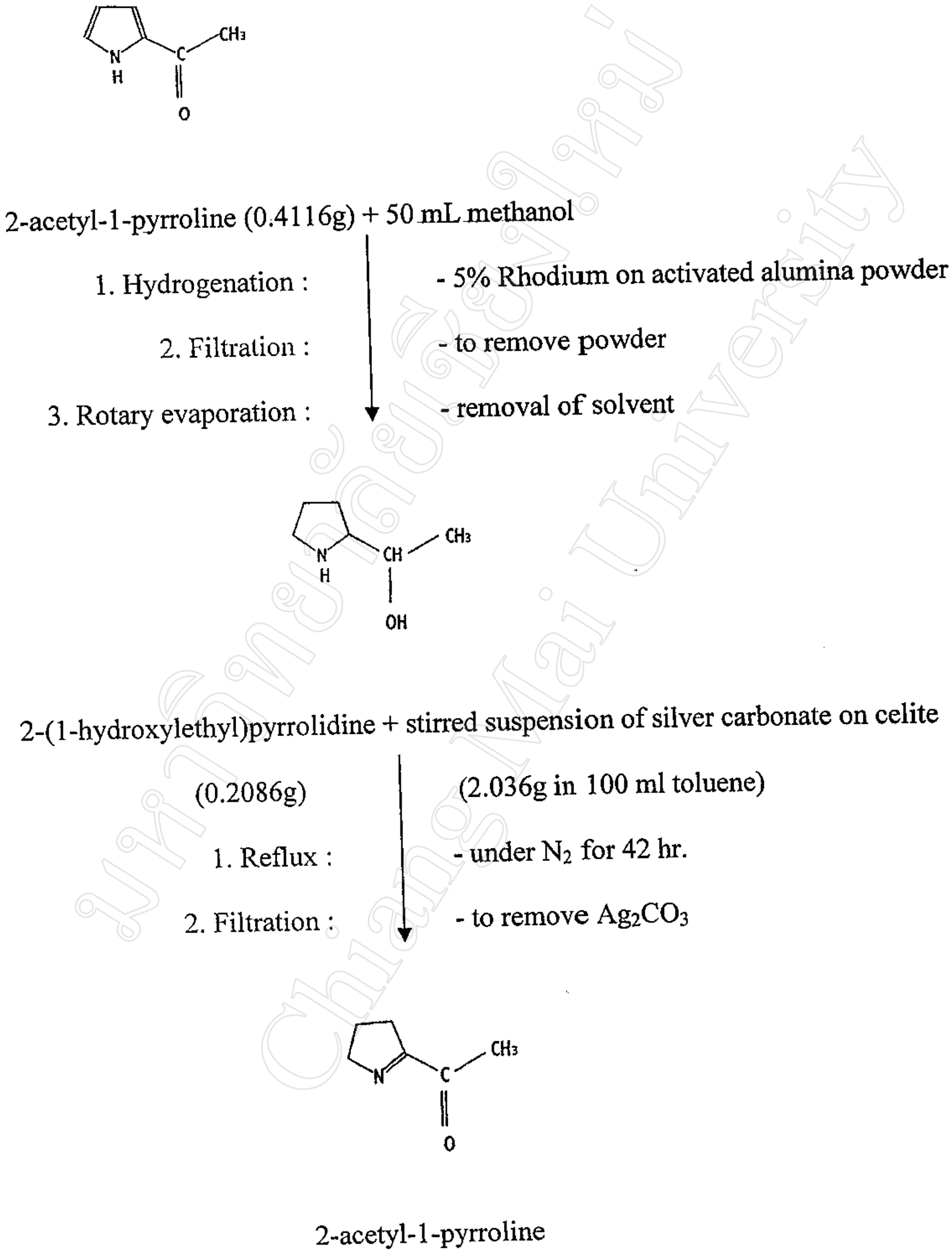
APPENDIX A

Synthesis of 2-acetyl-1-pyrroline (2-AP)

A synthetic standard of 2-AP was obtained using the method outlined by Buttery and Wuest [6]. There were two steps carried out according to the scheme in Figure A1. 2-Acetylpyrrole was used as a starting material. In the first step, it was converted into 2-(1-hydroxyethyl) pyrrolidine by hydrogenation reaction catalized by 5% rhodium on activated alumina. Then, this product was oxidized to yield 2-AP in the second step.



2-acetyl-1-pyrroline (0.4116g) + 50 mL methanol

1. Hydrogenation : - 5% Rhodium on activated alumina powder
2. Filtration : - to remove powder
3. Rotary evaporation : - removal of solvent

2-(1-hydroxylethyl)pyrrolidine + stirred suspension of silver carbonate on celite

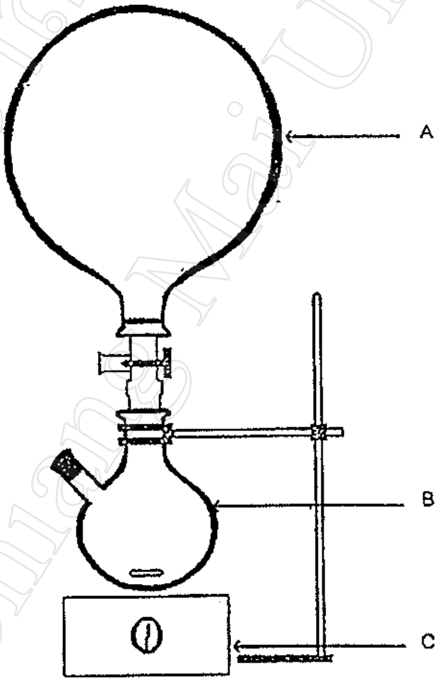
(0.2086g) (2.036g in 100 ml toluene)

1. Reflux : - under N_2 for 42 hr.
2. Filtration : - to remove Ag_2CO_3

2-acetyl-1-pyrroline

Figure A1 Scheme for synthesis of 2-AP

In this experiment, 2-acetylpyrrole weighted 0.4116 g was dissolved in 50 ml methanol. The solution was then hydrogenated by hydrogen (H_2) using 5% rhodium on alumina 0.556 g as catalyst at room temperature with constant stirring. According to the scheme in Figure A2, after filtration and removal of the solvent by rotary evaporation, 0.2806g of crude intermediate 2-(1-hydroxyethyl) pyrrolidine was yielded. The 2-(1-hydroxyethyl) pyrrolidine was dissolved in 100 ml toluene. The mixture was refluxed under nitrogen (N_2) for 42 hrs. Removal of the silver carbonate by filtration gave a toluene solution of 2-acetyl-1-pyrroline.



A = Gas-tight balloon containing N_2

B = Reaction bottle containing hydrogenation solution

C = Magnetic strirrer

Figure A2 Scheme of apparatus for hydrogenation reaction

APPENDIX B

1. Preparation of 100 ppm TMP ($C_8H_{11}N$, MW = 121.18 g/mol) in 250 ml solution

The density of stock solution is 0.914 g/ml. The % assay is 99. This means that TMP stock solution 100 ml contained TMP 99 ml.

Weight of TMP is calculated using equation,

$$D = wt / V \qquad \text{(B-1)}$$

Where V is volume of solution, wt is weight of TMP and D is density of solution.

Thus, weight of TMP in 99 ml of stock solution = 0.194 g/ml x 99 ml

= 90.486 g

There is 90.486 g of TMP in TMP stock solution 100 ml.

Thus, concentration of TMP stock solution is 904860 ppm (mg/L)

Then 100 ppm of TMP was prepared using TMP stock solution. Volume of TMP stock solution is calculated using equation,

$$C_1V_1 = C_2V_2 \qquad \text{(B-2)}$$

V_1 is the volume of TMP stock solution needed to prepare 250 ml (V_2) of the 100 ppm solution. C_1 and C_2 are the concentrations of TMP stock solution and 100 ppm solution, respectively.

$$V_1 = C_2V_2 / C_1$$

Thus, volume of TMP stock solution needed (V_2) = (100 ppm x 250 ml)/904860 ppm

= 27.63 μl

Pipetted TMP stock solution 27.63 μl into 250 ml volumetric flask and adjusted volume to 250 ml with 0.1 M HCl.

2. Preparation 1000 ml of 0.25 ppm of TMP

0.25 ppm of TMP was prepared using 100 ppm TMP solution. Volume of 100 ppm TMP solution needed is calculated using equation (B-2),

$$C_1V_1 = C_2V_2$$

Let V_1 be the volume of 100 ppm TMP solution needed to prepare 1000 ml (V_2) of the 0.25 ppm solution; C_1 and C_2 are the concentration of TMP solution in 100 ppm and 0.25 ppm, respectively.

$$V_1 = C_2V_2 / C_1$$

Therefore, volume of 100 ppm TMP solution needed (V_2) = (0.25 ppm x 1000 ml) / 100 ppm

= 2.50 ml

Pipetted 100 ppm TMP solution 2.50 ml into 1000 ml volumetric flask and adjusted volume to 1000 ml with 0.1 M HCl.

3. Preparation of 2-AP standard solution

3.1 Calculation of concentration of synthetic 2-AP solution

There were two steps for synthesis of 2-AP. 2-Acetylpyrrole (2A) was used in the first step (C_6H_7NO, MW = 109.135 g/mol). 2A was converted into hydrogenation product, 2-(1-hydroxyethyl)pyrrolidine ($C_6H_{13}NO$, MW = 115.189). In this reaction, only one product was yield and weight of hydrogenation product was 0.2806 g.

Mole of hydrogenation product is calculated using equation.

$$n = g / MW \qquad \text{(B-3)}$$

Thus, mole of hydrogenation product = 0.2806 / 115.189 mole

= 0.00244 mole

In the second step, hydrogenation product, 2-(1-hydroxyethyl) pyrrolidine, was oxidized. It was converted into oxidation product, 2-AP,(C_6H_9NO, MW = 111.153)

In this step, many products were obtained in toluene solution. All oxidation products were analyzed by GC for determination percentage of 2-AP in the reaction solution. It was calculated to be 40.14% according to percentage of peak area.

hydrogenation product 100 mole converted into 2-AP 40.14 mole

hydrogenation product 0.00244 mole converted into 2-AP $\frac{40.14 \times 0.00244}{100}$ mole

= 0.0009794 mole

From equation (B-3)

Weight of 2-AP is calculated using equation.

$$g = n \times MW \qquad \text{(B-4)}$$

therefore, weight of 2-AP = 0.0009794 x 111.153 g.

= 0.1089 g.

therefore, concentration of 2-AP in 100 ml toluene = 0.1089 g / 100 ml

= 0.001089 g/ml

= 1089 μg/l (ppm)

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

10 ppm of 2-AP stock solution was prepared using 1089 ppm synthetic 2-AP solution. Volume of 1089 ppm synthetic 2-AP solution needed is calculated using equation (B-2),

$$C_1V_1 = C_2V_2$$

V_1 is the volume of 1089 ppm synthetic 2-AP solution needed to prepare 50 ml(V_2) of the 10 ppm 2-AP stock solution. C_1 and C_2 are the concentrations of 1089 ppm synthetic 2-AP solution and 10 ppm stock solution.

$$V_1 = C_2V_2 / C_1$$

Thus, volume of 1089 ppm synthetic 2-AP solution = (10 ppm x 50 ml) / 1089 ppm

= 0.4591 ml

= 549.10 μl

Ten ppm of 2-AP stock solution can be prepared by pipetting 1089 ppm synthetic 2-AP solution 459.10 μl into volumetric flask and adjusting volume to 50 ml with toluene.

This stock solution was diluted again to 0.50-8.00 ppm solutions with toluene in order to made a standard calibration curve. It was prepared as listed in Table 2.1.

APPENDIX C

1. Percent of relative standard deviation (% R.S.D.)

Percent of relative standard deviation (% R.S.D.) can be calculated from the following equation. [39]

$$\% \text{R.S.D.} = (s / \bar{X})100 \qquad \text{(C-1)}$$

where % R.S.D. = percent of relative standard deviation

s = standard deviation

$\bar{X}$ = mean meassured value

s can be calculated from this equation

$$s = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})}{n-1}} \qquad \text{(C-2)}$$

where x_i = individual meassured value

n = number of meassurements

2. Recovery assay

2.1 Recovery assay of standard extract solution

The data for calculation of the recovery assay is shown in Table 3.16. The recovery assay was calculated based on peak area.

For example, the calculation of recovery assay in replicate number 1 is shown as follow.

peak area of 2-AP before extraction = 1154

peak area of 2-AP after extraction = 276

therefore, peak area of 2-AP which was extracted = 1154-276

2-AP before extract 1154, recovery assay 100 %

2-AP was extracted 1154-276, recovery assay $= \frac{1154-276}{1154} \times 100 = 76.08\ \%$

% Recovery in replicate number 2 and 3 can be calculated as same as the calculation in replicate number 1.

2.2 Recovery assay of rice extract solution

The data for calculation of the recovery assay is shown in Table 3.17. The recovery assay was calculated based on peak area.

For example, the calculation of recovery assay of KDML 105 Chaing mai extraction is shown as follow.

peak area of 1st extraction = 739

peak area of 2nd extraction = 152

therefore, 2-AP was extracted = 739-152

peak area of 2-AP 739, recovery assay 100 %

peak area of 2-AP 739-152, recovery assay $= \frac{739 - 152}{739} \times 100 = 79.43\ \%$

% Recovery of KDML 105 Tungkularonghai, Hom Supanburi and Hom Patumtani can be calculated as same as the calculation in KDML 105 Chiang mai.

2. Limit of Detection (LOD) calculation

The limit of detection was calculated from the linear regression line of the calibration curve.

$y = a + bx$ (C-3)

y = instrument signals

x = concentrations

a = intercept

b = slope of the straight line

$Y_L = Y_B + kS_B$ (C-4)

Y_L = lowest detectable instrument signals

Y_B = blank signal

$\cong$ intercept, a

k = constant depending on definition such as $k = 3, 1.5$ or 10 according to IUPAC, in calculation of LOD, $k = 3$ was normally used and was also used in this work.

S_B = blank signal standard deviation

$\cong S_{y/x}$

$S_{y/x}$ can be calculated from the equation

$S_{y/x} = \{\Sigma(Y_i - \hat{Y}_i)^2 / (n-2)\}^{1/2}$ (C-5)

Y_i = response value from the instrument corresponding to the individual x – values

$\hat{Y}_i$ = value of y on the calculated regression line corresponding to the individual x – values

n = number of points on the calibration line

From equation (A-9) and (A-10)

$$Y_L = a + 3\,S_{y/x} \qquad \text{(C-6)}$$

$$Y_L = a + bC_L \qquad \text{(C-7)}$$

Thus, $$a + 3\,S_{y/x} = a + bC_L$$

$$C_L = 3\,S_{y/x}\,/b \qquad \text{(C-8)}$$

Calculation of limit of detection (LOD) in term of the least amount of analyte, 2-AP, was performed as shown in Table C2. The linear regression equation is $Y = 0.1973x + 0.181$

Table C1 Calculation data of concentration on the linear regression equation

Conc (ppm)	Y_i	$\hat{Y}_i$	$\lvert Y_i - \hat{Y}_i \rvert$	$\lvert Y_i - \hat{Y}_i \rvert^2$
0.37	0.237	0.254	0.017	2.89×10^{-4}
0.74	0.326	0.327	0.001	1.00×10^{-6}
1.48	0.490	0.473	0.017	2.89×10^{-4}
2.97	0.775	0.767	0.008	6.40×10^{-5}
5.93	1.344	1.351	0.007	4.90×10^{-5}
			$\Sigma\lvert Y_i - \hat{Y}_i \rvert^2$	6.92×10^{-4}

$$S_{y/x} = [6.92 \times 10^{-4} / (5-2)]^{1/2} = 0.015$$

$$C_L = 3(0.015) / 0.1973$$

$$= 0.23 \text{ ppm}$$

APPENDIX D

1. Calibration curve

Series of 2-AP standard solution with different concentrations within the ranges of 0.5-8.0 ppm were used for construction of calibration curve. Recovery assay of standard extraction was 74.13 %. Thus the concentration of standard solutions calculated based on %recovery for each standard solution were found to be in the range of 0.37-5.93 ppm. (see Table D1)

Table D1 Data for calculation of concentration of 2-AP

Concentration (ppm) of 2-AP in toluene solution	Concentration of 2-AP in acidic solution
0.50	0.37
1.00	0.74
2.00	1.48
4.00	2.97
8.00	5.93

2. Quantity of 2-AP in Rice Samples

The quantity of 2-AP in rice samples was determined based on the calibration curve in Figure 3.20. Concentration ranges were in the range 0.37 – 5.93 ppm with a correlation coefficient of 0.9991 and the regression equation is $y = 0.1973X + 0.181$.

First, 2AP was calculated based on regression equation, $y = 0.1973X + 0.181$, (y = peak area ratio of 2-AP and TMP, x = concentration of 2-AP). Then the concentration of 2-AP in rice sample (x) must be calculated based on %recovery of rice extraction.

For example, the calculation of 2-AP in KDML 105 Chiang mai is shown as follows:

First, the concentration of 2-AP (x) was calculated based on regression equation and it was found to be 0.938 ppm. The recovery of 2-AP in acidic solution of KDML 105 Chiang mai extract was found to be 79.27%.

Thus, concentration of 2-AP in rice extract sample was calculated to be

$$= \frac{(0.938 \times 100)}{79.27} \text{ ppm}$$

$$= 1.18 \text{ ppm}$$

therefore, 2-AP was determined in KDML 105 Chiang mai at the concentration of 1.18 ppm.

The other samples can be calculated by the same method as demonstrated above. The data for calculation of concentration of 2-AP in rice samples is shown in Table D2.

Table D2 Data for calculation of concentrations of 2-AP in rice samples

Samples	P_A Ratio of 2-AP / TMP	Conc. of 2-AP in acidic solution (ppm) (x)	Conc. of 2-AP in rice seeds (ppm)
KDML 105 Chiang mai	0.336	0.938	1.18
KDML 105 Tungkularonghai	0.545	1.845	2.41
Howm Supanburi	0.243	0.314	0.41

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