CHAPTER 2

EXPERIMENT

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1) Gas chromatograph, HP 5890 series II plus, manufactured by Hewlett Packard, U.S.A. consisting of
   a) Headspace sampler, HP 7694
   b) Flame ionization detector (FID)
   c) Data processing system, Hewlett Packard Vectra 486/33M
   d) Capillary column;
      - DB-1701, 15m × 0.32 mm I.D., 0.15 μm film thickness, J&W Scientific, U.S.A.
      - DB-17MS, 30m × 0.32 mm I.D., 0.25 μm film thickness, J&W Scientific, U.S.A.
2) Vacuum ratory evaporator, B-169, manufactured by Buchi, Switzerland
3) Magnetic stirrer and Magnetic bar
4) Blender, manufactured by Maulinex, Ireland
5) Gas-tight ballon
6) Autopipette, manufactured by BRAND, Germany
7) Hand crimper, 20 mm cap, manufactured by Supelco, U.S.A.
8) Headspace vial 22 ml, Hewlett Packard, U.S.A.

9) Septa (PTFE faced silicone) 22 mm, Supelco, U.S.A.

10) Centrifuge, Cole-Parmer, U.S.A.

2.1.2 Chemicals

1) 2-Acetylpyrrole, >98.0%, Fluka, Switzerland

2) 5% Rhodium on activated alumina (5%Rh on Al), AR grade, Fluka, Switzerland

3) Silver nitrate, 99.5%, AR grade, BDH, England

4) Celite, Fluka, Switzerland

5) Sodium carbonate, 99.5%, AR grade, Merck, Germany

6) Methanol, 99.0% v/v, AR grade, J.T. Baker, USA

7) Hydrochloric acid, 37.0% w/v, BDH, England

8) Toluene, > 99.0% v/v, BDH, England

9) Sodium hydroxide, AR grade, Merck, Germany

10) Nitrogen gas, 99.99% (HP grade), TIG, Thailand

11) Air, Air-zero grade, TIG, Thailand

12) Hydrogen gas, 99.99% (HP grade), TIG, Thailand

2.2 Preparation of 1.0 M sodium hydroxide (NaOH)

1.0 M NaOH was prepared by dissolving 4.00 g of sodium hydroxide in water. Then the solution was adjusted to 100 ml in volumetric flask.

2.3 Preparation of 0.1 M Hydrochloric acid

37% w/w of hydrochloric acid solution was pipetted 8.28 ml into a volumetric flask. Then the solution was adjusted to 1000 ml by water.

2.4 Preparation of the internal standard, 2,4,6-trimethylpyridine (TMP), solution

2.4.1 Preparation of TMP 100 ppm solution

TMP 100 ppm was prepared by pipetting 27.63 µl of TMP into 250 ml volumetric flask. This solution was adjusted to 250 ml by 0.1 M HCl.

2.4.2 Preparation of TMP 0.25 ppm solution

TPM 0.25 ppm was prepared by pipetting 2.50 ml of 100 ppm in 1000 ml volumetric flask. Then the solution was adjusted to 1000 ml by 0.1 M HCl. This solution was used for the extraction of samples and standard solutions.

2.5 Preparation of standard solutions

2.5.1 Preparation of 2-AP stock solution (10 ppm)

2-AP stock solution was prepared by diluting synthetic 2-AP solution with toluene. The synthetic 2-AP solution was calculated, according to appendix B, to contain 1089 mg/l of 2-AP. It was pipetted 459.10 µl and adjusted to 50 ml in a volumetric flask.

2.5.2 Preparation of 2-AP standard solution (0.50-8.00 ppm)

Stock solution was diluted to 0.50 ppm, 1.00 ppm, 2.00 ppm, 4.00 ppm, and 8.00 ppm solution with toluene in order to made a standard calibration curve. It was prepared as listed in table 2.1.

Table 2.1 Composition of the standard solutions

Concentration of 2-AP (ppm)	Volume of stock solution B (ml)
0.50	0.50
1.00	1.00
2.00	2.00
4.00	4.00
8.00	8.00

These solutions were adjusted to 10 ml in a 10 ml volumetric flask.

2.6 Extraction of 2-AP in standard solutions by acidic solvent

Before subjecting to analysis by HS-GC, 2-AP in each standard solutions, 0.50 to 8.00 ppm, were extracted by 0.1 M HCl containing 0.25 ppm TMP. The extraction procedure was shown in Figure 2.1.

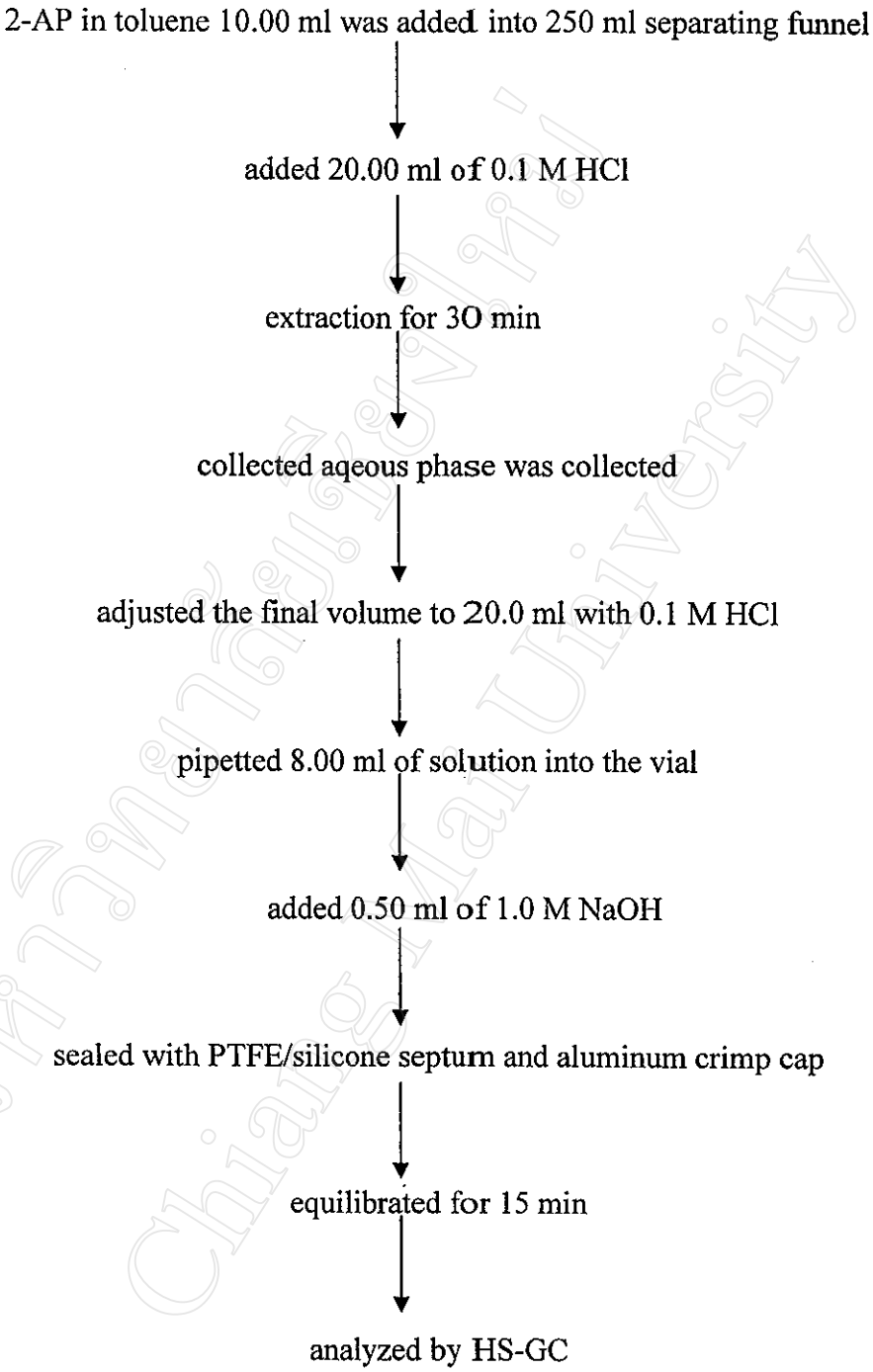

Figure 2.1 Scheme for extraction of standard solutions.

2.7 Extraction of 2-AP in rice seed extract solution by acidic solvent

2-AP was extracted from brown rice seed by 0.1 M HCl containing 0.25 ppm TMP. The extraction procedure was shown in Figure 2.2.

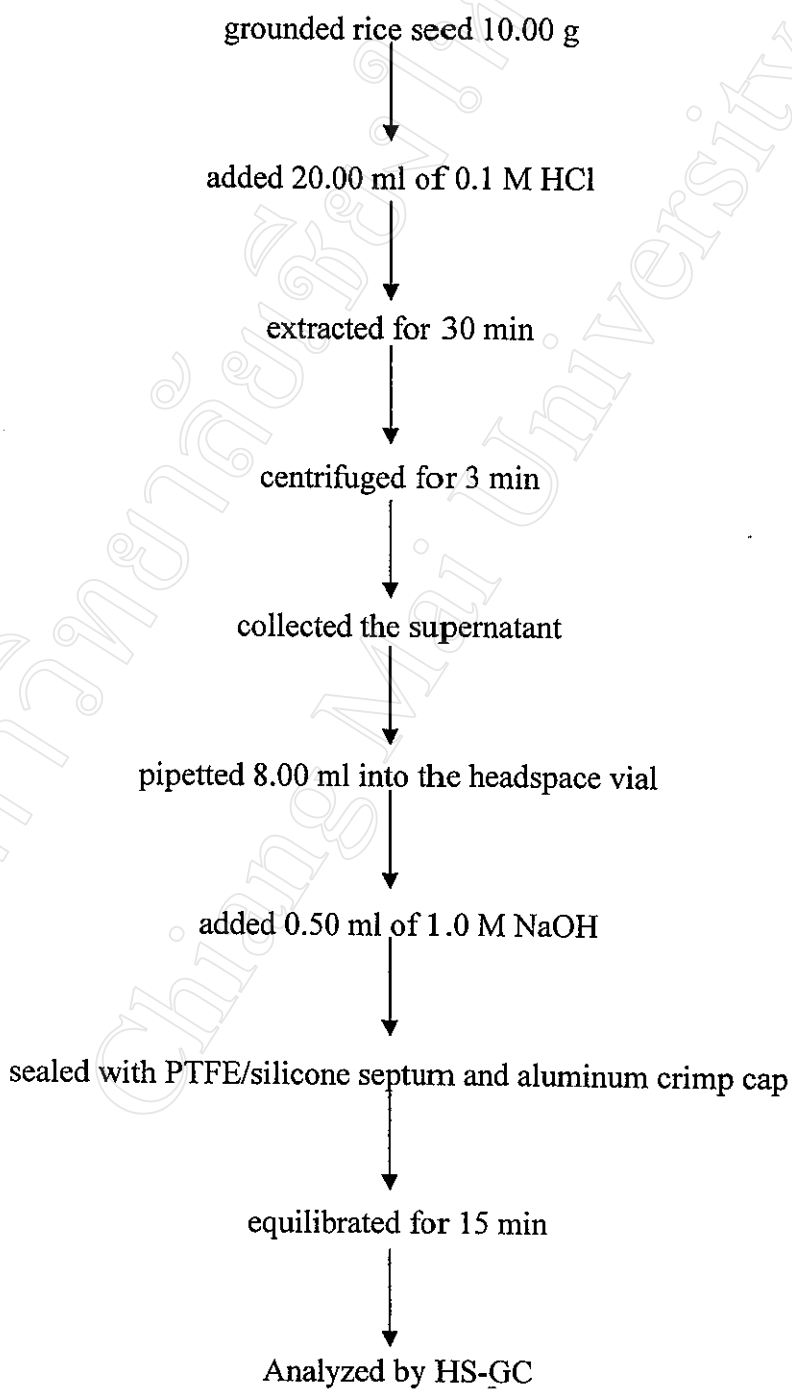

Figure 2.2 Scheme for extraction of rice seed.

2.8 Identification of 2-AP and internal standard (TMP) in rice seed extract

In this work, DB-1701 and DB-17MS capillary column were used for optimization of HS-GC conditions and quantification of 2-AP, respectively. The identification of 2-AP and TMP in the rice seed extract chromatogram obtained by HS-GC was performed on both columns.

The rice seed extract solution was prepared according to the scheme in Figure 2.2 and standard 2-AP solution was prepared according to scheme in Figure 2.1. Under the optimal extraction and automated headspace sampler conditions, identification of 2-AP and TMP was investigated by comparing the retention times with those of standard 2-AP and TMP. Spiking standard 2-AP solution into the rice seed extract in order to confirm its identity was also preformed. The extract was then analyzed by HS-GC using DB-1701 and DB-17MS column.

2.9 HS-GC instrumental conditions

In this work, 0.1 M HCl was used to extract 2-AP from rice seeds. The extract was then made alkaline and subjected to analysis by HS-GC. Automated headspace sampler conditions are shown in Table 2.2

Table 2.2 Conditions of automated headspace sampler

Operation	Conditions
1. Temperature (vial, sample loop and transfer line)	120,140,160 °C
2. Vial equilibration time	10 min
3. Shaking (mixing) speed	High
4. Loop filling time	0.3 min
5. Loop equilibration time	0.2 min
6. Pressurization time	0.5 min
7. Loop injection time	0.2 min

Gas chromatographic conditions for separation of 2-AP and TMP were investigated. It was carried out in order to obtain adequate separation of peaks on the chromatogram with both reasonably short analysis time and good resolution.

Gas chromatographic separation was achieved on a gas chromatograph equipped with a flame ionization detector. The conditions of GC are shown in Table 2.3.

Table 2.3 Conditions of GC

Operation	Conditions
1. Column	DB-1701, 15 mx0.32 mm I.D., 0.15 μm film thickness DB-17MS, 30 mx0.32 mm I.D., 0.25 μm film thickness
2. Temperature program	45 °C hold 2 min, the ramped up to 80 °C
3. Injection temperature	200 °C
4. Detector temperature	230 °C
5. Injection mode	Splitless
6.Carrier gas	N_2 at 5.5 ml/min (for DB-1701) N_2 at 3.0 ml/min (for DB-17MS)

2.10 Optimization

The rice seed extract solutions were used for optimization of both extraction conditions and automated headspace sampler parameters. The HS-GC employed DB-1701 capillary column was run for 11 min.

2.10.1 Optimization of extraction conditions

The rice seed extract solutions were prepared according to the scheme in Figure 2.2 with each of extraction conditions varying as following:

2.10.1.1 Extraction time

When 2-AP in rice seeds was extracted by 0.1 M HCl, extraction time was varied from 5 to 40 min with 5 min increment. The other conditions were maintained as the same as listed in Figure 2.2. HS-GC conditions used was listed in Table 2.2-2.3.

2.10.1.2 Volume of 1.0 M NaOH added into the extract solution

The rice seed extract solution was made alkaline using 1.0 M NaOH. Initially, 0.50 ml of NaOH was added into the rice seed extract before it was subjected to analysis by HS-GC. Then, the volume of 1.0 M NaOH was varied from 0.10 to 1.00 ml with 0.10 ml increment. The extraction time was 30 min. The other conditions were maintained as the same as listed in Figure 2.2. HS-GC conditions used was used listed in Table 2.2-2.3.

2.10.1.3 Equilibrium time after 1.0 M NaOH was added into rice seed extract

After the rice seed extract solution was made alkaline using 1.0 M NaOH. The extracts were allowed to equilibrate with equilibrium time varied from 5 to 40 min with 5 min increment whereas the extraction time was 30 min and volume of 1.0 M NaOH was 0.50 ml. The other conditions were maintained as the same as listed in Figure 2.2. HS-GC conditions used was listed in Table 2.2-2.3.

2.10.2 Optimization of automated headspace sampler parameters

There are many instrument parameters of headspace sampler that can affect the sensitivity, precision and accuracy of headspace analysis. These include

temperature of oven, sample loop and transfer line and time for vial equilibration, loop filling, loop equilibration, pressurization and injection.

2.10.2.1 Optimization of temperature of vial, transfer line and sample loop

The rice seed extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The series of temperature of vial, sample loop and transfer line was varied from 70, 90, 110 to 120, 140, 160 °C, respectively with 10 °C increment whereas the other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.10.2.2 Optimization of vial equilibration time

The rice seed extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The vial equilibration time was varied from 1 to 8 min with 1 min increment whereas the temperature of vial, sample loop and transfer line were set to 120, 140 and 160 °C, respectively. The other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.10.2.3 Optimization of pressurizing time

The rice extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The pressurization time was varied from 0.1 to 0.5 min with 0.1 min increment whereas the temperature of vial, sample loop and transfer line were set to 120, 140 and 160 °C, respectively and vial equilibration time was 3 min. The other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.10.2.4 Optimization of loop filling time

The rice seed extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The loop filling time was varied from 0.5 to 3.0 min with 0.5 min increment whereas the temperature of vial, sample loop and transfer line were set to 120, 140 and 160 °C, respectively, vial equilibration time was 3 min and pressurizing time was 0.3 min. The other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.10.2.5 Optimization of loop equilibration time

The rice seed extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The loop fill time was varied from 0.01 to 0.08 min with 0.01 min increment whereas the temperature of vial, sample loop and transfer line were set to 120, 140 and 160 °C, respectively, vial equilibration time was 3 min, pressurizing time was 0.3 min and loop filling time was 2 min. The other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.10.2.6 Optimization of loop injection time

The rice seed extract solution was prepared according to scheme in Figure 2.2. The headspace of solution was analyzed by HS-GC. The loop injection time was varied from 0.1 to 0.7 min with 0.1 min increment whereas whereas the temperature of vial, sample loop and transfer line were set to 120, 140 and 160 °C, respectively, vial equilibration time was 3 min, pressurizing time was 0.3 min, loop filling time was

2 min and loop equilibration time 0.04 min. The other parameters were maintained as the same conditions as listed in Table 2.2-2.3.

2.11 Construction of calibration curve

For quantification of 2-AP released in rice seeds, the internal standard method was used for construction of calibration curve. A series of standard 2-AP solution was prepared as shown in Table 2.1. 2-AP in each standard solution was extracted into aqueous acidic solution. Extraction steps followed the scheme shown in Figure 2.1.

In this part of experiment, DB-17MS capillary column was used for quantification of 2-AP in headspace of the scented rice extract. The carrier gas was nitrogen at constant flow rate of 3.0 ml/min.

2.12 Validation

After establishing the optimum conditions of extraction and HS-GC, the detection limit, linearity and precision were investigated.

2.12.1 Detection limit

The detection limit for amount of rice seeds which were extracted by 0.1M HCl at optimum conditions was calculated by comparing the integrated peak height of 2-AP in the GC-FID chromatogram. The rice seeds used were KDML 105 from Chiang mai harvested in 2001. The criteria was signal/noise (S/N) ratio of a

minimum 3:1. The detection limits in term of the lowest amount of rice seed sample required are shown in Table 3.13.

Limit of detection (LOD) in term of the least amount of analyte, 2-AP, that yields the minimal detectable signal and gives clearly defined peak signal greater than a blank signal (signal-to-noise ratio ca. 3) [38] can be calculated from the linear regression line of the calibration curve as described in Appendix C.

In the analysis of rice seed extracts, calculation of the limit of detection was based on the internal standard calibration curve in the concentration range 0.50-8.00 ppm.

2.12.2 Linearity

The linearity plot was constructed for 2-AP standard solution in the concentration range 0.50-15.00 ppm. 2-AP in each standard solution was extracted into aqueous acidic solution as shown in Figure 2.1. The result of initial concentrations of standard 2-AP, the adjusted concentration which calculated based on % recovery and peak area ratio of 2-AP to TMP are shown in Table 3.14. Calibration curve in the range of 0.37-11.57 ppm are shown in Figure 3.21.

2.12.3 Precision

The precision is reproducibility. It gives a measure of error in the development methodology and usually reported as a percent of relative standard deviation (%R.S.D.). The precision was determined by analyzing rice extract solution

once a day for seven days for reproducibility. The results of reproducibility is shown in Table 3.15. %R.S.D. can be calculated as described in Appendix C.

2.12.4 Recovery assay

Under the optimal conditions of the research work, extraction efficiency of the method was investigated by recovery test. It was done by using standard solution extract at 2-AP concentration of 8.00 ppm and the rice seed extract. The percent recovery was calculated based on peak area. The results are shown in Table 3.16-3.17.

2.12.4.1 Preparation of standard solution for recovery assay

2-AP standard toluene solution at 0.50-8.00 ppm was analyzed by GC/FID. This solution was extracted by 0.1 M HCl. Then, organic phase was collected and analyzed by GC/FID. The percentage recovery was calculated based on peak area of 2-AP as described in Appendix C.

2.12.4.2 Preparation of rice sample for recovery assay

Ground KDML 105 Chiang mai, KDML 105 Tungkularonghai, Hom Supanburi and Hom Patumtani seeds were extracted by 0.1 M. HCl (1^{st} extraction). The supernatant, rice seed extract solution, and solid were separated. The rice seed extract solution was analyzed by HS-GC. The remaining solid was extracted again by 0.1 M HCl (2^{nd} extraction) and the extract solution was analyzed by HS-GC. The percentage recovery was calculated based on peak area of 2-AP as described in Appendix C.

2.13 Analysis of real samples

In this work, the optimized analytical method was applied for the determination of 2-AP in rice seed samples. The rice extract was prepared according to the scheme in Figure 2.2. The headspace of the extract was subjected to analysis by HS-GC. Four samples of rices were selected for determination of 2-AP as shown in Table 2.4.

Table 2.4 Information of rice samples

Sample no.	Rice Sample
1	KDML 105 Chiang mai, brown rice, harvested in 2001
2	KDML 105 Tungkularonghai, brown rice, harvested in 2002
3	Hom Supanburi, brown rice, harvested in 2001
4	Hom Patumtani, brown rice, harvested in 2001