CHAPTER 3

RESULTS AND DISCUSSION

Quantification of aroma compound, 2-acetyl-1-pyrroline (2-AP), in rice seed extracts by HS-GC was attempted in this work. 2-AP was extracted from rice seeds by acidic solvent extraction. The rice seed extract was made alkaline using NaOH in order to make the compound less dissolved in the extract solution. Then, the headspace of the rice seed extract was subjected to analysis by HS-GC. This method provided shorter analysis time. Thus number of samples to be analyzed can be increased compared with all the conventional methods. The conventional methods for extraction aroma compound that employ steam distillation and solvent extraction, resulting in longer time for sample preparation, high solvent consumption and degradation of aroma compounds of interest. Apart from these, HS-GC offers advantages over the direct injection technique. In headspace analysis, only volatile components are introduced into the GC system, it provides superior sensitivity.

In this work, the headspace of rice seed extract was analyzed by GC-FID. Quantification was preformed by internal standard method. DB-1701 capillary column was used for optimization of extraction and automated headspace sampler conditions and DB-17MS capillary column was used for quantification of 2-AP in rice seed extract.

3.1 Identification of 2-AP and internal standard (TMP) on chromatogram of the rice seed extract using optimization of extraction method.

3.1.1 Identification of 2-AP and TMP in headspace of the rice seed extract using DB-1701 column

2-AP and TMP peaks on chromatogram obtained by HS-GC using DB-1701 column of the rice seed extract were identified by means of comparing the retention time of 2-AP in the rice seed extract and the synthetic 2-AP and standard solution of TMP chromatographed under the same conditions. It was found that the retention time of 2-AP and TMP were 2.45 min and 3.32 min, respectively. HS-GC chromatographic patterns of the rice seed extract containing 2-AP and internal standard TMP is shown in Figure 3.1.

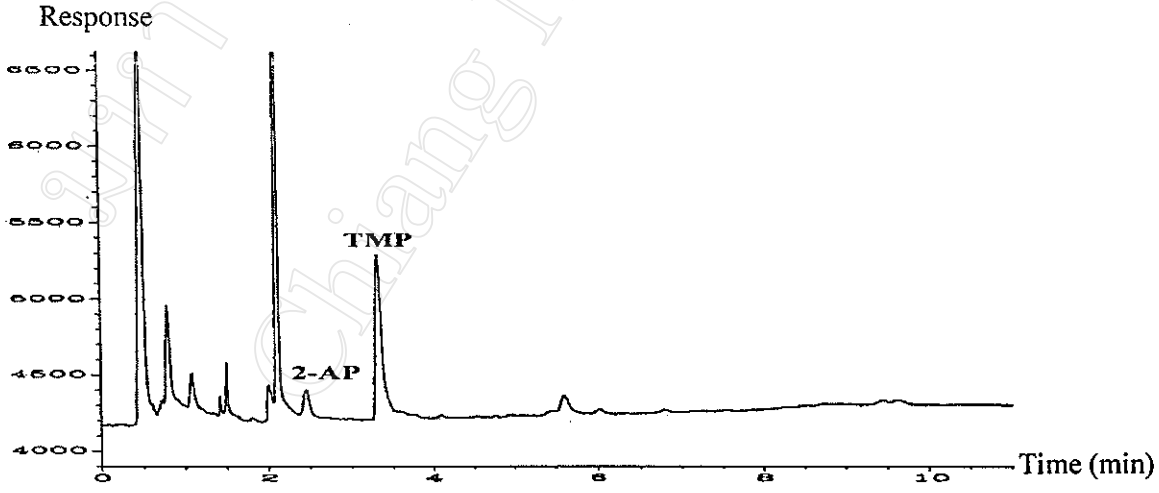

Figure 3.1 HS-GC chromatographic pattern of rice seed extract containing 2-AP and TMP.

Under the optimal conditions, identification of 2-AP and TMP in the rice seed extract containing TMP was also investigated by spiking standard solution of 2-AP and TMP in rice seed extract. The rice extract was then analyzed by HS-GC using DB-1701 column. HS-GC chromatogram of the nonspiked and spiked rice seed extract are shown in Figures 3.2-3.3. It was found that peak area of 2-AP and TMP in HS-GC chromatogram of the spiked rice seed extract were increased compared with peak area of 2-AP and TMP in HS-GC chromatogram of the nonspiked rice seed extract.

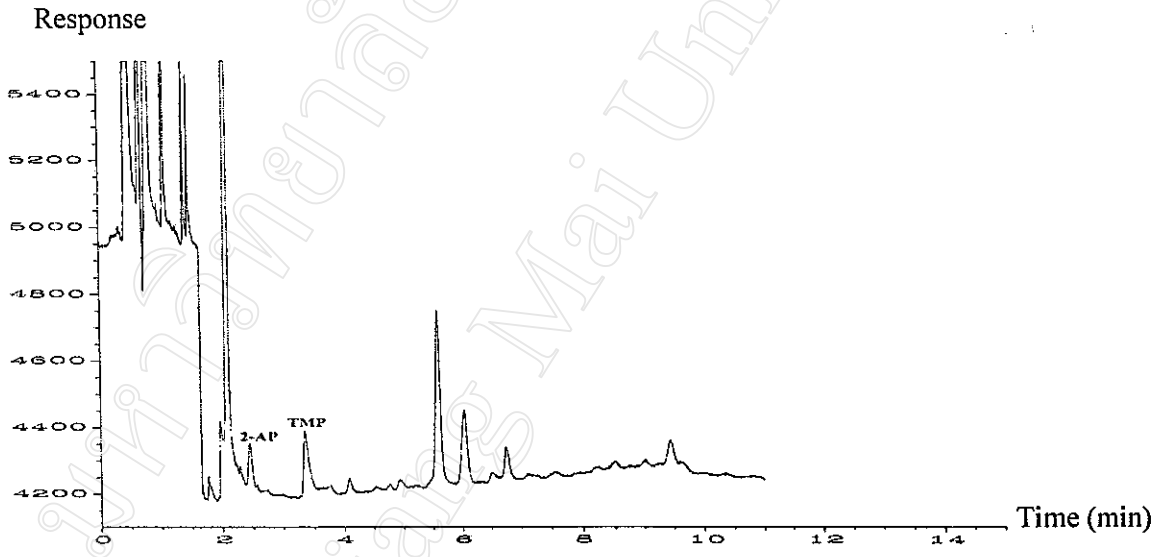

Figure 3.2 HS-GC chromatogram of the nonspiked rice seed extract.

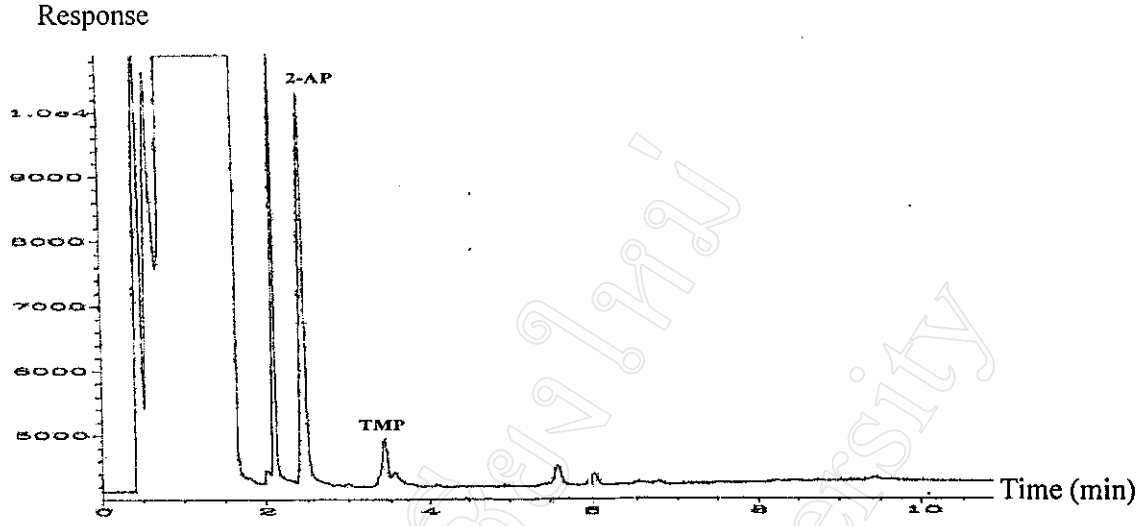

Figure 3.3 HS-GC chromatogram of the spiked rice seed extract.

3.1.2 Identification of 2-AP and TMP in headspace of the rice seed extract using DB-17MS column

2-AP and TMP peaks on chromatogram obtained by HS-GC using DB-17MS column of the rice seed extract were identified by means of comparing the retention times of 2-AP and TMP in the rice seed extract with the synthetic 2-AP and standard solution of TMP chromatographed under the same conditions. It was found that the retention time of 2-AP and TMP were 7.35 min and 8.65 min, respectively. HS-GC chromatographic patterns of the rice seed extract containing 2-AP and internal standard TMP is shown in Figure 3.4.

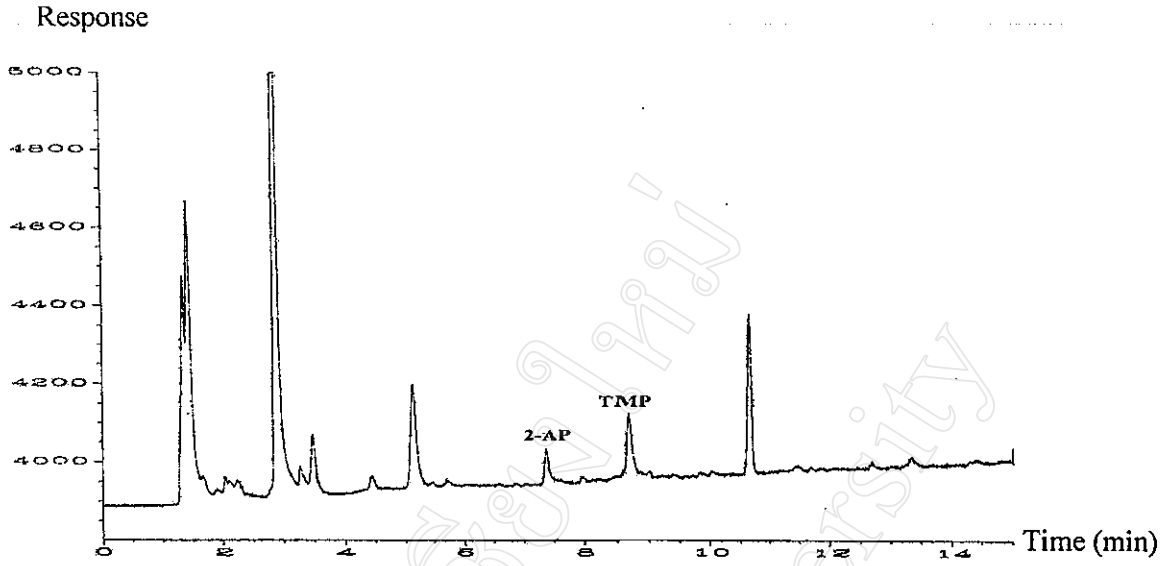

Figure 3.4 HS-GC chromatographic pattern of rice seed extract containing 2-AP and TMP.

Under the optimal conditions, identification of 2-AP and TMP in the rice seed extract containing TMP was also investigated by spiking standard solution of 2-AP and TMP in rice seed extract. The rice extract was then analyzed by HS-GC using DB-17MS column. HS-GC chromatograms of the nonspiked and spiked rice extract are shown in Figure 3.5-3.6. It was found that peak area of 2-AP and TMP in HS-GC chromatogram of the spiked rice seed extract were increased compared with peak area of 2-AP and TMP in HS-GC chromatogram of the nonspiked rice seed extract.

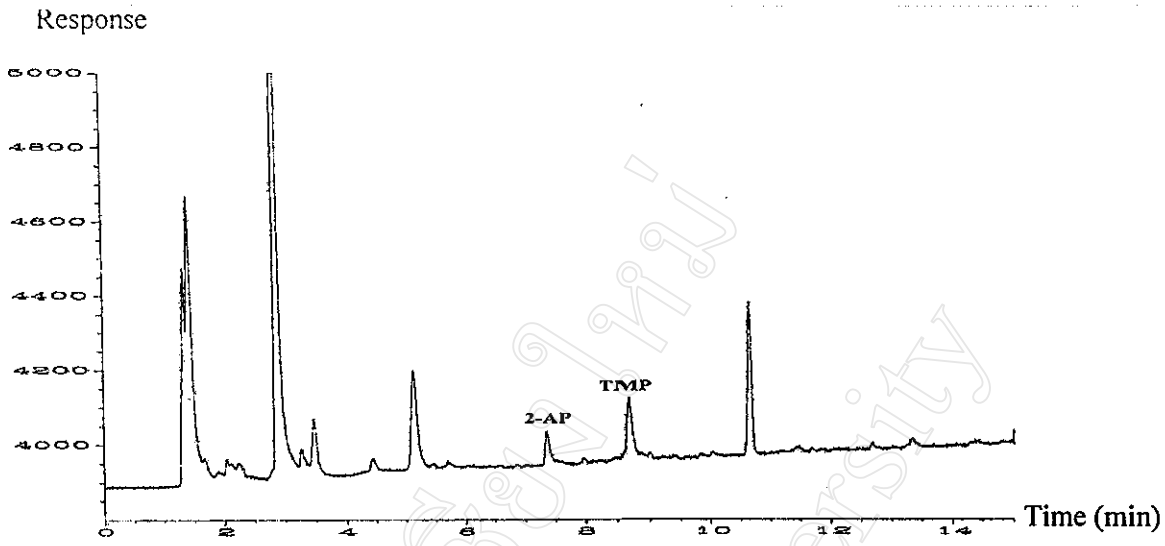

Figure 3.5 HS-GC chromatogram of the nonspiked rice seed extract.

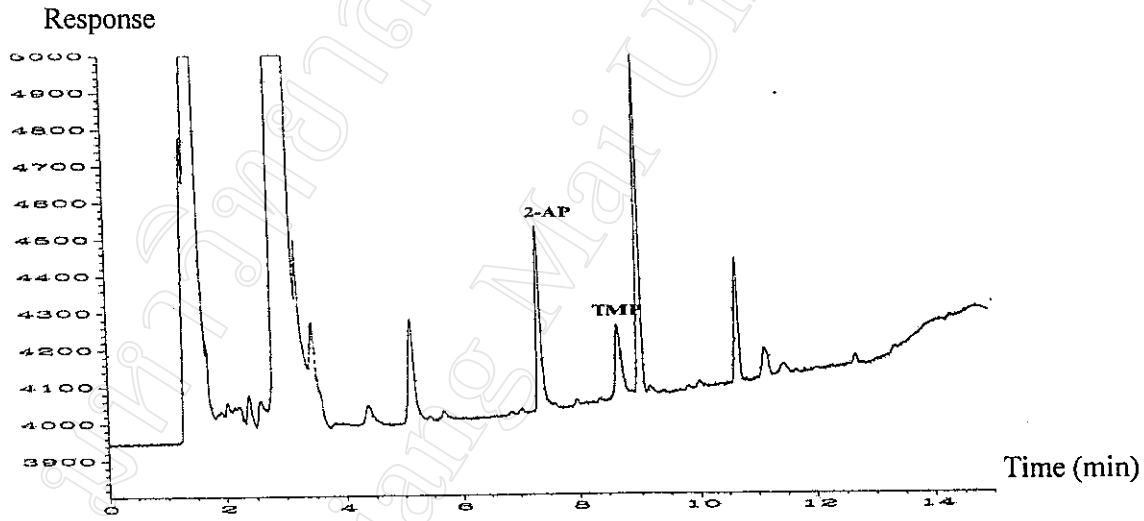

Figure 3.6 HS-GC chromatogram of the spiked rice seed extract.

3.2 Optimization of extraction conditions

Before subjecting to analysis by HS-GC, the aroma compound, 2-AP was extracted from rice seed by acidic solvent extraction. The rice seed extract solution were prepared according to the scheme in Figure 2.2. The extraction parameters effecting sensitivity and separating by HS-GC consists of extraction time, volume of 1.0 M NaOH

used for making alkaline solution and equilibrium time. Rice seed extract solution of KDML 105 was used for optimization. The GC employed capillary DB-1701 column. HS-GC conditions used were listed in Tables 2.2-2.3. 2-AP in rice seeds was extracted by 0.1 M HCl which was then made alkaline. The headspace of the extract solutions with varied conditions were analyzed by HS-GC. The results of extraction parameters are shown in Tables 3.1-3.3 and Figures 3.7-3.9. Results on the optimization of extraction conditions are shown in Table 3.4.

3.2.1 Effect of extraction time

Extraction time was varied from 5 to 40 min with 5 min increment whereas the other conditions were maintained as the same as listed in Figure 2.2. HS-GC conditions used were listed in Tables 2.2-2.3. The result of peak areas of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.1 and the plot of extraction time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.7.

Table 3.1 Retention times and peak areas of 2-AP and TMP at various extraction times

Extraction time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
5	2.554	823	3.378	1427	0.577
10	2.556	855	3.383	1359	0.629
15	2.558	896	3.383	1379	0.650
20	2.554	999	3.384	1509	0.662
25	2.558	1030	3.384	1588	0.648
30	2.559	994	3.393	1456	0.683
35	2.561	942	3.385	1410	0.668
40	2.558	983	3.381	1781	0.552

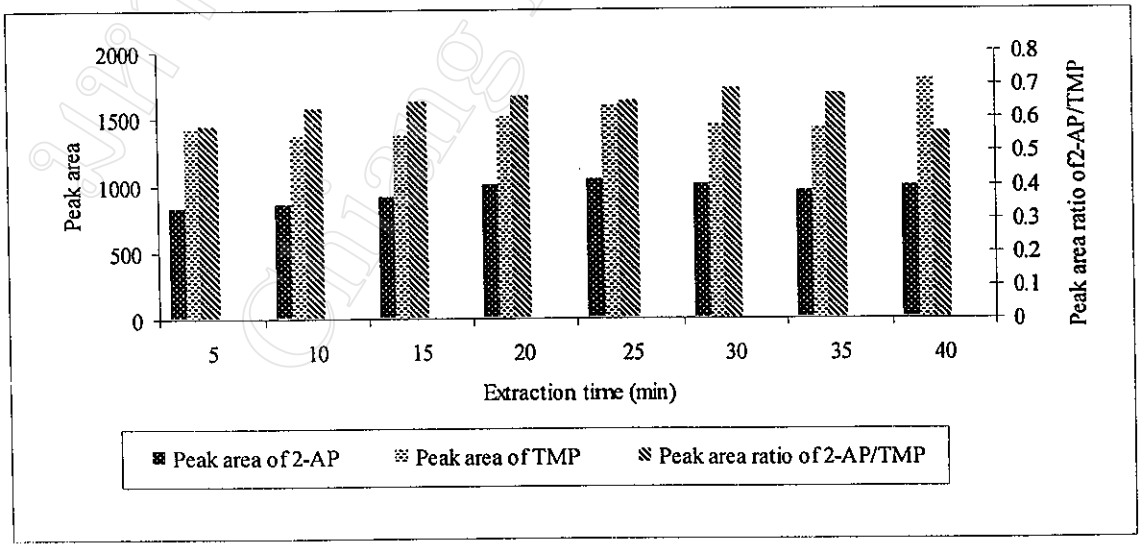

Figure 3.7 Peak area of 2-AP and TMP and ratio of peak areas of 2-AP/TMP obtained at various extraction times.

The effect of extraction time was investigated from 5 to 40 min. 2-AP was extracted by acid solvent because of its basicity. Then rice extract was made alkaline by 1.0 M NaOH in order to make the compound less dissolved in the extract. The vapour headspace above a rice extract was analyzed by HS-GC. The results presented in Table 3.1 and Figure 3.7 indicated that the optimum extraction time was 30 min. Increasing extraction time from 5 to 30 min increased the peak area ratio of 2-AP to TMP. Above 30 min, the peak area ratio of 2-AP to TMP were much poorer. The optimal extraction time chosen was 30 min because it provided more the peak area ratio of 2-AP to TMP than other extraction times.

3.2.2 Effect of volume of 1.0 M NaOH

Volume of 1.0 M NaOH was varied from 0.10 to 1.00 ml with 0.10 ml increment whereas the extraction time was 30 min. The other conditions were maintained as the same as listed in Figure 2.2. HS-GC conditions used were listed in Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.2 and the plot of volume of 1.0 M NaOH against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.8.

Table 3.2 Retention times and peak areas of 2-AP and TMP at various volumes of 1.0 M NaOH

Volume of NaOH (ml)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
0.3	2.550	424	3.386	804	0.527
0.4	2.558	823	3.382	1323	0.622
0.5	2.561	980	3.385	1184	0.828
0.6	2.559	919	3.383	1326	0.693
0.7	2.560	716	3.379	1304	0.549
0.8	2.558	608	3.380	1377	0.442
0.9	2.551	447	3.377	1359	0.329
1.0	2.552	447	3.376	1530	0.292

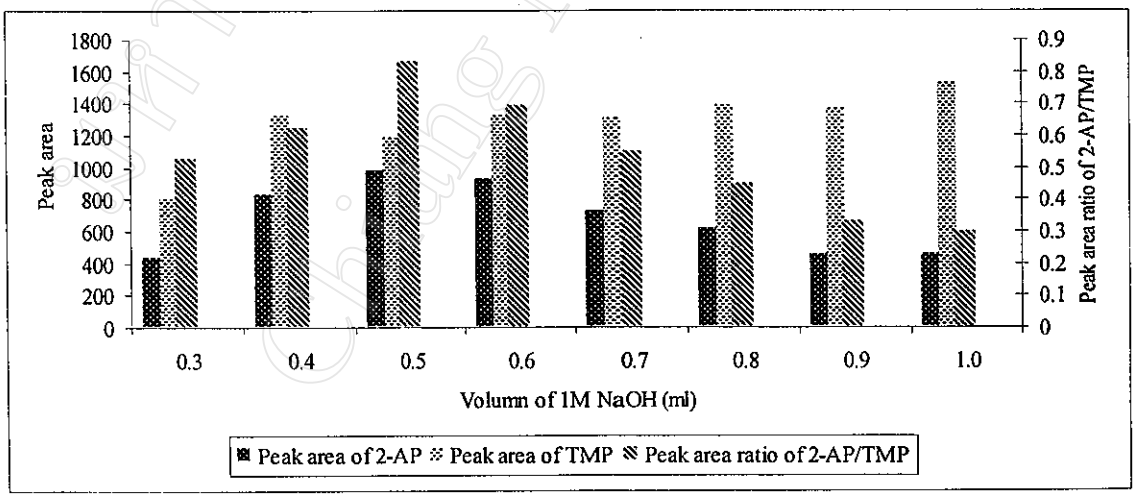

Figure 3.8 Ratio of peak areas of 2-AP/TMP obtained at various volumes of 1.0 M NaOH.

The effect of volume of 1.0 M. NaOH was optimized. After 2-AP was extracted by acidic solvent extraction, the rice seed extract solution which contained in a closed vial was made alkaline using 1.0 M NaOH. The vapour headspace above a rice seed extract was analyzed by HS-GC. Since 2-AP in rice extract behaved basicity it was volatilized by making the solution alkaline. In this work, rice extract was made alkaline using 1.0 M NaOH. The volume of 1.0 M NaOH was varied from 0.10 to 1.00 ml. The results are presented in Table 3.2 and Figure 3.8. It was found that the optimum volume of NaOH was 0.50 ml. Volume of 1.0 M NaOH less than 0.20 ml, 2-AP could not be detected because the rice extract was not alkaline. Increasing volume of 1.0 M NaOH from 0.30 to 0.50 ml increased peak area of 2-AP and peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP. Above 0.50 ml, peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP was decreased. The optimal volume of 1.0 M NaOH chosen was 0.50 ml because it provided the highest peak area of 2-AP and the peak area ratio of 2-AP to TMP as well as detection sensitivity of 2-AP than other extraction times.

3.2.3 Effect of equilibrium time

Equilibrium time after 1.0 M NaOH was added to the extract solution was 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 were listed in Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.3 and the plot of equilibrium time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.9.

Table 3.3 Retention times and peak areas of 2-AP and TMP at various equilibrium times

Equilibrium time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
5	2.546	906	3.396	693	1.307
10	2.548	900	3.395	572	1.573
15	2.554	951	3.382	532	1.788
20	2.553	927	3.396	583	1.723
25	2.555	916	3.399	650	1.409
30	2.557	927	3.392	744	1.246
35	2.550	952	3.390	791	1.204
40	2.560	954	3.392	767	1.244

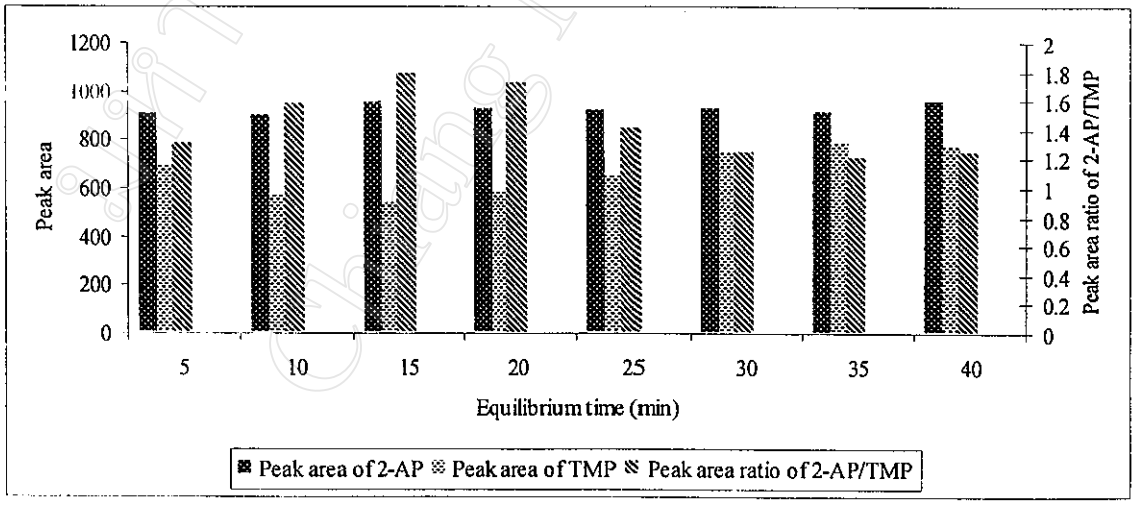

Figure 3.9 Ratio of peak areas of 2-AP/TMP obtained at various equilibrium times.

The effect of equilibrium time was investigated in range 5 to 40 min. After the rice seed extract was made alkaline, 2-AP volatilized into headspace above solution. Then, the headspace of solution was subjected to analysis by HS-GC. The equilibrium time refers to incubation time for evaporation of 2-AP into headspace of rice seed extract solution. The results are presented in Table 3.3 and Figure 3.9. It was found that the optimum of equilibrium time was 15 min. Increasing equilibrium time from 5 to 15 min increased the peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP. Above equilibrium time 15 min, the peak area ratio of 2-AP to TMP were much poorer due to the increase in peak area of TMP. The optimal of equilibrium time chosen was 15 min because it provided higher peak area of 2-AP and the peak area ratio of 2-AP to TMP than other equilibrium times.

3.3 Summary of optimized extraction conditions

The optimized extraction conditions for acidic solvent extraction of 2-AP in rice seed are summarized in Table 3.4.

Table 3.4 Optimized extraction conditions for extract 2-AP in rice seeds

Operation	Optimal conditions
Extraction time	30 min
Volume of 1.0 M NaOH	0.5 ml
Equilibrium time	15 min

3.4 Optimization of the automated headspace sampler conditions

The automated headspace sampler parameters consists of temperature of oven, loop and transfer line, vial equilibrium time, pressurizing time, loop fill time, loop equilibrium time and loop injection time. Rice seed extract solution of KDML 105 was used for optimization. The GC employed capillary DB-1701 column. 2-AP in rice seeds was extracted by 0.1 M HCl for 30 min, then the solution was made alkaline by adding 0.50 ml of 1.0 M NaOH and allowed to equilibrate for 15 min. The other conditions were maintained as the same as listed in Figure 2.2. The headspace of extract solution was analyzed by HS-GC. HS-GC conditions used were listed in Tables 2.2-2.3. The results of optimization of automated headspace sampler parameters are shown in Tables 3.5-3.10 and Figures 3.10-3.18. Results on the optimization of automated headspace sampler parameters are shown in Table 3.11.

3.4.1 Effect of temperature of vial, loop and transfer line

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, vial, loop sample and transfer line was varies from 70, 90, 110 to 120, 140, 160 °C, respectively with 10 °C increment whereas the other parameters were maintained at the same conditions as listed in Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.5 and the plots of temperature against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.10. Figures 3.11-3.13 illustrated chromatograms of temperature of vial, loop, transfer line: 100,120,140 °C and 110,130,150 °C and 120, 140,160 °C, respectively.

Table 3.5 Retention times and peak areas of 2-AP and TMP at various temperatures

Series of temperature (°C)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
70,90,110	2.478	387	3.360	1032	0.375
80,100,120	2.481	690	3.349	1371	0.503
90,110,130	2.480	460	3.379	851	0.541
100,120,140	2.474	480	3.439	322	1.491
110,130,150	2.474	627	3.401	516	1.215
120,140,160	2.469	893	3.348	1499	0.596

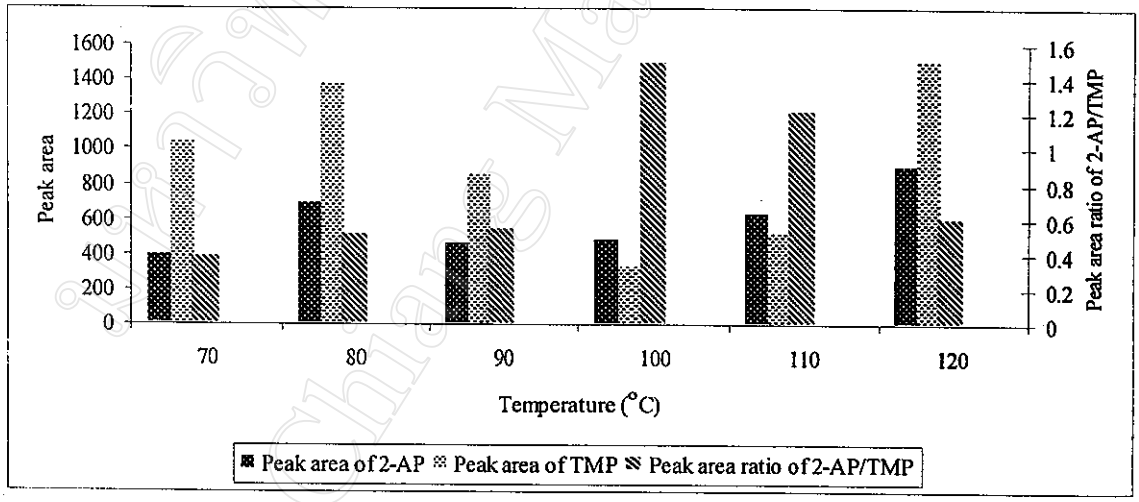

Figure 3.10 Ratio of peak areas of 2-AP/TMP obtained at various temperatures.

The effect of series of temperatures was investigated in temperature range 70, 90, 110 °C to 120, 140, 160 °C. As temperature influenced volatility, the series of temperatures were oven temperature, loop temperature, and transfer line temperature were optimized. The vial which contained rice extract was thermostatted for equilibration time until equilibrium is reached. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are presented in Table 3.5. The plots of temperatures against peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Figure 3.10. The results presented in Figure 3.10 indicated that temperature at 100, 120, 140 °C provides higher peak area ratio of 2-AP to TMP than other temperature. Although this temperature was optimum in term of peak area ratio, it provides lower detection sensitivity of 2-AP than temperature at 120, 140, 160 °C. The chromatograms are shown in Figures 3.11-3.13. The optimal series temperatures chosen were 120, 140, 160 °C because it provided higher detection sensitivity of 2-AP than other temperatures. The effect of temperature for headspace sensitivity can be explained via the following equation:[16]

$$\text{Log } K = (B'/T) - C' \qquad (3.1)$$

Where K is the partition coefficient, T is temperature, B′ and C′ are substance-specific constants.

$$A \propto C_G = C_O / (K + \beta) \qquad (3.2)$$

Where A is the peak area, C_G is the analyte's concentration in the headspace, C_O is the analyte's concentration in the original sample, β is phase ratio. It is obvious from equation 3.1 that raising the temperature will decrease K. According to equation 3.2, headspace sensitivity depends on the combined effect of K and β. Decreasing K will increase A (peak area). Therefore the higher temperature provided higher sensitivity.

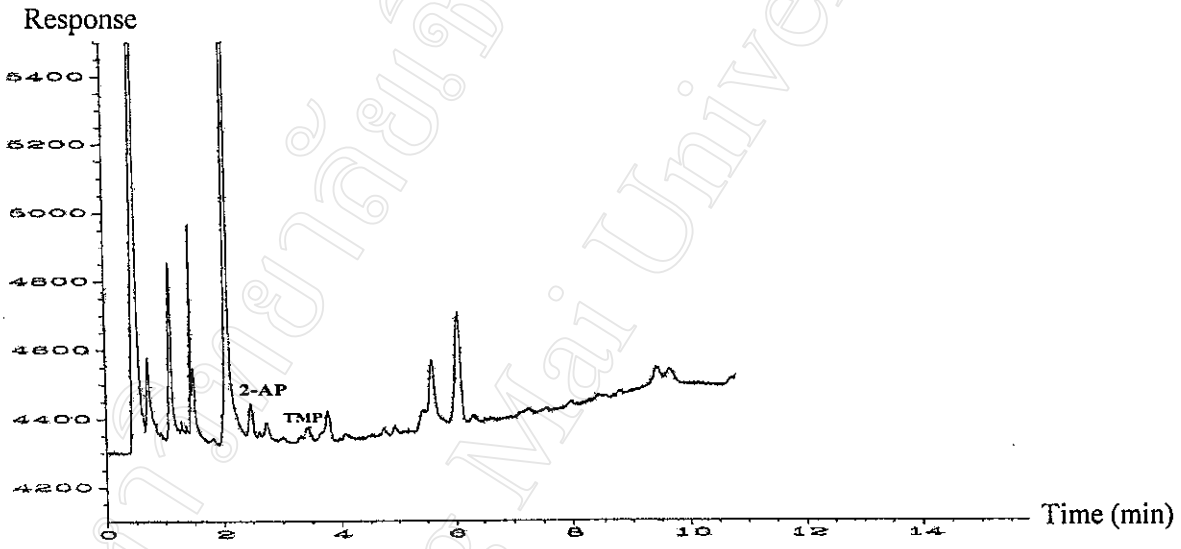

Figure 3.11 Chromatogram of temperature of oven, loop and transfer line was 100,120,140 °C.

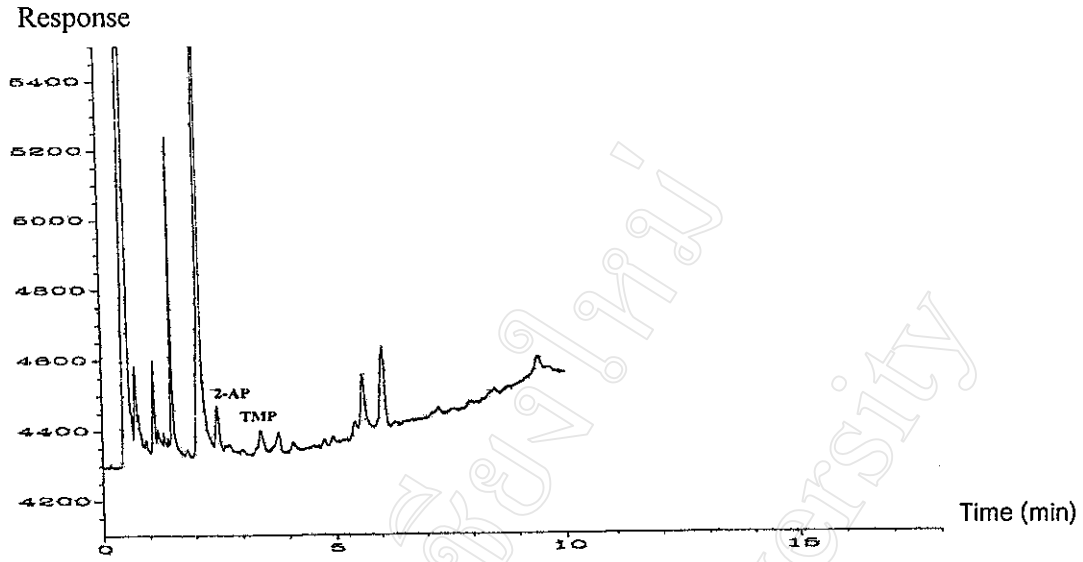

Figure 3.12 Chromatogram of temperature of oven, loop and transfer line was 110,130,150 °C.

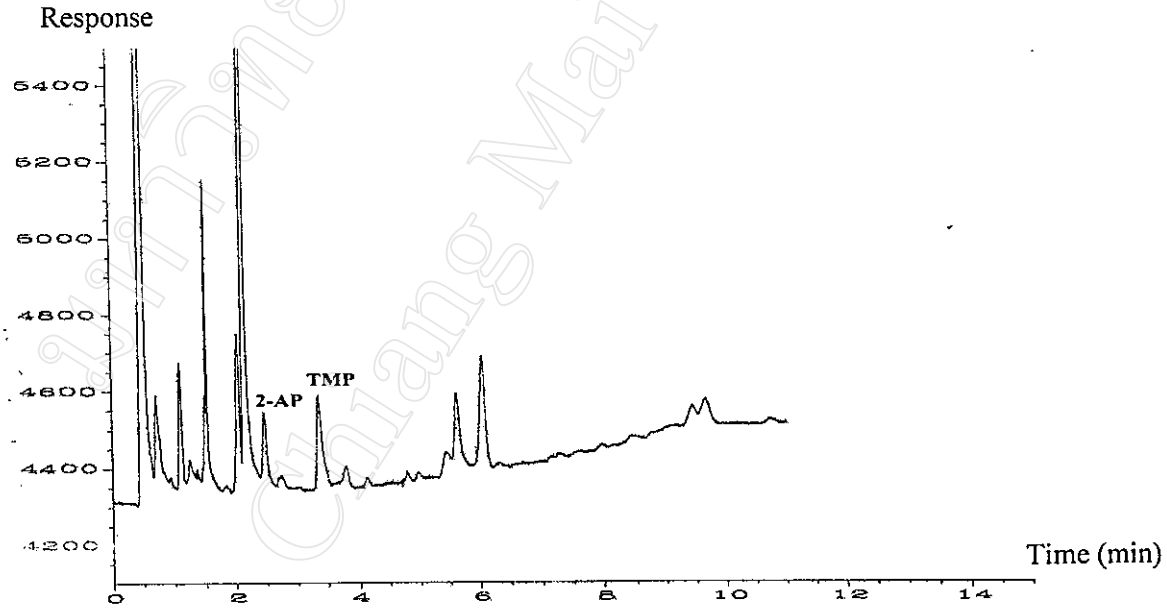

Figure 3.13 Chromatogram of temperature of oven, loop and transfer line was 120,140,160 °C.

3.4.2 Effect 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 7 with 1 min increment whereas the temperature of vial, loop sample and transfer line were set to 120, 140 and 160 °C, respectively. The other parameters were maintained as the same conditions as listed in Tables 2.2-2.3.The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.6 and the plots of vial equilibration time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.14.

Table 3.6 Retention time and peak areas of 2-AP and TMP at various vial equilibrium times

Vial equilibrium time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
1	2.542	306	3.358	656	0.466
2	2.549	318	3.387	480	0.663
3	2.535	473	3.367	603	0.784
4	2.551	396	3.378	600	0.660
5	2.549	426	3.380	793	0.537
6	2.547	488	3.375	974	0.501
7	2.553	412	3.382	891	0.462

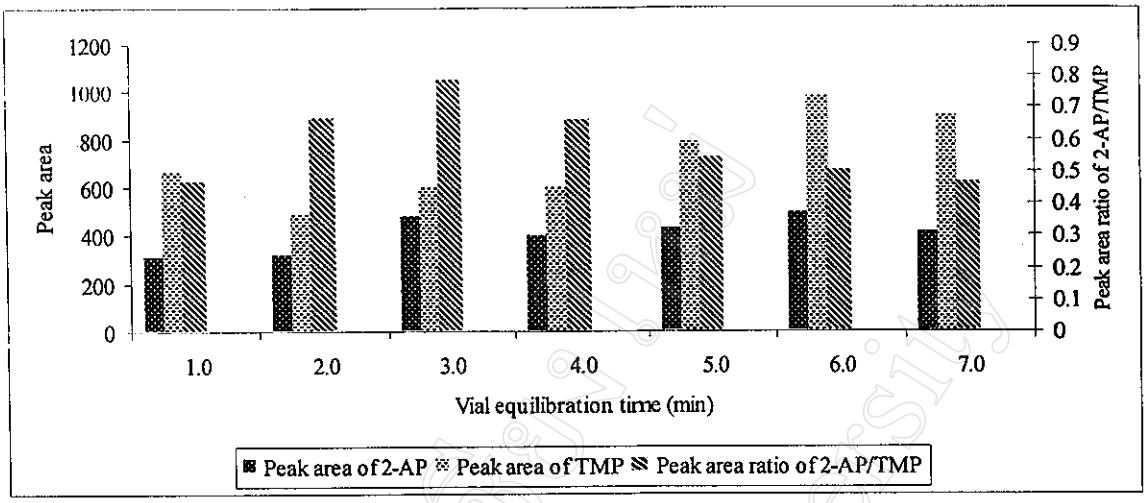

Figure 3.14 Ratio peak areas of 2-AP/TMP obtained at various equilibrium times.

The effect of vial equilibration time was investigated from 1 to 7 min. Table 3.6 and Figure 3.14 show peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP obtained and the plot of vial equilibration time against peak area and peak area ratio of 2-AP to TMP under various vial equilibration times, respectively. After vial is thermostatted at temperature 120 °C, 2-AP will be distributed between the rice seed extract and gas phase above the solution. After equilibrium between rice seed extract and gas phase has been reached, agitation was stopped. Then, the headspace of solution was subjected to GC system. The equilibration time refers to the thermostatting time in oven. The results indicated that the equilibration time range 1 to 2 min provided less peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP than equilibration time of 3 min because the system of vial was not still reach the equilibrium. At equilibration time of 3 min, the equilibrium of the system was reached. Therefore, this vial equilibration time of 3 min, provided higher peak area ratio of 2-AP to TMP than other vial equilibration time. At vial equilibration time above 4 min, the peak area ratio of 2-AP to TMP decreased due

to the increase in amount of TMP in gas phase. Although the vial equilibration time of 6 min gave the highest detection sensitivity of 2-AP, the vial equilibration time of 3 min was chosen for further study because it provided shorter analysis time.

3.4.3 Effect of pressurization 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 pressurization time was varied from 0.1 to 0.5 min with 0.1 min increment whereas the temperature of vial, loop sample 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 Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.7 and the plots of pressurization time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.1.

Table 3.7 Retention times and peak areas of 2-AP and TMP at various pressurization times

Pressurization time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
0.1	2.516	400	3.363	485	0.887
0.2	2.509	462	3.367	645	0.794
0.3	2.520	633	3.375	486	0.976
0.4	2.518	539	3.373	632	0.878
0.5	2.509	469	3.373	654	0.928

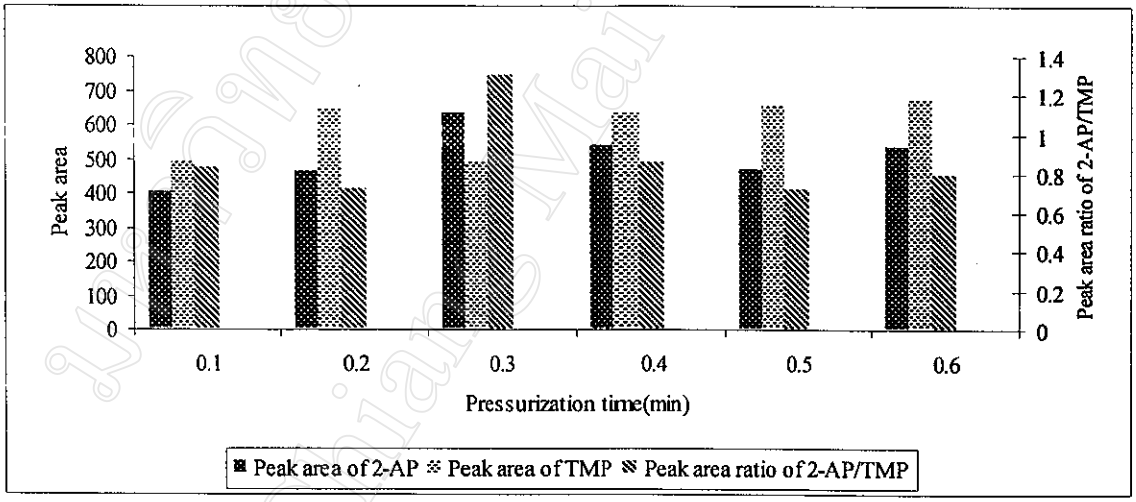

Figure 3.15 Ratio of peak areas of 2-AP/TMP obtained at various pressurization times.

The effect of pressurization time was investigated within the range of 0.1 to 0.5 min. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.7 and the plots of pressurization time against peak area and

peak area ratio of 2-AP to TMP are shown in Figure 3.15. It was found that the optimal pressurization time was 0.3 min. After the rice extract solution was heated for 3 min, the equilibrium was reached. To achieve the flow of headspace gas into the injector and into the column of GC, the vial must be pressurized with nitrogen gas in order to increase pressure of the sampling system. In this case, the vial pressure was higher than inlet pressure. Thus, headspace gas can flow into the column of GC [16]. The optimal pressurization time chosen was 0.3 min because it provided higher peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP than other pressurization times. Above 0.3 min of pressurization time, the peak area ratio of 2-AP to TMP was poorer due to a dilution of gas phase at longer time of pressurization.

3.4.4 Effect 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 with 0.5 min increment whereas the temperature of vial, loop sample 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 Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.8 and the plots of loop filling time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.16.

Table 3.8 Retention times and peak areas of 2-AP and TMP at various loop filling times

Loop filling time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
0.5	2.510	507	3.373	687	0.854
1.0	2.512	1038	3.362	1267	0.819
1.5	2.512	1142	3.363	1321	0.864
2.0	2.515	1144	3.364	1231	0.929
2.5	2.512	1118	3.365	1222	0.915
3.0	2.510	1036	3.363	1329	0.780

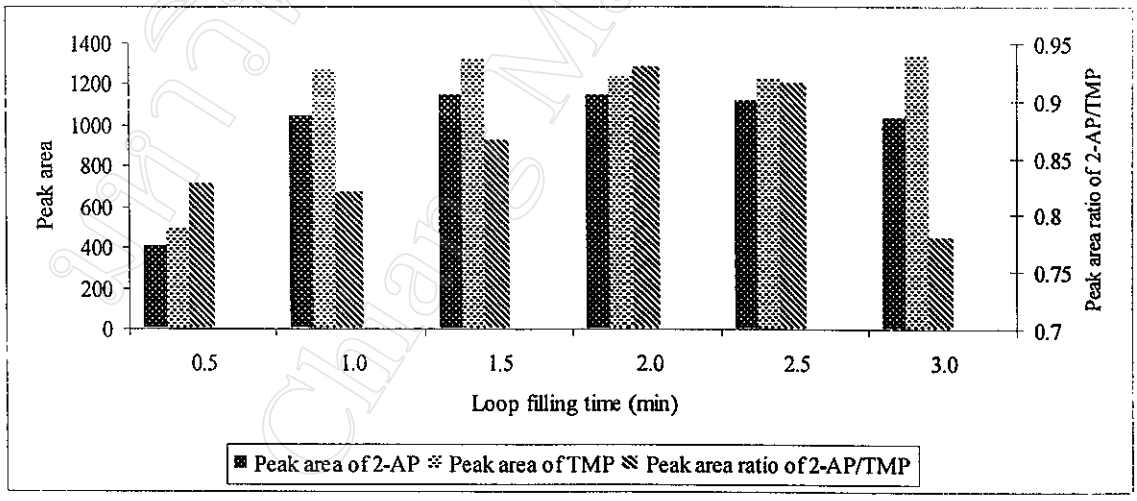

Figure 3.16 Ratio of peak areas of 2-AP/TMP obtained at various loop filling times.

The effect of loop filling time was investigated in the range of 0.5 to 3.0 min. The results are shown in Table 3.8 and Figure 3.16. It was found that the optimum loop filling time was 2 min. Varying the loop filling time from 0.5 to 1.5 min gave increment of peak area ratio of 2-AP to TMP. The loop filling time at 2 min provided higher peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP than other loop filling times. Above loop filling time of 2 min, detection sensitivity of 2-AP and peak area ratio of 2-AP to TMP were poorer. The possible reason for this is that a short loop filling time leaves pressure in the loop closing to the vial pressure, this allows more analyte to be injected and some of them were splitted off during injection.

3.4.5 Effect 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 equilibration time was varied from 0.01 to 0.08 min with 0.01 min increment whereas the temperature of vial, loop sample 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 Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.9 and the plots of loop equilibration time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.17.

Table 3.9 Retention times and peak areas of 2-AP and TMP at various loop equilibrium times

Loop equilibrium time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
0.01	2.475	805	3.358	1080	0.745
0.02	2.4814	935	3.365	1195	0.782
0.03	2.481	935	3.372	1121	0.834
0.04	2.482	1166	3.368	1212	0.962
0.05	2.481	982	3.369	1260	0.779
0.06	2.485	1097	3.368	1301	0.843
0.07	2.481	1102	3.370	1220	0.903
0.08	2.481	1060	3.366	1201	0.883

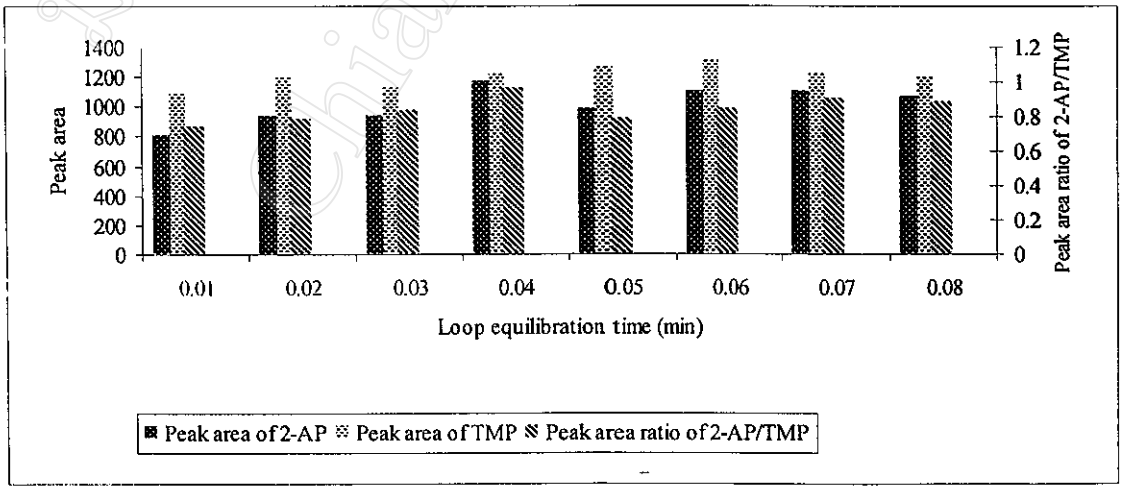

Figure 3.17 Ratio of peak areas of 2-AP/TMP obtained at various loop equilibrium times.

The effect of loop equilibration time was investigated in the range of 0.01 to 0.08 min. After the headspace gas was filled into loop sample, the gas was maintained to equilibrium in loop sample. The results are shown in Table 3.9 and Figure 3.17. It was found that the optimum loop equilibration time was 0.04 min. Increasing loop equilibration time from 0.01 to 0.04 min increasing peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP because equilibrium of loop sample was not reached. At loop equilibration time 0.04 min, equilibrium of loop sample was reached. At loop equilibration time 0.05 min, peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP were much poorer. Although loop equilibration time of 0.06 to 0.08 min and 0.04 min gave high detection sensitivity of 2-AP and peak area ratio of 2-AP to TMP, loop equilibration time of 0.04 min provided shorter analysis time. The optimal of loop equilibration time chosen was 0.04 min because it provided more the peak area ratio of 2-AP to TMP and higher detection sensitivity of 2-AP than other loop equilibration times.

3.4.6 Effect 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 the temperature of vial, loop sample 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 Tables 2.2-2.3. The results of peak area of 2-AP and TMP as well as the peak area ratio of 2-AP to TMP are shown in Table 3.10 and the

plots of loop equilibration time against peak area and peak area ratio of 2-AP to TMP are shown in Figure 3.18.

Table 3.10 Retention time and peak areas of 2-AP and TMP at various loop injection times

Loop injection time (min)	2-AP		TMP		P_A Ratio (2-AP/TMP)
	t_R (min)	P_A	t_R (min)	P_A	
0.1	2.468	524	3.376	607	0.863
0.2	2.470	1090	3.351	1212	0.899
0.3	2.469	1122	3.353	1220	0.920
0.4	2.467	1148	3.357	1133	1.010
0.5	2.466	960	3.355	1172	0.819
0.6	2.472	1085	3.348	1274	0.852
0.7	2.469	980	3.353	1260	0.778

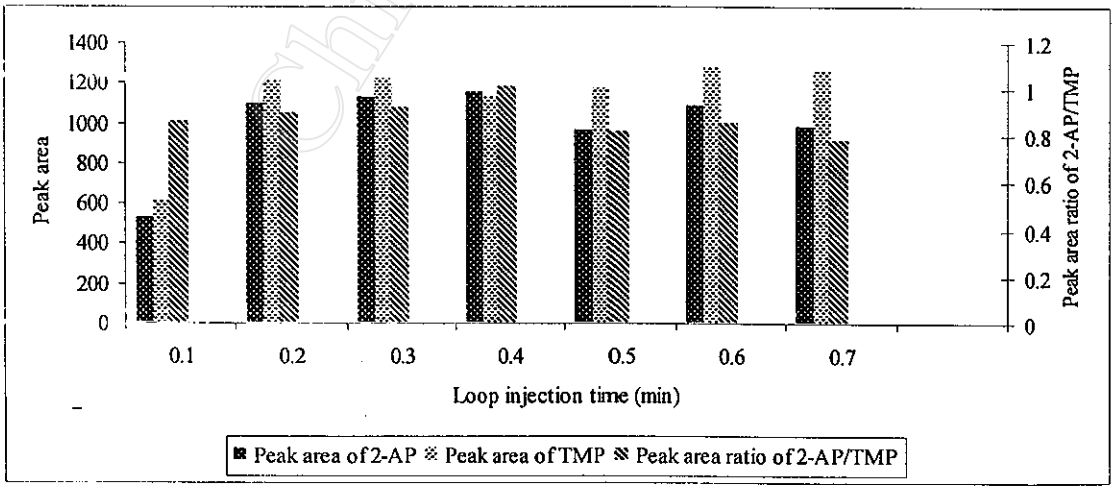

Figure 3.18 Ratio peak areas of 2-AP/TMP obtained at various loop injection times.

The effect of loop injection time was investigated in the range of 0.1 to 0.7 min. At the end of loop equilibrium time, the sample valve brings the sample loop in line with carrier gas flow. The carrier gas flows though the sample loop and transfer line into the GC inlet port. This sweeps the sample into the GC. The loop injection time refers to time of injection gas from loop sample to GC. The results are shown in Table 3.10 and Figure 3.18. Varying the loop injection time gave different peak area ratio of 2-AP to TMP. Increasing loop injection time from 0.1 to 0.4 min increased peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP. It was found that the optimum loop injection time was 0.4 min because it provided higher peak area ratio of 2-AP to TMP and detection sensitivity of 2-AP than other loop injection times.

3.5 Summary of optimized automated headspace sampler conditions

The optimized automated headspace sampler conditions for introduction of headspace volatile of the rice seed extract to GC are summarized in Table 3.11. Chromatograms of the rice seed extract obtained with HS-GC under optimum conditions are shown in Figure 3.19.

Table 3.11 Optimized automated headspace sampler conditions for analysis of 2-AP in rice seed extract

Operation	Optimal conditions
Temperature (vial, loop, transfer line)	120,140,160 °c
Vial equilibrium time	3 min
Pressurization time	0.3 min
Loop fill time	2.0 min
Loop equilibrium time	0.04 min
Loop injection time	0.4 min

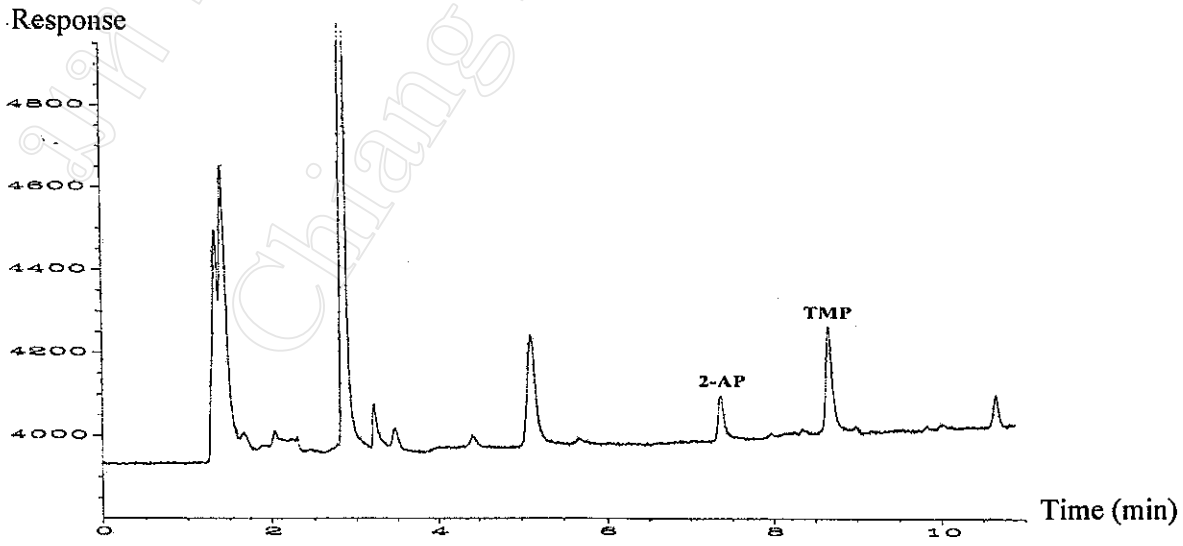

Figure 3.19 Chromatogram of rice extract obtained with HS-GC under optimum conditions.

3.6 Construction of Calibration Curves

In this work, the internal standard method was used in construction of calibration curve. The calibration curve was constructed by plotting concentration of standard 2-AP against peak area ratio of 2-AP to TMP. A series of standard 2-AP solution was prepared as shown in Table 2.1. 2-AP in each standard solutions was extracted by 0.1 M HCl containing 0.25 ppm TMP for 30 min, then the solution was made alkaline by adding 0.5 ml of 1.0 M NaOH and allowed to equilibrate for 15 min. Soon after that, the standard extract solution was analyzed by HS-GC. The automated headspace sampler conditions used were listed in Table 3.11. In this part of experiment, DB-17MS capillary column with 30m x 0.321mm dimension and 0.25 μm film thickness was used. GC conditions were listed in Table 2.3. Recovery assay of standard extraction was 74.1%, thus the concentrations of standard were calculated to be 0.37-5.93 ppm (see appendix D). The results 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 3.12. Calibration curve is shown in Figure 3.20.

Table 3.12 Peak area ratio of 2-AP to TMP of each standard solutions

Initial concentration (ppm)	Concentration based on %recovery	P_A Ratio (2-AP/TMP)
0.50	0.37	0.237
1.00	0.74	0.326
2.00	1.48	0.490
4.00	2.97	0.775
8.00	5.93	1.344

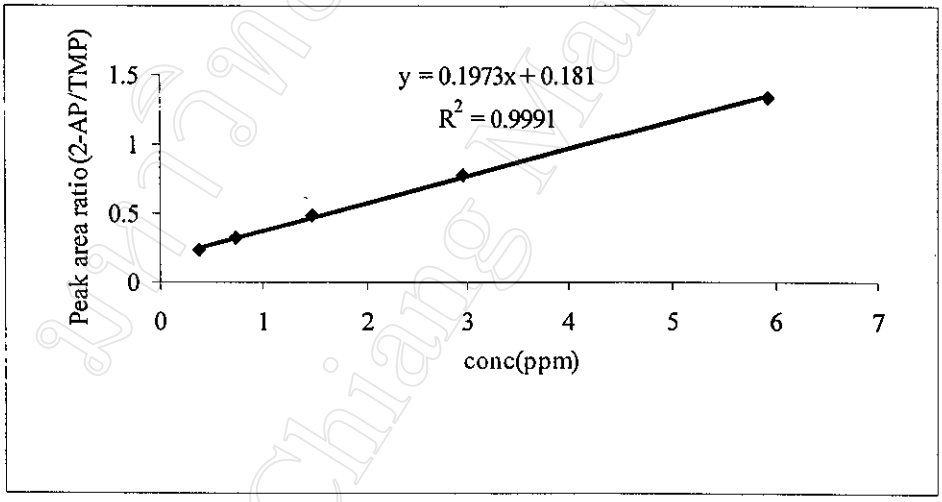

Figure 3.20 Calibration curve of 2-AP.

3.7 Validation

After establishing the optimized conditions of extraction and automated headspace sampler, detection limit, linearity and recovery assay were investigated.

3.7.1 Detection limit

The detection limit for 2-AP analysis of rice seed sample was selected based on the signal-to-noise (S/N) ratio at a minimum of 3:1. The results of S/N ratio obtained for 2-AP analysis of rice seeds with weights varied from 0.5-5.0 g are shown in Table 3.13. Detection limit in term of the lowest amount of rice seed sample required was 4.00 g.

Table 3.13 The detection limit of 2-AP in rice analysis

Weight of rice (g)	Mean S/N
0.5	1.48
1.0	1.62
3.0	2.33
4.0	3.14
5.0	4.41

In addition, calculation of the limit of detection was based on a method of Miller and Miller. The calibration curve was constructed by plotting peak area ratio of 2-AP to TMP against concentration of standard 2-AP in the range 0.37-5.93 ppm. The linear regression equation was $Y = 0.1973X + 0.181$. Detection limit in term of the least amount of analyte, 2-AP, was 0.23 ppm (see appendix C).

3.7.2 Linearity

In order to investigate the linearity of calibration curve, plotting of concentrations of standard 2-AP against peak area ratio of 2-AP to TMP was performed in concentration range 0.50-15.00 ppm. 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. A linear detector response was found for standard solutions of 2-AP in the concentration range 0.50-8.00 ppm, as shown in Figure 3.20. The calibration curve in this range was used for quantification of 2-AP in rice seed extract.

Table 3.14 Peak area ratio of 2-AP to TMP of each standard solutions

Initial concentration (ppm)	Concentration based on %recovery	P_A Ratio (2-AP/TMP)
0.50	0.37	0.237
1.00	0.74	0.326
2.00	1.48	0.490
4.00	2.97	0.775
8.00	5.93	1.344
15.00	11.57	1.613

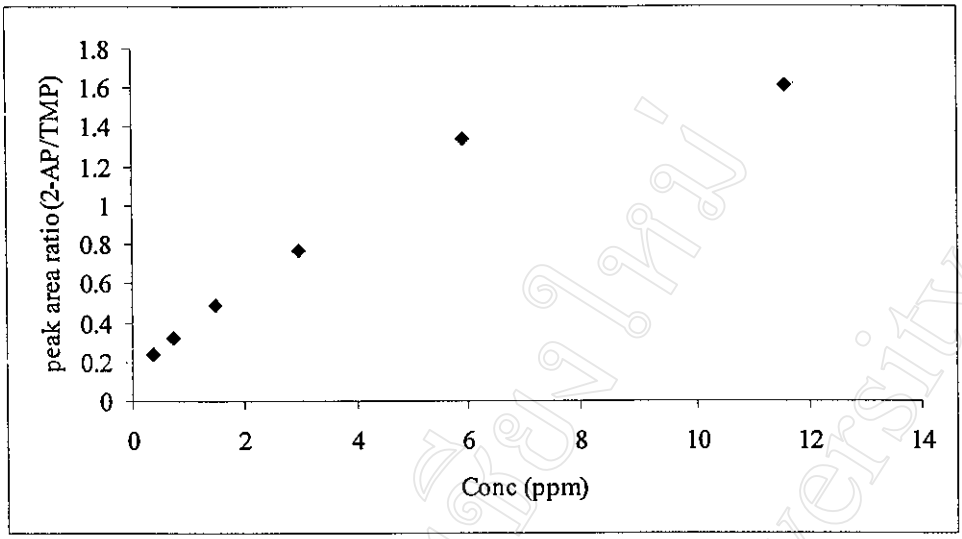

Figure 3.21 Calibration curve of 2-AP in the range 0.37-11.57 ppm.

3.7.3 Precision

The precision, reproducibility gives a measure of error in the development methodology and usually reported as a percentage of relative standard deviation (%R.S.D) [39]. In this work, the precision in term of reproducibility was performed by analyzing a rice seed extract solution in the same conditions for seven days.

Reproducibility of peak area ratio of 2-AP to TMP are shown in Table 3.15. R.S.D. value of peak area ratio of 2-AP to TMP was 7.5 %.

Table 3.15 Reproducibility of peak area ratio of 2-AP to TMP

Date	P_A ratio			Average P_A ratio
	1	2	3	
1	0.358	0.371	0.319	0.349
2	0.413	0.441	0.427	0.427
3	0.385	0.400	0.380	0.388
4	0.383	0.369	0.359	0.370
5	0.332	0.373	0.321	0.342
6	0.372	0.378	0.375	0.375
7	0.393	0.375	0.404	0.390
			%R.S.D.	7.5

3.7.4 Recovery assay

Under the optimal conditions of the research work, extraction efficiency of the method was investigated by recovery test. It was done standard solution extract at 2-AP concentration of 8.00 ppm and rice seed extract.

3.7.4.1 Recovery assay of standard extract solutions

2-AP standard-toluene solution at 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 percent recovery was calculated based on peak area. The

recovery assay was resulted as shown in Table 3.16. The percent recovery of standard extraction was 74.1% (see appendix C).

Table 3.16 Recovery assay of standard extract solutions

P_A of 2-AP before extraction	P_A of 2-AP after extraction	% Recovery
1154	276	76.08
974	248	74.04
944	262	72.26
	average	74.1

3.7.4.2 Recovery assay of rice extract solutions

KDML 105 Chiang mai, KDML 105 Tungkularonghai, Howm Supanburi and Howm Patumtani seeds were extracted by 0.1 M. HCl (1^{st} extraction).The rice seed extract was analyzed by HS-GC. Then, the remaining solid from the 1^{st} extraction was again extracted (2^{nd} extraction) and analyzed by HS-GC. The percentage recovery was calculated based on peak area. The recovery assay obtained was shown in Table 3.17. The average percentage recovery of rice extraction was found to be 77.4% (see appendix C).

Table 3.17 Recovery assay of rice extract solutions

Sample	% Recovery
KDML 105 Chiang mai	79.27
KDML 105 Tungkularonghai	76.58
Howm Supanburi	76.28
Howm Patumtani	ND
average	77.4

ND = Not Detceted

3.8 Analysis of Real Samples

In this part of experiment, DB-17MS capillary column with 30m x 0.32mm dimension and 0.25 μm film thickness was used for quantitation. The optimized analytical method was applied for the determination of 2-AP in rice seed extract. 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 rice samples were analyzed by HS-GC. The quantity of 2-AP in rice sample was determined by comparison with the calibration curve in Figure 3.20. In this work, % recovery of rice extraction was 79.27. Thus the concentration of 2-AP in rice sample must be calculated based on % recovery of rice extraction (see appendix D). The corresponding chromatograms are shown in Figures 3.22-3.25. The results of HS-GC determination of 2-AP in rice samples are shown in Table 3.18.

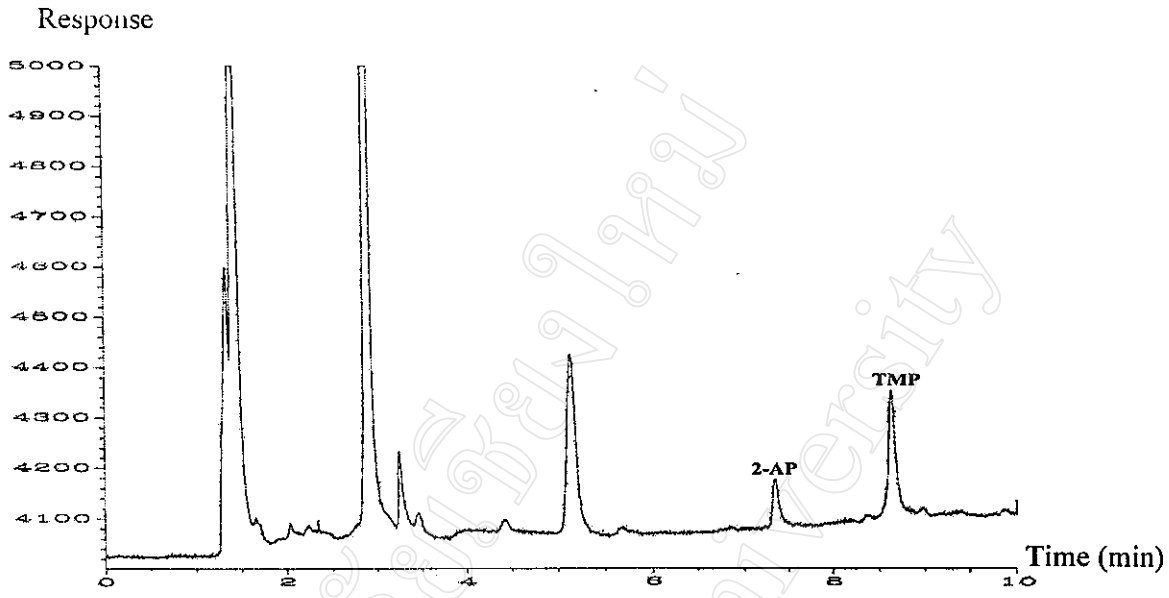

Figure 3.22 Chromatogram of rice extract of KDML 105 Chiang mai.

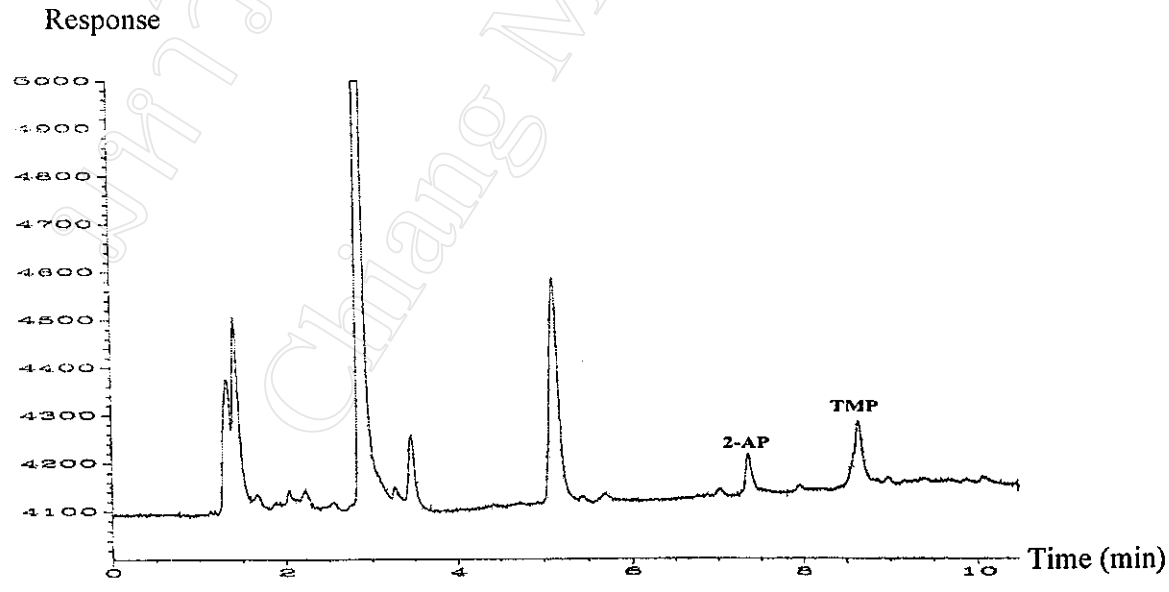

Figure 3.23 Chromatogram of rice extract of KDML 105 Tungkularonghai.

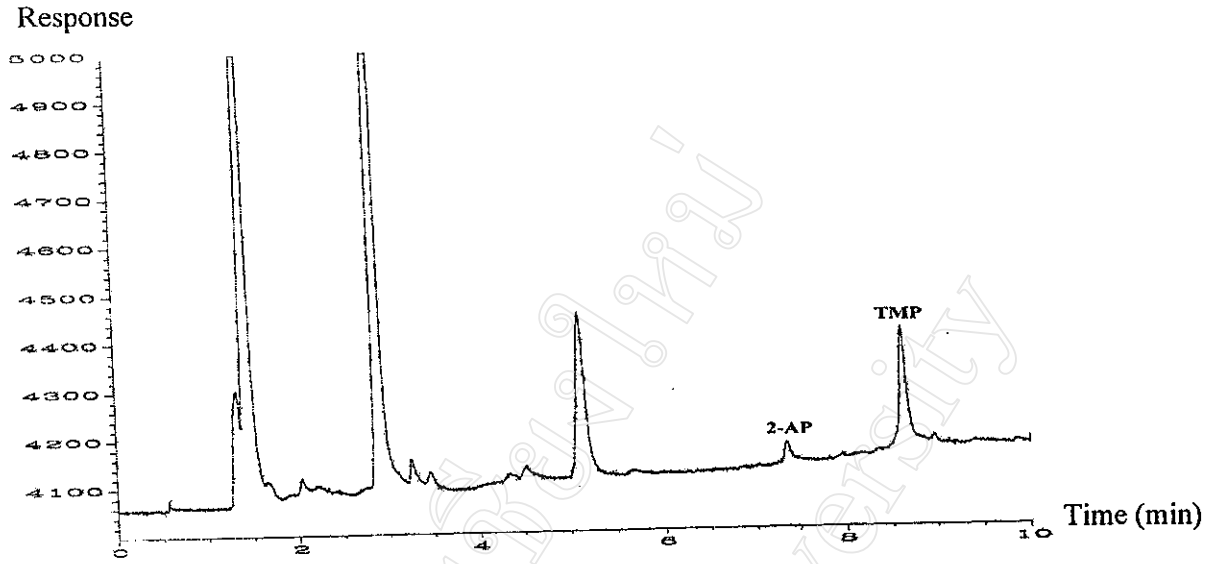

Figure 3.24 Chromatogram of rice extract of Howm Supanburi.

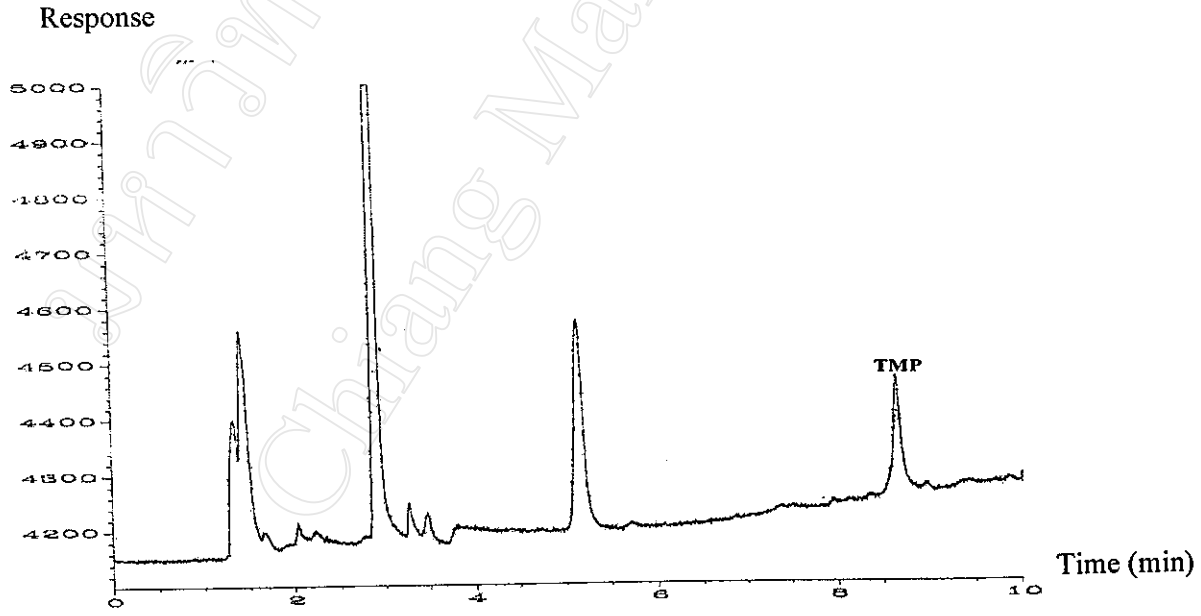

Figure 3.25 Chromatogram of rice extract of Howm Patumtani.

Table 3.18 Results of HS-GC determination of 2-AP in rice samples

sample	%Recovery	Conc 2-AP (ppm)
KDML 105 Chiang mai	79.27	1.18
KDML 105 Tungkularonghai	76.58	2.41
Howm Supanburi	76.28	0.41
Howm Patumtani	ND	ND

ND = Not Detceted