Chapter I

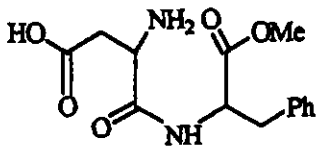
Introduction

Aspartame (N-L-α-aspartyl-L-phenylalanine methyl ester) (Figure 1.1a), is a non-carbohydrate artificial sweetener first discovered in 1965 by J. Schlatter and commercialized by Searle & Co. under a brand name of Nutrasweet. It is also known as Equal, Canderel, Sanecta or Tri-sweet. Aspartame is about 200 times sweeter than sucrose (1). It is widely used as a substitute for sugar and saccharin in table top sweeteners, chewing gums, breakfast cereals, dessert mixes and soft drinks.

Aspartame is digested and metabolized as the corresponding dipeptide, phenylalanine-aspartic acid. The energy content of aspartame is 17 kJ/g, similar to that of protein and carbohydrate. Because aspartame is much sweeter than sucrose, the amount of aspartame used in food is small compared to the amount of sucrose used to obtain the same degree of sweetness. As a result, the calories caused by aspartame are negligible and this makes the compound suitable for low caloric drinks/foods.

Ingestion of aspartame can elevate plasma phenylalanine concentration. Therefore, a person with phenylketonuria (PKU) should avoid this sweetener. The joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives has approved the use of aspartame in 1982, to which acceptable daily intake of 0-40 mg/kg body weight was allocated. Nevertheless, there

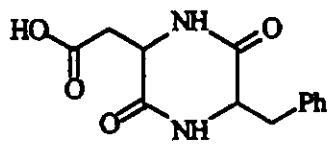
are a number of studies which show quite dangerous health effects from aspartame (2).



ASPARTAME

(MW=294)

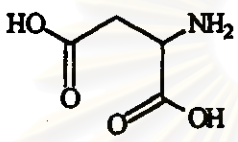
a



DIKETOPIPERAZINE

(MW=262)

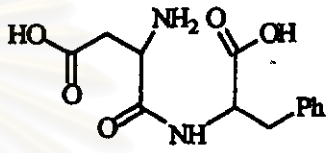
b



ASPARTIC ACID

(MW=133)

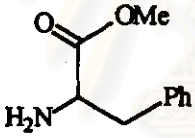
c



ASPARTYL PHENYLALANINE

(MW=280)

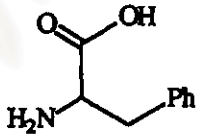
d



PHENYLALANINE METHYL ESTER

(MW=179)

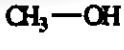
e



PHENYLALANINE

(MW=165)

f



METHANOL

(MW=32)

g

Figure 1.1 Structure of aspartame and its degradation products.

Previous studies on stability of aspartame in dry-product applications showed that the compound is relatively stable (1). This is in agreement with the fact that the hydrolysis of ester and peptide bonds usually requires H_2O molecules. However, one should keep in mind that these studies were done where the temperature and moisture

is quite different from the condition in Thailand. Degradation of aspartame usually occurs in liquid products (1,3). Since aspartame is the methyl ester of the dipeptide aspartyl phenylalanine, its stability should, therefore, be dependent on pH of aqueous solution and temperature, as these two parameters affect directly to the rate of ester bond and peptide bond hydrolysis reactions. It has been shown that, either the ester linkage or the peptide bond (or both) in aspartame molecule will be hydrolyzed slowly at low pH range, yielding sweetless degradation products. Known degradation products of aspartame are 5-benzyl-3,6-dioxo-2-piperazineacetic acid (Diketopiperazine, DKP) (Figure 1.1b), aspartic acid (Figure 1.1c), aspartyl phenylalanine (Figure 1.1d), phenylalanine (Figure 1.1e), phenylalanine methyl ester (Figure 1.1f) and methanol (Figure 1.1g) (1-4). Many analytical methods were proposed to determine aspartame and its degradation products. Tsang, Clarke and Parrish (3) determined aspartame and its degradation products in carbonated soft drinks by reverse-phase high performance liquid chromatography (HPLC) with UV detection at 214 nm. They found that aspartame is relatively unstable in carbonated soft drinks over long periods of storage time. However, they did not state the condition of storage such as temperature, in the study. They could identify four degradation products including DKP, phenylalanine, phenylalanine methyl ester and aspartyl phenylalanine. This study shows that aspartame level in soft drinks will drop to about 90 % if kept for only 1 month and will drop to about 40 % if kept for 6 months.

The only study of aspartame by LC-MS was done by Duchateau, Munsters, Kwakkenbos and Van Leuken (5) in which, they studied effects of various buffer

solutions on the analysis of ionic compounds with liquid chromatography-thermospray-mass spectrometry (LC-TSP-MS). This study concentrated on finding out the appropriate salts required for ionization of aspartame in LC-TSP-MS. They have shown that ammonium salts of formate, acetate and bicarbonate were suitable buffers for the LC-TSP-MS analysis of the three ionic degradation products of aspartame including aspartyl phenylalanine, phenylalanine and DKP. It has been known recently, however, that the TSP-MS technique is much less sensitive than electrospray-MS (ESI-MS). The ESI-MS is now the most recent and one of the most sensitive of ionization technique.

Study by Prodolliet and Bruelhart (1) showed that aspartame and the three degradation products, aspartyl phenylalanine, phenylalanine and DKP, were observed in most foods tested, using HPLC with UV detection at 214 nm. Although the method could not be used for detecting the other two degradation products, aspartic acid and methanol, the result showed the instability of aspartame in foods.

In addition to HPLC and TSP-MS, spectrofluorimetry is another method used in determining aspartame quantitatively. Sanchez and Gallardo (6) used fluorescamine to derivatize aspartame in soups and carbonated soft drinks. The data shows that the aspartame detection limit of aspartame by LC-spectrofluorimetry is 0.04 μg/mL while that of LC-spectrophotometry is 0.22 μg/mL.

Villarta, Suleiman and Guilbault (7) used amperometric enzyme electrode for the determination of aspartame in protein-free samples. The data shows that the detection limit is 1.5×10^{-4} M. The method was very sensitive but required quite pure sample.

In this study, we have used atmospheric pressure ionization-mass spectrometry (API-MS) to determine aspartame and its degradation products quanlitatively and quantitatively for the first time. The advantage of API-MS technique is that we can simultaneously detect aspartame and its degradation products in the same injection. Since aspartame and various degradation products can be differentiated from one to another by the differences in mass per charge (M/Z) values. The analysis, therefore, can be done directly without the prior separation by HPLC column. As a result, the time of each analysis is very short (~1-5 min). The method is also quite sensitive for most of the degradation products including aspartyl phenylalanine, phenylalanine, phenylalanine methyl ester, DKP and aspartic acid.

Although study of aspartame degradation has been done by several groups (with different methodology), none of the study was done with the consumer products distributed in Thailand. The condition of quite high temperature with lot of sunshine (UV ray) and high humidity can, very likely, elevate the degradation of aspartame. It will, therefore, be very useful to study degradation of aspartame under room temperature in Bangkok. The result will either insure the harmless use of aspartame or reveal the unsafe use of this sweetener in the hot and humid region. Moreover, since the degradation products of aspartame bare no sweetness, this leads to the change in degree of sweetness of stored aspartame-modified foods/drinks. This affects directly to the quality control of the food/drink industry. The result of this study will, therefore, help to estimate the shelf-life of the products and encourage the establishment of appropriate safe-life-time of aspartame-modified foods/drinks. This study, therefore, will at least yield an immediately usefulness to the society. It is also a

basis of a technology transfer-screening process which is in demand for our society. More importantly, this study also includes the study of effects of pH and temperature on degradation pathways of aspartame in which nobody has ever studied before. This will yield a better understanding of aspartame in term of its degradation chemistry. The results can also be applied directly to minimize aspartame degradation in various products.

Mass Spectrometry

Mass spectrometry (MS) is the most specific technique for the detection and identification of organic compounds. Mass Spectrometry can provide not only molecular weight information but also a wealth of structural detail which together give a unique finger-print for each analyte. Mass spectrometer is an instrument that ionizes the incoming sample molecules then detects the mass over charge ratio (M/Z) of those ionized particles. Result of sample analysis is in the form of mass spectrum which is a plot of ion abundance versus M/Z ratio. Besides molecular ions resulted from the ionization upon sample molecule, mass spectrum usually contains many fragmented ions those produced through fragmentation of sample molecules.

Mass spectrometry analysis involves 4 steps:

1. Introduction of sample into the mass spectrometer. This sample inlet part can be used to either directly inject the sample or connected to other separation techniques such as gas chromatograph (GC) and liquid chromatograph (LC).

2. Conversion of the analyte molecules into ions in gaseous phase. This step occurs in ion source. Fragmentation of molecular ion usually occurs during this step. At present, there are several methods (>15) of ionization. Six important and widely used ionization methods include:

2.1 **Electron Impact (EI) Source** generates ions by bombarding the gaseous sample molecules with a beam of energetic electrons.

2.2 **Chemical Ionization (CI) Source** generates ions through collision among gaseous sample and reagent ions produced by electron bombardment of an excess of a reagent gas. The common reagent gas includes methane, isobutane and ammonia. Usually CI gives less fragmentation than EI.

2.3 **Fast Atom Bombardment (FAB) Source** Sample in a glycerol matrix is ionized by bombardment with energetic xenon or argon atoms. Positive and negative ions are sputtered from the surface of the sample by desorption process. FAB is used for high molecular weight biochemical compounds.

2.4 **Laser Desorption (LD) Source** Sample molecules are simultaneously ionized and desorped into gaseous ions by the use of laser beam.

2.5 **Electrospray Ionization (ESI) Source** The sample solution is atomized into tiny droplets by the high voltage (~2-4 kV) at the tip of the electrospray probe. Preformed ions in the solution are then become ions in gaseous phase by the evaporation of solvent molecules.

2.6 **Atmospheric Pressure Chemical Ionization (APCI) Source** generate ions by high voltage from corona discharge pin. Sample solution is sprayed into the ion source where high electrical voltage needle is located. The high voltage of

the needle causes ionization of solvent molecules. Collisions among solvent ions-solvent molecules and solvent ions-sample molecules generate sample ions.

Different ionization methods usually yield different mass spectrum. In this study the atmospheric pressure ionization (API) is used which includes ESI and APCI as the ionization methods (see details on page 10).

3. Separation of ions according to their mass to charge ratio by the mass analyzer. There are several types of mass analyzers, e.g., magnetic sector, quadrupole, time of flight and ion cyclotron resonance. The instrument used in this study is the quadrupole type. A quadrupole mass analyzer uses an electrostatic field to separate ions according to their M/Z values.

4. Detection of separated ions by detector. The detector of the instrument used in this study is photomultiplier type. Ions from mass analyzer is accelerated and directed to hit the photoemissive cathode. This cathode when hit by ions will generate electrons. These ejected electrons are accelerated to hit the plate called dynode. This dynode when hit by electrons will emit more electrons out (usually at least double in number of the hit electrons). The secondary emitted electrons are directed towards another dynode as to multiply the number of electrons. The process is repeated until enough amplification of number of electrons is obtained and then converted to electrical signal.

The schematic representation of mass spectrometer components process is shown in Figure 1.2

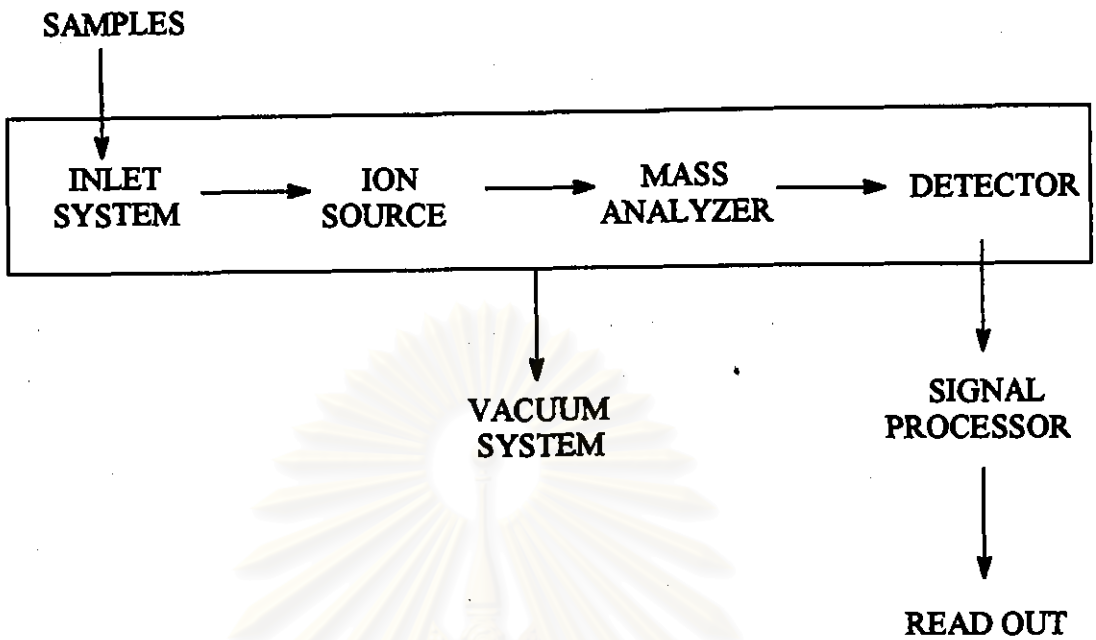


Figure 1.2 Mass spectrometer components.

In addition to the 4 major components namely sample inlet, ion source, mass analyzer and detector, described above, a mass spectrometer also contains another two important features:

1. **Ion Optical System** This system is usually consisted of many electromagnetic lens and acceleration voltages. They are distributed through out the machine as to direct ions into the right path.

2. **Vacuum system** This system is usually consisted of several vacuum pumps including rotary type and diffusion type. Low pressure is usually required in all 4 major parts of the MS except the ion source of API type in which the ionization occurs at atmospheric pressure.

Atmospheric Pressure Ionization (API)

Most ion sources such as EI, CI, FAB and LD operate at low pressure ($<10^{-4}$ torr) as to minimize the collision among ions and molecules of normally high internal energy. Limitation of these low pressure ion sources is that it can not be connected directly to liquid chromatography (LC). This is because, the introduction of liquid effluences from LC will disrupt the low pressure due to the evaporation and expansion of solvents. To overcome this limitation, ion source operated at atmospheric pressure has been invented and was given the name atmospheric pressure ionization (API). This high pressure source can still be connected with mass analyzer which required very low pressure ($<10^{-8}$ torr) by the use of very small hole (skimmer) which permits only charged species to be accelerated and passed into the mass analyzer (neutral solvent molecules are pumped out). Atmospheric pressure ionization can be divided into electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

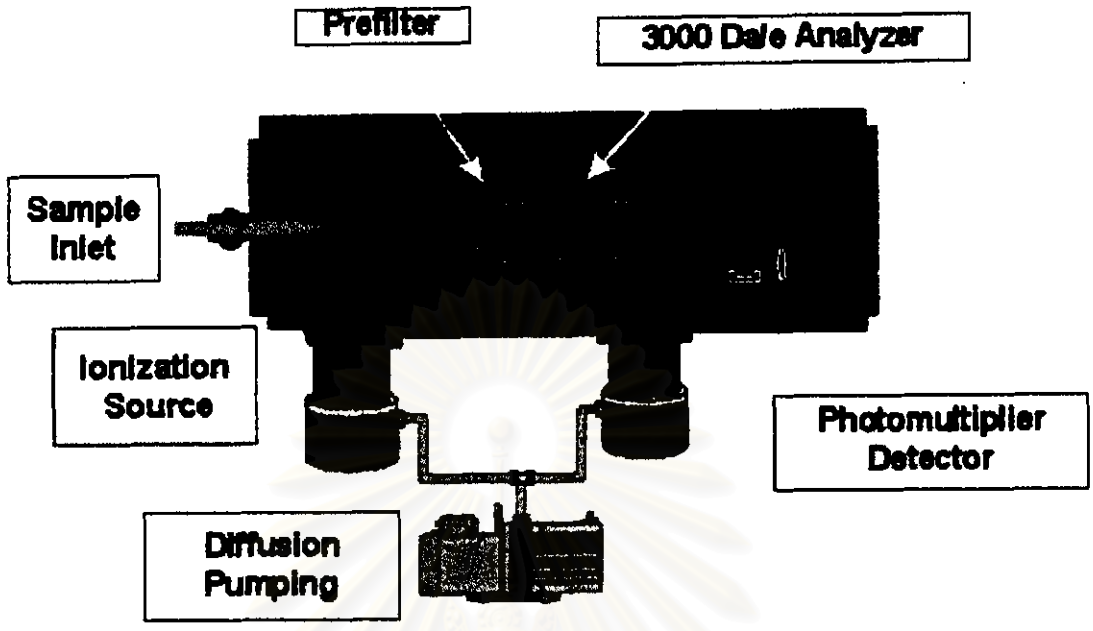


Figure 1.3 Schematic of liquid interface and mass spectrometer. The mass analyzer is quadrupole type.

1. **Electrospray Ionization (ESI)** allows rapid, accurate and sensitive analysis of a range of analytes, from low molecular weight (less than 200 Da) polar compounds to biopolymers with molecular weight larger than 100 kDa.

The sample of preformed ions in solution, emerged from a high voltage capillary tube into a strong electrostatic field at atmospheric pressure produces an aerosol of highly charged droplets. Evaporation of solvent from these droplets results in sample ions in gaseous phase. These ions are, subsequently, passed through the sample cone (which acts also as a counter electrode) and accelerated through the first and second skimmer into the mass analyzer (Figure 1.5). The electrospray source also allows further ion fragmentation in the region between sample cone and the skimmers. Degree of fragmentation can be partially controlled by adjusting the voltage gradient

between the tip of the capillary and the counter electrode and the accelerating voltage between the counter electrode and sample cone and between the sample cone and the skimmers (as the voltage induces changes in ion velocities, which affects collision and results in fragmentations).

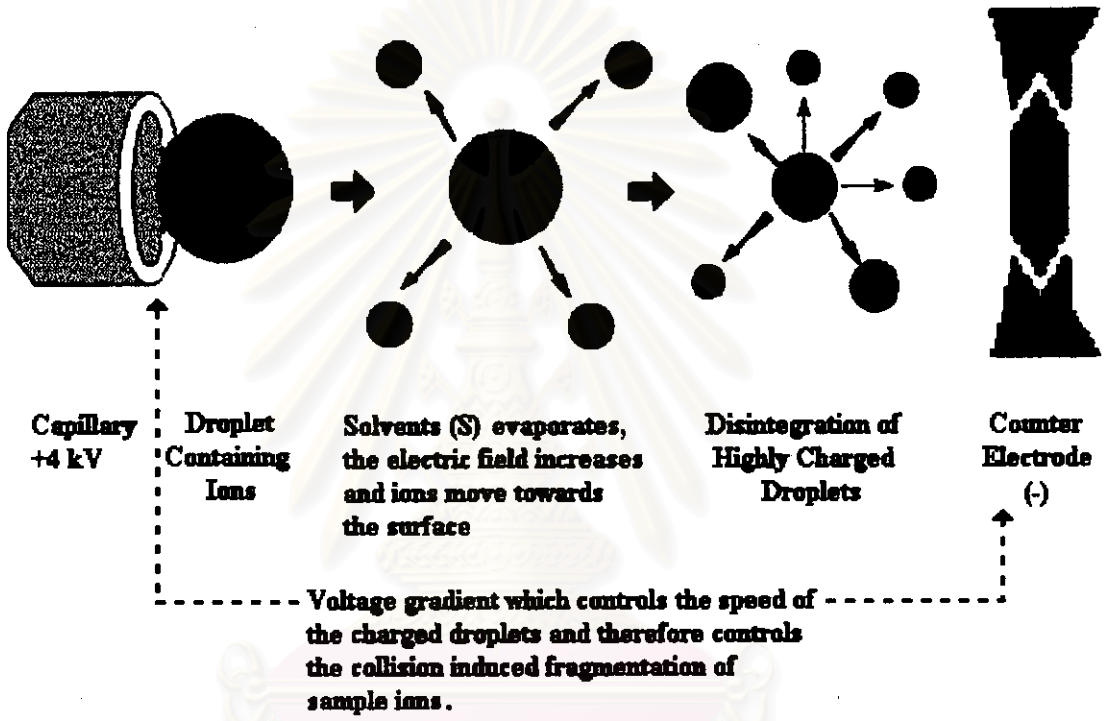


Figure 1.4 Ion evaporation mechanism in the API source.

This ionization method can, therefore, be adjusted to obtain minimum (none in many cases) fragmentation and reveal molecular weight of samples or it can be adjusted to a high accelerating voltage which will induce some fragmentation and provides valuable structural information for analytes. The method, however, is considered the softest ionization (among all ionization methods available today) when

the voltage is adjusted correctly. So, molecular weight information can be obtained easily, even for labile molecules. Generally, in ESI ion source, compounds of less than 1 kDa produce singly charged protonated molecular ions ($M+H^+$) in positive ion mode while high molecular weight compounds, for example peptides, proteins and oligonucleotides, produce a series of multiply charged ions and, therefore, reduce their M/Z values into the range that mass analyzer can handle. This makes it possible to analyse extremely high molecular weight material such as proteins and carbohydrates. The acquired data can be transformed by the data system to give a molecular weight profile of the biopolymer.

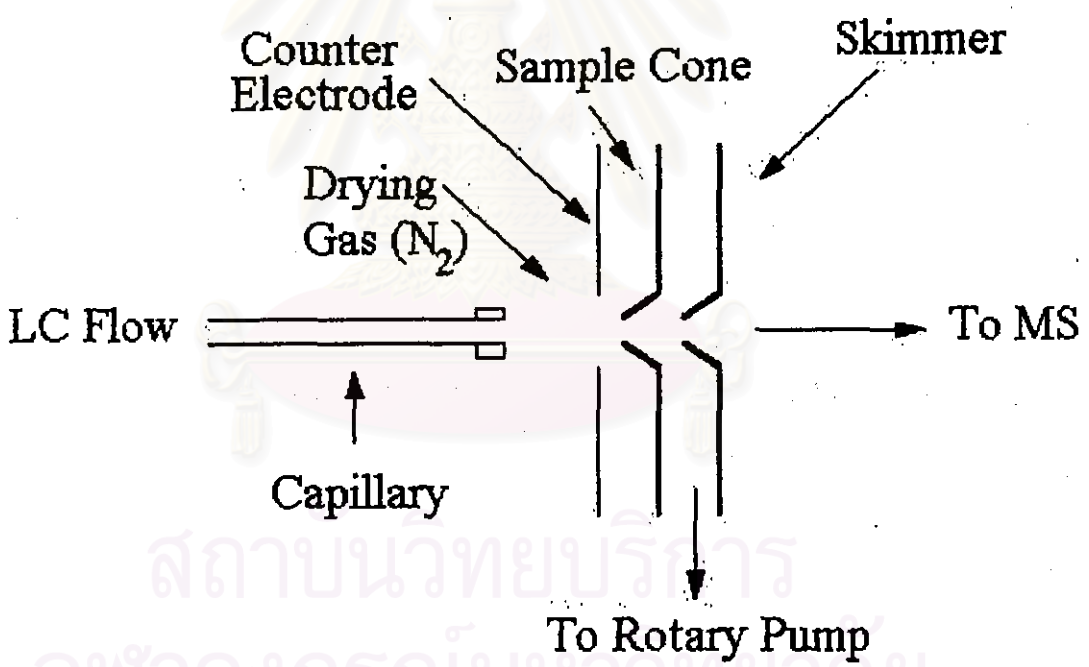


Figure 1.5 Schematic representation of electrospray source.

A typical ESI flow rate is about 10 μL/min and the method is considered very sensitive for polar compounds. A megaflow electrospray (Figure 1.6) enables flow

rates in excess of 200 μL/min to be accommodated and, therefore, possible to connect directly with HPLC.

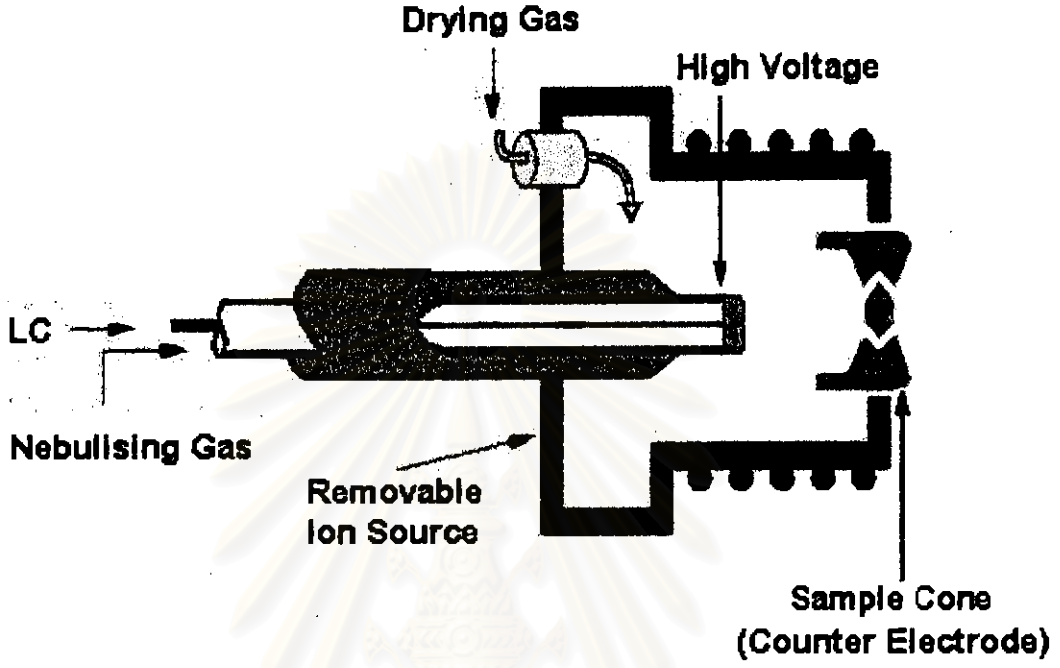


Figure 1.6 Megaflow inlet.

The mobile phase commonly used in ESI is 1:1 mixture of acetonitrile and water. Two major roles of mobile phase during ionization process are 1) ionizing the uncharged sample molecules into charged ions in the solution. These ions in solution are called preformed ions and 2) evaporating themselves away from the electrosprayed droplets leaving sample ions in gaseous phase. This explains why the mobile phase for ESI is usually the mixture of water (helps dissolving and forming the preformed ions) and volatile, low surface tension organic solvent such as acetonitrile. Usually the lower surface tension of the mobile phase, the better spray (small uniform liquid droplets) can be obtained. Moreover, the volatile mobile phase results in faster evaporation of solvent from the droplets yielding more sample ions in gaseous phase

and less cluster ions of sample and solvents. Practically, appropriate mobile phase depends on the nature of the sample.

2. **Atmospheric Pressure Chemical Ionization (APCI)** is a soft ionization technique. It is an easy to use LC-MS interface which produces singly-charged protonated molecular ions for a broad range of polar and thermally labile analytes. This ionization can also be used alone without LC connection.

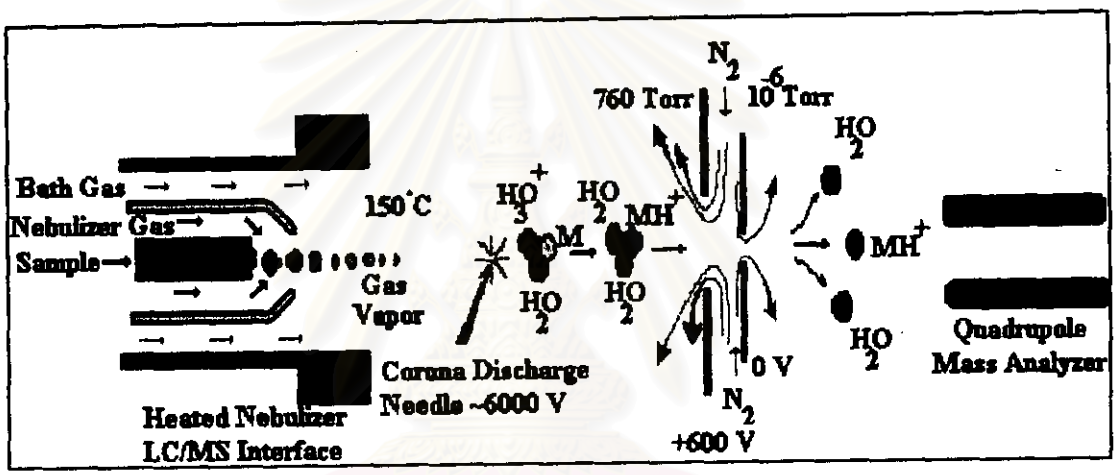


Figure 1.7 APCI ion generation mechanism.

Mobile phase from LC column (or from direct injection) enters the probe where it is pneumatically converted into an aerosol and rapidly heated into the vapor phase at the probe tip. Hot sample droplets evaporate upon entering the heated volume of the source equipped with the corona discharge pin typically maintained at 3 kV. Mobile phase molecules rapidly react with ions from the corona discharge to produce stable reagent ions. Analyte molecules contained in the mobile phase react with reagent ions (through charge transfer reaction such as proton transfer) at atmospheric pressure and

typically become protonated (in positive ion mode). The sample ions are subsequently directed through the skimmer into the mass analyzer.

The ability to operate with 100% organic or 100% aqueous mobile phases at flow rates of up to 2 mL/min makes APCI an ideal technique for analyzing eluent from standard normal phase or reverse phase analytical column (4.6 mm i.d.). The method is not limited to analytes which can form the preform ions in the mobile phase as required in the ESI. The method, however, produces more fragmentation than ESI and usually is less sensitive than the ESI.

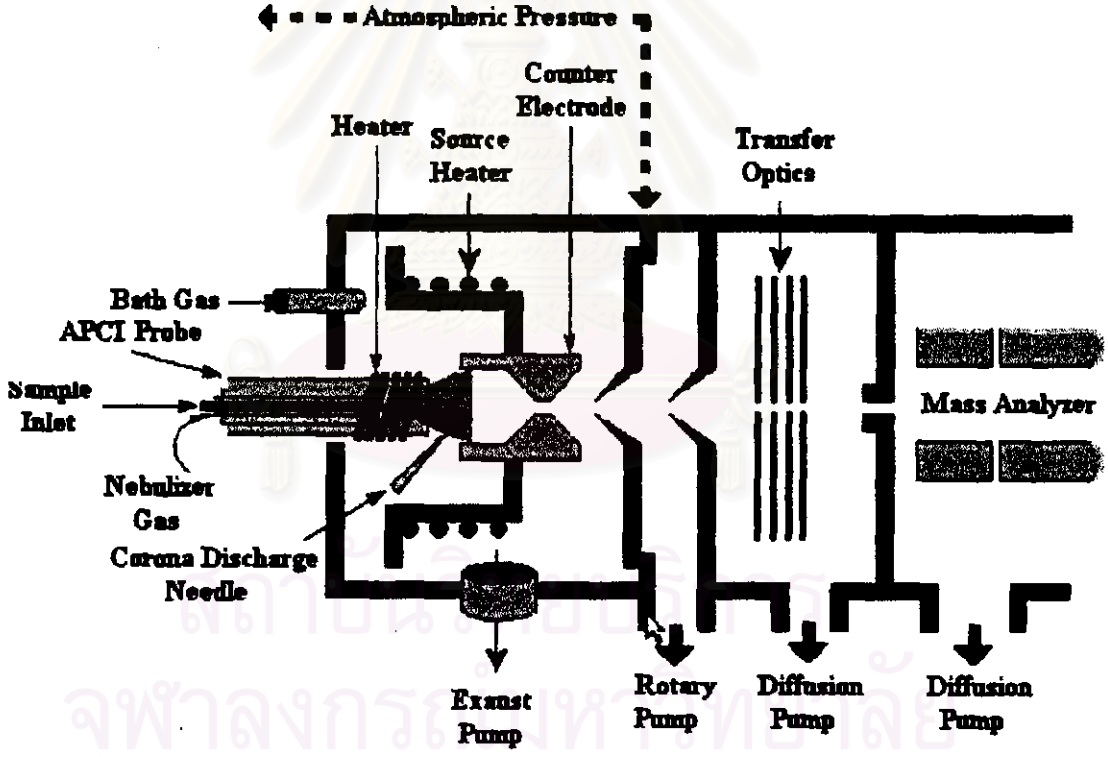


Figure 1.8 Schematic representation of APCI source.

Objective

As mentioned earlier, this work was concentrated on studying of aspartame stability in various environments such as those of different pH values and storage times. Attention was also be paid on elucidation of possible degradation pathways occurred when kept at different conditions. To achieve these purposes, firstly, analytical technique to quanlitate and quantitate aspartame and its degradation products must be established (available techniques do not permit the quantitation of all required degradation products). After obtaining the analytical technique, then the aspartame stabilty study can be done. From the results of the experiments the proposed degradation pathways detected at various conditions were drawn out. And finally, the technique was applied to look into some soft drinks distributed in Thailand. The scope of this study could be concluded as follows:

1. To study the qualitative and quantitative analysis of aspartame by API-MS technique.

2. To study the stability and degradation of aspartame at various pH, temperature and storage time.

3. To determine aspartame and its degradation products in some soft drinks and some dry products.