CHAPTER 3

RESULTS AND DISCUSSION

3.1 Synthesis of 2AP

In this study, 2AP was synthesized as a standard solution and was used for constructing a standard calibration curve. The synthesis of2AP was performed using conditions presented in section 2.3.

2-Acetypyrrole was used as a starting material of synthesis reaction. The hydrogenation and oxidation products were 2-(1-hydroxyethyl)pyrrolidine and 2AP, respectively.

Figure 3.1, 3.2 and 3.3 show GC-MS chromatograms and mass spectra of reactant, hydrogenation product and oxidation product, respectively. The was found that the yields of the reactant, hydrogenation product, 2-(1-hydroxyethyl)pyrrolidine and the oxidation product, 2AP, were 99.55, 97.35 and 54.26%, respectively. The mass spectra in Figure 3.1 (B), 3.2 (B) and 3.3 (B) could confirm structures of the reactant, hydrogenation product and oxidation product. They were compared with those complied in the mass spectral Wiley275 library that the high percent matching were yielded.

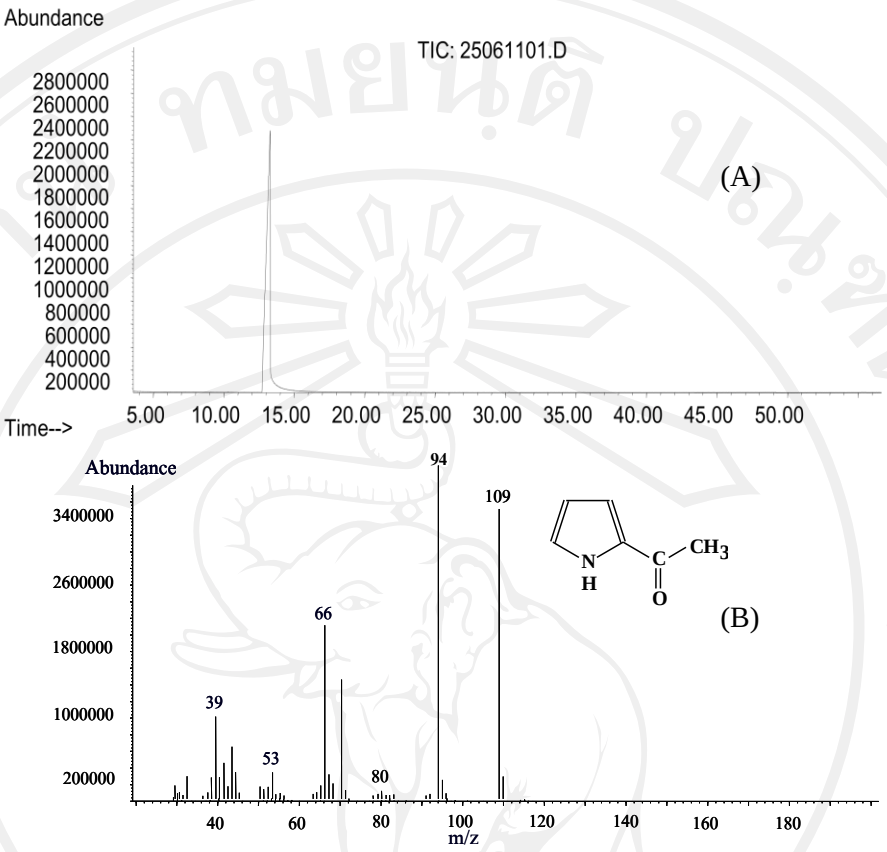

Figure 3.1 GC-MS chromatogram (A) and mass spectrum (B) of the reactant, 2-acetylpyrrole.

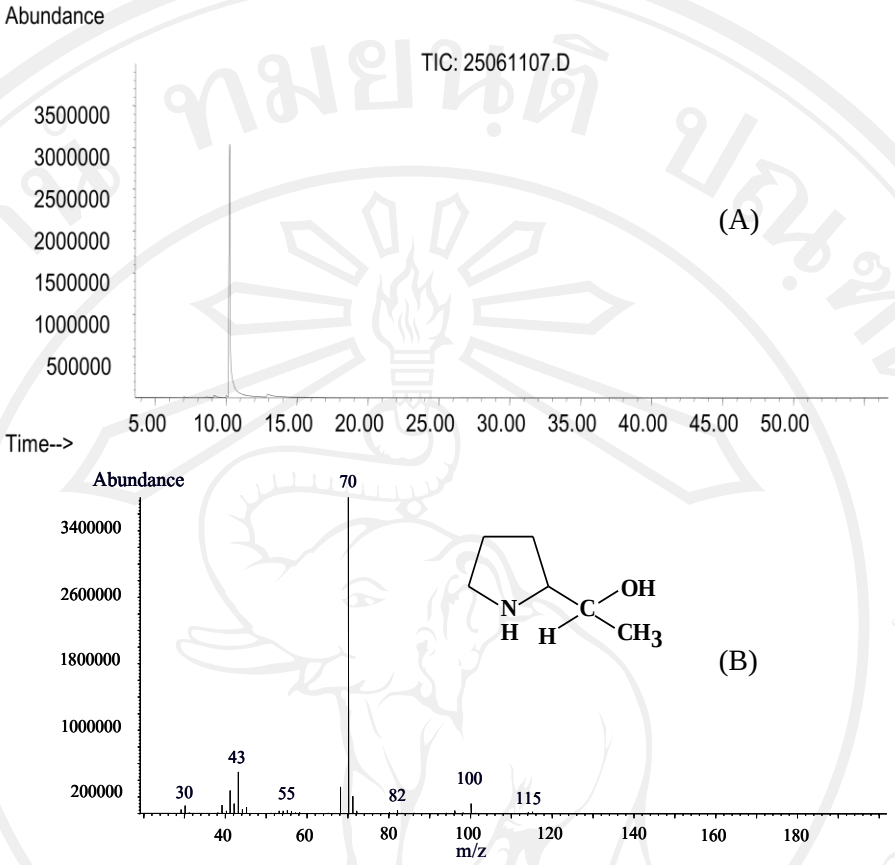

Figure 3.2 GC-MS chromatogram (A) and mass spectrum (B) of the hydrogenation product, 2-(1-hydroxyethyl)pyrrolidine.

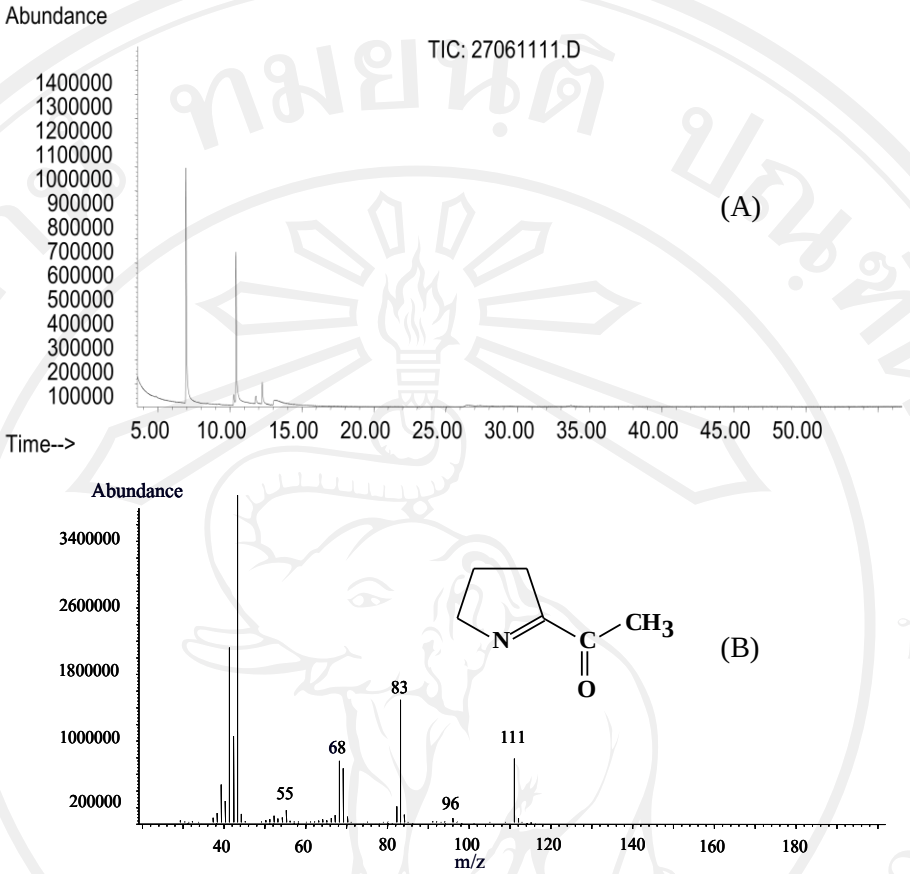

Figure 3.3 GC-MS chromatogram (A) and mass spectrum (B) of the oxidation product, 2AP.

2AP as a final product was analyzed by GC-MS for confirmation of its identity. The final product was then analyzed by NMR for its quantity in the solution. The concentration of 2AP in the product solution was 1596.18 ppm, the detail of which was shown in appendix B.

3.2Relative content of 2AP in rice leaves at different ages

The amounts of2AP in KDML 105rice leaves at different ages of rice plant between 10 to 120 days were determined using the developed SHS-GC/NPD method having conditions as previously optimized and shown in Table 2.1.

The results in Figure 3.4and Table 3.1 show the amounts of 2AP in rice leaves at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 days of age determined in terms of area under peak of 2AP were 354, 472, 485, 590, 900, 532, 540, 459, 463, 459, 320 and316pA, respectively. The amount of 2AP in the rice grain of 1.000 g was 280pA.2AP content continuously increased after 10 days of leaf age and the highest amount was found at 50 days (900pA). After that, the content of 2AP decreased down to 316 at 120 days of leaf age.

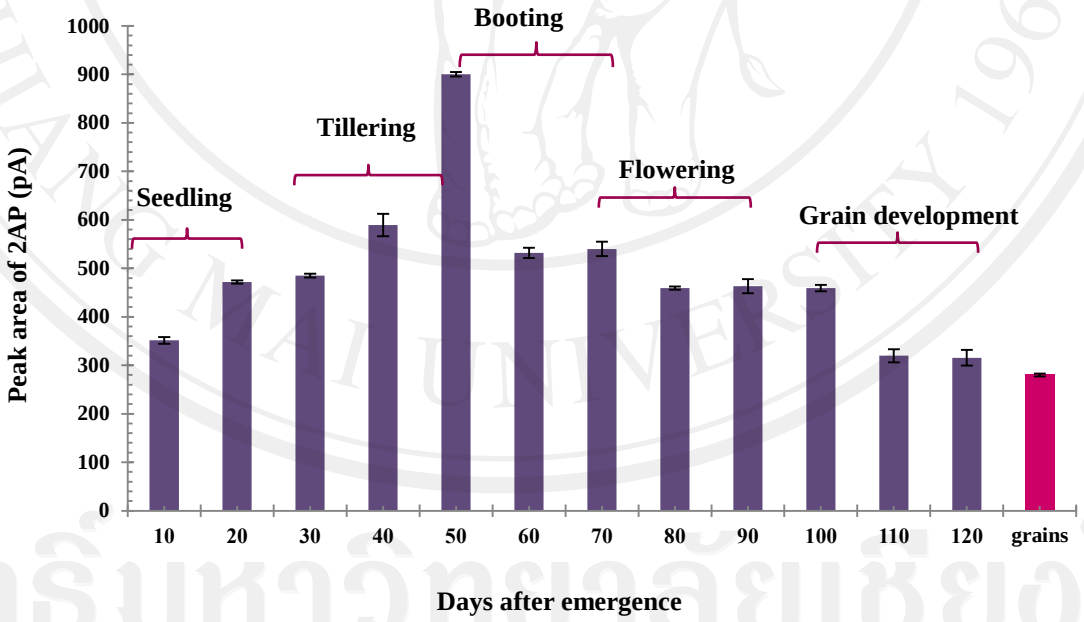

Figure 3.4 Peak areas of 2AP in KDML 105 rice leaves at different ages and rice grains.

Table 3.1 Quantitative data was obtained by SHS-GC/NPD of 2AP in KDML 105 rice leaves at different ages and rice grain.

Age (Days)	Amount of rice leaf sample, g	Peak area of 2AP (pA)	Average of peak area of 2AP	SD	%RSD
10	0.200	362	354	6.70	1.89
	0.200	350			
	0.200	350			
20	0.200	470	472	3.07	0.65
	0.200	470			
	0.200	475			
30	0.200	481	485	3.84	0.79
	0.200	486			
	0.200	489			
40	0.200	611	590	23.12	3.92
	0.200	592			
	0.200	565			
50	0.200	904	900	4.60	0.51
	0.200	895			
	0.200	902			
60	0.200	524	532	10.42	1.96
	0.200	528			
	0.200	544			
70	0.200	557	540	15.02	2.78
	0.200	533			
	0.200	530			
80	0.200	461	459	3.31	0.72
	0.200	460			
	0.200	455			
90	0.200	448	463	14.59	3.15
	0.200	477			
	0.200	464			
100	0.200	457	459	6.60	1.44
	0.200	467			
	0.200	455			
110	0.200	331	320	13.41	4.19
	0.200	323			
	0.200	305			
120	0.200	334	316	16.03	5.08
	0.200	306			
	0.200	307			
grains	1.000	269	280	13.61	4.87
	1.000	295			
	1.000	275			

The different amounts of 2AP in rice leaves showed tendency of increasing from seedling (10-20 days) to booting stage that the highest amount of 2AP was found in this stage (50 days). The amounts of 2AP in KDML 105 rice leaves at 10 to 120 days were higher than its grains. This is in agreeable with the literature that secondary metabolites are mostly produced in rice plant during booting stage. The decreasing peak area of 2AP until the end of grain development stage (100-120 days) is probably due to the translocation of 2AP from leaves to grain as well as the lower rate of secondary metabolite production during these lately stages of rice plant.

From the results, the age of rice leaves having the highest amount of 2AP was at 50. However, KDML105 rice leaves at 20 days of age was selected to be used in further experiments. Although 2AP content was lower in this age of rice leaves compared with that at 50 days of age, the peak area of 2AP was high enough to provide good sensitivity. Moreover, this age of rice can reduce the time for sample preparation (planting) that would make the method more convenient.

3.3Optimization of parameters for sample preparation

3.3.1 Effect of amount of rice leaf sample

The developed SHS-GC/NPD method was used for determination of 2AP in KDML 105 rice leaves at different amounts of rice leaf samples which were 0.200, 0.500, 1.000, 1.500, 2.000, 2.500 and 3.000 g. The quantitative data of 2AP in different amounts of rice leaf sample which was obtained by SHS-GC/NPD were listed in Table 3.2, which shows that the peak area of 2AP in rice leaves at 0.200,

0.500, 1.000, 1.500, 2.000, 2.500 and 3.000 g were 402, 510, 485, 383, 325, 224 and 196 pA, respectively. It was found that the peak area of 2AP continuously increased when the sample amount was higher than 0.200 g and the highest peak area of 2AP was found at 0.500 g which was 510 pA. After that the peak area of 2AP decreased down to 196 pA at 3.000 g.

Table 3.2 Quantitative data of 2AP in KDML 105 rice leaves at different amounts of leaf sample obtained by SHS-GC/NPD.

Amount of rice leaf sample, g	Peak area of 2AP (pA)	Average of peak area of 2AP	SD	%RSD
0.200	417 383 406	402	16.90	4.20
0.500	529 497 502	510	17.25	3.38
1.000	492 487 476	485	8.39	1.73
1.500	373 369 409	383	21.90	5.71
2.000	327 326 322	325	2.38	0.73
2.500	230 228 215	224	7.78	3.47
3.000	191 196 201	196	4.81	2.45

In headspace analysis, the peak area obtained when analyzing an aliquot of the headspace is directly proportional to the concentration of the analyte in the headspace. This follows the expression 3.1.

$$A \propto C_G = C_O/(K + \beta) \quad (3.1)$$

where C_G is concentration of the analyte in the headspace and C_O is its original concentration in the sample, K is the partition coefficient of the analyte between the sample and the headspace, and β is the phase ratio relating the volume of the gas phase in the vial to the volume of the condensed phase or the rice leaf sample.[40]

According to the equation 3.1, sensitivity of SHS-GC/NPD method depends on the combined effect of K and β. For this reason, the experiment was carried out by varying the rice sample amount. The influence of the phase ratio on the analytical result was investigated using a constant temperature. The size of the analyzed rice sample was limited at 3.000 g to prevent the needle of the headspace sampling system from protruding into the rice sample.

Figure 3.5 shows the relationship between peak areas of 2AP at different rice sample amounts up to 3.000 g. With the increase in sample amount, the peak area of 2AP increased exponentially, while the phase ratio values decreased in the same manner. The most suitable amount of rice sample will be the amount that provides the highest percentage recovery of 2AP in the rice headspace. Thus, the optimal of sample amount is 0.500 g. However, at this amount, the percentage of water is too high that can disturb or make damage to the NPD detector. Whereas, the amount at 0.200 g could produce sufficient signal of 2AP and that high sensitivity can

be obtained. Thus, in this experiment, rice leaf amount of 0.200 g was used for further optimization of other parameters.

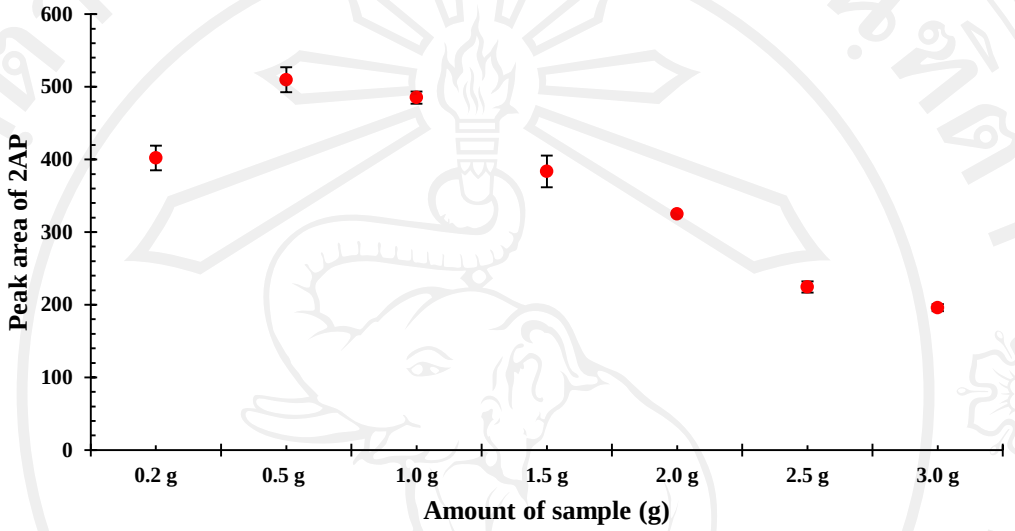

Figure 3.5 Peak areas of 2AP in KDML 105 rice leaves at different amounts of sample.

3.3.2Effect of concentration of internal standard solution

The concentration of internal standard solution used for determination of 2AP by SHS-GC/NPD was investigated using conditions presented in Table 2.1.

Figure 3.6 shows the GC chromatograms of rice leaf sample with added internal standard, 2,6-DMP, at different amounts. The concentration at 500 ppm of 2,6-DMPwas selected because it provided the highest detection sensitivity and was in the same range with the concentration of 2AP.

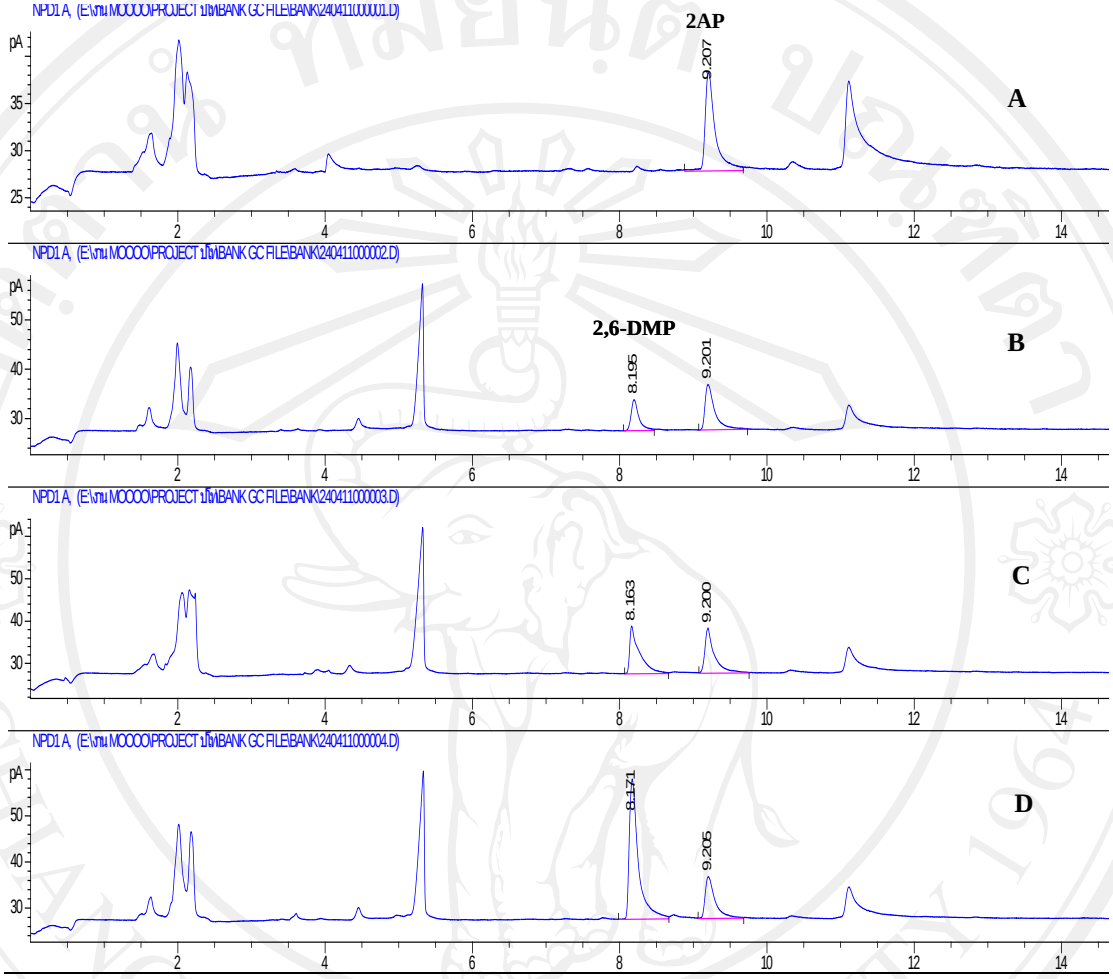

Figure 3.6 GC chromatograms of the headspace volatiles in KDML 105 rice leaves with added 2,6-DMP: (A) none, (B) 100ppm, (C) 250 ppm and (D) 500 ppm.

3.4 Optimization of static headspace autosampler parameters (SHS)

In this study, the appropriate conditions of SHS for determination of 2AP in rice leaves were investigated. Generally, SHS parameters included oven temperature, loop temperature, transfer line temperature, vial equilibration time, vial pressurization time, loop equilibration time and inject time.

3.4.1Effect of temperature of headspace oven and vial equilibration time

The temperature of headspace oven was optimized in the range of90 to 130°C at 10°C increment and the vial equilibration time was varied from 1 to 28min. The other SHS-GC/NPD conditions for this experiment are shown in Table 2.1.

3.4.1.1 Oven temperature at 90 °C

At headspace oven temperature 90°C, the equilibration time was varied in the range of 1 to 28 min. The results in Table 3.3 showed that the peak area ratios of 2AP/2,6-DMPin KDML105 rice leaves at 1, 7, 10, 14, 21 and 28min of the equilibration time at oven temperature 90 °C were 0.28, 0.38, 0.38, 0.46, 0.36 and0.27, respectively.

Table 3.3 Quantitative data of peak area ratios of 2AP/2,6-DMPat oven temperature 90 °C and varied equilibration times at 1, 7, 10, 14, 21 and 28 min obtained by SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	43.9	11.1	0.25			
1	15.5	4.6	0.30	0.28	0.02	8.06
	25.2	7.1	0.28			
	86.0	32.4	0.38			
7	81.2	31.4	0.39	0.38	0.01	1.40
	104.1	39.4	0.38			
	107.5	38.5	0.36			
10	98.7	39.4	0.40	0.38	0.02	5.80
	98.6	38.8	0.39			
	106.9	47.1	0.44			
14	99.3	45.7	0.46	0.46	0.02	4.00
	88.2	42.1	0.48			
	120.5	39.3	0.33			
21	93.8	33.9	0.36	0.36	0.03	8.81
	91.2	35.5	0.39			
	96.6	26.6	0.28			
28	106.3	29.4	0.28	0.27	0.00	1.32
	93.4	25.2	0.27			

Figure 3.7 shows that the peak area ratio of 2AP/2,6-DMP continuously increased after 1 min and the highest peak area ratio was found at equilibration time 14 min, which was 0.46. After that the peak area ratio decreased down to 0.27 at 28 min. Thus, the oven temperature at 90 °C and equilibration time14 min provided efficient extraction and good sensitivity of the 2AP analysis.

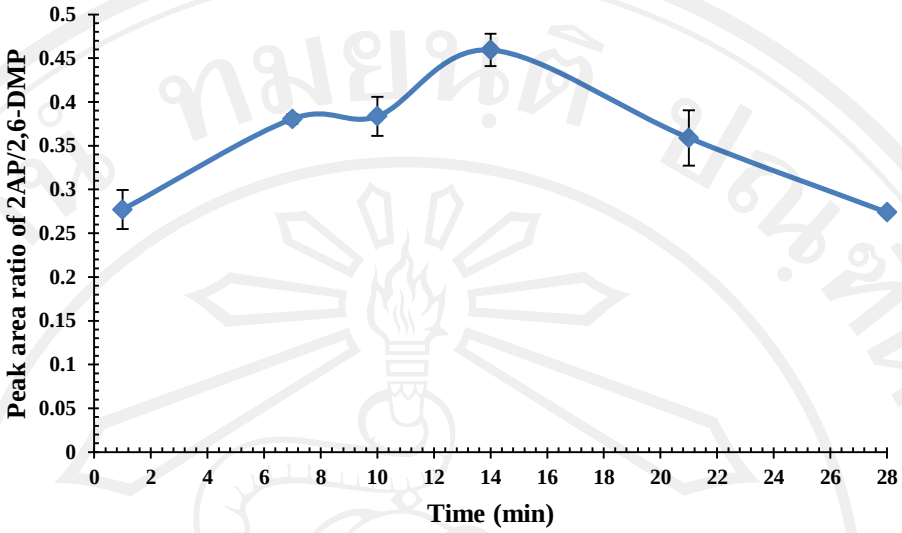

Figure 3.7 Effect of equilibration times on peak area ratio of 2AP/2,6-DMP obtained at oven temperature at 90 °C.

3.4.1.2 Oven temperature at 100 °C

The equilibration times of oven temperature at 100°C was varied in the range of 1 to 20 min. The peak area ratios of 2AP/2,6-DMP in KDML105 rice leaves at 1, 5, 10, 15 and 20 min of the equilibration time at oven temperature 100 °C were 0.30, 0.55, 0.73, 0.59 and 0.46, respectively that show in Table 3.4.

Table 3.4 Quantitative data of peak area ratios of 2AP/2,6-DMP at oven temperature 100°C and varied equilibration times at 1, 5, 10, 15 and 20 min obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	138.9	45.3	0.33			
1	113.7	31.1	0.27	0.30	0.03	8.84
	109.5	32.4	0.30			
	124.1	64.3	0.52			
5	129.8	68.8	0.53	0.55	0.04	7.82
	138.2	82.6	0.60			
	132.8	96.6	0.73			
10	112.1	83.3	0.74	0.73	0.01	1.77
	104.4	74.9	0.72			
	123.3	75.5	0.61			
15	124.0	74.2	0.60	0.59	0.03	5.51
	136.2	75.0	0.55			
	116.0	53.6	0.46			
20	124.6	56.3	0.45	0.46	0.01	1.39
	110.0	51.0	0.46			

Figure 3.8shows that the peak area ratio of 2AP/2,6-DMP and the highest peak area ratio at oven temperature 100°C was found at equilibration time of 10 min, whichwas0.73. After that the peak area ratio of 2AP/2,6-DMP decreased down to 0.46 at 20 min. The oven temperature at 100°C and equilibration time 10 min provided efficient extraction and good sensitivity of 2AP analysis.

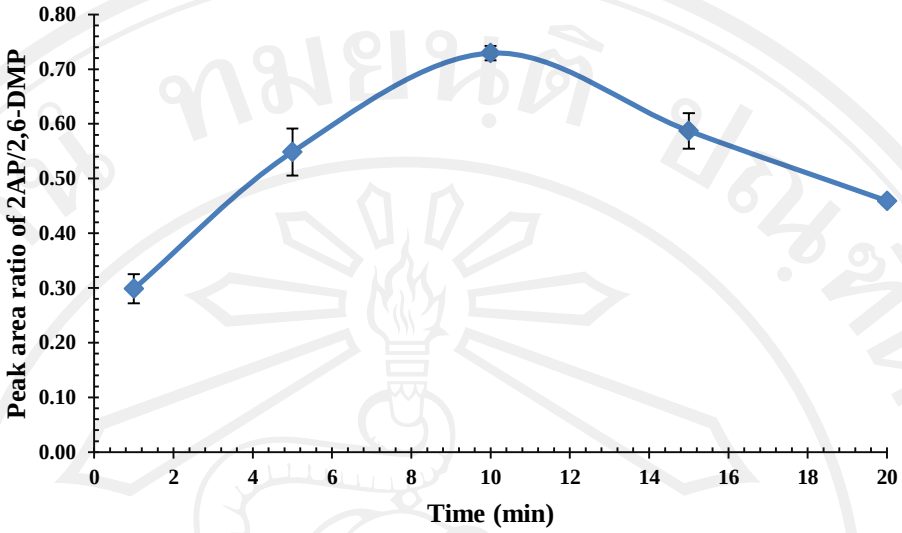

Figure 3.8 Effect of equilibration times on peak area ratio of 2AP/2,6-DMP obtained at oven temperature at 100 °C.

3.4.1.3 Oven temperature at 110 °C

The equilibration time at oven temperature 110°C was varied in the range of 1 to 13 min. The peak area ratio data of 2AP/2,6-DMP found in different equilibration times of headspace oven temperature at 110°C obtained by SHS-GC/NPD were listed in Table 3.5.The results showed that the peak area ratios in KDML105 rice leaves at 1, 3,5, 7, 9,11 and 13min of equilibration time were 0.37, 0.70, 0.92, 1.00, 0.90, 0.84 and 0.73, respectively.

Table 3.5 Quantitative data of peak area ratios of 2AP/2,6-DMP at oven temperature 110°C and varied equilibration times at 1, 3, 5, 7, 9, 11 and 13 min obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	178.3	68.3	0.38			
1	224.2	85.0	0.38	0.37	0.03	7.28
	224.4	75.2	0.34			
	206.2	143.9	0.70			
3	168.4	118.3	0.70	0.70	0.01	0.83
	190.3	135.0	0.71			
	134.5	126.5	0.94			
5	169.2	142.5	0.84	0.92	0.07	7.20
	185.7	179.7	0.97			
	183.5	193.9	1.06			
7	200.2	195.3	0.98	1.00	0.05	4.93
	207.0	200.3	0.97			
	190.8	172.8	0.91			
9	195.6	171.6	0.88	0.90	0.02	2.36
	146.6	134.7	0.92			
	168.3	137.0	0.81			
11	180.9	160.8	0.89	0.84	0.05	5.38
	147.9	119.5	0.81			
	177.0	124.4	0.70			
13	184.5	136.2	0.74	0.73	0.02	3.31
	190.1	142.4	0.75			

Figure 3.9 shows that the peak area ratios of 2AP/2,6-DMP continuously increased after 1 to 7 min and the highest peak area ratio was found at equilibration time 7 min, which was 1.00. The peak area ratio decreased down to 0.73 at 13 min. The oven temperature at 110 °C and equilibration time 7 min provided good sensitivity and efficient extraction of 2AP analysis.

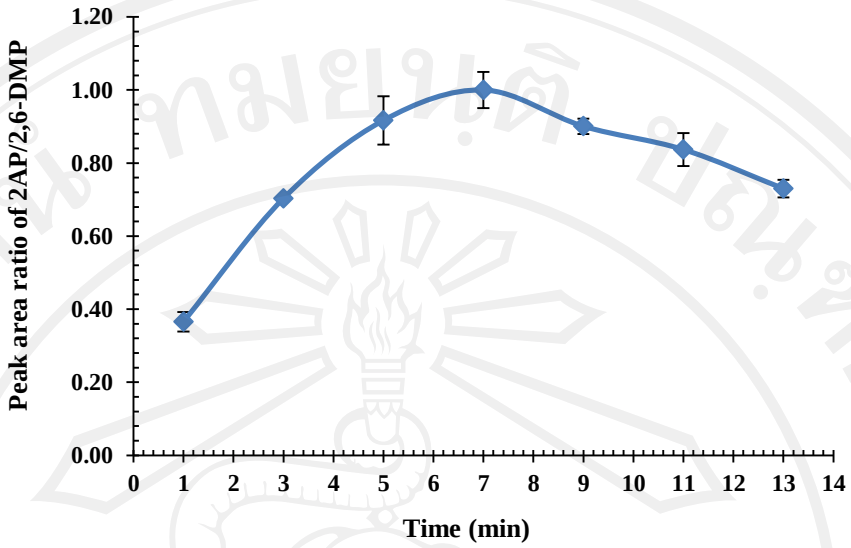

Figure 3.9 Effect of equilibration times on peak area ratio of 2AP/2,6-DMP obtained at oven temperature at 110 °C.

3.4.1.4 Oven temperature at 120 °C

The equilibration times of oven temperature 120°C was varied in the range of 1 to 7 min. The peak area ratios data of 2AP/2,6-DMP found in different times of headspace oven temperature at 90°C obtained by SHS-GC/NPD were listed in Table 3.6. It was found that the peak area ratios in KDML105 rice leaves at equilibration times 1, 2, 3, 4, 5, 6 and 7min were 0.57, 0.86, 1.61, 1.79, 2.10, 1.74 and1.65, respectively.

Table 3.6 Quantitative data of peak area ratios of 2AP/2,6-DMPat oven temperature 120°C and varied equilibration times at 1, 2, 3, 4, 5, 6 and 7 min obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	215.9	118.1	0.55			
1	201.3	116.9	0.58	0.57	0.02	3.80
	195.2	114.7	0.59			
	120.2	109.6	0.91			
2	159.4	138.6	0.87	0.86	0.05	5.99
	153.9	124.5	0.81			
	126.1	209.7	1.66			
3	118.9	183.2	1.54	1.61	0.06	4.00
	103.2	169.0	1.64			
	126.7	220.1	1.74			
4	59.6	115.9	1.94	1.79	0.14	7.65
	85.7	144.5	1.69			
	128.9	269.0	2.09			
5	130.5	272.0	2.08	2.10	0.02	0.95
	115.0	243.8	2.12			
	126.2	225.1	1.78			
6	121.1	213.8	1.77	1.74	0.06	3.41
	127.2	212.8	1.67			
	91.1	148.0	1.62			
7	121.9	204.4	1.68	1.65	0.03	1.59
	113.8	187.5	1.65			

Figure 3.10 shows that the peak area ratios of 2AP/2,6-DMP continuously increased after 1 min and the highest peak area ratio was found at equilibration time 5 min, which was 2.10. After that the peak area ratios decreased down to 1.65 at 7 min. The oven temperature at 120 °C and equilibration time 5 min provided efficient extraction and good sensitivity of the 2AP analysis.

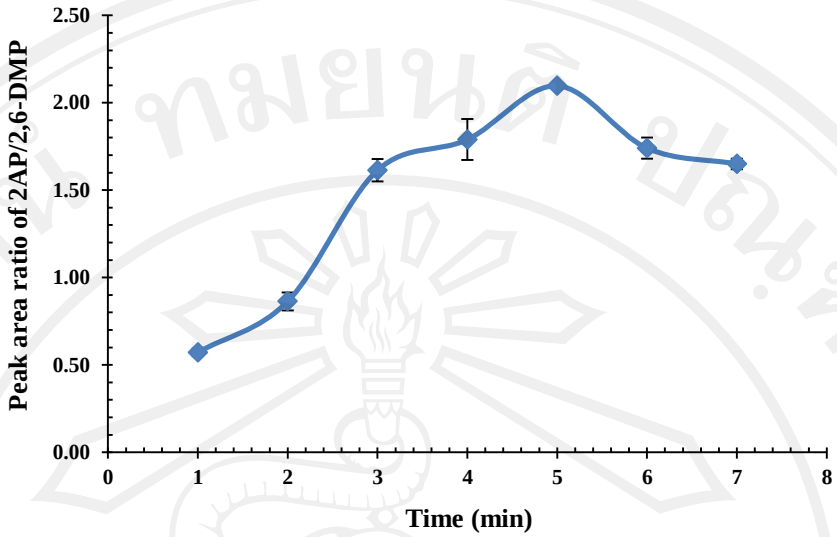

Figure 3.10 Effect of equilibration times on peak area ratio of 2AP/2,6-DMP obtained at oven temperature at 120 °C.

3.4.1.5 Oven temperature at 130 °C

The equilibration times of oven temperature at 130°C was varied in the range of 1 to 5 min. The results in Table 3.7 showed that the peak area ratio of 2AP/2,6-DMPin KDML105 rice leaves at 1, 2, 3, 4 and 5min of the equilibration times at oven temperature 130 °C were 0.34, 0.78, 1.50, 1.84 and1.70, respectively.

Table 3.7 Quantitative data of peak area ratios of 2AP/2,6-DMP at oven temperature 130°C and varied equilibration times at 1, 2, 3, 4 and 5 min obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	51.2	16.5	0.32			
1	143.4	52.4	0.37	0.34	0.02	6.62
	198.1	65.9	0.33			
	152.7	116.5	0.76			
2	170.8	124.3	0.73	0.78	0.07	9.02
	144.3	124.7	0.86			
	111.3	187.0	1.68			
3	113.6	163.9	1.44	1.50	0.16	10.54
	120.7	166.6	1.38			
	124.4	246.2	1.98			
4	134.0	220.8	1.65	1.84	0.17	9.40
	114.6	217.9	1.90			
	103.9	170.7	1.64			
5	77.3	138.2	1.79	1.70	0.08	4.65
	94.8	157.5	1.66			

Figure 3.11 shows that the highest peak area ratios of 2AP/2,6-DMP was found at equilibration time 4 min, which was 1.84. After that the peak area ratio of 2AP/2,6-DMP decreased down to 1.70 at 5 min. The oven temperature at 130 °C and equilibration time 4 min provided good sensitivity of 2AP analysis in rice leaf sample.

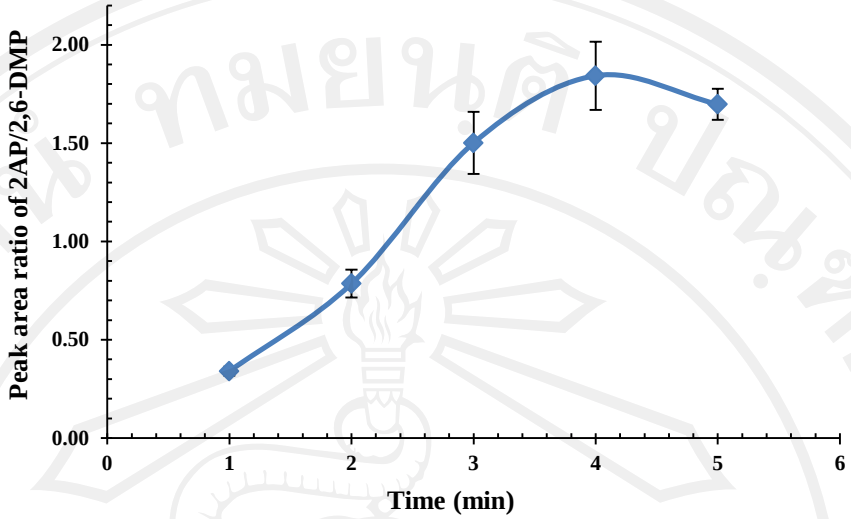

Figure 3.11 Effect of equilibration times on peak area ratio of 2AP/2,6-DMP obtained at oven temperature 130 °C.

The important parameters on SHS-GC/NPD analysis are both oven temperature and the equilibration time that influence the method sensitivity and repeatability.[55] Heating changes the solubility of the analyte and drives its equilibrium to the gas phase.[56] Thus, the oven temperature was firstly optimized in this study parallel with the equilibration time.

In this part of experiment, the oven temperature and vial equilibration time were optimized in range of 90 to 130°C and 1 to 28 min, respectively. The results are presented in Figure 3.12. It was found that each oven temperature gave the highest peak area ratio of 2AP/2,6-DMP at different vial equilibration times, which were 14, 10, 7, 5 and 4 min for 90, 100, 110, 120 and 130°C, respectively. Although the easiest way to increase the peak intensity is to rise the oven temperature, heating the rice sample at 130°C yielded increasing complexity of the rice headspace volatiles

as compared to those at lower temperatures. This complexity form heating sample may be caused by thermal degradation of some rice components resulting in consequent products eluted as interferences in the analysis.

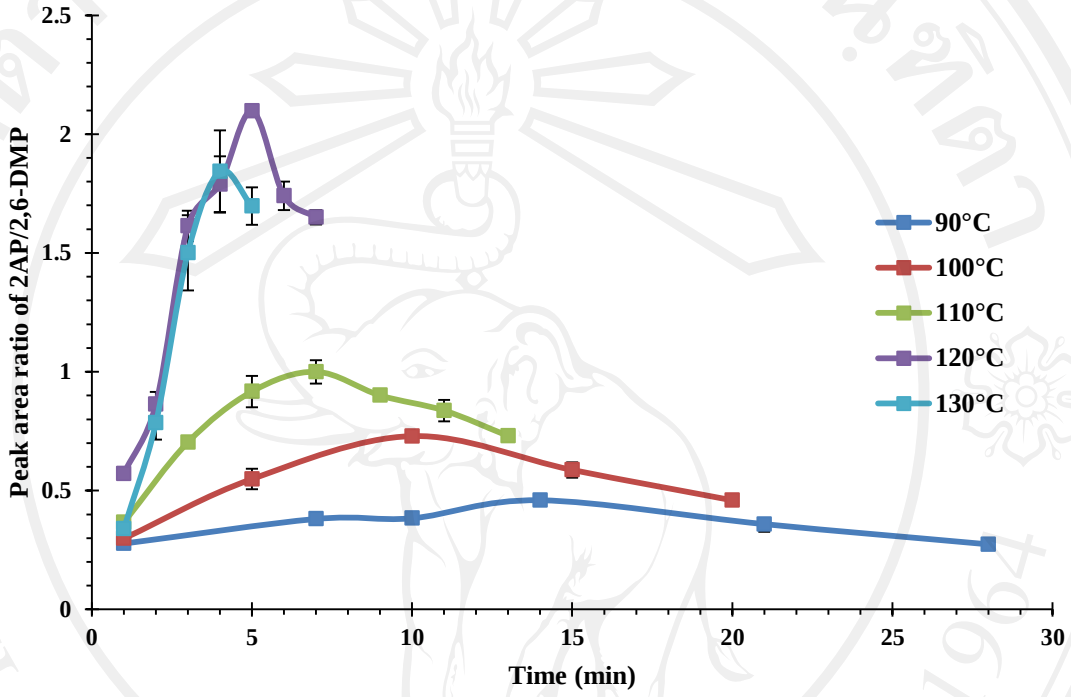

Figure 3.12 Effect of oven temperatures and equilibration times on peak area ratio of 2AP/2,6-DMP.

So, the optimal oven temperature and vial equilibration time that yielded the highest peak area ratio of all were 120°C and 5 min, respectively. At these optimum parameters, 2AP was analyzed efficiently and good sensitivity which was suitable for quantitative analysis of 2AP in rice leaf samples could be obtained.

3.4.2 Effect of pressurizing time

The optimization of pressurizing time of automatic headspace extractor was investigated using conditions presented in section 2.9.3. The other SHS-GC/NPD conditions in this experiment were listed in Table 2.1, whereas the temperature of headspace oven, sample loop and transfer line were set to 120, 130 and 140°C, respectively and vial equilibration time was 5 min. The peak area ratio data of 2AP/2,6-DMP at different pressurizing times obtained by SHS-GC/NPD were listed in Table 3.8.

Table 3.8 Quantitative data of peak area ratios of 2AP/2,6-DMP at different pressurization times obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	148.7	83.3	0.56			
0.01	148.8	87.2	0.59	0.56	0.02	3.57
	118.6	64.8	0.55			
	173.2	106.7	0.62			
0.05	142.3	94.2	0.66	0.63	0.03	4.27
	149.7	92.0	0.61			
	149.6	80.9	0.54			
0.10	184.8	99.0	0.54	0.54	0.00	0.52
	155.2	83.2	0.54			
	171.8	85.0	0.49			
0.15	171.7	90.9	0.53	0.51	0.02	3.39
	169.7	87.4	0.52			
	191.8	86.4	0.45			
0.20	239.5	117.6	0.49	0.49	0.03	6.97
	171.7	88.9	0.52			

The effect of pressurization time was investigated within the range 0.01 - 0.20 min. After the end of the vial equilibration time, the helium gas was allowed to enter the vial through the sample needle to build up additional pressure in the vial, which helped transferring volatiles in the sample headspace to the sample loop. The results of peak area ratio of 2AP/2,6-DMP are shown in Figure 3.13. The optimal pressurization time at 0.05 min was chosen for further optimization of other parameters because it provided the highest peak area ratio of the 2AP/2,6-DMP and thus, good sensitivity could be obtained for the analysis.

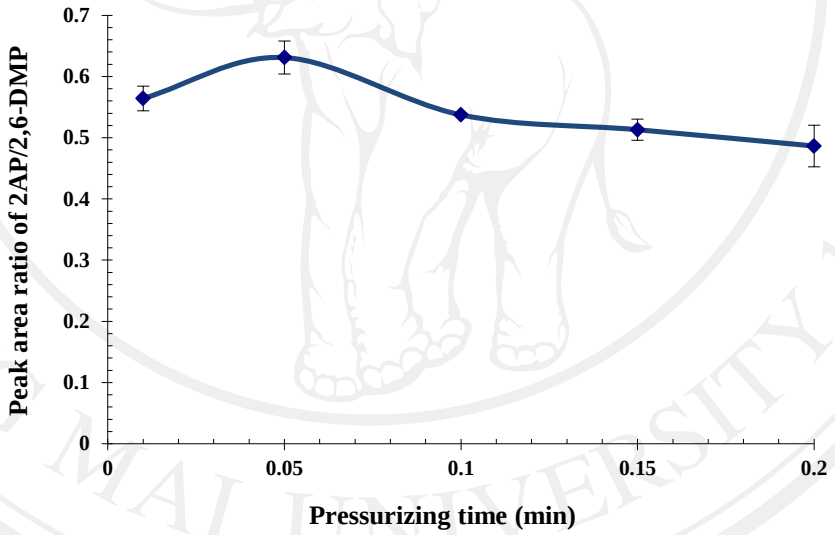

Figure 3.13 Effect of pressurization time on peak area ratio of 2AP/2,6-DMP.

3.4.3 Effect of loop filling time

The loop filling time of automatic headspace extractor was varied in the range of 0.01- 0.50 min. The other SHS-GC/NPD conditions in this experiment was listed in Table 2.1, whereas the temperature of headspace oven, sample loop and transfer line were set at 120, 130 and 140°C, respectively, vial equilibration time was 5 min and pressurization time was 0.05 min. The peak area ratio data of 2AP/2,6-DMP at different loop filling times obtained by SHS-GC/NPD were listed in Table 3.9.

The results in Table 3.9 shows that the peak area ratio of 2AP/2,6-DMP in KDML105 rice leaves at 0.01, 0.02, 0.03, 0.04 and 0.05 min of the loop filling time continuously decreased from 0.49 down to 0.25.

Table 3.9 Quantitative data of peak area ratios of 2AP/2,6-DMPat different loop filling times obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	84.0	42.0	0.50			
0.01	129.4	62.6	0.48	0.49	0.01	1.80
	113.6	55.2	0.49			
	77.7	31.5	0.41			
0.02	77.0	30.1	0.39	0.40	0.01	1.82
	119.1	47.4	0.40			
	113.9	38.8	0.34			
0.03	80.0	28.3	0.35	0.35	0.01	3.05
	122.4	44.3	0.36			
	92.0	25.9	0.28			
0.04	78.5	21.3	0.27	0.27	0.01	3.28
	76.6	20.2	0.26			
	60.7	15.9	0.26			
0.05	87.9	21.8	0.25	0.25	0.01	2.82
	92.0	23.2	0.25			

The rice volatiles inside the vial were kept at a high pressure and it tended to equilibrate to atmospheric pressure during the filling process. Loop filling time must be long enough to allow a complete filling of the sample loop. On the other hand, short filling times allow the vapor in the loop to be at high pressure. At this high pressure, the volatiles were more concentrated with the subsequent improvement of the analytical sensitivity. In this experiment, it was found that the optimal loop filling time was 0.01 min as shown in Figure 3.14. Varying the loop filling time from 0.01 to 0.05 min gave reduction in peak area ration of 2AP/2,6-DMP. Thus, the loop filling time at 0.01 min should provide the highest detection sensitivity and was selected.

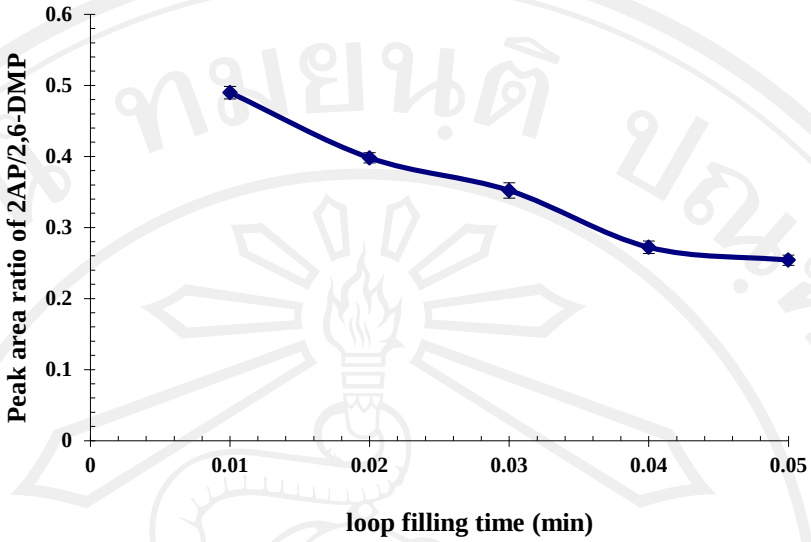

Figure 3.14 Effect of loop filling time on peak area ratio of 2AP/2,6-DMP.

3.4.4 Effect of loop equilibration time

The loop equilibration time of automatic headspace extractor was varied in the range of 0.20- 1.00 min. The other SHS-GC/NPD conditions in this experiment was listed in Table 2.1, whereas the temperature of headspace oven, sample loop and transfer line were set at120, 130 and 140°C, respectively, vial equilibration time was 5 min, pressurization time was 0.05 min and loop filling time was 0.01 min. The peak area ratio data of 2AP/2,6-DMP at different loop equilibration times obtained by SHS-GC/NPD were listed in Table 3.10.

Table 3.10 Quantitative data of peak area ratios of 2AP/2,6-DMP at different loop equilibration times obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	124.5	47	0.38			
0.20	149.3	53.4	0.36	0.37	0.01	2.83
	151.7	55	0.36			
	167.1	65	0.39			
0.40	123.4	48.4	0.39	0.39	0.00	0.54
	102.3	40.2	0.39			
	152.9	49.8	0.33			
0.60	125.6	43.6	0.35	0.33	0.01	3.97
	156.9	50.7	0.32			
	188.8	53	0.28			
0.80	179.7	46.4	0.26	0.27	0.01	4.35
	163.2	45	0.28			
	171.6	42.3	0.25			
1.00	170.1	42.6	0.25	0.25	0.00	1.08
	117.4	28.8	0.25			

The effect of loop equilibration time was carried out in the range from 0.20 to 1.00 min. After the headspace gas filled into sample loop, the gas was maintained to equilibrium in the sample loop for a particular time. Figure 3.15 shows that the optimum loop equilibration time was 0.40 min as the peak area ratio of 2AP/2,6-DMP had tendency of increasing from 0.20 to 0.40 min of loop equilibration time that the highest peak area ratio of 2AP/2,6-DMP was found at 0.40 min. The loop equilibration time of 0.40 min should yield efficient analysis of 2AP with good sensitivity.

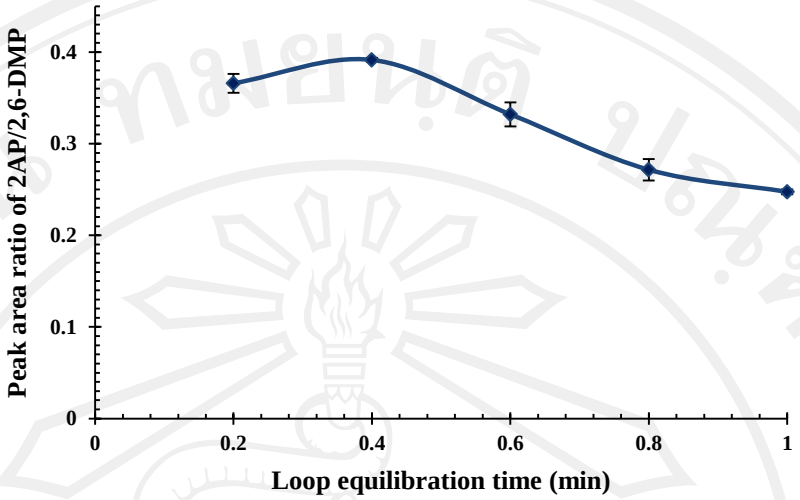

Figure 3.15 Effect of loop equilibration time on peak area ratio of 2AP/2,6-DMP.

3.4.5 Effect of injection time

The optimization of injection time that was obtained from automatic headspace extractor was investigated using other SHS-GC/NPD conditions that were listed in Table 2.1. The temperature of vial, sample loop and transfer line were set to 120, 130 and 140°C, respectively, vial equilibration time was 5 min, pressurizing time was 0.05 min, loop filling time was 0.01 min and loop equilibration time 0.40 min. The peak area ratio data of 2AP/2,6-DMP at different injection times obtained by SHS-GC/NPD were listed in Table 3.11.

Table 3.11 Quantitative data of peak area ratios of 2AP/2,6-DMP at different injection times obtained from SHS-GC/NPD.

Time (min)	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of Peak area ratio	SD	% RSD
	2,6-DMP	2AP				
	24.6	3.6	0.15			
0.20	30.1	4.4	0.15	0.14	0.00	2.40
	22.1	3.1	0.14			
	175.9	109.5	0.62			
0.40	151.8	96.2	0.63	0.62	0.02	3.15
	146.0	87.0	0.60			
	159.4	85.0	0.53			
0.60	174.3	90.3	0.52	0.54	0.03	6.09
	158.2	92	0.58			
	181.2	108.2	0.60			
0.80	159.2	80.0	0.50	0.53	0.06	10.69
	167.6	83.0	0.50			
	213.8	115	0.54			
1.00	115.5	67.6	0.59	0.51	0.09	18.30
	141.6	57.4	0.41			

Figure 3.16 shows the effect of injection time that was investigated in the range of 0.20-1.00 min. After the sample vial was heated until the rice headspace volatiles reached the equilibrium in the gas phase above the solid phase. At the end of the vial equilibration time, the sample valve was opened and the gases that were transferred to the sample loop were swept by the carrier gas flow through the transfer line into the GC port. The injection time is the time which the gases in the sample loop were injected into the GC. This time must be sufficient to complete sample transfer. If the time is too short, the analysis will lose sensitivity because not all the gas sample is transferred. The results of injection time effect on peak area ratio of 2AP/2,6-DMP indicated that increasing the injection time could increase peak area

ratio of 2AP/2,6-DMP. The optimum injection time was 0.40 min since it provided, in average, the highest peak area ratio than other injection times.

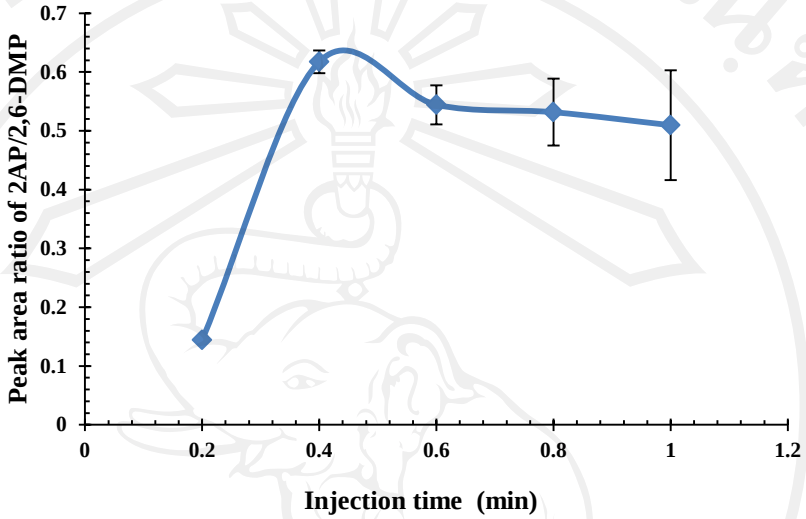

Figure 3.16 Effect of injection time on peak area ratio of 2AP/2,6-DMP.

From the results in section 3.4.1 – 3.4.5, it can be concluded that the optimum condition of SHS indicated temperature of oven 120 °C; temperature of loop 130 °C; temperature of transfer line 140 °C; vial equilibration time 5 min; pressurizing time 0.05 min; loop filling time 0.01 min; loop equilibration time 0.40 min and injection time 0.40 min. These optimum parameters were listed in Table 3.12

Table 3.12 The optimum condition of SHS for analysis of the rice leaves sample.

Parameter	Value
1. Temperature (headspace oven, sample loop, transfer line)	120, 130, 140 °C
2. Vial equilibration time	5 min
3. Vial pressurization time	0.05 min
4. Loop fill time	0.01 min
5. Loop equilibration time	0.40 min
6. Injection time	0.40 min
7. Carrier gas flow	He

3.5 Effect of gas flow rate on GC/NPD

The gas flow rate on GC/NPD was investigated using the optimum headspace condition shown in Table 3.12 and the GC/NPD parameters were listed in Table 2.1.

Figure 3.17 shows the chromatograms of KDML105 rice leaves at the different flow rates of GC carrier gas, which were varied in the range of 3-11 °C/min. It was found that sensitivity of detection was increased by using high flow rates. In this experiment, an increase in flow rate of GC carrier gas also resulted in the bad separation, as the plasma of NPD detector was extinguished at 11 °C/min of the gas flow rate (Figure 3.17(I)). The selected gas flow rate of GC carrier gas was 8 °C/min. At this optimal gas flow rate, relatively high sensitivity and good separation were yielded for determination of 2AP in the rice leaf samples.

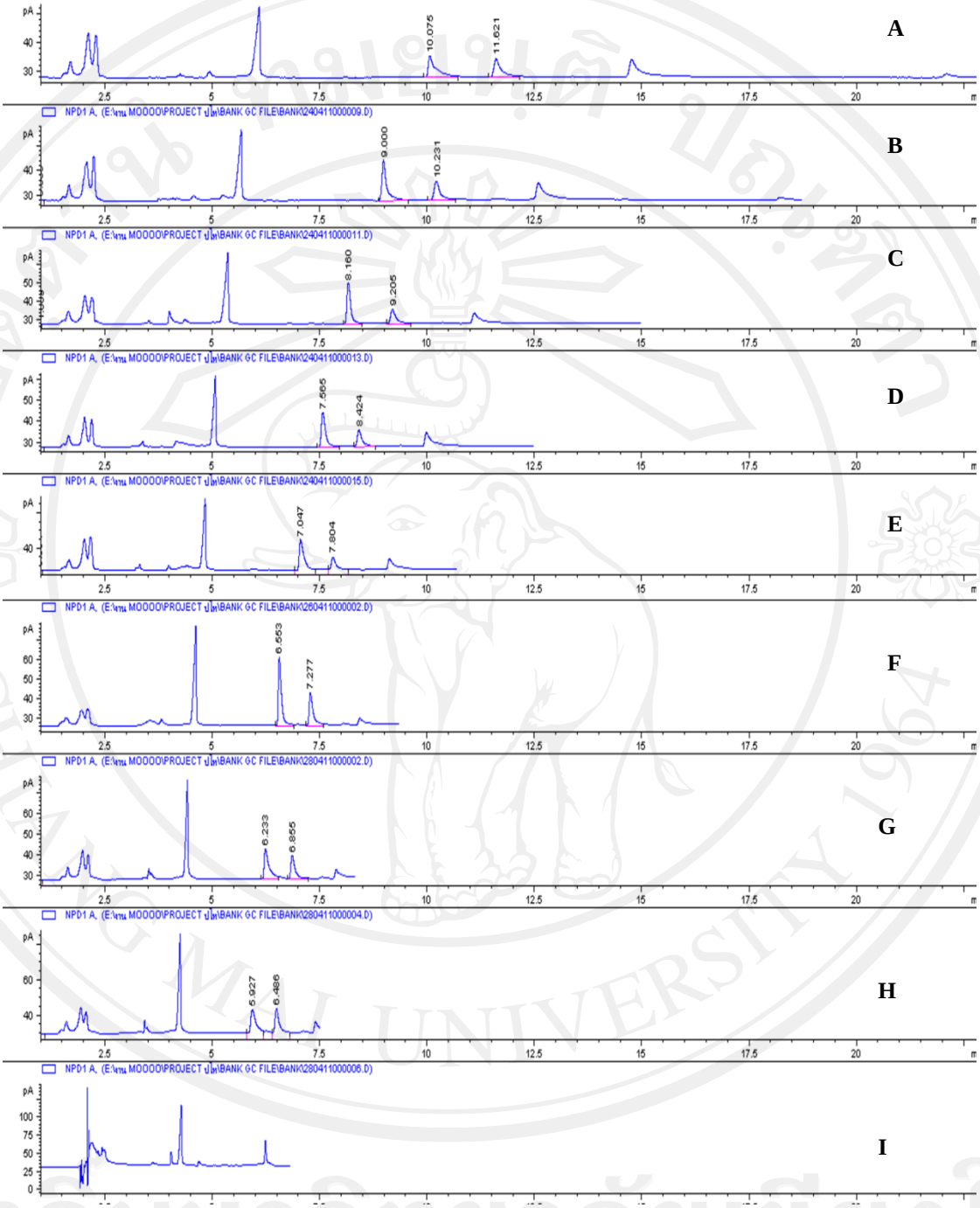

Figure 3.17 GC chromatograms of KDML10 rice leaf sample show the effect of gas flow rate of GC carrier gas that was varied in the range of 3 to 11 °C/min with 1 °C increment (A-I).

Thus, the results in section 3.4 and 3.5 had led to the conclusion of the optimum conditions of SHS-GC/NPD as those shown in Table 3.13. In these optimum conditions were applied to the analysis of 2AP in leaves of different rice varieties.

Table 3.13 The optimum conditions of SHS-GC/NPD for analysis the rice leaf samples.

Parameter	Value
Optimum condition of static headspace autosampler	
1. Temperature (headspace oven, sample loop, transfer line)	120, 130, 140°C
2. Vial equilibration time	5 min
3. Vial pressurization time	0.05 min
4. Loop fill time	0.01 min
5. Loop equilibration time	0.40 min
6. Injection time	0.40 min
7. Carrier gas flow	He
Gas chromatography condition	
1. Injection temperature	230°C
2. Oven initial temperature	50°C
3. Final temperature	125°C
4. Heater NPD	300 °C
5. Program rate	8°C/min
6. Post run	250°C, 10 min
7. Injection Mode	Splitless
8. Column	HP-5 (30 m × 5.3 mm × 1.5 µm)
9. Carrier gas flow	He

3.6 Percentage of extraction of 2AP from rice leaves by SHS-GC/NPD

For the determination of percentage of extraction that was obtained from automatic headspace extractor, the optimum SHS-GC/NPD conditions were listed in Table 3.13.

In this experiment, MHE was performed on KDML105 rice leaf samples and peak areas of 2AP were calculated. The percentage of extraction of 2AP from rice leaves by SHS-GC/NPD was shown in Table 3.14 and Figure 3.18. The percentage recovery of 2AP in the first headspace extraction step, or the peak area of 2AP relative to the sum of peak areas in the consecutive MHE measurements, was determined for KDML105 rice leaf sample. Results show that the highest recovery was 65.84% was obtained when a rice sample amount of 0.200 g was used.

Table 3.14 Quantitative data and percentage of extraction was obtained by SHS-GC/NPD of 2AP in KDML 105 rice leaves using multiple headspace extraction (MHE).

No.	Peak area of 2AP					Average	% extraction	Average of % extraction	SD	%RSD
	Number of extraction*									
	1	2	3	4	5					
1	330.9	86.6	37.3	26.2	18.2	499.2	66.29			
2	323.9	87.0	37.8	28.9	17.6	495.2	65.41	65.84	0.439	0.67
3	324.9	85.9	37.8	23.6	21.3	493.5	65.84			

* Triplicate determinations were performed.

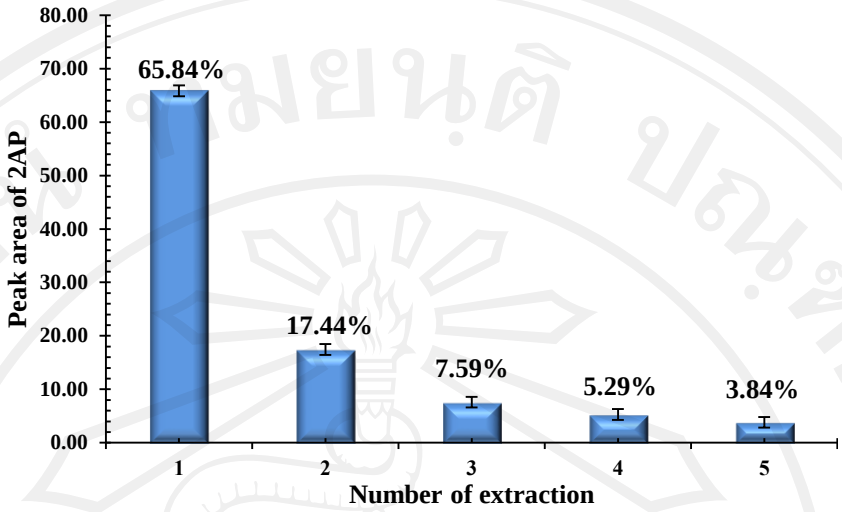

Figure 3.18 The decrease in percentages of extraction of 2AP from KDML 105 rice leaves by 5 times headspace extraction.

The use of SHS-GC which is the direct analysis of headspace volatiles from rice leaf samples using NPD as detector was more specific to the detection of 2AP, which had made the quantification more accurate, sensitive and required less amount of sample.

3.7 Calibration curve

The calibration curve that was obtained from automatic headspace extraction of 2AP in leaves of non-aromatic rice, Supanburi2, by SHS-GC with NPD detector was investigated using conditions that presented in section 2.12. The SHS-GC/NPD conditions for this experiment were listed in Table 3.13.

The standard 2AP calibration graph exhibits excellent linearity in the range of 0.50 - 50.00 µg/g with a correlation coefficient of 0.9998 as shown in Figure 3.19.

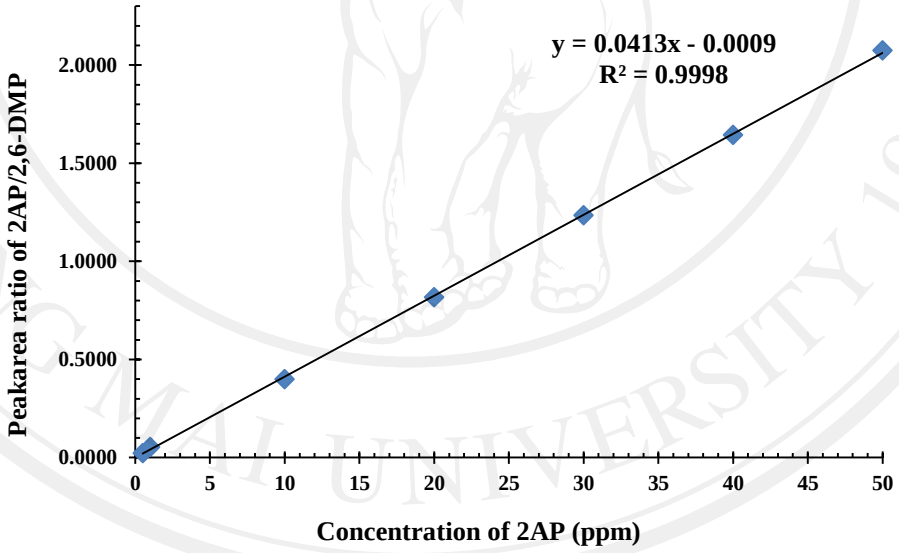

Figure 3.19 Calibration graph of 2AP obtained by using SHS-GC/NPD.

3.8 Validation of the developed SHS-GC/NPD method

3.8.1Limit of Detection (LOD)

In this experiment for the determination of LOD, the SHS-GC/NPD conditions for were listed in Table 3.13.

LOD value is defined as the concentration at which signal to noise ratio (S/N) higher than or equal 3 in terms of the least amount of 2AP standard.

Results in Figure 3.20 shows SHS-GC/NPD chromatograms of non-aromatic rice, Supanburi 2, added with different amounts of 1000 ppm 2AP standard solution. The peak height of 2AP continuously decreased from the volume of 8 µl down to 0.1 µl of 1000 ppm 2AP standard solution. At the volume of 1 µl of 2AP standard solution, the signal to noise ratio (S/N) equal to 3 was yielded. Thus, the LOD for this system was determined at 0.1 µg of 2AP in 0.200 g of rice leaves.

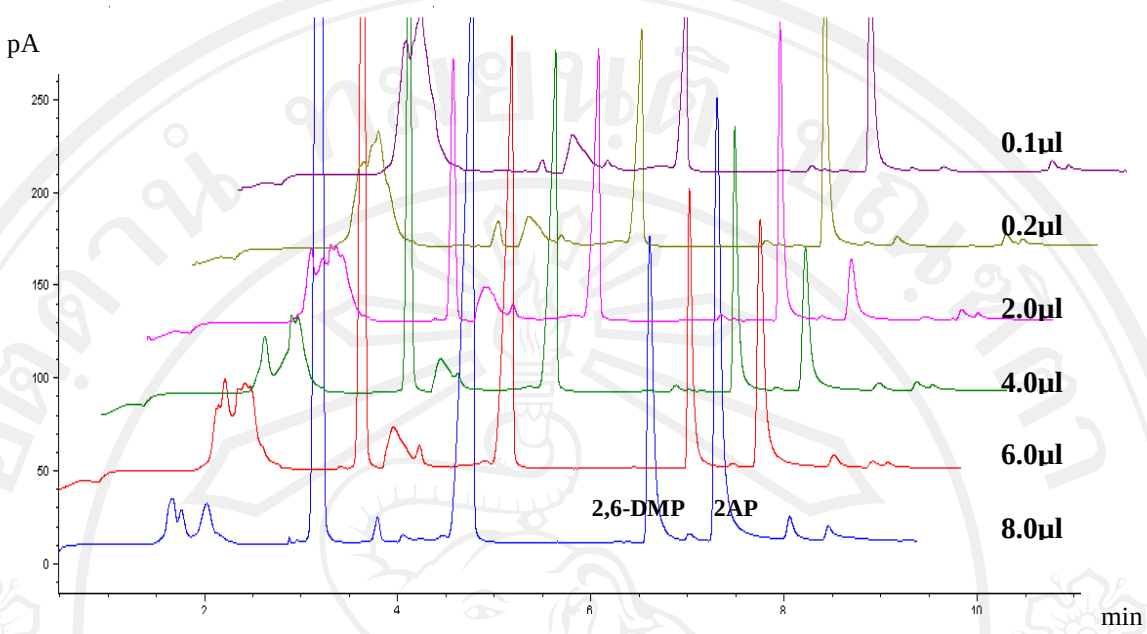

Figure 3.20 SHS-GC/NPD chromatograms obtained by adding 1000 ppm of 2AP standard solution into headspace vial containing 0.2000 g of the non-aromatic rice(cv.Supanburi 2)at 8.0, 6.0, 4.0, 2.0, 0.2 and 0.1 µl.

3.8.2 Limit of Quantitation (LOQ)

The experiment for determination of LOQ by using the SHS-GC with NPD detector was presented in section 2.13.2 and the SHS-GC/NPD conditions for this experiment were listed in Table 3.13.

LOQ value is defined as the concentration of the analyte at which signal to noise ratio (S/N) higher than or equal 10 in terms of the least amount of rice leaf sample.

Figure 3.21 shows the SHS-GC/NPD chromatograms of aromatic rice, KDML105, at different amounts of rice leaf sample, which were in the range of 0.0080 to 0.3000 g. It was found that at the amount of 0.0100 g of rice leaf sample

gave the signal to noise ratio (S/N) equal to 10. Thus, the LOQ for this system was determined at 0.0100 g of KDML105 rice leaves.

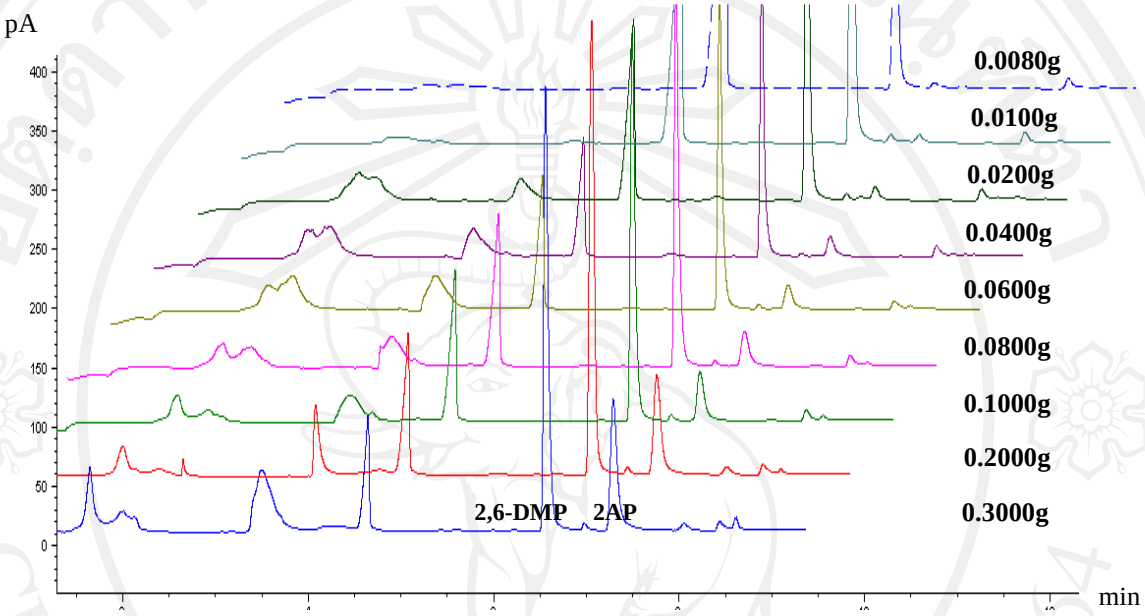

Figure 3.21 SHS-GC/NPD chromatograms obtained by varying the amounts of KDML105 rice leaf sample at 0.3, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01 and 0.008 g.

3.8.3 Repeatability (Intraday)

For quantitative analysis of 2AP in rice leaves by SHS-GC/NPD, the intraday repeatability was investigated using conditions presented in section 2.13.3 and the SHS-GC/NPD conditions were those listed in Table 3.13.

Repeatability expresses the precision under the same operating conditions over a short interval of time and usually performed in the same day of experiment. Repeatability is also termed intra-assay precision.

The results in Table 3.15 and Figure 3.22 shows that analysis of 2AP in KDML105 rice leaf samples for 10 replications which were performed in 1 day gave the intraday coefficient of variation of 1.93% RSD (n =10). This validation data confirmed good efficiency in terms of precision and accuracy of the SHS-GC/NPD system.

Table 3.15 Quantitative data obtained by SHS-GC/NPD of 2AP in KDML 105 rice leaves analysed 10 replication within 1 day.

Repeat	Peak area		Peak area ratio	Average	SD	%RSD
	2,6-DMP	2AP				
1	1186.8	489.5	0.4125	0.4161	0.0080	1.93
2	1031.8	433.1	0.4198			
3	1168.2	475.8	0.4073			
4	1053.5	442.1	0.4196			
5	1098.4	446.6	0.4066			
6	941.9	384.3	0.4080			
7	1031.7	431.6	0.4183			
8	1001.3	418.7	0.4182			
9	747.0	323.7	0.4333			
10	964.7	402.9	0.4176			

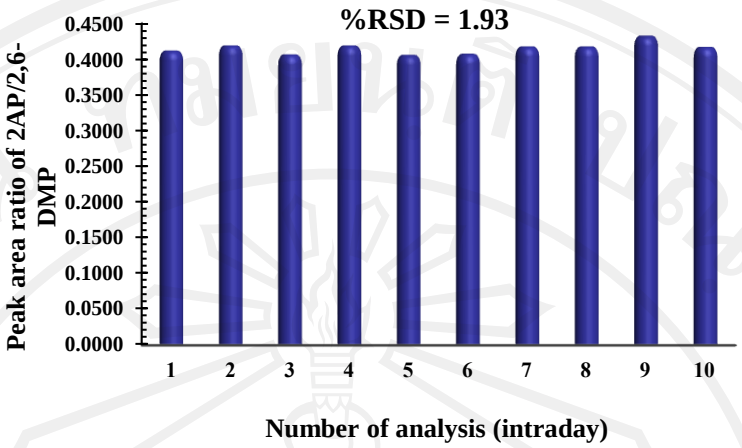

Figure 3.22 Peak area ratios of 2AP/2,6-DMP in KDML105 rice leaves obtained by 10 replication of SHS-GC/NPD analysis within 1 day.

3.8.4 Reproducibility(Interday)

For quantitative analysis of 2AP in rice leaves by SHS-GC/NPD, the interday reproducibility was investigated using conditions presented in section 2.13.4 and the SHS-GC/NPD conditions listed in Table 3.13.

Reproducibility is a concept of precision relating to measurements made under reproducible conditions, i.e. same method, different operator, different laboratories, different equipment and long time period.

The results in Table 3.16 and Figure 3.23 shows that the repeated analysis of 2AP in KDML105 rice leaf samples for 5 times which were performed once a day in 5 days gave the interday coefficient of variation of 2.40% RSD (n =25). This validation data also confirmed good efficiency in terms of precision and accuracy of the SHS-GC/NPD system.

Table 3.16 Quantitative data obtained by SHS-GC/NPD analysis of 2AP in KDML 105 rice leaves once a day for 5 times in 5 days.

repeat	Peak area		Peak area ratio of 2AP/2,6-DMP	Average of peak area ratio	SD	%RSD
	2,6-DMP	2AP				
1	782.2	307.0	0.3925	0.3907	0.0087	2.22
	779.0	314.0	0.4031			
	729.6	285.9	0.3919			
	770.3	292.2	0.3793			
	1492.1	577.2	0.3868			
2	1186.8	489.5	0.4125	0.4131	0.0064	1.55
	1031.8	433.1	0.4198			
	1168.2	475.8	0.4073			
	1053.5	442.1	0.4196			
	1098.4	446.6	0.4066			
3	964.4	385.8	0.4000	0.4042	0.0063	1.56
	869.2	349.4	0.4020			
	868.9	347.0	0.3994			
	476.3	197.6	0.4149			
	870.2	352.4	0.4050			
4	610.4	245.3	0.4019	0.3944	0.0133	3.38
	803.7	309.0	0.3845			
	764.9	299.5	0.3916			
	753.6	311.5	0.4133			
	794.3	302.3	0.3806			
5	688.1	283.6	0.4121	0.4107	0.0056	1.35
	669.7	271.7	0.4057			
	768.8	312.3	0.4062			
	553.6	232.2	0.4194			
	720.7	295.6	0.4102			

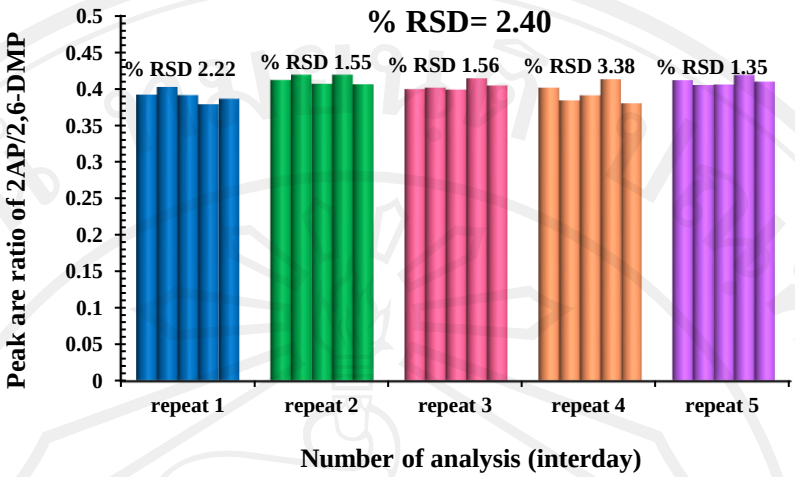

Figure 3.23 Peak area ratios of 2AP/2,6-DMP in KDML105 rice leaves obtained by 5 replications of SHS-GC/NPD analysis within 5 days.

3.9 Application of the developed SHS-GC/NPD method for determination of 2AP in grains and leaves of some Thai rice varieties

As the main objective of this study was to develop SHS-GC/NPD technique in order to use for the estimation of aroma compound, 2AP, in rice grains by determination its concentration in leaves. The developed SHS-GC/NPD method was then applied to quantitative analysis of 2AP in grains and leaves of some Thai rice varieties which were KDML105 (organic), KDML105, RD15, RD33 and RD6.

The quantitative data obtained as peak area ratio of 2AP/2,6-DMP of leaves and grains were presented in Table 3.17 and Table 3.18, respectively and a bar graph represented these quantitative data is shown in Figure 3.24. The five rice varieties were cultivated until 20 days. Then, their leaves were picked up for analysis

of the aroma compound. At the same time, 2AP analysis was also performed in the grains of these five varieties. The amounts of 2AP in 20-day rice leaves and rice grains were compared and the results showed higher concentrations of 2AP in rice leaves than their rice grains which were found in ranges of 24.58-40.83 and 5.58-13.38 ppm, respectively. These differences were approximately 3.7 times in average. The reason for the differences of 2AP concentrations in both rice samples may be due to the higher production of secondary metabolites in rice leaves. These results are in agreeable with the results shown in section 3.2.

Results in section 3.2 showed that the amount of 2AP in KDML 105 rice leaves has a tendency of increasing from seedling (10-20 days) to booting stage that the highest amounts of 2AP was found in this stage (50 days). The decreased concentration of 2AP in KDML105 leaves from booting stage down to the end of grain in grain development stage was probably due to the lower rate of secondary metabolite production during these lately stages of rice plant. In overall, the amounts of 2AP in KDML 105 rice leaves at 10 to 120 days were higher than that in their grains.

Table 3.17 Quantitative data of 2APconcentrationsin rice leaf samples of some Thai rice varieties obtained by SHS-GC/NPD.

Sample	Amount, g	Peak area		Peak area ratio of 2AP/2,6-DMP	Concentration of 2AP, ppm	Average of Concentration of 2AP, ppm	SD	%RSD
		2,6-DMP	2AP					
KDML 105 (organic)	0.2000	295.3	487.3	1.65	39.98			
	0.2000	226.3	394.2	1.74	42.20	**40.83**	1.195	2.93
	0.2000	281.1	467.9	1.66	40.33			
KDML 105	0.2000	280.4	323.4	1.15	27.95			
	0.2000	278.1	319.8	1.15	27.87	**28.00**	0.163	0.58
	0.2000	271.3	315.5	1.16	28.18			
RD 15	0.2000	280.5	295.5	1.05	25.53			
	0.2000	296.8	298.0	1.00	24.33	**24.58**	0.853	3.47
	0.2000	275.3	271.3	0.99	23.88			
RD 33	0.2000	259.4	293.0	1.13	27.37			
	0.2000	254.3	288.2	1.13	27.46	**27.88**	0.801	2.87
	0.2000	239.1	284.2	1.19	28.80			
RD 6	0.2000	217.7	363.2	1.67	40.42			
	0.2000	259.8	414.0	1.59	38.61	**39.97**	1.200	3.00
	0.2000	210.7	355.5	1.69	40.87			

Table 3.18 Quantitative data of 2APconcentrationsin rice grain samples of some Thai rice varieties obtained by SHS-GC/NPD.

Sample	Amount, g	Peak area		Peak area ratio of 2AP/2,6-DMP	Concentration of 2AP, ppm	Average of Concentration of 2AP, ppm	SD	%RSD
		2,6-DMP	2AP					
KDML 105 (organic)	1.0000	452.3	1002.0	2.22	13.05	**13.32**	0.381	2.86
	1.0000	439.5	1013.8	2.31	13.59			
KDML 105	1.0000	455.4	547.1	1.20	7.06	**6.96**	0.146	2.09
	1.0000	484.9	565.6	1.17	6.86			
RD 15	1.0000	468.1	440.5	0.94	5.53	**5.58**	0.067	1.20
	1.0000	456.4	436.8	0.96	5.62			
RD 33	1.0000	468.2	535.6	1.14	6.73	**6.71**	0.017	0.25
	1.0000	484.6	552.4	1.14	6.70			
RD 6	1.0000	398.7	881.8	2.21	13.02	**13.38**	0.499	3.73
	1.0000	380.7	887.5	2.33	13.73			

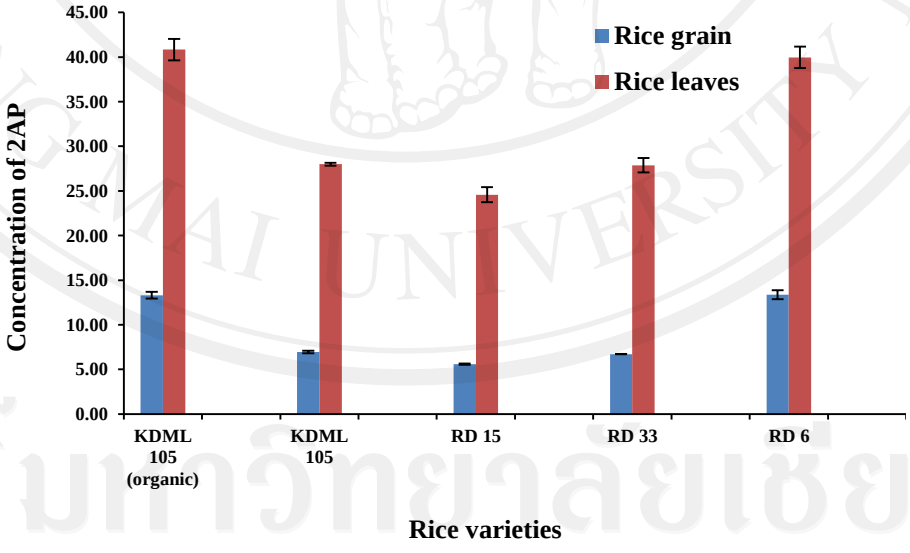

Figure 3.24 Comparison of 2AP concentrations in grains and leaves of some Thai rice varieties.

The RSDs of each analysis of rice leaf and grain samples were presented in ranges of 0.58-3.47% and 0.25-3.73%, respectively. The 2AP concentrations in grains and leaves of these five rice varieties were different because of the different genetic factors controlling aroma production.[57]However,the gaps between 2AP concentrations in grains and leaves among these five rice samples were relatively consistent. Thus, the analysis of aroma compound, 2AP, in rice leaves yields the results that could be used to predict the amount of 2AP in rice grains. The method can reduce time required for sample preparation in terms of planting which has made it more attractive and convenient than that used for analysis of 2AP in rice grains. The only caution to this developed method was that error in sample preparation process can be high because of moisture in rice leaves. So, attention must be paid carefully for the age and time during the day when the leaf samples are collected. Regardless of this limitation, the method can be a good choice for 2AP analysis in aromatic rice breeding program.