Chapter II

Experiments

Chemicals

Aspartame (L-(+)-aspartyl-L-phenylalanine methyl ester), aspartic acid and phenylalanine methyl ester hydrochloride, analytical grade, were purchased from Sigma Chemical Co., Ltd., St.Louis, MO, USA.

Aspartyl phenylalanine and Diketopiperazine ((2s-cis)-(-)-5-benzyl-3,6-dioxo-2-piperazineacetic acid), analytical grade, were purchased from Aldrich Chemical Co., Ltd. Milwakee, WI, USA.

Methanol, acetonitrile and sodium hydroxide, analytical grade, were purchased from J.T.Baker Inc., Phillipsberg, NJ, USA.

Phenylalanine, analytical grade, was purchased from Fluka Chemie AG, Buchs, Switzerland.

Formic acid, analytical grade, was purchased from Unilab Ajax Chemical Ltd., Auburn, Australia.

Hydrochloric acid, analytical grade, was purchased from BDH Laboratory Supplies, Poole, England.

Nitrogen gas, high purity (99.99 %), was purchased from Thai Industrial Gas Public Co.,Ltd., Chachungthrau, Thailand.

Equipments and Instruments

High Performance Liquid Chromatograph-Mass Spectrometer (HPLC-MS) High performance liquid chromatography was done on a Water 626 Pump and Water 600 S Controller (Waters Corp., Milford, MA, USA). All mass spectra were obtained with a quadrupole mass spectrometer Model Trio 2000 equipped with atmospheric pressure chemical ionization or electrospray ionization source (VG Biotech, Altrincham, UK).

Methodology

Firstly, LC parameters of ESI and APCI were optimized to get the optimal condition for aspartame detection. By comparing results of ESI and APCI obtained, the ionization method that gave the best sensitivity and repeatibility was chosen for the rest of the study. The stability of aspartame was then studied at various pH and temperature by quantitating the level of aspartame and its degradation products at various storage time. Finally, the real samples, i.e. softdrinks and some dry products (sugar substitute such as Equal), were tested by the established method. The concentrations of aspartame and its degradation products were calculated and results were interpreted.

Experiments

1. **Preparation of standard solutions and samples**

Each *standard solution* was separately prepared into a 50 mL volumetric flask with distilled water. Amounts of standards used were as followed:

1.1 Aspartame, 50 mg, concentration of standard solution = 1000 ppm.

1.2 Phenylalanine, 50 mg, concentration of standard solution = 1000 ppm.

1.3 Aspartic acid, 50 mg, concentration of standard solution = 1000 ppm.

1.4 Phenylalanine methyl ester hydrochloride, 60 mg, concentration of standard solution = 1000 ppm.

1.5 Aspartyl phenylalanine, 10 mg, concentration of standard solution = 200 ppm.

1.6 Diketopiperazine, 10 mg, concentration of standard solution = 200 ppm.

1.7 Methanol, absolute methanol was used as standard solution.

Mobile phase for ESI and APCI was a mixture of acetonitrile and water added with formic acid to give 10 % (V/V) formic acid concentration. The mobile phase was prepared by mixing acetonitrile, water and formic acid into an appropriate ratio, then degassed by ultrasonic bath.

2. Mass Spectrometry

2.1 Optimization of MS parameters for aspartame analysis

Parameters for both ESI and APCI technique were optimized in order to obtain the molecular peak representing aspartame at the highest sensitivity with the least fragmentation. For APCI technique, various parameters were adjusted in the following order; cone voltage, APCI discharge needle voltage, counter electrode voltage, lens2 voltage, lens3 voltage, source temperature, mobile phase composition and flow rate. Optimization of ESI parameters were carried out in a similar manner except that APCI discharge needle voltage was replaced by ESI capillary tip voltage. Mass spectra were obtained under the scan mode from M/Z of 30 to 350 by injecting of 50 μL 100 ppm aspartame solution. After the peak representing molecular ion was identified, the selected ion recording (SIR) mode was used in the parameter-optimization-experiments in which each parameter was varied in order to obtain the maximum peak area. In this case, 50 μL of the 100 ppm aspartame solution was injected for each analysis.

2.2 Determination of Fragmentation Patterns

Fragmentation patterns under optimized condition (obtained from section 2.1) for both ESI and APCI were determined for the following standards;

2.2.1 Aspartame

2.2.2 Aspartic acid

2.2.3 Aspartyl phenylalanine

2.2.4 Diketopiperazine

2.2.5 Phenylalanine

2.2.6 Phenylalanine methyl ester

2.2.7 Methanol

2.2.8 Mobile phase (optimal composition of the mobile phase obtained from section 2.1)

Mass spectra of the standards were obtained under scan mode of appropriated mass range using standard solution of each standard.

2.3 **Detection Limit Experiment**

Detection limit is the minimum concentration or minimum weight of analyte that can be detected at a known confidence level. The number also indicates sensitivity of the technique towards the particular compounds tested, i.e., the lower detection limit for compound X means the higher sensitivity towards this compound. This limit depends upon the ratio of the magnitude of the analytical signal to the size of the statistical fluctuations of the blank signal. For this research, it is the minimum concentration that gives the signal to noise ratio of three. Detection limit of each compound was determined using the signal of molecular ion obtained by SIR mode. This was done by injecting of 50 μL of standard solutions at various concentration and obtaining its SIR spectrum. The experiment was repeated with the more diluted solution until the SIR spectrum gave the S/N of about 3.

Since it is possible that the detection limit might be varied with the pH of the solution, therefore, detection limit at various pH were determined. This was, therefore, tested by adjusting the pH of the standard solution immediately before the run as to minimize the degradation of the compounds.

2.4 Repeatibility Experiment

Ten ppm aspartame solution was used in this study. This solution was prepared by diluting the standard solution with distilled water. The solution was analyzed repeatedly by ESI-MS and APCI-MS using the optimal condition obtained earlier under SIR mode (at M/Z of 295). Each analysis was done by injecting of 50 μL aspartame solution. Peak area from each analysis was recorded and repeatibility of the peak area was calculated.

2.5 Stability of aspartame in different pH solutions

Aspartame solutions were prepared by diluting the aspartame standard solution with distilled water and adjusting the pH to 2 ,4 ,6 , 7, 8 and 10 using HCl and NaOH solutions. The final concentration of the aspartame was 10 ppm. These solutions were then kept at room temperature (about 30 ^{0}C). The pH of the solutions was periodically checked to assure the correct pH of the kept solutions. Fifty microlitres of the kept solutions were withdrawn for analyses at appropriate times. The analysis was done by monitoring the SIR signals at M/Z of 33, 134, 166, 180, 263, 281 and 295 which representing molecular ion of methanol, aspartic acid, phenylalanine, phenylalanine methyl ester, diketopiperazine, aspartyl phenylalanine and aspartame, respectively.

2.6 Stability of aspartame solutions at different temperature

Aspartame solutions were prepared by diluting the aspartame standard solution with distilled water. The final concentration of the aspartame was 10 ppm. These solutions were kept at 4, 30 and 80 ^{0}C. Fifty microlitres of the kept solutions were withdrawn for analyses at appropriate times. The analysis was done by

monitoring the SIR signals at M/Z of 33, 134, 166, 180, 263, 281 and 295 which representing molecular ion of methanol, aspartic acid, phenylalanine, phenylalanine methyl ester, diketopiperazine, aspartyl phenylalanine and aspartame, respectively.

2.7 Stability of aspartame in soft drinks and dry products

Soft drinks (in cans) were bought and stored at room temperature for certain periods. After appropriate storage time, the sample was opened and liquid was transfered into a beaker and then degasified by ultrasonic bath for 15-30 minutes before diluted into an appropriate concentration with distilled water. The sample was analysed by the established method in which 50 μL of the solution were injected into the ESI-MS and SIR was done at M/Z of 33, 134, 166, 180, 263, 281 and 295.

Solid sweeteners were purchased from the stores and separately dissolving in 25 ^{0}C and 100 ^{0}C distilled water to give the concentration of about 190 ppm. This solution was then degassed and analyzed by ESI-MS under SIR at M/Z of 33, 134, 166, 180, 263, 281 and 295.