Green seaweeds

Red seaweeds

Fig 1.2 Basic colours of seaweeds

1.2.1 Classification

Seaweeds belong to a rather ill-defined assemblage of plants known as the algae. The term ‘seaweed’ itself does not have any taxonomic value, but it is rather a popular term used to describe the common large attached (benthic) marine algae found in the groups Chlorophyceae, Rhodophyceae, Phaeophyceae or green, red and brown algae, respectively.

1.2.1.1 Phaeophyceae: Brown Algae

The brown colour of these algae results from the dominance of the xanthophyll pigment fucoxanthin, which masks the other pigments, Chlorophyll a and c (no Chlorophyll b), beta-carotene and other xanthophylls. Food reserves are typically complex polysaccharides, sugars and higher alcohols. The principal carbohydrate reserve is laminaran and true starch is absent (compare with the green algae). The walls are made of cellulose and alginic acid, a long-chained heteropolysaccharide.

There are no known unicellular or colonial representatives; the simplest plant form is a branched, filamentous thallus. The kelps are the largest (up to 70 m long) and perhaps the most complex brown algae, and they are the only algae known to have internal tissue differentiation into conducting tissue; there is, however, no true xylem tissue as found in the 'higher' plants.

Most brown algae have an alternation of haploid and diploid generations. The haploid thalli form isogamous, anisogamous or oogamous gametes and the diploid thalli form zoospores, generally by meiosis. The haploid (gametangial) and diploid (sporangial) thalli may be similar (isomorphic) or different (heteromorphic) in appearance, or the gametangial generation may be extremely

reduced (Fucales). The brown Giant Kelp *Macrocystis pyrifera* is harvested off the coasts of California for feeding abalone. It used to be used for alginate extraction, but this now mostly comes from Atlantic *Ascophyllum nodosum* and *Laminaria hyperborea*. Alginates, derivatives of alginic acids, are used commercially for toothpaste, soap, ice cream, tinned meats, fabric printing and a host of other applications. It forms a stable viscous gel in water and its primary function in the above applications is as a binder, stabilizer, emulsifier, or moulding agent. *Saccharina japonica*, formerly *Laminaria*, and other species of the genus are grown on ropes in China, Korea and Japan for food and alginate production. *Undaria pinnatifida* is also cultivated in Japan, Korea and China for production of Wakame, a valuable food kelp. Small amounts are also grown in Atlantic France for the European market.

About 16,000 tonnes of *Ascophyllum nodosum* (*Feamainn bhuí* in Irish, referring to the yellow colour in summer) are harvested each year in Ireland, dried and milled in factories at Arramara Teo., Cill Chiaráin (Kilkerrin), Co. Galway; and some 3,000 tonnes of the resulting seaweed meal is exported and processed in Scotland for the production of alginic acid. *Laminaria hyperborea* stipes (sea rods) are harvested in Norway and used to be collected in drift in Scotland and Ireland. The rods are used for the manufacture of high-grade alginates. Other brown algae are used for the extraction of agricultural sprays ('liquid seaweed extracts'). These extracts are used at low concentrations on crops and their hormone-like activities are thought to be due to betaines, cytokinenins, etc.

1.2.1.2 Chlorophyta: Green Algae

Green colour from chlorophyll a and b in the same proportions as the 'higher' plants; beta-carotene (a yellow pigment); and various characteristic xanthophylls (yellowish or brownish pigments). Food reserves starch, some fats or oils like higher plants. Some green algae are to be the progenitors of the higher green plants but there is currently some debate on this point.

Green algae may be unicellular (one cell), multicellular (many cells), colonial (living as a loose aggregation of cells) or coenocytic (composed of one large cell without cross-walls; the cell may be uninucleate or multinucleate). They have membrane-bound chloroplasts and nuclei. Most green are aquatic and are found commonly in freshwater (mainly Charophytes) and marine habitats (mainly chlorophytes); some are terrestrial, growing on soil, trees, or rocks. Some are symbiotic with fungi giving lichens. Others are symbiotic with animals, e.g. the freshwater coelentrate *Hydra* has a symbiotic species of *Chlorella* as does *Paramecium bursaria*, a protozoan. *Chlorella* is now grown and sold as a health supplement and *Dunaliella* is grown as a source of beta-carotene. A number of freshwater green algae (charophytes, desmids and *Spirogyra*) are now included in the Charophyta (charophytes), a phylum of mostly freshwater and terrestrial algae, which are more closely related to the higher plants than the marine green algae belonging to the Chlorophyta (known as chlorophytes).

Asexual reproduction may be by fission (splitting), fragmentation or by zoospores (motile spores). Sexual reproduction is very common and may be isogamous (gametes both motile and same size); anisogamous (both motile and different sizes - female bigger) or oogamous (female non-motile and egg-like; male

motile). There have an alternation of haploid and diploid phases. The haploid phases form gametangia (sexual reproductive organs) and the diploid phases form zoospores by reduction division (meiosis). Some do not have an alternation of generations, meiosis occurring in the zygote. There are about 8,000 species of green algae, about 1000 of which are marine chlorophytes and the remainder freshwater charophytes. Unfortunately, just because algae are green it no longer means that they are closely related: two major aggregation of green algae, the Chlorophyta and the Charophyta have turned out not be closely related.

1.2.1.3 Rhodophyta: Red algae

The red colour of these algae results from the pigments phycoerythrin and phycocyanin; this masks the other pigments, Chlorophyll a (no Chlorophyll b), beta-carotene and a number of unique xanthophylls. The main reserves are typically floridean starch, and floridoside; true starch like that of higher plants and green algae is absent. The walls are made of cellulose and agars and carrageenans, both long-chained polysaccharide in widespread commercial use. There are some unicellular representatives of diverse origin; more complex thalli are built up of filaments.

A very important group of red algae is the coralline algae, which secrete calcium carbonate onto the surface of their cells. Some of these corallines are articulated with flexible erect branches; others are crustose. These corallines have been used in bone-replacement therapies. Coralline algae were used in ancient times as vermifuges, thus the binomial *Corallina officinalis*.

Several red algae are eaten: best known amongst these is dulse (*Palmaria palmata*) and Carrageen Moss (*Chondrus crispus* and *Mastocarpus stellatus*).

However, Nori, popularised by the Japanese is the single most valubable marine crop grown by aquaculture with a value in excess of US$1 billion.

The red algae *Kappaphycusand Betaphycus* are now the most important sources of carrageenan, a commonly used ingredient in food, particuarly yoghurts, chocolate milk and repared puddings. *Gracilaria, Gelidium, Pterocladia* and other red algae are used in the manufacture of the all-important agar, used widely as a growth medium for microorganisms and for biotechnological applications.

There are about 6000 described species of red algae, the vast majority of which are marine. These are found in the intertidal and in the subtidal to depths of up to 40, or occasionally, 250 m. The main biomass of red algae worldwide is provided by the Corallinaceae and Gigartinaceae.

1.2.2 Seaweed uses and utilization

Seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertiliser. The major utilisation of these plants as food is in Asia, particularly Japan, Korea and China, where seaweed cultivation has become a major industry. In most western countries, food and animal consumption is restricted and there has not been any major pressure to develop seaweed cultivation techniques. This present and potential uses of seaweeds. Industrial utilisation is at present largely confined to extraction for phycocolloids and, to a much lesser extent,

certain fine biochemicals. Fermentation and pyrolysis have not been carried out on an industrial scale at present but are possible options for the 21st century.

The present uses of seaweeds at present are as human foods, cosmetics, fertilisers, and for the extraction of industrial gums and chemicals. They have the potential to be used as a source of long- and short-chain chemicals with medicinal and industrial uses. Marine algae may also be used as energy-collectors and potentially useful substances may be extracted by fermentation and pyrolysis.

1.2.3 Nutritional value

The principal components of the edible algae are carbohydrates (sugars or vegetablegums), small quantitiet of protein and fat, ash, which is largely composed of sodium and potassium [14] and 80-90% of water. **Table 1.1** gives the composition of selected representative seaweeds, some of which are currently used for food or have been used as food in the past.

In regard to the amount of protein, the convention is to convert the total nitrogen to protein by multiplying by 6.25. This should be treated with some caution as, for example, the amount of free nitrate will affect the total nirtogen level. Free nitrates are found in varying amounts in red and brown algae.

Table 1.1 Nutritional values of Irish Sea-vegetables and Irish Sea-vegetable aquaculture [13].

Nutritional value (g/ 100g dry matter)	*Ascophyllum nodosum* (Brown)	*Laminaria digitata* (Brown)	*Alaria esculenta* (Brown)	*Palmaria palmata* (Red)	*Porphyra yezoensis* (Red)	*Ulva* species (Green)
Water*	70-85	73-90	73-86	79-88	n.a.	78
Ash	15-25	73-90	73-86	15-30	7.8	13-22
Total carbohydrates	-	-	-	-	44.4	42-46
Alginic acid	15-30	20-45	21-42	0	0	0
Xylans	0	0	0	29-45	0	0
Laminaran	0-10	0-18	0-34	0	0	0
Mannitol	5-10	4-16	4-13	0	0	0
Fucoidan	4-10	2-4	n.a.	0	0	0
Floridoside	0	0	0	2-20	n.a.	0
Protein	5-10	8-15	9-18	8-25	43.6	15-25
Fat	2-7	1-2	1-2	0.3-0.8	2.1	0.6-0.7
Tannins	2-10	1	0.5-6.0	n.a.	n.a.	n.a.
Potassium	2-3	1.3-3.8	n.a.	7-9	2.4	0.7
Sodium	3-4	0.9-2.2	n.a.	2.0-2.5	0.6	3.3
Magnesium	0.5-0.9	0.5-0.8	n.a.	0.4-0.5	n.a.	n.a.
Iodine	0.01-0.1	0.3-1.1	0.05	0.01-0.1	n.a.	n.a.

* expressed as percentage

n.a.: not available

Marine algae extracts have been demonstrated to have strong antioxidant properties [15, 16], protective effects against liver injury caused by carbon tetrachloride [17], antiproliferative activity towards HeLa cells [16], antimicrobial activity [18] and antiviral properties [19]. *Padina antillarum* (previously known as *Padina tetrastromatica*) is a brown algae which proliferates in tropical waters. It was used as seasoning in dried flake form and as table salt replacement for high blood pressure patients [20]. There were studies which show that it contained alginic acid, a major polysaccharide which show high anticoagulant [21] and antiviral properties [19]. *Caulerpa racemosa* is a green algae which mainly grows in tropical regions, although some varieties may be found in subtropical regions [22]. In South East Asian countries, it is usually served raw as salad or eaten cooked. In addition, it is used as animal feed and in folk medicine to reduce blood pressure and to treat rheumatism [20, 22]. *Kappaphycus alvarezzi*, formerly termed as *Eucheuma cottonii* [23], is a red algae which was cultivated in many tropical countries. It is a popular species for aquaculture, being farmed at places with strong wave action such as on the reef edge [22]. Its main product of commercial importance is carrageenan.

Santoso, *et al*. [24] studied the distribution and antioxidative activities of polyphenolic compounds of twelve species of Indonesian and Japanese seaweeds. Peroxide value (POV), scavenging effect and Fe^{2+} chelating were determined as oxidation markers. The separation and determination of the phenolic compounds, such as catechin, caffeic acid, rutin and quercetin extracted from seaweed samples were investigated. Seaweed samples were extracted with methanol and determined by high performance liquid chromatography (HPLC). The experiments were carried out on octadecylsilyl column using acetonitrile/ethyl acetate/0.1% phosphoric acid

(8.5/2.0/89.5 v/v) as mobile phase, flow rate at 1 ml min^{-1} and analyzed at 280 nm with UV detector. The studies revealed that extracts of *Hizikia fusiformis* had the best antioxidant power in both absence and presence of Fe^{2+} in fish oil emulsion. The highest of scavenging effects was found in morin, followed by rutin and extract of seaweeds (*H. fusiformis and U. pinnatifida*). Catechin and gallic acid had the highest effects of Fe^{2+} chelating, and the smallest Fe^{2+} chelating was found in both of extracts seaweeds (*H. fusiformis and U. pinnatifida*). *Eisenia bicyclis* contained catechin and its isomers, whereas *Caulerpa racemosa, Kappaphycus alvarezii, Monostroma nitidum, Undaria pinnatifida* and *Laminaria religiosa* did not contain catechin and its isomers. Catechol was found in all Japanese seaweed samples, except *E. bicyclis*. The highest concentrations of flavonoids were found in *M. nitidum*, whereas almost all of flavonoids could be found in *U. pinnatifida*.

Suzuki, *et al*. [25] studied about Indonesian seaweeds. In this study, Indonesian green, brown and red algae were used as experimental materials. Their mineral components have been analyzed by using an atomic absorption spectrophotometry. The catechins and flavonoids of these seaweeds were extracted with methanol and analyzed by HPLC; the antioxidant activities of these seaweeds were evaluated in a fish oil emulsion system. The mineral components of tropical seaweeds were dominated by calcium, potassium and sodium, as well as small amounts of copper, iron and zinc. A green algae usually contained epigallocateehin, gallocatechin, epigallocatechin gallate and catechin. However, catechin and its isomers were not found in some green and red algae. In the presence of a ferrous ion catalyst, all the methanol extracts from the seaweeds show significantly lower peroxide values of the emulsion than the control, and that of a green algae shows the strongest antioxidant

activity. The highest chelating efficiency on ferrous ions was also found in the extract of this algae, which was significantly different from the other methanol extracts in both 3 and 24 h incubations.

Ganesan, *et al*. [26] studied about antioxidant activities of three selected Indian red seaweeds, including *Euchema kappaphycus, Gracilaria edulis* and *Acanthophora spicifera* were evaluated. Total phenolic content and reducing power of crude methanol extract were determined. The antioxidant activities of total methanol extract and five different solvent fractions, namely petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), butanol (BuOH) and aqueous were also evaluated. EA fraction of *A. spicifera* exhibited higher total antioxidant activity (32.01 mg ascorbic acid equivalent/g extract) among all the fractions. Higher phenolic content (16.26 mg gallic acid equivalent/g extract) was noticed in PE fraction of *G. edulis*. Reducing power of crude methanol extract increased with increasing concentration of the extract. Reducing power and hydroxyl radical scavenging activity of *E. kappaphycus* were higher compared to a standard antioxidant (α-tocopherol). The total phenol content of all the seaweeds was significantly different ($P < 0.05$). In vitro antioxidant activities of methanol extracts of the three seaweeds exhibited dose dependency; and increased with increasing concentration of the extract.

Mezadri, *et al.* [27] reported that development of HPLC method for determination of phenolic acids and flavonoids of acerola samples. Samples were crushed and filtered through 0.22 μm membrane filter before determined by HPLC. The experiments were carried out on Merck Superspher 100 RP-18 column. The mobile phase consisted of two solvents A (acetic acid in water adjust to pH 2.46) and B (20% A + 80% acetonotrile) in gradient elution program. By means of HPLC and

diode-array detection five phenolic compounds, including procyanidin, chlorogenic acid, epigallocatechin gallate, epicatechin and rutin have been identified in the samples analysed.

As these three species of seaweeds are used by humans as food and in view of the potential benefits described above, it is therefore of great interest to study and determine the phenolic compounds in seaweeds.

1.3 High Performance Liquid Chromatography

High performance liquid chromatographic (HPLC) techniques are now widely used for separation and quantitation of phenolic compounds. Various supports and mobile phases are available for the analysis of anthocyanins, procyanidins, flavonones and flavonols, flavan-3-ols, flavones and phenolic acids [5]. Introduction of reversed phase columns has considerably enhanced the HPLC separation of different classes of phenolic compounds.

Liquid chromatography is a separation technique based on a different distribution rate of sample components between a stationary and a liquid mobile phase. High performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column containing stationary phase. HPLC is widely used for separating non-volatile and thermally labile compounds because of its sensitivity, its ready adaptability to accurate quantitative determinations and its applicability to substances regarding to industry and many fields of science [28-30].

In reversed-phase HPLC, a non-polar stationary phase is used in conjunction with polar mobile phase. Almost 80% of all HPLC applications utilized this technique. Its popularity is based largely on ease of use, fast equilibration time, reproducible retention times and the basic principles of the retention mechanism can be understood easily [31].

1.3.1 Chromatographic theory [28-30, 32]

Chromatography is a separation technique where component molecules (solutes) in a sample mixture are transported by a mobile phase over a stationary phase. Each component or solute is distributed between the two phases with an

equilibrium established defined by the distribution or partition coefficient (K). The distribution equation for each component between two phases is given as follows:

$$K = C_S/C_M \quad (1.1)$$

where C_S and C_M are the concentrations of the solute in the stationary phase and in the mobile phase, respectively.

Three basic terms are used to express the key parameters in all the chromatographic experiments, namely retention, resolution and efficiency.

1) Retention

This term is expressed as retention time (t_R) and/or in terms of capacity factor (k'). The retention time is a time for elution at the peak maximum. The capacity factor is a direct measurement of the strength of the interaction of the sample with the packing material and is defined by the expression:

$$k' = \left(\frac{t_R - t_0}{t_0}\right) \quad (1.2)$$

where t_0 is the time the analyte spends in the mobile phase (holdup time) as depicted in **Fig. 1.3**. Typically, k' values between 2 and 5 represent a good balance between analysis time and resolution; however, k' values between 1 and 10 are usually acceptable.

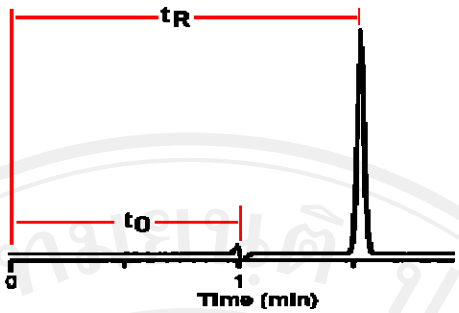


Fig.1.3 Generalized chromatogram used to calculate chromatographic parameters [33].

2) Resolution

The resolution (R_s) of a column refers to its ability to separate two components of a sample as expressed in equation 1.3.

$$R_s = \frac{2(t_{R2}-t_{R1})}{(W_1+W_2)} \qquad (1.3)$$

where W_1 and W_2 are the peak widths of components 1 and 2, respectively. A resolution of 1.5 (baseline resolution) is ideal for quantification of particular sample peaks [30]. Yet even with a resolution of 1.0, quantification is possible because only 2% of the peak overlapping. Chromatograms with resolutions of less than 1.0 should not be used for quantitative analysis. On the other hand, $R_s > 1.5$ is not necessary, due to the long analysis time.

In liquid chromatography, factors affecting the resolution can be expressed in equation 1.4 because it combines the chromatographic parameters, i.e. separation factor (α), capacity factor (k′) and plate number (N). The effect of these parameters on resolution is illustrated in **Fig. 1.4**. The most effective way to alter resolution is to change the separation factor and the capacity factor of the column.

Column length or flow rate velocity is less significant, as resolution increases proportionally as the square root of the plate number. Thus, doubling the plate number by adding a second column increases resolution by only a factor of equation 1.4. If increased resolution is required, a column with a higher capacity factor is often choice. However, increasing the capacity factor will increase the analysis time, so a compromise must be reached between resolution and analysis time.

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{k\prime}{k'+1}\right)\left(\frac{\propto-1}{\propto}\right) \qquad (1.4)$$

where

$$\propto = \frac{t_{R2}-t_0}{t_{R1}-t_0} = \frac{k\prime_2}{k\prime_1} \qquad ; k'_2 > k'_1 \qquad (1.5)$$

The value for α can range from unity (1), when the retention times of two components are identical ($t_2 = t_1$), to infinity if the first component of interest is eluted in the void volume. If α = 1, no separation is possible, no matter how high the separation efficiency may be. The most powerful approach to increase α is to change the composition of the mobile phase.

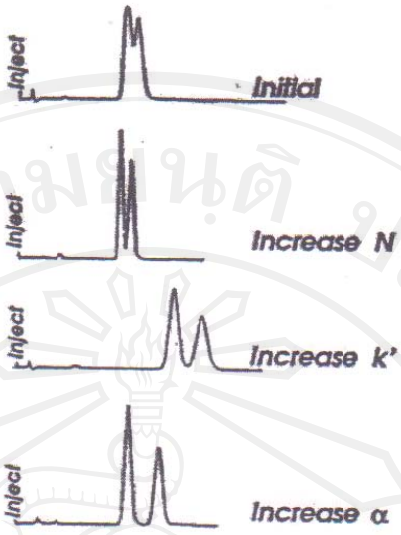


Fig. 1.4 Effects of separation factor, capacity factor and efficiency on resolution [29]

3) Column efficiency

The column efficiency is described in terms of the number of theoretical plates (N) that can be calculated from the following equations.

$$N = 16\left(\frac{t_R}{W}\right)^2 \quad (1.6)$$

or $$N = 5.545\left(\frac{t_R}{W_{1/2}}\right)^2 \quad (1.7)$$

where, W and $W_{1/2}$ are peak widths at its base and half-height, respectively. A large plate number system is highly efficient. In order to obtain a large plate number, the height equivalent to a theoretical plate (HETP) of plate height (H) should be the lowest value. These terms are related as follows:

$$H = \frac{L}{N} \qquad (1.8)$$

where, L is the length of the column. Thus, the smaller the height equivalent to a theoretical plate (HETP) or plate height (H), the greater is the efficiency of the column. In general, the H value is smaller for small stationary phase particle sizes, low mobile phase flow rates, less viscous mobile phases, higher separation temperatures and smaller solute molecule sizes.

1.3.2 Instrumentation

The basic components of a HPLC system are shown schematically in **Fig.1.5**. The instrument consists of solvent reservoir to contain the mobile phase, pump to move the eluent and sample through the system, injection port to allow sample introduction, column to provide solute separation, detector to visualize the separated components and data processing unit to assist in interpretation and storage of results.

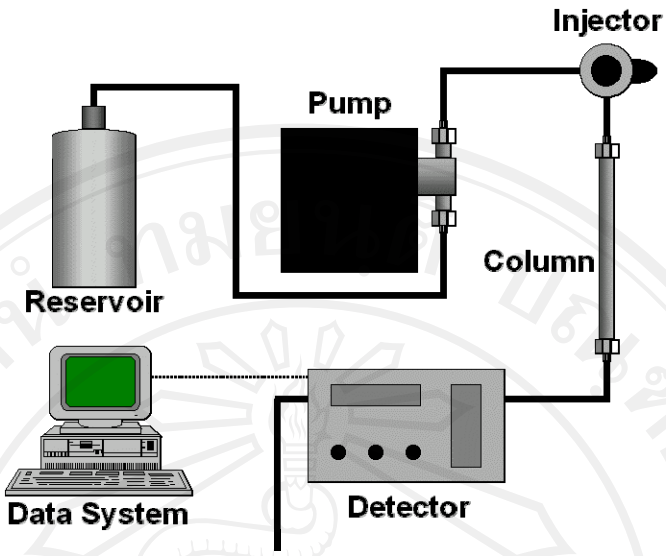


Fig. 1.5 Schematic diagram of HPLC system

1.3.2.1 Mobile phase [28, 29, 32]

Retention in reversed-phase chromatography is a function of sample hydrophobicity whereas selectivity arises from the combined action of mobile phase and stationary phase on the solutes. A suitable solvent will preferably have low viscosity, be able to completely dissolve the sample without reacting with analytes and be compatible with the detection system. The mobile phase used in reversed-phase chromatography is based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. Properties of some mobile phases used in LC are illustrated in **Table 1.2.**

Table 1.2 Properties of some mobile phases in LC [28]

Solvent	UV cut-off (nm)	Polarity Index (P')	Solvent strength (ε°)	Viscosity at 25°C (cP)
Heptane	195	0.2	0.01	0.40
Hexane	190	0.1	0.01	0.30
Pentane	195	0.0	0.00	0.22
Cyclohexane	200	-0.2	0.04	0.90
Toluene	285	2.4	0.29	0.55
Ethyl ether	218	2.8	0.38	0.24
Benzene	280	2.7	0.52	0.60
Methylene chloride	233	3.1	0.42	0.41
1,2-Dichloroethane	228	3.5	0.44	0.78
Butanol	210	3.9	0.70	2.60
Propanol	240	4.0	0.82	1.90
Tetrahydrofuran	212	4.0	0.57	0.46
Ethylacetate	256	4.4	0.58	0.43
Isopropanol	205	3.9	0.82	1.90
Chloroform	245	4.1	0.40	0.53
Dioxane	215	4.8	0.56	1.20
Acetone	330	5.1	0.56	0.30
Ethanol	210	4.3	0.88	1.08
Acetonitrile	190	5.8	0.65	0.34
Metthanol	205	5.1	0.95	0.54
Nitromethane	380	6.0	0.64	0.61
Water	<200	10.2	Large	0.89

1.3.2.2 Stationary phase [28, 29, 34]

Reversed-phase stationary phases appear to be the most generally useful of the stationary liquid phases. The most common stationary phases in reversed-phase chromatography are those in which a functional group is chemically attached to a silica support (bonded phases). The most popular bonded phases are the alkyl groups, such as $–CH_3$, $–C_4H_9$, $–C_8H_{17}$ and $–C_{18}H_{37,}$ phenyl (C_6H_5) groups, cyano [$(-CH_2)_3CN$] groups and amino [$(-CH_2)_3NH_2$] groups, with retention increasing exponentially with chain length. These packing materials, with some restrictions on the pH of the eluent used, provide good hydrolytic stability and are resistant to solvent stripping within normal column operating pressures.

Recently, chemically bonded phases are widely used in reversed-phase HPLC. They are usually made by derivatization of the silanol groups of silica. It is not possible for all the silanol groups on the silica surface to react with the functional groups, and usually only about 45% of the silanols will be bonded. Remaining unreacted acidic silanol groups can cause tailing of basic solutes such as amines. Thus, unreacted silanols are often removed by treatment with a small silating agent such as trimetylchlorosilane [$Si(CH_3)_3Cl$], a process known as end-capping. The functional group affect both of column selectivity and efficiency. The nature of the functional group controls selectivity while the chain length controls column efficiency. A list of commonly used bonded phases is given in **Table 1.3**

Table 1.3 Commonly used bonded phases [34]

Functional group	Structure	Principal use
Octadecyl	$-(CH_2)_{17}-CH_3$	Reversed-phase
Octyl	$-(CH_2)_7-CH_3$	Reversed-phase
Propyl	$-C_3H_7$	Reversed-phase
Phenyl	$-C_6H_5$	Reversed-phase
Aminoalkyl	$-(CH_2)_n-NH_2$	Normal and Reversed-phase
Cyanopropyl	$-(CH_2)_3-CN$	Normal and Reversed-phase
Diol	$-CH(OH)-CH_2(OH)$	Normal-phase
Sulfonic acid	$-(CH_2)_n-SO_3H$	Strong cation exchanger
Quaternary amine	$-(CH_2)_n-N^+(CH_3)_3$	Strong anion exchanger

1.3.2.3 Detectors for HPLC [29, 32, 34]

The purpose of the detector in an HPLC system is to detect the compounds of interest in the eluent from the HPLC column. Detectors are classified as selective or universal depending on the property measurement, as shown in **Table 1.4.** Selective (solute property) detectors such as ultraviolet (UV)-visible absorption detectors measure a physical or chemical property that is characteristic of the solute(s) in the mixture. Universal (bulk property) detectors such as refractive index (RI) detectors measure a physical property of the eluent. All solutes which possess a refractive index different from that of the eluent will be detected. Selective detectors tend to be more sensitive than universal detectors and they are more widely used. The choice of detector is often dictated by the chemical characteristics of the

analyte species and this choice may subsequently determine which eluent is used and also possibly which stationary phase and mode of chromatography. The detector response will be related to the amount of the analyte in the column effluent though different analyte will respond to differing extents and hence the detector must be calibrated with respect to each of the analytical species of interest.

The ideal HPLC detector should have the following characteristics [32].

- high sensitivity
- good stability and reproducibility
- linear response over several orders of magnitude
- small internal volume minimizing zone broadening
- a short response time independent of flow rate
- insensitive to changes in temperature and pressure
- high reliability and ease to use
- similar response to analytes or selective response to analyte classes

Table 1.4 Characteristics of chromatographic detectors [32]

Detector	Type	Gradient possible	Detection limit (g ml^{-1})	Linear range
UV-VIS	Selective	Yes	$5x\ 10^{-10}$	10^4-10^5
Photo-diode	Selective	Yes	$>2x\ 10^{-10}$	10^4-10^5
Fluorescence	Selective	Yes	$\sim10^{-12}$	10^3-10^4
Refractive index (RI)	Universal	No	$5x10^{-7}$	10^3-10^4
Infrared (IR)	Selective	Yes	10^{-6}	$\sim10^3$
Conductometric	Selective	No	10^{-8}	10^3-10^4
Amperometric	Selective	No	10^{-12}	10^4-10^5
Mass spectrometry (MS)	universal	Yes	10^{-10}	10^4

UV-VIS detector [28, 29, 35]

The UV-VIS detector is the most frequently used detector in HPLC. It is nondestructive and responds only to substances that absorb radiation at the wavelength of the light source.

With direct UV detection the eluent exhibits little or no absorbance at the wavelength to be monitored. When a solute is exposed to UV radiation, the radiation is absorbed by particular electronic configurations of the compound. The wavelength and the intensity of absorption depend on the presence chromophoric groups in the molecules. The common chromophores in organic compounds are unsaturated functional groups such as carbonyl, nitrile, aromatic and olefinic. These compounds absorb in the UV region (190-350 nm) and the intensity of absorption increases when these groups are conjugated. Colored compounds with highly

conjugated chromophores and which contain easily excitable electrons, absorb visible light (350-700 nm). The intensity of absorption is proportional to the concentration of the analyte in the mobile phase according to Lambert-Beer law which is defined as follows [28]:

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon l \qquad (1.9)$$

where A is absorbance, ε the molar extinction coefficient, l the optical path length, C the concentration of analyte in the mobile phase, I_o and I the intensity of the light beam passing through the detector cell without and with the sample, respectively.

If a solute of interest does not contain a chromophore, it may be detected by indirect UV detection. Indirect detection is a technically simple and sensitive method for detection of compounds with little inherent detector response [28, 29]. In indirect UV detection a chromophore is added to the mobile phase so a continuous, positive baseline signal is generated. If an analyte having no chromophore passes the detector cell, the absorption of the mobile phase is decreased and a negative peak is recorded. The advantage of this method is that analytes without chromophores can be detected. Whereas the disadvantage is its relatively poor detection limit (between 0.1 and 1 ppm).

Mass spectrometric detector

Mass spectrometry is an analytical technique that measures the mass-to-charge ratio (*m/z*) of charged particles. It is most generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components. The mass spectrum is measured by a mass spectrometer.

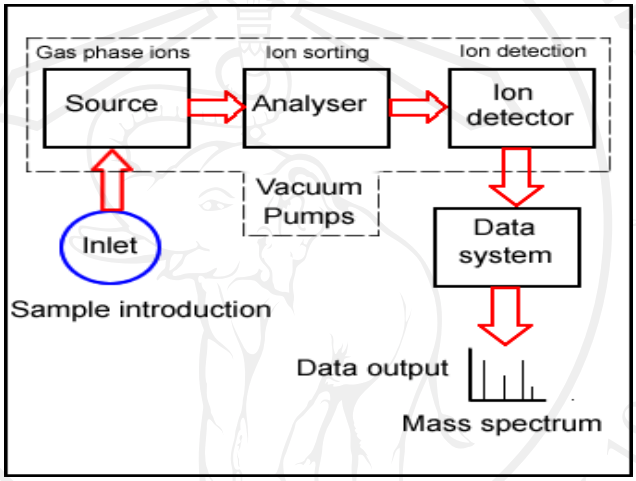


Fig. 1.6 Schematic diagram of a mass spectrometer [36]

Mass spectrometers consist of three basic parts: an ion source, a mass analyzer, and a detector system, as shown in **Fig. 1.6**. The stages within the mass spectrometer are:

1. Production of ions from the sample
2. Separation of ions with different masses
3. Detection of the number of ions of each mass produced
4. Collection of data to generate the mass spectrum

The technique is applicable in:

- identifying unknown compounds by the mass of the compound molecules or their fragments
- determining the isotopic composition of elements in a compound
- determining the structure of a compound by observing its fragmentation
- quantifying the amount of a compound in a sample using carefully designed methods (mass spectrometry is not inherently quantitative)
- studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in vacuum)
- determining other important physical, chemical, or even biological properties of compounds with a variety of other approaches.

Different chemicals have different masses, and this fact is used in a mass spectrometer to determine what chemicals are present in a sample. For example, table salt (NaCl), may be vaporized (turned into gas) and ionized (broken down) into electrically charged particles (Na^+ and Cl^-), called ions, in the first phase of the mass spectrometry. The sodium ions are monoisotopic, with mass 23Da. Chloride ions have two isotopes of mass 35 Da (~75%) and mass 37 Da (~25%). They also have a charge, which means that the speed and direction may be changed with an electric or magnetic field. An electric field accelerates the ions to a high speed. After this, they are directed into a magnetic field which applies a force to each ion perpendicular to the plane defined by the particles direction of travel and the magnetic field lines. This force deflects the ions (makes them curve instead of traveling in a straight line) to varying degrees depending on their *m/z*. Lighter ions get deflected more than the

heavier ions. This can be explained by Newton's second law of motion. The acceleration of a particle is inversely proportional to its mass. Therefore, the magnetic field deflects the lighter ions more than it does the heavier ions. The detector measures the deflection of each resulting ion beam. From this measurement, the *m/z* of all the ions produced in the source can be determined. From this information, the chemical composition of the original sample (i.e. that both sodium and chlorine are present in the sample) and the isotopic compositions of its constituents (i.e. whether the ratio of ^{35}Cl to ^{37}Cl has been changed by some process) can be determined. Techniques for ionization have been key to determining what types of samples can be analyzed by mass spectrometry. Electron ionization and chemical ionization are used for gases and vapors. In chemical ionization sources, the analyte is ionized by chemical ion-molecule reactions during collisions in the source. Two techniques often used with liquid and solid biological samples include electrospray ionization and matrix-assisted laser desorption-ionization (MALDI). Inductively coupled plasma sources are used primarily for metal analysis on a wide array of sample types. Others include glow discharge, field desorption (FD), fast atom bombardment (FAB), thermospray, desorption-ionization on silicon (DIOS), direct analysis in real time (DART), atmospheric pressure chemical ionization (APCI), secondary ion mass spectrometry (SIMS), spark ionization and thermal ionization. Ion attachment ionization is a newer soft ionization technique that allows for fragmentation free analysis [37].

1) Fast atom bombardment (FAB)

There are a number of fast particle beam desorption ionization methods. Fast particle desorption ionization superseded the earlier field desorption method, which always suffered from complex source design and sample preparation. The techniques of FAB is very similar in concept and design as they both involve the bombardment of a solid spot of the analyte-matrix mixture on the end of a sample probe by a fast particle beam as shown in **Fig. 1.7**. The matrix (a small organic species like glycerol or 3-nitrobenzyl alcohol) is used to keep a homogenous sample surface. The particle beam is incident onto the surface of the analyte-matrix spot, where it transfers its energy bringing about localized collisions and disruptions. Some species are ejected (sputtered) from the surface as secondary ions by this process. These ions are then extracted and focused before passing to the mass analyzer. The polarity of ions produced depends on the source potentials. **Fig. 1.7** shows a positive ion beam being formed.

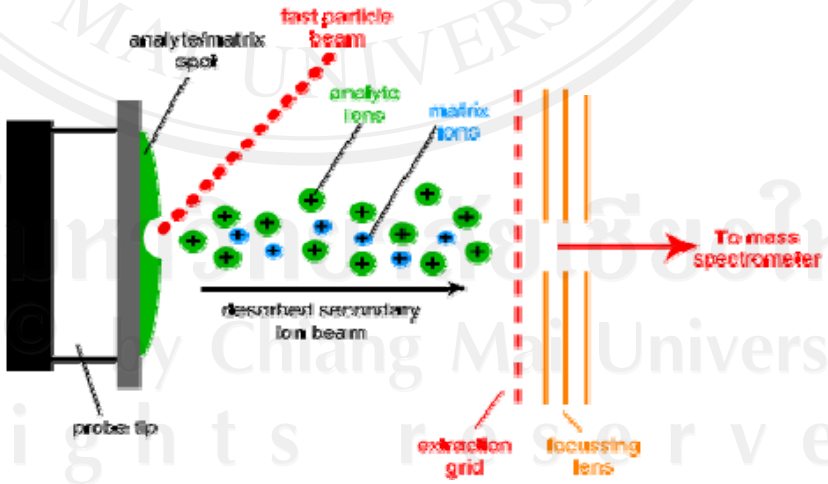


Fig. 1.7 Schematic of the mechanism of fast atom bombardment ionization mass spectrometry (FAB) [38]

2) MALDI mass spectrometry

MALDI mass spectrometry as depicted in **Fig.1.8** demonstrates that the irradiation of low-mass organic molecules with a high-intensity laser pulse lead to the formation of ions that could be successfully mass analyzed. This was the origins of laser desorption (LD) ionization. Other the next few decades, the technique underwent substantial development, culminating in the extension of the technique to the volatilizations of non-volatile biopolymers and organic macromolecules. There was, however, a sharp cut of in mass at about 5-10 kDa, limiting the technique's application. The other main limitation was that ions were created in bursts which prevented the technique from being coupled to scanning mass analyzers. In fact LD was only really successful when coupled to TOF-mass analyzers.

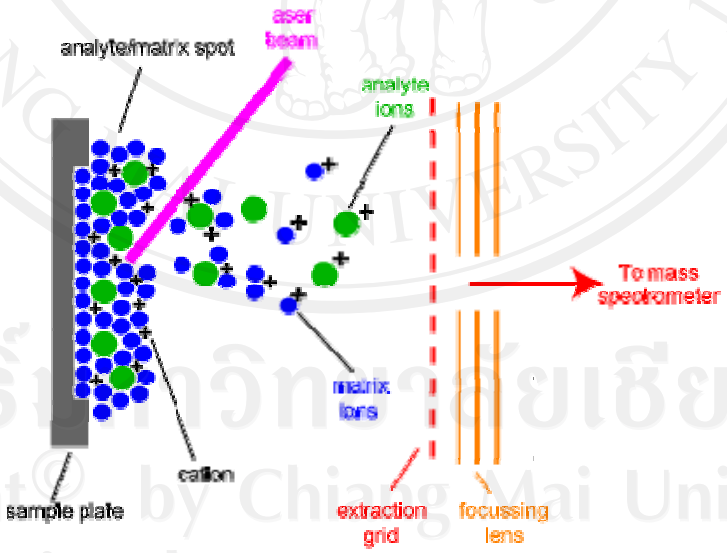


Fig. 1.8 A schematic diagram of the mechanism of MALDI. [39]

The mechanism of MALDI is believed to consist of three basic steps [39]:

(I) Formation of a 'solid solution': It is essential for the matrix to be in excess thus leading to the analyst molecules being completely isolated from each other. This eases the formation of the homogenous 'solid solution' required to produce a stable desorption of the analyst.

(II) Matrix Excitation: The laser beam is focused onto the surface of the matrix-analyst solid solution. The chromophore of the matrix couples with the laser frequency causing rapid vibrational excitation, bringing about localized disintegration of the solid solution. The clusters ejected from the surface consist of analyst molecules surrounded by matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyst in the gas-phase.

(III) Analyte Ionisation: The photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. It is in this way that the characteristic $[M+X]^+$ (X= H, Na, K etc.) analyte ions are formed. These ionization reactions take place in the desorbed matrix- analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for analysis.

3) Atmospheric-pressure ionization

Two different sample introduction approaches are used in combination with atmospheric pressure ionization (API) device, *i.e.* atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) as shown in **Fig. 1.9**. They primary differ in the nebulization principle and in the application range they cover. For both types, the solvent evaporation is almost completed using a heated tube (quartz or stainless steel).

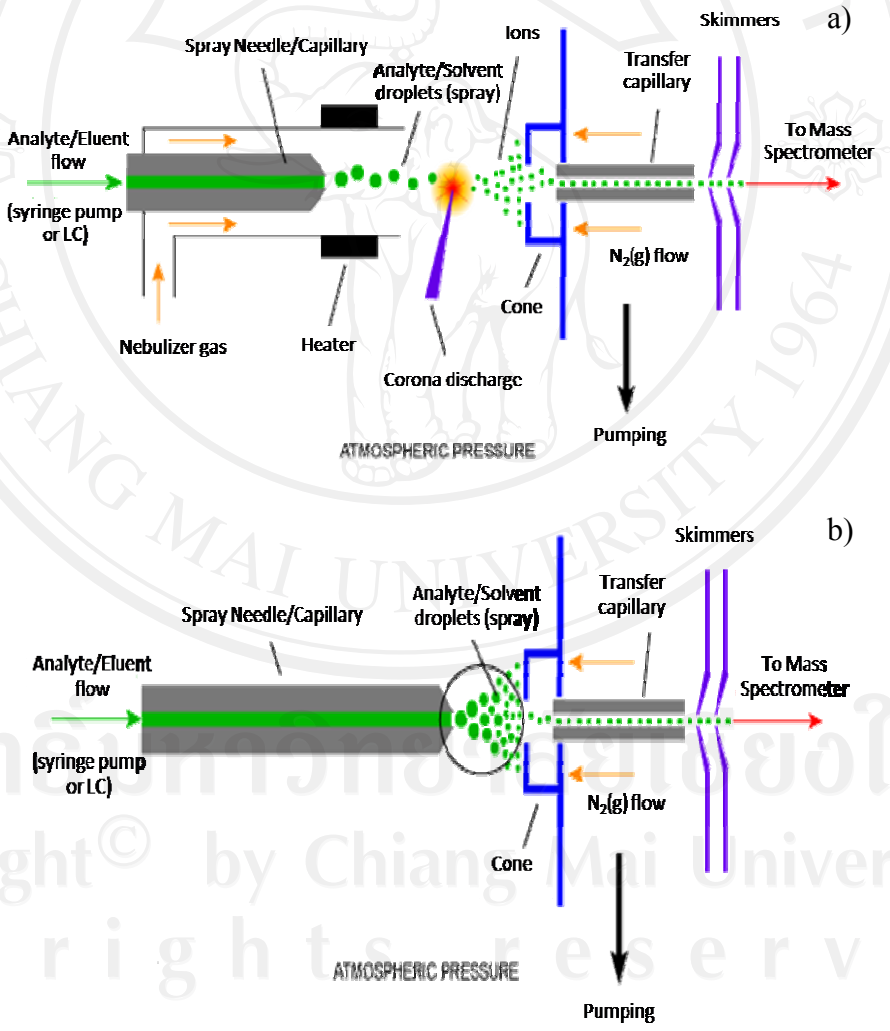


Fig. 1.9 Schematic of atmospheric pressure ionization devices: (a) APCI and (b) ESI [40]

Atmospheric pressure chemical ionization is an analogous ionization method to chemical ionization (CI). The significant difference is that APCI occurs at atmospheric pressure and has its primary applications in the areas of ionization of low mass compounds APCI is not suitable for the analysis of thermally labile compounds. The general source set-up as depicted in **Fig. 1.9 (a)** shares a strong resemblance to ESI. Where APCI differs to ESI, is in the way ionization occurs. In ESI, ionization is bought about through the potential difference between the spray needle and the cone along with rapid but gentle desolvation. In APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions. These primary ions collide with the vaporized solvent molecules to form secondary reactant gas ions e.g. H_3O^+ and $(H_2O)_nH^+$ as shown in **Fig. 1.10**. These reactant gas ions then undergo repeated collisions with the analyte resulting in the formation of analyte ions. The high frequency of collisions results in a high ionization efficiency and thermalization of the analyte ions. These results in spectra of predominantly molecular species and adduct ions with very little fragmentation. Once the ions are formed, they enter the pumping and focusing stage in much the same as ESI.

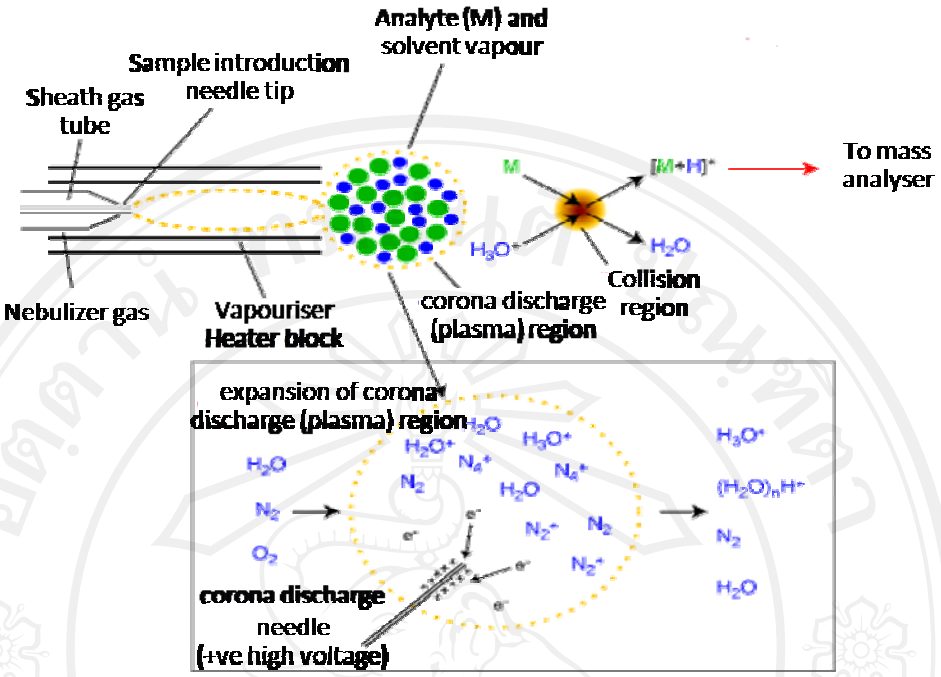


Fig. 1.10 A schematic of the mechanism of ion formation in of APCI interface [40]

In electrospray ionization, the analyst is introduced to the source at flow rates typically of the order of 1 µl min^{-1}. The analyst solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode. As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs. This is circled on **Fig. 1.9 (b)** and enlarged upon in **Fig. 1.11**. As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a *"Coulombic explosion"* occurs and the droplet is ripped apart. This produces smaller droplets that can repeat

the process as well as naked charged analyst molecules. These charged analyst molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionization as very little residual energy is retained by the analyst upon ionization. It is the generation of multiply charged molecules that enables high molecular weight components such as proteins to be analyzed since the mass range of the mass spectrometer is greatly increased since it actually measures the *mass to charge ratio (m/z)* rather than mass *per sec.* The major disadvantage of the technique is that very little (usually no) fragmentation is produced although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS or MS^n [40].

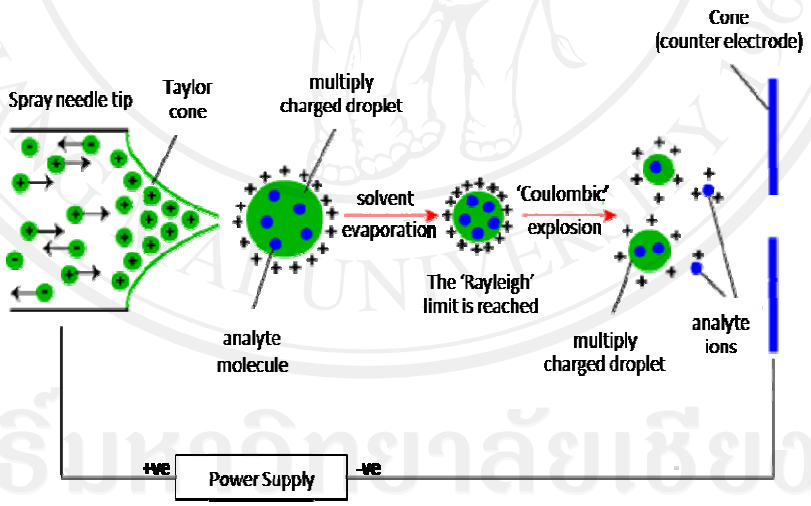


Fig. 1.11 A schematic of the mechanism of ion formation in ESI interface [40]

Many researches found that electrospray ionization mass spectrometry (ESI-MS) has been employed for structural confirmation of phenolics in plant.

All ions in an ionization chamber are to be analyzed according to *m/z*. Ions have an electrical charge that permits them to be controlled by various electrical fields. They are then, separated by their *m/z* values in a mass analyzer. Combination with chromatography requires a mass spectrometer that can produce mass spectra at a rapid rate, such as the ion-trap, quadrupole, or time of flight (TOF) mass spectrometers.

4) Ion trap mass spectrometer

A schematic of the basic set up of a quadrupole ion tap (QIT) mass analyzer is shown in **Fig. 1.12**. The ions, produced in the source of the instrument, enter into the trap through the inlet and are trapped through action of the three hyperbolic electrodes; the ring electrode and the entrance and exit end cap electrodes. Various voltages are applied to these electrodes which results in the formation of a cavity in which ions are trapped. The ring electrode RF potential, an AC. potential of constant frequency but variable amplitude, produces a 3D quadrupolar potential field within the trap. This traps the ions in a stable oscillating trajectory. The exact motion of the ions is dependent on the voltages applied and their individual *m/z* ratios. For detection of the ions, the potentials are altered to destabilize the ion motions resulting in ejection of the ions through the exit end cap. The ions are usually ejected in order of increasing *m/z* by a gradual change in the potentials. This stream of ions is focused onto the detector of the instrument to produce the mass spectrum.

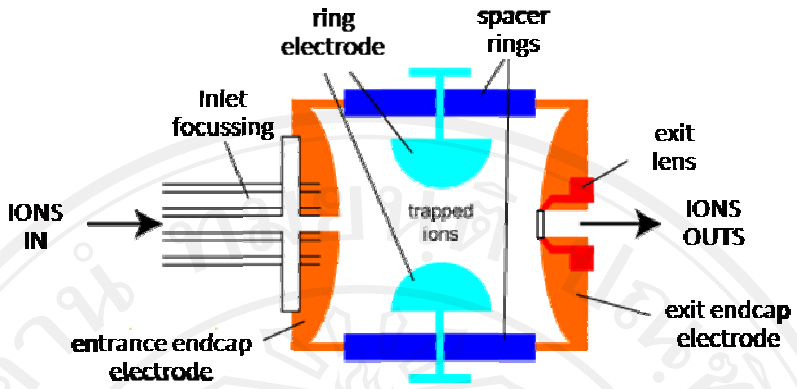


Fig. 1.12 A schematic of a quadrupole ion trap mass analyzer [41]

The nature of trapping and ejection makes a quadrupolar ion trap especially suited to performing MS^n experiments in structural elucidation studies. It is possible to selectively isolate a particular *m/z* in the trap by ejecting all the other ions from the trap. Fragmentation of this isolated precursor ion can then be induced by CID experiments. The isolation and fragmentation steps can be repeated a number of times and is only limited by the trapping efficiency of the instrument.

5) Sector mass spectrometer

The sector mass spectrometer is one of the most common types of mass analyzer and probably the most familiar to the everyday scientist. In the 1950's, the first commercial mass spectrometers were sector instruments. They consist of some combination of a large electromagnetic (B sector) and some kind of electrostatic focusing device (E sector) (different manufactures use differing geometries). **Fig. 1.13** shows a schematic of a standard BE geometry double focusing instrument that is a dual sector instrument consisting of a magnetic sector followed by an electrostatic sector.

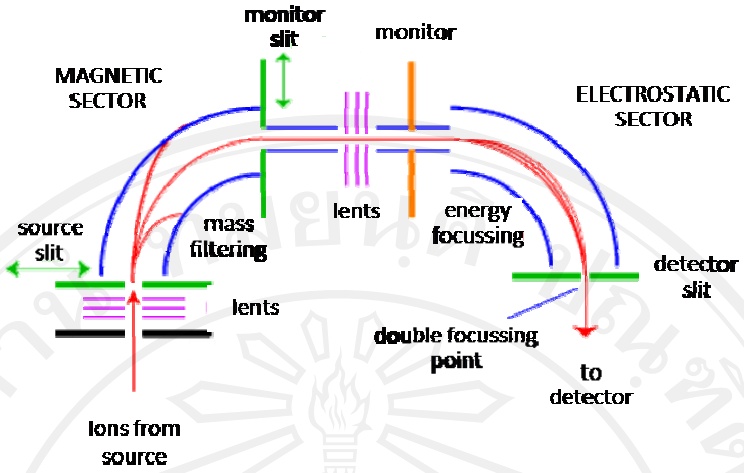


Fig. 1.13 A schematic of a sector mass spectrometer [42]

Ions enter the instrument from the source (bottom left) where they are initially focused. They enter the magnetic sector through the source slit where they are deflected according to the left-hand rule. Higher-mass ions are deflected less than lower-mass ions. Scanning the magnet enables ions of different masses to be focused on the monitor slit. At this stage, the ions have been separated only by their masses. To obtain a spectrum of good resolution where all ions with the same *m/z* appear coincident as one peak in the spectrum, ions have to be filtered by their kinetic energies. After another stage of focusing, the ions enter the electrostatic sector where ions of the same *m/z* have their energy distributions corrected for and are focused at the double focusing point on the detector slit.

6) Quadruple mass spectrometer

A quadrupole mass analyzer consists of four parallel rods as shown in **Fig. 1.14** that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focused and passed along the middle

of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular *m/z* will be in resonance and thus pass through to the detector. The RF is varied to bring ions of different *m/z* into focus on the detector and thus build up a mass spectrum. The trajectory of the ions through the quadrupole is actually very complex.

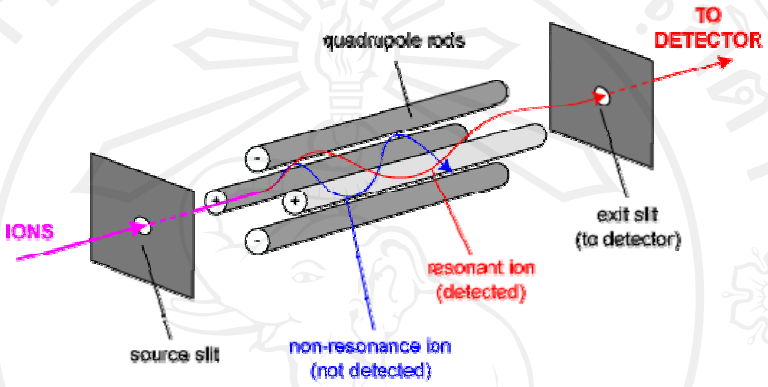


Fig. 1.14 Schematic of a quadrupole mass analyzer [43]

The two opposite rods in the quadrupole have a potential of +(U+Vcos(θt)) (labeled + on the **Fig. 1.14**) and the other two -(U+Vcos(θt)) where U is the fixed potential and Vcos(θt) is the applied RF of amplitude V and frequency θ. The applied potentials on the opposed pairs of rods varies sinusoid ally as cos (θt) cycles with time t. This results in ions being able to traverse the field free region along the central axis of the rods but with oscillations amongst the poles themselves. These oscillations result in complex ion trajectories dependent on the *m/z* of the ions. Specific combinations of the potentials U and V and frequency will result in specific ions being in resonance creating a stable trajectory through the quadrupole to the detector. All other *m/z* values will be non-resonant and will hit the quadrupoles and not be detected as depicted in **Fig. 1.14**. The mass range and resolution of the instrument is determined by the length and diameter of the rods.

7) Time of flight mass spectrometer

The time of flight (TOF) mass spectrometer involves measuring the time required for an ion to travel from an ion source to a detector located 1-2 m from the source. All the ions receive the same kinetic energy during instantaneous accelerations (e.g. 3000 eV), but because they may have different *m/z* values, they separate into groups according to velocity (and hence *m/z)* as they traverse the field-free region between the ion source and detector. The ions sequentially strike the detector in order of increasing *m/z* value, creating a time-based waveform, or simply a transient. Ions of low *m/z* reach the detector before those of high *m/z* because the later have velocity, as indicated schematically in **Fig. 1.15** [44].

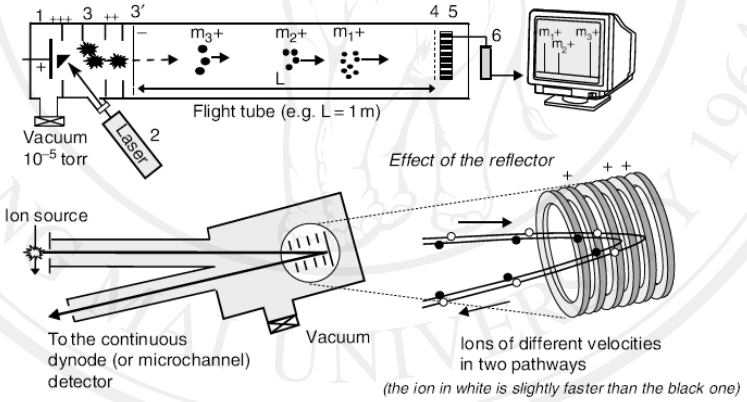


Fig. 1.15 A simplified schematic of a time of flight spectrometer and the principle of the ion reflector (reflectron). (1) sample and sample holder, (2) MALDI ionization device by pulsed laser bombardment, (3) and (3') ions are formed between a repeller plate and an extraction grid (PD 5000V) then accelerated by another grid, (4) control grid, (5) microchannel collector plate and (6) signal output. Below, a reflectron, which is essentially an electrostatic mirror that is used to time-focus ions of the same mass but which have initially different energies. The widths of the peaks are of the order of 10^{-9}s and the resolution ranges between 15 to 20,000 [44].

8) Mass spectrometry-mass spectrometry

Mass spectrometry-mass spectrometry or tandem mass spectrometry (MS/MS) involves multiple steps of mass selection or analysis, usually separated by some forms of fragmentation. A tandem mass spectrometer is one capable of multiple rounds of mass spectrometry. A conceptdual representation of MS/MS is illustrated in **Fig. 1.16**. The conventional approach of ionization and fragmentation of a given molecule is represented at the top with mass selection to obtain a conventional mass spectrum. One can then choose ion of a particular (*m/z*) value as precursor ions for dissociation process for propose of characterizing the precursor ion. Because of the equipment of two mass selective processes, the name MS/MS was an obvious name for the technology [45].

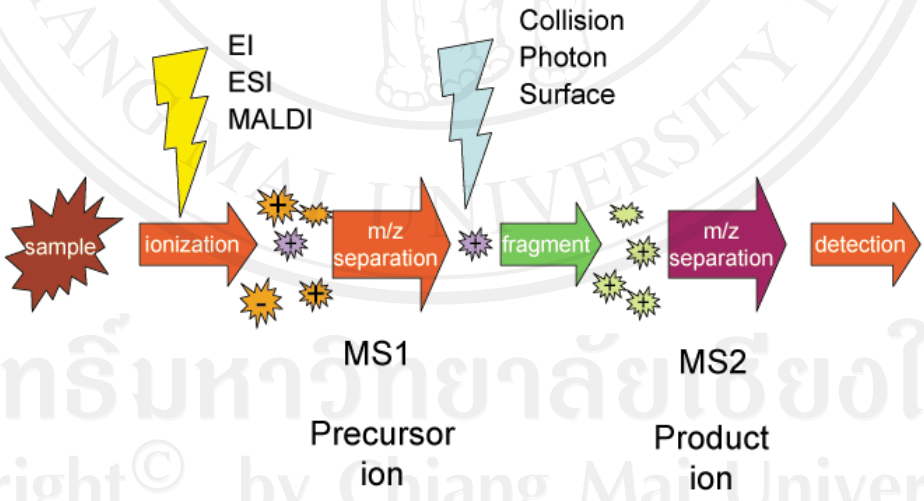


Fig. 1.16 Schematic of a MS/MS mass analyzer [45]

A collision cell is a small chamber mounted in the ion path of the mass spectrometry. The collision cell has two small opening, one to let the precursor ions in and the second to let the product ions and surviving precursor ions out. The chamber can be pressurized, usually to 10^{-4}-10^{-3} torr, with a collision cell is differentially pumped so that the collision gas does not interfere with proper operation of other parts of the mass spectrometer. The collision cell is mounted in an appropriate field-free region that is, between the mass-selective devices.

A precursor ion is any ions selected for analysis by collisionally activated dissociation (CAD). This has been called the parent ion. The precursor ion can be a fragment ion from the first mass spectrometer. A molecular ion also can be chosen as a precursor ion for a CAD experiment. Product ions are those fragment ions produced upon decomposition of a precursor ion in the collision cell during CAD; in the recent past, these were termed daughter ions.

A product ion spectrum is an array of product ions produced by decomposition of a given precursor.

A neutral-loss spectrum is an array of ions that undergo a common loss, such as expulsion of H_2O or glucoside.

Applications of different LC-MS techniques are versatility for the analysis of natural compounds in food. Specific examples of substances are liquid, oligosaccharides, flavonoids, anthocyanins, and related substances. LC-MS is a powerful technique in food analysis and especially for analysis of complex mixtures. The relative applicability of LC-MS techniques is shown in **Fig. 1.17**.

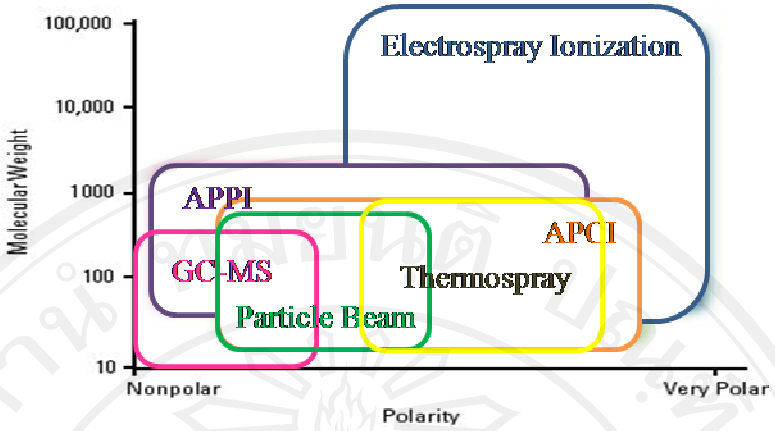


Fig. 1.17 Schematic diagram of relative applicability of LC-MS techniques compared with that of GC-MS.

1.4 The Scope and Aims of This Research

Six phenolic compounds, namely gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin, were selected to be analyzed in this work. The main objective of this research is the development of HPLC method, particularly in respect to short analysis time and efficient analysis of these phenolic compounds. Solvent extraction was used to extract these phenolic compounds in seaweeds sample prior to HPLC-UV analysis.

The aims of this research work can be summarized as follows:

1. Optimization of HPLC condition for separation of phenolic compounds such as detection wavelength, type and mobile phase composition, flow rate of the mobile phase, type and concentration of common acids
2. Investigation of seaweed sample pretreatment
3. Identification of phenolic compounds (gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin) using electrospray ionization-mass spectrometry (ESI-MS)
4. Determination of individual phenolic compounds in seaweeds using the developed method