# CHAPTER IV
# RESULTS AND DISCUSSION

## 1. Aluminium content assay

The percentage of aluminium content in AH calculated triplicately by back titration method (USP 25) was 2.26 $\pm$ 0.01 %Al.

## 2. Adsorptive capacity of single antigen on adjuvant

The concentration unit of DT, TT and JE were transformed from Lf (diphtheria, tetanus) and antigen unit (a.u.) (JE) to µg. The measurement as µg was calculated by BCA analysis and referred to the original concentration as Lf or antigen unit (Table 19, in Appendix B). The results were used to calculate the amount of aluminium adjuvant for adsorption each antigen from the adsorptive capacity. They are shown by the following.

| | | | | |
|---|---|---|---|---|
| Diphtheria toxoid | ; | 1 Lf | = | 3.99 µg |
| Tetanus toxoid | ; | 1 Lf | = | 5.21 µg |
| JE antigen | ; | 1 a.u. | = | 161.93 µg |

Basically vaccine preparation processes of adsorbed antigens on adjuvants are formulated in cold room. In many studies, the mixing time was 20 to 60 minutes and the temperature was at ambient, 25 °C, 37 °C (Al-Shakhshir et al., 1994; Al-Shakhshir et al., 1995; Rinella et al., 1995; Heimlich et al., 1999; Chang et al., 2001; Shi et al., 2002; Iyer et al., 2003; Morefield et al., 2004; Morefield et al., 2005; Seeber, White and Hem, 1991; Sripongsarn, 2005) so in this experiment, the samples were well mixed at 120 rpm, at 37 °C and for 30 minutes.

Figures 7-9 show the adsorption isotherm of DT, TT and JE by AH. The data in Figure 7 was plotted according to the linearized adsorption pattern and the adsorptive capacity of DT was 0.46 mg/mg Al. TT showed linear relation between concentration of antigen and adsorption, so the adsorptive capacity was 0.6 mg/mgAl.

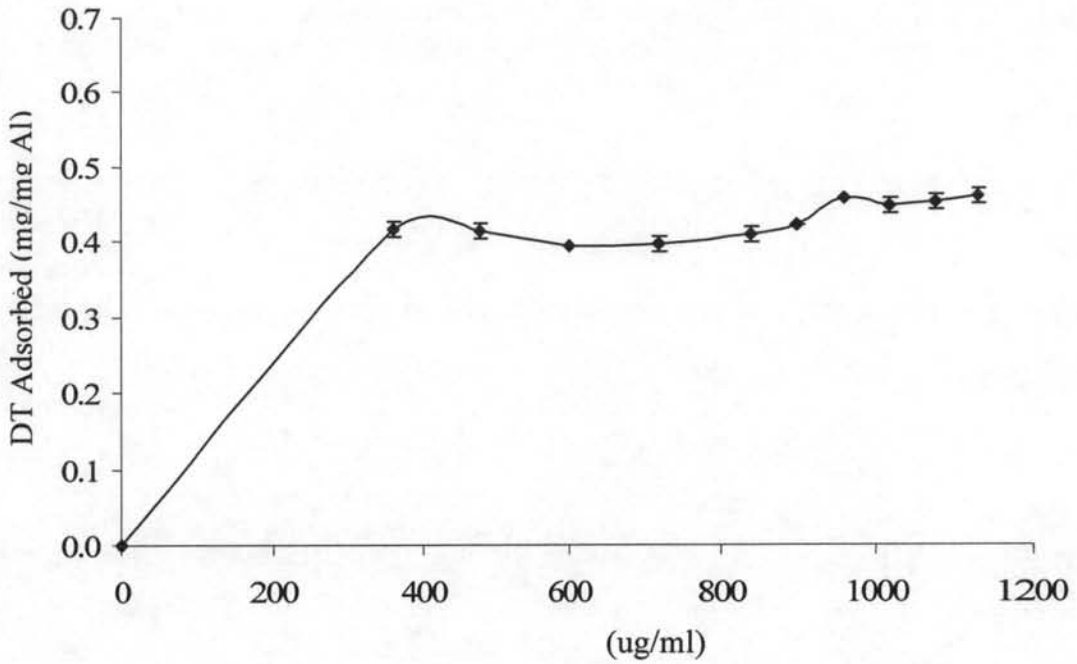



**Figure 7** Adsorption isotherm of diphtheria toxoid on aluminium hydroxide

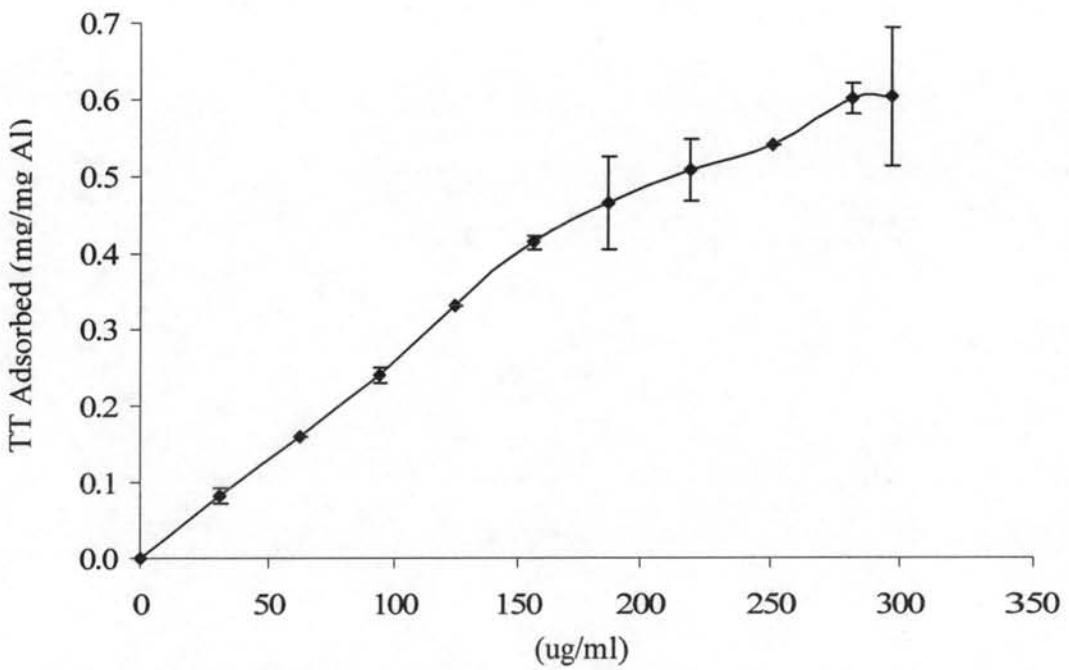



**Figure 8** Adsorption isotherm of tetanus toxoid on aluminium hydroxide

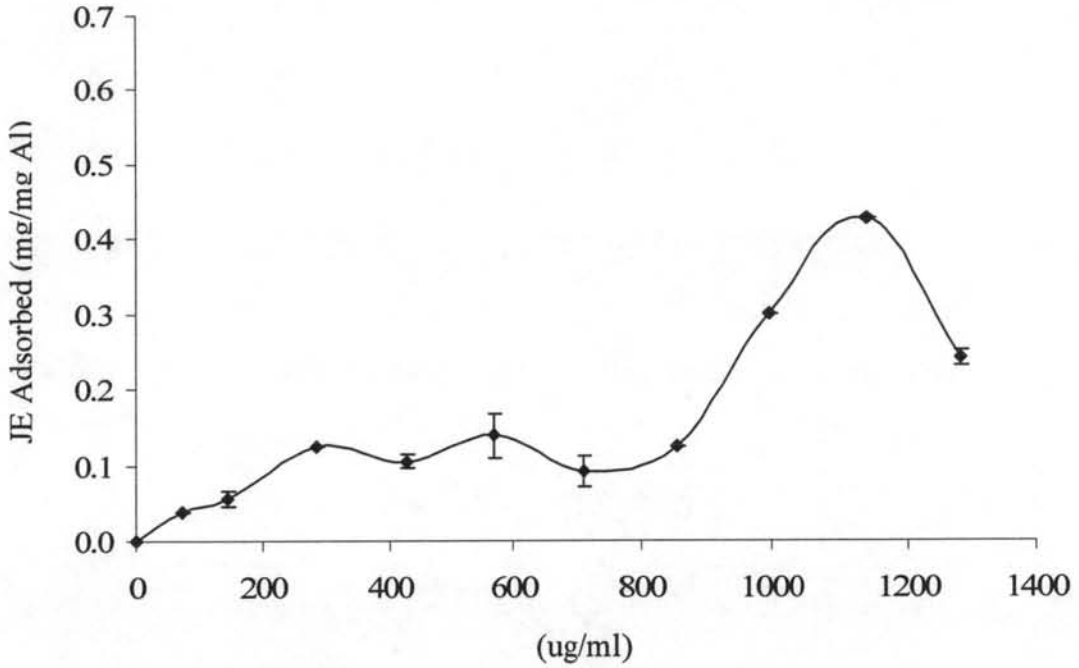



**Figure 9** Adsorption isotherm of JE antigen on aluminium hydroxide

The adsorption of JE by AH was not linear, the fluctuated adsorption pattern. Thus, the adsorptive capacity could not be determined. However, the maximal adsorption was at 0.43 mg/mgAl.

The study of Sripongsarn (2005) had shown that at pH 6.0-7.4 AH had positive charge, DT and TT expressed negative charge while JE antigen expressed slightly positive or neutral surface charge. Therefore, AH was a good adsorbent for DT and TT but not for JE. The aforementioned adsorptive capacity results of DT, TT and JE may be due to the electrostatic attraction has a major role in adsorption of proteins by aluminium-containing adjuvants (Al-shakhshir et al., 2005; Seeber, White and Hem, 1991) and the real surface charge of antigen and adsorbent are important for the adsorption process (Matheis et al., 2002). These results were according to the studies of Seeber, White and Hem (1991). It was shown that the adsorption data of albumin (negative charge) by boehmite (positive charge) was plotted according to the linearized Langmuir isotherm but the adsorption of lysozyme (positive charge) by boehmite was not linear, thus the adsorptive capacity can not be determined. Moreover, boehmite is a good adsorbent for albumin but not for lysozyme. The

adsorption data can be explained on the basis of electrostatic adsorption forces. Extensive adsorption between protein and aluminium-containing adjuvant was seen when they were oppositely charged. These linearized isotherms also followed the Langmuir equation. In this study, DT and TT had opposite charge with AH so the adsorption data of they were plotted according to the linearized Langmuir isotherm.

The quantities of AH were calculated from the adsorptive capacity with the amount of antigens which were used in combined preparation (equivalent to adsorbed DT 30 Lf, adsorbed TT 6 Lf and adsorbed JE 0.35 a.u.). Consideration from the adsorptive capacity values indicated that the amount of 1.67%w/v AH for adsorption of each antigens were 0.33 mg for DT, 0.08 mg for TT and because the total amount of AH was not more than 0.85 mg Al/dose as per USFDA allowance, thus for JE was 0.44 mg of 1.67%w/v AH.

**3. Adsorption of single antigen on adjuvant at various processing variables**

The adsorption of each antigen (equivalent to adsorbed DT 30 Lf, adsorbed TT 6 Lf and adsorbed JE 0.35 a.u.) on 1.67% w/v AH (equivalent to adsorptive capacity of each antigen) by mixing were performed at investigated various processing variables (e.g. temperature, mixing speed and mixing time). The mean percentages of adsorption (% adsorption) of DT, TT and JE at various processing variables are presented in Table 13 and Figure 10-12. The result showed that DT has maximal % adsorption at every various processing variables and JE was minimal.

In order to easy investigation, the effect of processing variables on %adsorption of each antigen are performed in Table 13 and Figure 10-12, the separate result of each processing variable affected the adsorption of each antigen were presented.

Table 13 and Figure 10-12 illustrate the effect of temperature at 5 °C, 15 °C, 25 °C and37 °C on %adsorption of each antigen. The %adsorption value pattern of DT was similar to TT which was slightly decreased as the temperature was raised. On the other hand, the %adsorption value pattern of JE was gradually increased as the

temperature was raised. It indicated that the temperature was a factor which slightly influenced the adsorption of each antigen on AH which same as the studies of Sripongsarn (2005). It reported that the adsorption pattern of antigens (DT, TT and JE) on AH at 9 °C showed the similar pattern with at 37 °C and the temperature was not a major factor which influenced the adsorption of antigen on AH. According to the report of Gupta et al. (1998), they had shown that adsorption of antigens on aluminium salts depended heavily on electrostatic forces between adjuvant and antigen, besides that the temperature was one of physical conditions affecting adsorption of antigen on aluminium adjuvants which may affect the rate of interaction between the gel and the antigen.

There were statistically significant differences between %adsorption of DT at 5 °C with 15, 25 and 37 °C ($p<0.05$) but not between %adsorption of DT at 15 °C with 25 °C. Moreover, the %adsorption of DT was highest at 5 °C and lowest at 37 °C. It could be concluded that DT had the maximal adsorption at 5 °C. There was no statistically significant difference between %adsorption of TT at 5 °C with 15 °C ($p>0.05$). At 15 °C, the maximal %adsorption of TT was close to at 5 °C but %adsorption of TT at 25 and 37 °C were lower than at 5 and 15 °C. It showed that at 5 and 15 °C, the maximal adsorption of TT occurred. There were statistically significant differences between %adsorption of JE at 37 °C with 5, 15 and 27 °C ($p<0.05$). In addition, JE had the maximal %adsorption at 37 °C so the adsorption was conducted at 37 °C. These results of temperature study which affected to the adsorption of antigens showed that DT was same but TT and JE were in contrast to the previous studies of Sripongsarn (2005) who reported that the adsorption value of DT and JE on AH at 9 °C was more than at 37 °C but TT was inverse or DT and JE could be adsorbed on AH more than TT during formulation at low temperature.

The case of JE had the maximal %adsorption at 37 °C was contrary with the study of Chaetanachan et al. (2001). They reported that JE particles at 37 °C appeared to aggregate, hence, JE was preferentially adsorbed at low temperature than at high temperature. In order to get optimal adsorption of JE, high shear force having at high mixing speed for prolonged mixing time was performed for well mixing. These optimal processing variables were discussed below.

In brief, the optimal temperature for adsorption of DT and TT was at 5 °C because it had the highest % adsorption value for DT and the maximal %adsorption of TT at 15 °C was close to 5 °C. On the other hand, at 37 °C was the optimal temperature for adsorption of JE.

The effect of mixing speed to the %adsorption of DT, TT and JE on AH are shown in Table 13 and Figure 10-12. The %adsorption value of DT showed the similar pattern at every various mixing speed but TT and JE showed the %adsorption patterns which were slightly increased as the mixing speed was raised. It indicated that mixing speed was not a major factor which influenced the adsorption of DT and it had slightly effect to the adsorption of TT and JE. DT showed the highest %adsorption value at 400 rpm. There were no statistically significant differences between %adsorption of TT and JE at 400 rpm with 500 rpm. Both TT and JE had the maximal %adsorption value at 400 rpm which were higher than 500 rpm.

The results suggested that the optimal mixing speed for adsorbed DT, TT and JE on AH was at 400 rpm because it had the maximal %adsorption value for every antigen.

Table 13 and Figure 10-12 present the effect of mixing time to the adsorption of each antigen on AH. The %adsorption value pattern of DT was similar to TT, which seemed to be approximately constant among every various mixing time. The effect of various mixing time to JE revealed the slightly fluctuated adsorption pattern on AH. It showed that mixing time was not the parameter which affected the adsorption of DT and TT but it had little effect to the adsorption of JE. There were statistically significant differences between the %adsorption value of DT at 5 hr with 1, 12 and 24 hr; in addition, DT had the highest % adsorption at 5 hr.

There was statistically significant difference between the %adsorption value of TT at 5 hr with 24 hr, but not with 1 hr and 12 hr and there were statistically significant differences between the %adsorption value of JE at 5 hr with 1, 12 and 24 hr. Both TT and JE had the maximal %adsorption at 5 hr. Therefore, DT, TT and JE preferred adsorption on AH for 5 hr than the other durations of mixing time.

**Table 13** The percentage of adsorption of each antigen on adjuvant at various processing variables.

| Temperature (° C) | Mixing speed (rpm) | Mixing time (hr) | Mean percentage of adsorption (%) ± SD | | |
|---|---|---|---|---|---|
| | | | DT | TT | JE |
| 5 | 200 | 1 | 97.13 ± 0.72 | 90.13 ± 1.12 | 21.12 ± 0.98 |
| | | 5 | 97.71 ± 0.65 | 90.15 ± 1.49 | 23.70 ± 0.64 |
| | | 12 | 97.16 ± 0.55 | 90.59 ± 0.56 | 13.37 ± 0.72 |
| | | 24 | 96.72 ± 0.62 | 88.55 ± 1.44 | 10.91 ± 0.61 |
| | 300 | 1 | 96.46 ± 0.54 | 90.58 ± 1.61 | 13.34 ± 0.89 |
| | | 5 | 97.69 ± 0.54 | 91.60 ± 0.69 | 15.49 ± 0.99 |
| | | 12 | 97.00 ± 0.31 | 89.24 ± 0.68 | 14.58 ± 0.87 |
| | | 24 | 97.03 ± 0.68 | 91.20 ± 1.51 | 10.58 ± 0.95 |
| | 400 | 1 | 97.29 ± 0.02 | 90.98 ± 0.76 | 25.65 ± 0.99 |
| | | 5 | 97.72 ± 0.53* | 91.07 ± 0.74* | 33.10 ± 1.00* |
| | | 12 | 97.90 ± 0.24 | 87.96 ± 0.94 | 22.11 ± 0.99 |
| | | 24 | 96.77 ± 0.28 | 87.87 ± 0.89 | 11.49 ± 0.88 |
| | 500 | 1 | 96.75 ± 0.67 | 90.93 ± 0.77 | 12.89 ± 0.88 |
| | | 5 | 97.15 ± 0.26 | 90.25 ± 0.66 | 39.98 ± 0.96 |
| | | 12 | 98.39 ± 0.25 | 89.79 ± 0.55 | 19.48 ± 0.81 |
| | | 24 | 96.70 ± 0.81 | 89.67 ± 0.98 | 14.88 ± 0.90 |

* the mean percentage of adsorption of each antigen at optimal processing variables

**Table 13** The percentage of adsorption of each antigen on adjuvant at various processing variables.

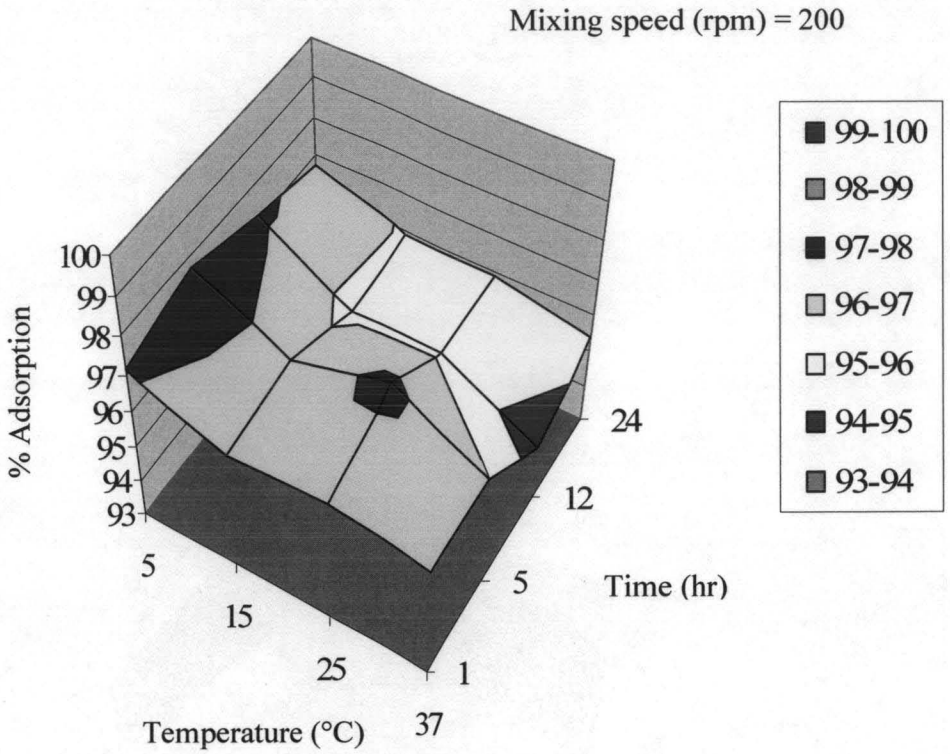
| Temperature (° C) | Mixing speed (rpm) | Mixing time (hr) | Mean percentage of adsorption (%) ± SD | | |
|---|---|---|---|---|---|
| | | | DT | TT | JE |
| 15 | 200 | 1 | 96.21 ± 0.50 | 91.42 ± 0.21 | 12.82 ± 1.73 |
| | | 5 | 96.54 ± 0.61 | 90.07 ± 1.55 | 21.65 ± 1.61 |
| | | 12 | 95.72 ± 0.75 | 91.27 ± 1.69 | 20.36 ± 1.97 |
| | | 24 | 95.88 ± 0.67 | 88.87 ± 1.58 | 21.53 ± 1.92 |
| | 300 | 1 | 94.63 ± 0.67 | 89.03 ± 1.27 | 10.32 ± 1.83 |
| | | 5 | 96.57 ± 0.90 | 90.67 ± 0.91 | 29.15 ± 1.98 |
| | | 12 | 97.07 ± 0.69 | 91.70 ± 0.17 | 19.06 ± 1.93 |
| | | 24 | 96.50 ± 0.35 | 89.91 ± 0.90 | 15.98 ± 1.70 |
| | 400 | 1 | 96.64 ± 0.48 | 91.25 ± 1.46 | 27.78 ± 1.93 |
| | | 5 | 97.53 ± 0.50 | 91.70 ± 1.26 | 31.09 ± 1.98 |
| | | 12 | 97.39 ± 0.87 | 91.01 ± 0.44 | 12.51 ± 1.93 |
| | | 24 | 96.06 ± 0.64 | 89.65 ± 1.35 | 19.69 ± 1.96 |
| | 500 | 1 | 95.80 ± 0.42 | 91.04 ± 1.94 | 20.85 ± 1.78 |
| | | 5 | 97.02 ± 0.88 | 92.22 ± 1.86 | 33.00 ± 1.42 |
| | | 12 | 96.41 ± 0.77 | 90.09 ± 1.46 | 27.95 ± 1.77 |
| | | 24 | 96.23 ± 0.52 | 90.60 ± 0.97 | 20.70 ± 1.75 |

**Table 13** The percentage of adsorption of each antigen on adjuvant at various processing variables.

| Temperature (° C) | Mixing speed (rpm) | Mixing time (hr) | Mean percentage of adsorption (%) ± SD | | |
|---|---|---|---|---|---|
| | | | DT | TT | JE |
| 25 | 200 | 1 | 96.32 ± 0.37 | 85.24 ± 1.28 | 16.19 ± 1.91 |
| | | 5 | 97.22 ± 0.28 | 86.19 ± 1.92 | 17.87 ± 1.54 |
| | | 12 | 95.83 ± 0.73 | 85.57 ± 1.53 | 15.80 ± 1.98 |
| | | 24 | 95.89 ± 0.56 | 83.72 ± 1.33 | 23.80 ± 2.00 |
| | 300 | 1 | 95.79 ± 0.63 | 82.84 ± 1.80 | 13.63 ± 1.32 |
| | | 5 | 96.46 ± 0.50 | 86.63 ± 2.00 | 15.31 ± 1.96 |
| | | 12 | 97.14 ± 0.97 | 85.31 ± 1.60 | 24.16 ± 1.85 |
| | | 24 | 96.42 ± 0.24 | 84.22 ± 0.87 | 19.24 ± 1.81 |
| | 400 | 1 | 96.92 ± 0.66 | 86.82 ± 1.81 | 21.93 ± 1.82 |
| | | 5 | 97.70 ± 0.57 | 86.94 ± 1.67 | 30.02 ± 1.85 |
| | | 12 | 97.26 ± 0.10 | 87.15 ± 0.98 | 28.15 ± 2.00 |
| | | 24 | 95.90 ± 0.53 | 84.77 ± 1.47 | 26.27 ± 1.60 |
| | 500 | 1 | 96.00 ± 0.75 | 86.38 ± 1.33 | 14.92 ± 1.88 |
| | | 5 | 96.48 ± 0.36 | 84.79 ± 1.32 | 28.52 ± 1.93 |
| | | 12 | 97.54 ± 0.15 | 89.88 ± 0.14 | 27.43 ± 1.82 |
| | | 24 | 96.43 ± 0.60 | 85.83 ± 0.91 | 23.56 ± 1.81 |

**Table 13** The percentage of adsorption of each antigen on adjuvant at various processing variables.

| Temperature (° C) | Mixing speed (rpm) | Mixing time (hr) | Mean percentage of adsorption (%) ± SD | | |
|---|---|---|---|---|---|
| | | | DT | TT | JE |
| 37 | 200 | 1 | 96.03 ± 0.85 | 73.11 ± 1.86 | 29.67 ± 0.40 |
| | | 5 | 96.03 ± 0.54 | 79.44 ± 1.06 | 31.80 ± 1.93 |
| | | 12 | 94.44 ± 1.18 | 80.55 ± 1.66 | 28.65 ± 1.62 |
| | | 24 | 95.39 ± 0.58 | 74.96 ± 1.02 | 26.61 ± 1.61 |
| | 300 | 1 | 95.82 ± 0.48 | 75.19 ± 1.85 | 32.15 ± 1.74 |
| | | 5 | 95.68 ± 0.59 | 80.59 ± 1.00 | 25.19 ± 1.58 |
| | | 12 | 94.52 ± 0.52 | 84.33 ± 1.62 | 22.16 ± 1.81 |
| | | 24 | 93.83 ± 1.27 | 75.23 ± 1.33 | 20.90 ±1.89 |
| | 400 | 1 | 96.01 ± 0.58 | 86.24 ± 1.83 | 34.30 ± 1.95 |
| | | 5 | 96.33 ± 0.63 | 87.53 ± 1.54 | 40.01 ± 1.45 |
| | | 12 | 96.17 ± 0.24 | 83.57 ± 1.67 | 22.65 ± 1.83 |
| | | 24 | 94.52 ± 0.50 | 85.34 ± 1.87 | 14.84 ± 1.63 |
| | 500 | 1 | 95.71 ± 0.28 | 84.32 ± 1.76 | 28.91 ± 1.95 |
| | | 5 | 95.75 ± 0.51 | 82.07 ± 1.88 | 31.43 ± 1.80 |
| | | 12 | 94.31 ± 1.26 | 83.37 ± 1.99 | 27.89 ± 1.73 |
| | | 24 | 93.69 ± 0.59 | 87.80 ± 1.21 | 20.69 ± 1.79 |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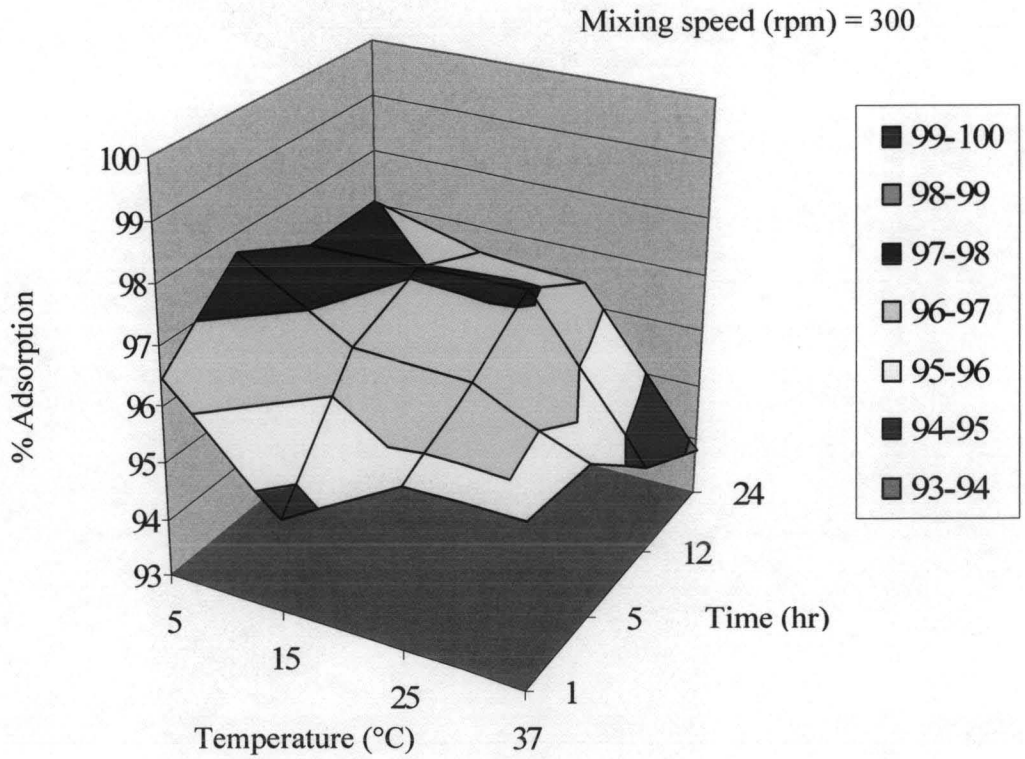
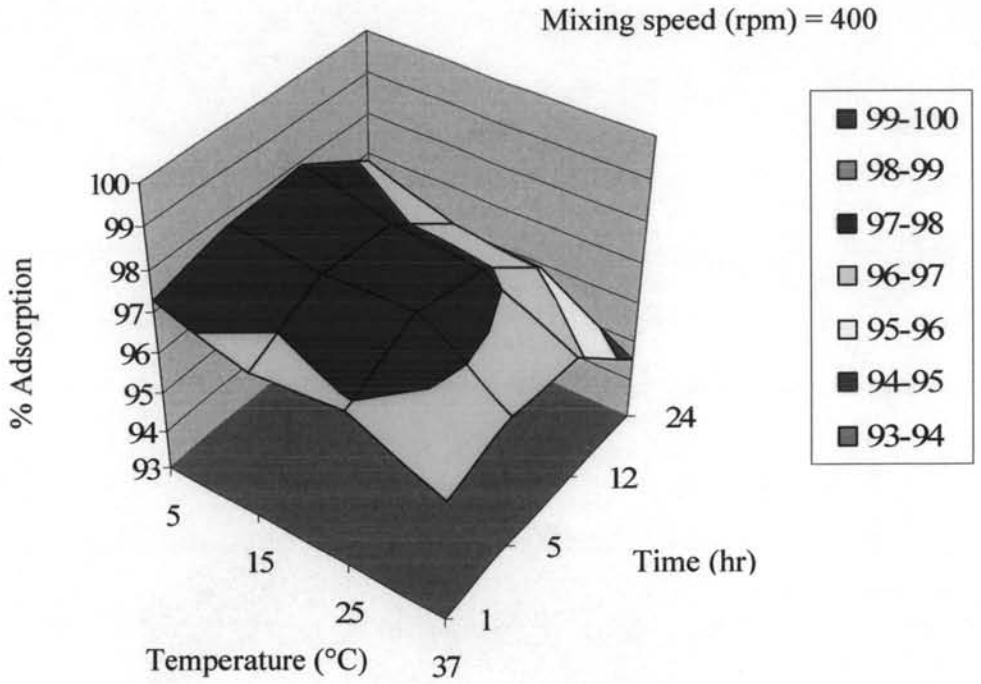
% Adsorption
100
99
98
97
96
95
94
93
Temperature (°C)
5
15
25
37
Time (hr)
1
5
12
24
99-100
98-99
97-98
96-97
95-96
94-95
93-94

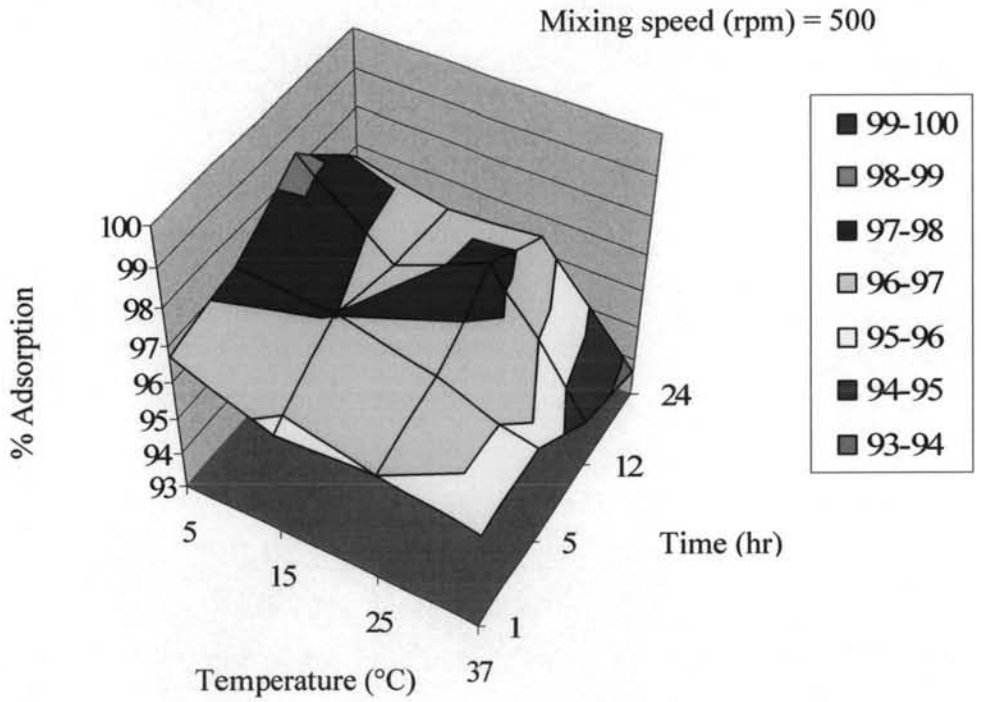
(A)


Mixing speed (rpm) = 300
% Adsorption
100
99
98
97
96
95
94
93
Temperature (°C)
5
15
25
37
Time (hr)
1
5
12
24
99-100
98-99
97-98
96-97
95-96
94-95
93-94

(B)

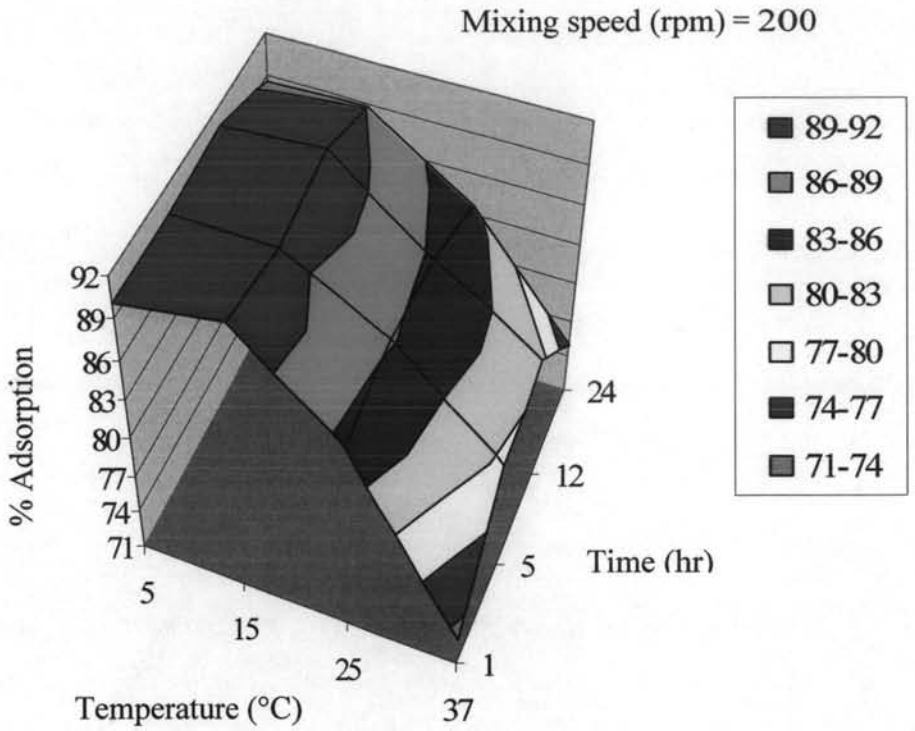



(C)

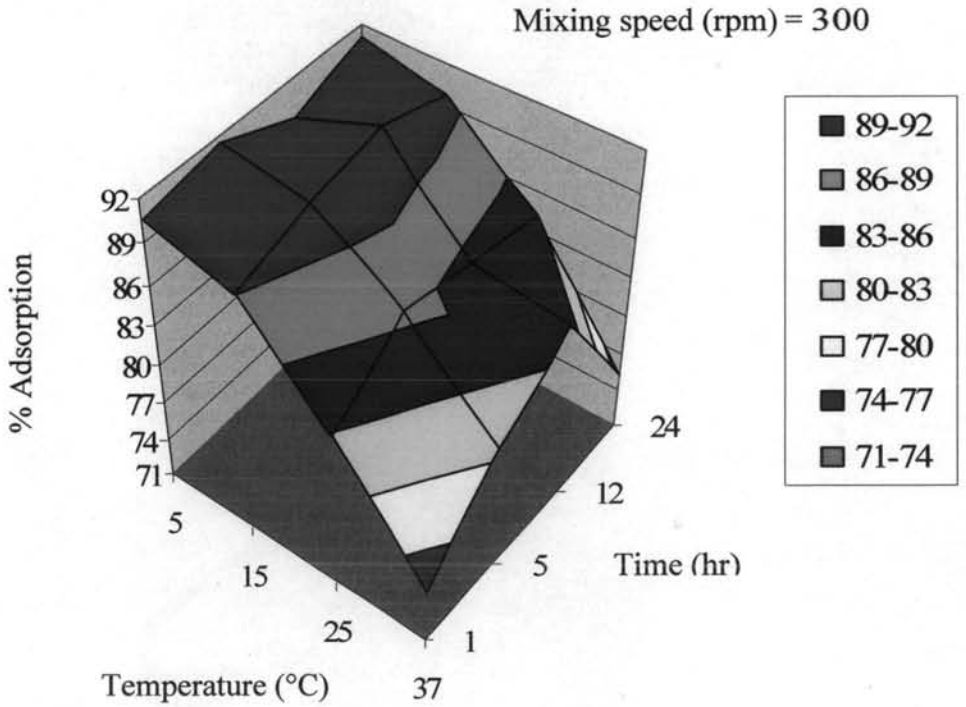



(D)

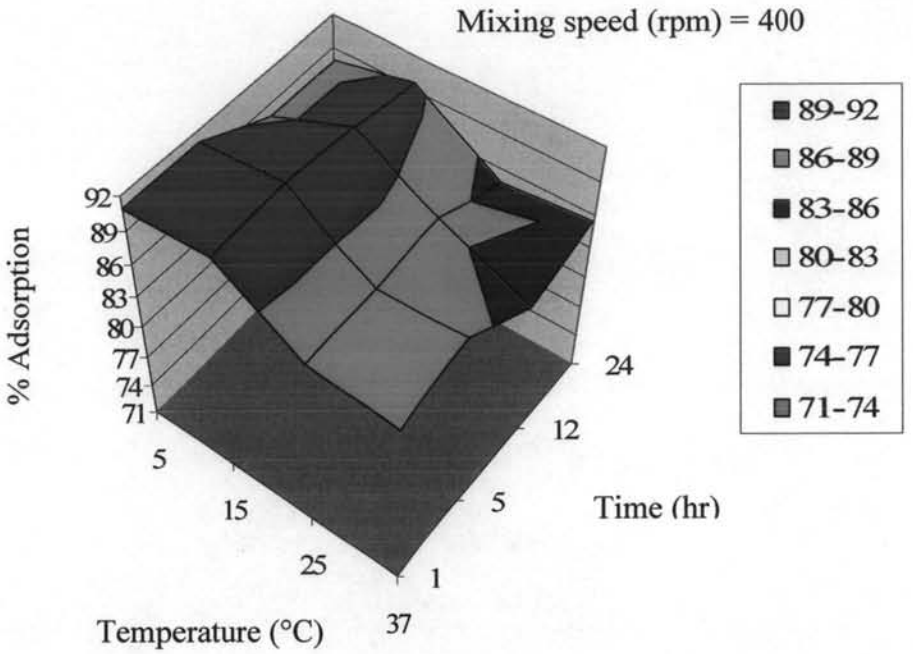
**Figure 10** The percentage of adsorption of diphtheria toxoid onto aluminium hydroxide at various temperature, various mixing time and (A) 200 rpm (B) 300 rpm (C) 400 rpm (D) 500 rpm.


Mixing speed (rpm) = 200
89-92
86-89
83-86
80-83
77-80
74-77
71-74
% Adsorption
92
89
86
83
80
77
74
71
5
15
25
37
Temperature (°C)
1
5
12
24
Time (hr)

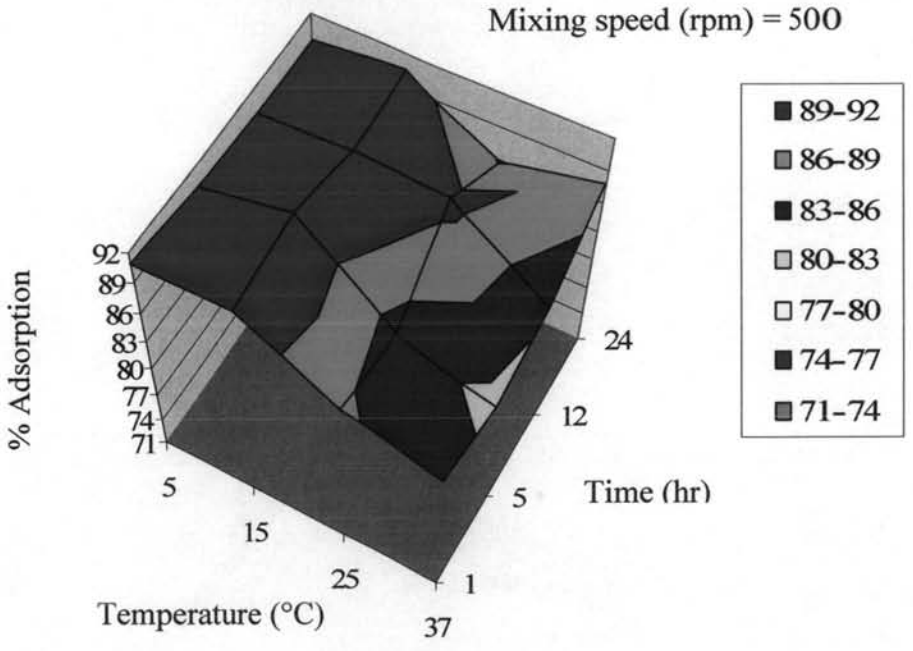
(A)


Mixing speed (rpm) = 300
89-92
86-89
83-86
80-83
77-80
74-77
71-74
% Adsorption
92
89
86
83
80
77
74
71
5
15
25
37
Temperature (°C)
1
5
12
24
Time (hr)

(B)

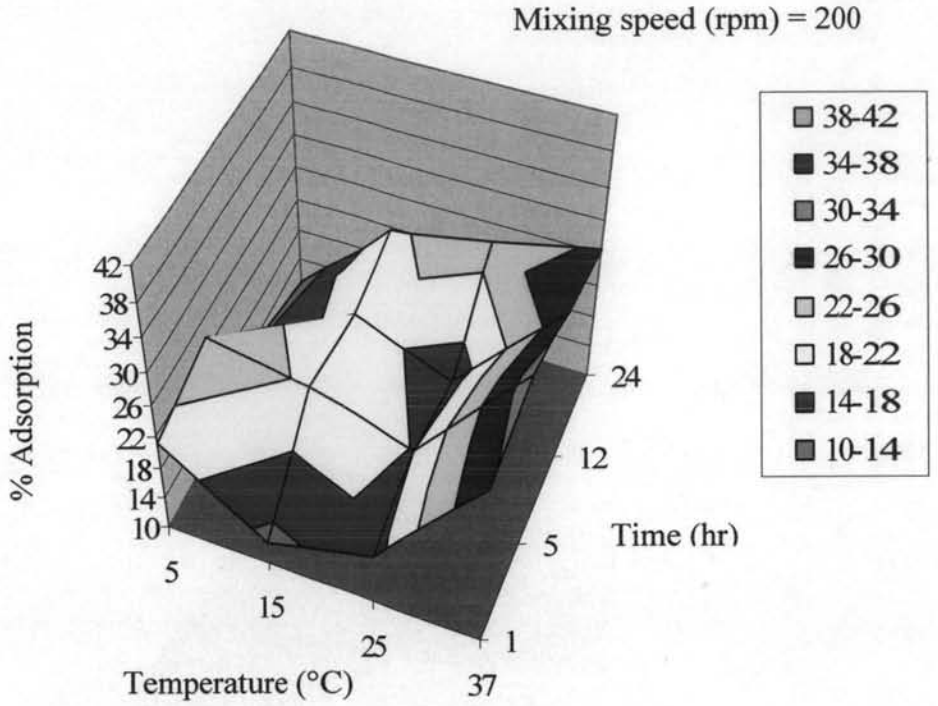



(C)

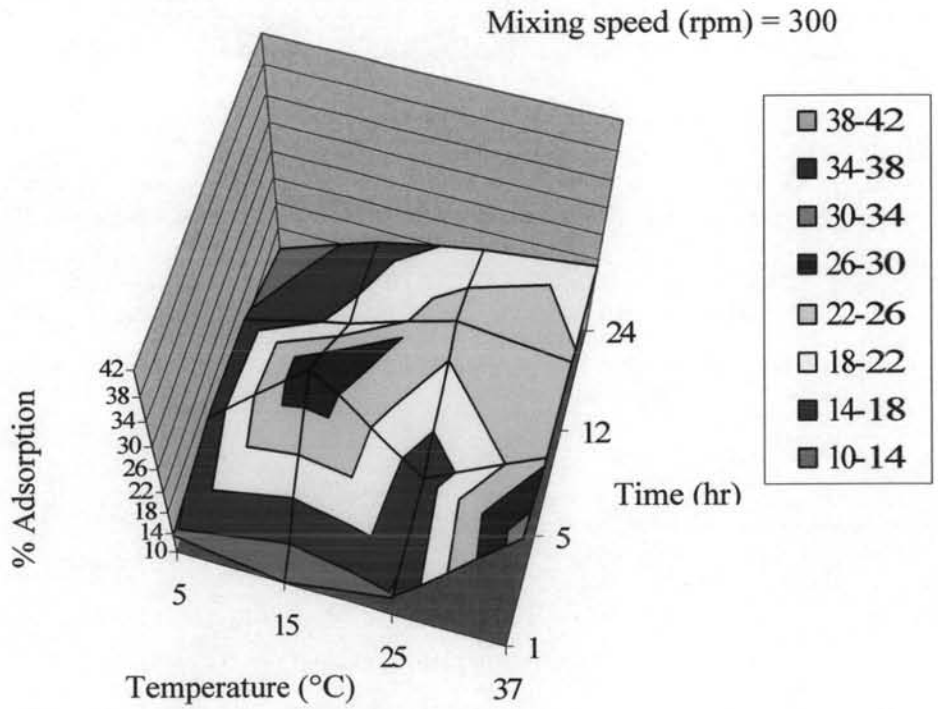



(D)

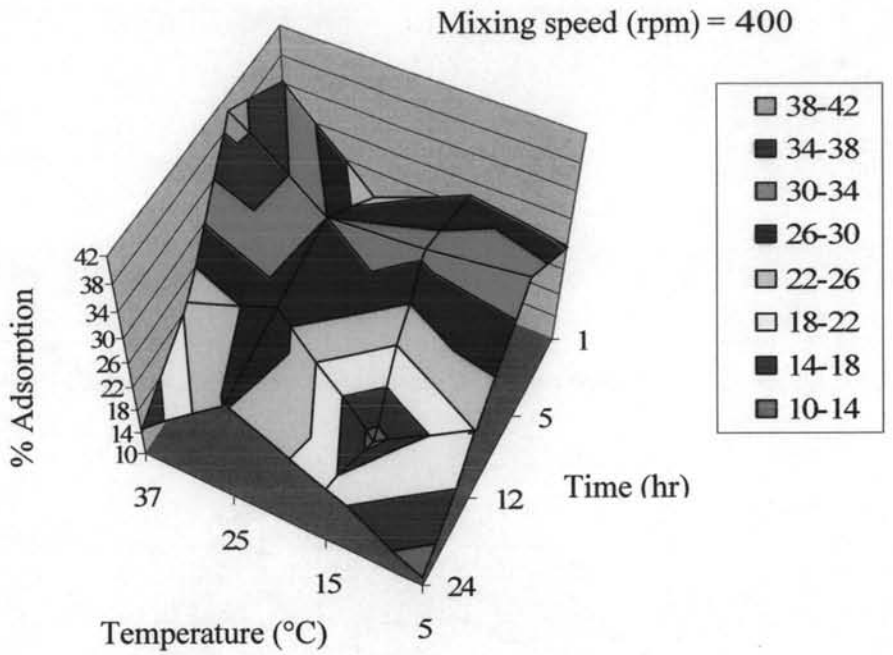
**Figure 11** The percentage of adsorption of tetanus toxoid onto aluminium hydroxide at various temperature, various mixing time and (A) 200 rpm (B) 300 rpm (C) 400 rpm (D) 500 rpm.


Mixing speed (rpm) = 200
% Adsorption
42
38
34
30
26
22
18
14
10
5
15
25
37
Temperature (°C)
1
5
12
24
Time (hr)
38-42
34-38
30-34
26-30
22-26
18-22
14-18
10-14

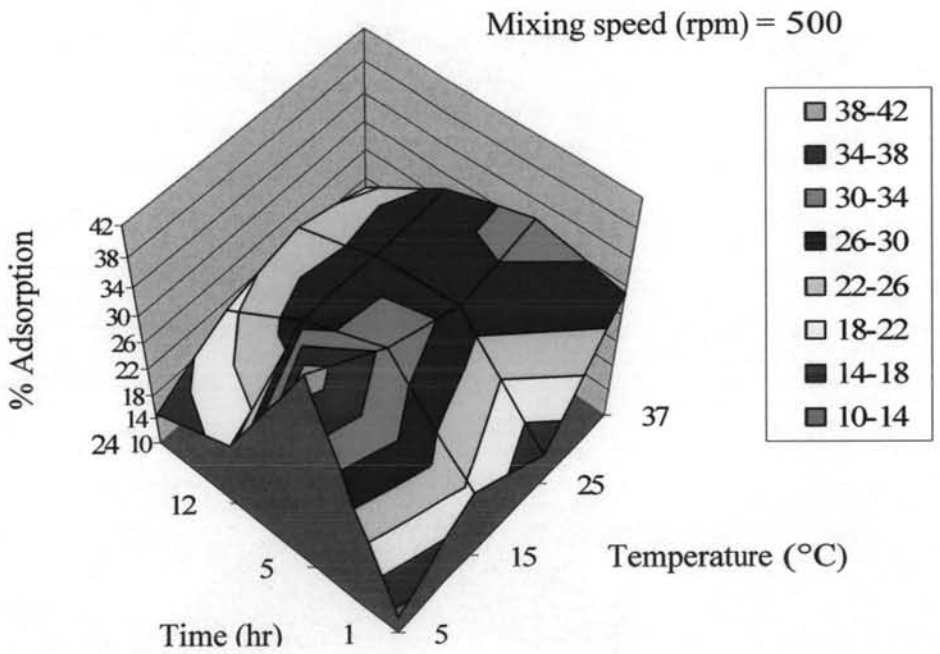
(A)


Mixing speed (rpm) = 300
% Adsorption
42
38
34
30
26
22
18
14
10
5
15
25
37
Temperature (°C)
1
5
12
24
Time (hr)
38-42
34-38
30-34
26-30
22-26
18-22
14-18
10-14

(B)




(C)




(D)

**Figure 12** The percentage of adsorption of JE antigen onto aluminium hydroxide at various temperature, various mixing time and (A) 200 rpm (B) 300 rpm (C) 400 rpm (D) 500 rpm.

The studies by Morefield et al. (2004) had shown that AH composed of very small primary particles. These particles formed irregularly shaped aggregates having diameters between 5 and 10 μm. The surface hydroxyls of AH adjuvant provided the basis for the major mechanism of adsorption of antigen: electrostatic adsorption when the antigen and adjuvant had opposite charges. Examination by flow cytometry after 1 min of mixing indicated that the AH adjuvant (strong positive charge) aggregates had bovine serum albumin (BSA) and recombinant protective antigen (rPA) (strong negative charge) adsorbed. Thus, the two antigens that adsorbed by strong electrostatic attraction, BSA and rPA, exhibited rapid and uniform adsorption by AH. When the fluorescent photomicrographs of myoglobin (small negative charge) mixed with AH, after 1 min indicated that the adjuvant aggregates had myoglobin adsorbed and myoglobin exhibited uniform adsorption after 60 min of mixing. They were compared to those of BSA and rPA. It was clear that antigens which adsorbed by weak attractive forces required a longer mixing time for uniform adsorption. Moreover, during mixing caused the well-defined region of fluorescence to become smaller and more highly dispersed throughout the adjuvant aggregates. This suggested that mixing caused the adjuvant aggregates to de-aggregate and then to re-aggregate by combining antigen-adsorbed aggregate fragments with aggregate fragments that were free of antigen. This cycle of de-aggregate and re-aggregate led to uniform adsorption of the antigen throughout the vaccine.

Sripongsarn (2005) showed that at pH 6.0-7.4 AH had positive charge while DT, TT had negative charge and JE antigen expressed slight positive or neutral surface charge. Hence, DT and TT adsorbed by strong electrostatic attraction but JE adsorbed by weak electrostatic attraction onto AH adjuvant. The result of % adsorption value of DT, TT, JE were affected by mixing speed and mixing time indicated that the optimal % adsorption value of DT and TT occurred at lower mixing speed and faster mixing time when compared with JE.

Adsorption of antigens on aluminium salts depends heavily on electrostatic forces between adjuvant and antigen. However, these forces may not suffice to cause adsorption of antigen if the same charge or electrostatic repulsive force is present on antigen and adjuvant. It is important to select the aluminium adjuvant carefully on the

basis of the charge of the antigen (Gupta et al., 1998). A lot of factors affecting adsorption of antigens on aluminium adjuvants include temperature, mixing speed and mixing time which were studied. For comparison of the adsorption of antigens at various processing variables are necessary in order to perform the optimal adsorption under optimal conditions.

In brief, the results from investigation of various processing variables could be concluded that the optimal processing variables for adsorption of DT, TT and JE on AH was temperature 5 °C; mixing speed 400 rpm and mixing time 5 hr because at this condition had the optimal %adsorption of DT, TT and JE. In case of JE had the maximal %adsorption at 37 °C but at this temperature, the %adsorption of DT and TT were lower than at 5 °C.

**4. Stability studies of combined preparations**

**4.1 Evaluations the content of each antigen by ELISA method**

The combined preparations were formulated by 2 processes; competitive adsorption (C) and separate adsorption (S). The physical appearances of the preparations were observed at initial and 4-month storage, 2-8 °C by SEM, FTIR, X-RD and particle size distribution analysis. The content of each antigens were evaluated by randomly sampling every 1 month interval for analysis by ELISA method. The mean amounts of DT, TT, PT and JE antigen are presented in Table14.

S had higher DT content than C throughout the storage periods but there was no statistically significant difference between at initial and 1 month ($p>0.05$). As seen in Figure 13, the stability patterns of DT during storage periods are shown. The pattern of C and S showed that their antigen contents were gradually decreased throughout the storage periods. Moreover, DT content of S was higher than C at every interval periods. The result suggested that the different process affected to DT content. The competitive adsorption (C) was not a good process for DT antigen. It might be due to adsorbed DT desorbed from adjuvant or DT was competed by others

could lose DT content. The separate adsorption was better than competitive adsorption because it showed higher stability.

**Table 14** The content of antigens in adsorbed preparations during storage at 2-8 °C for 4 months.

| Antigens | Formulations | Mean concentration of remained antigens at various time [a] (± SD) | | | | |
|---|---|---|---|---|---|---|
| | | initial | 1 month | 2 months | 3 months | 4 months |
| DT (Lf) | C | 27.03 ± 0.35 | 25.13 ± 0.07 | 21.48 ± 0.51 | 19.23 ± 0.41 | 15.80 ± 0.62 |
| | S | 28.61 ± 0.90 | 26.41 ± 1.69 | 23.98 ± 1.02 | 22.75 ± 0.53 | 20.51 ± 0.63 |
| TT (Lf) | C | 4.81 ± 0.14 | 5.48 ± 0.07 | 3.82 ± 0.28 | 2.56 ± 0.15 | 1.82 ± 0.18 |
| | S | 4.36 ± 0.12 | 5.56 ± 0.10 | 3.43 ± 0.99 | 2.95 ± 0.20 | 1.70 ± 0.11 |
| PT (o.u.) | C | 17.17 ± 1.57 | 15.05 ± 1.80 | 10.92 ± 0.87 | 7.37 ± 0.92 | 3.38 ± 1.75 |
| | S | 17.69 ± 1.87 | 16.00 ± 1.75 | 9.29 ± 1.75 | 6.53 ± 0.74 | 4.13 ± 0.80 |
| JE (a.u.) | C | 0.268 ± 0.005 | 0.177 ± 0.009 | 0.128 ± 0.012 | 0.103 ± 0.008 | 0.062 ± 0.008 |
| | S | 0.283 ± 0.006 | 0.194 ± 0.008 | 0.151 ± 0.001 | 0.093 ± 0.005 | 0.071 ± 0.005 |

[a]remaining amounts of DT and TT were presented as Lf, PT as o.u. and JE as a.u.

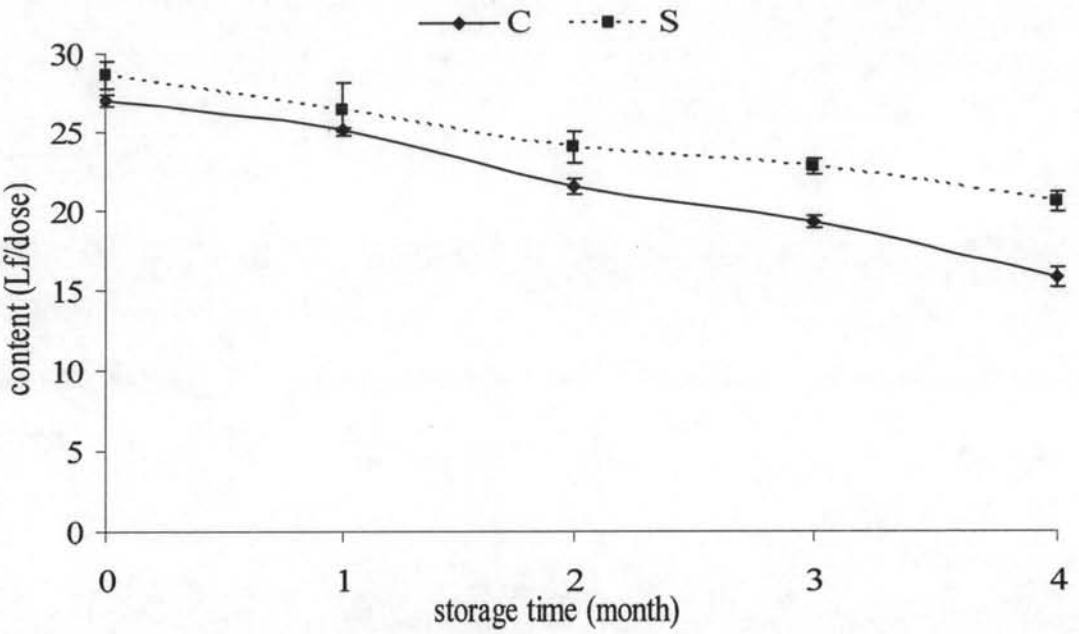



**Figure 13** The contents of diphtheria toxoid in combined formulations by different adsorption process during storage periods at 2-8 °C.

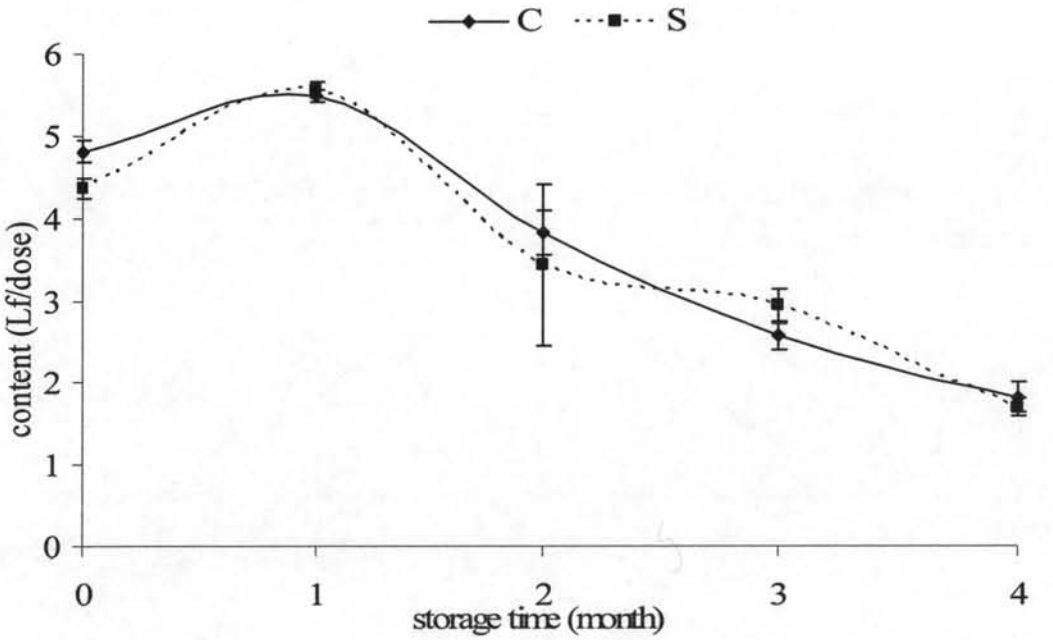



**Figure 14** The contents of tetanus toxoid in combined formulations by different adsorption process during storage periods at 2-8 °C.

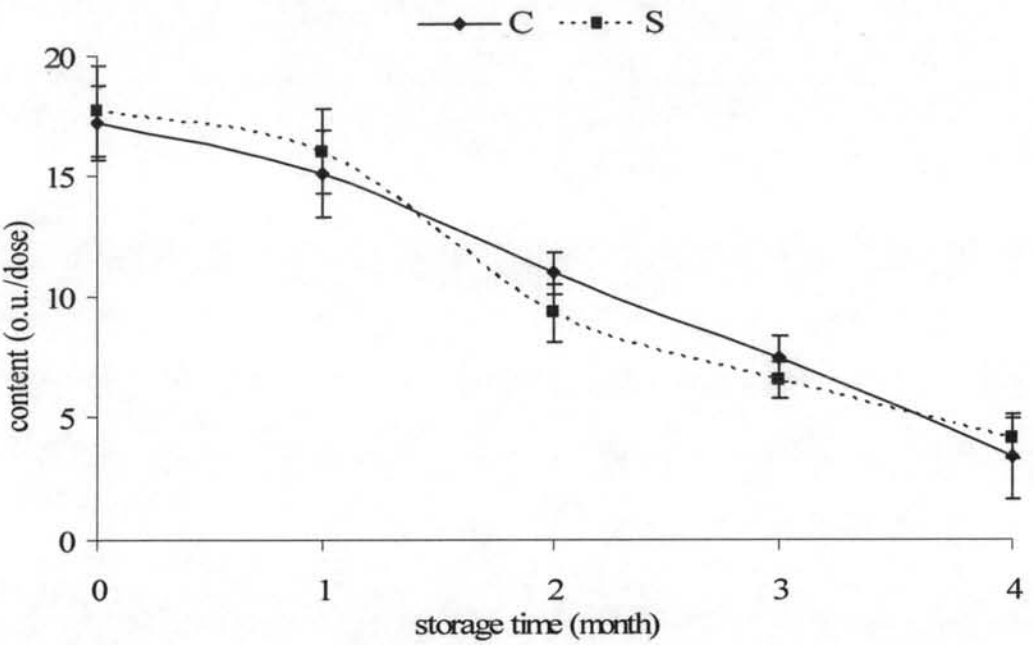



**Figure 15** The contents of *Bordetella pertussis* in combined formulations by different adsorption process during storage periods at 2-8 °C.

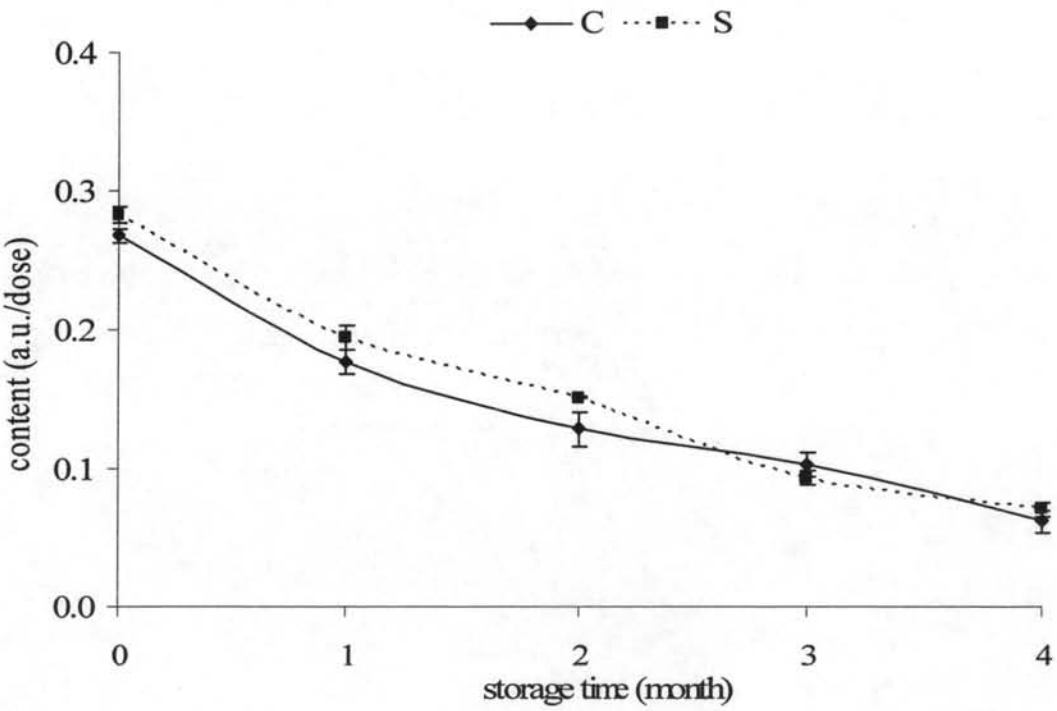



**Figure 16** The contents of JE antigen in combined formulations by different adsorption process during storage periods at 2-8 °C.

There was no statistically significant difference in TT content between C and S during 2 to 4 months ($p>0.05$). C had the antigen content close to S throughout the storage periods. Figure 14 shows the antigen content of TT. The stability patterns of C and S were all the same. The antigen content of C was higher than S at initial, 2 months and 4 months but S was higher than C at 1 month and 3 months. The TT contents of C and S at initial were lower than 1 month, so one of the causes might be TT was not completely adsorbed at initial. The result showed that the adsorption process had no effect to the TT content.

The PT contents of C and S were similarly and there was no statistically significant difference throughout storage periods ($p>0.05$) except at 2 months ($p=0.05$). Figure 15 shows the stability patterns of PT during storage periods. The PT content pattern of C was the same as S. The antigen content of C and S were gradually decreased and they were close in every interval periods of storage. From these result, PT contents were stabilized for both formulations. It concluded that the adsorption process had no influence to PT. This was similarly to the report of Callahan et al. (1991) that vaccines composed of killed whole virus or bacterial could be self-adjuvant.

At initial time, the content of JE antigen was very low in both formulations. There were statistically significant difference between C and S at initial and 2 months ($p<0.05$) except the others ($p>0.05$). The content of JE antigen during stability study shows in Figure 16. The antigen content of C and S were gradually decreased in every interval periods. The antigen content values of S were slightly higher than C throughout the storage periods except at 3 months. The result indicated that the adsorption method had low effect to JE content. Moreover, the separate adsorption process (S) showed the better stability of JE content than competitive adsorption process (C) after storage.

The result of Table 14 and Figure 13-16 indicated that after 4 months at 2-8°C, S had the maximal DT, PT and JE contents and the minimal TT content, whereas C had the maximal TT content only but the other antigens were maximal. The result could be concluded that S, which was formulated by separate adsorption, was the

optimal formulation for combining preparation. Due to the fact that the separate adsorption was individually mixed antigen with adjuvant and the antigen could be completely adsorbed under controlled conditions. Other adding ions or components were less likely to interfere the adsorption process. On the other hand, competitive adsorption was the process which all antigens were simultaneously mixed with adjuvant and other components in preparation at the same time. The antigen which the first adsorbed on the adjuvant might be partly or completely adsorbed and the later antigen might not be adsorbed completely. In addition, the antigen which adsorbed already might be desorbed from the adjuvant. The weakly adsorbed antigens might be desorbed during mixing and storage. The other ions or other components could interfere this adsorption process (Matheis et al., 2002). There is the study of adsorption process affecting the content of DT, TT, PT and JE of combined preparation by Sripongsarn (2005). It showed that the adsorption process had the effect to DT, TT and JE content, while PT was not be interfered by adsorption process and it could be self-adjuvant. Moreover, the stability patterns of each antigen could be concluded that separate adsorption was the best formulation for combining preparation under storage condition at 2-8 °C for 3 months.

Figure 17-18 present the antigen contents of combined formulations from C and S during storage periods, respectively. DT showed the same pattern between C and S. The DT content of C and S were decreased throughout storage time, but DT content of S was higher than C at every interval storage. It showed that the adsorption process was the controlling factor in the antigen content. In other words, the separate adsorption could keep the constant quantity of antigen better than the competitive adsorption.

The antigen content pattern of TT in C was similar to S and both were the fluctuation pattern during initial to 2 months. The antigen contents of C were close to S at every storage periods. It indicated that the adsorption process had not the effect on TT content.

The stability pattern of PT in C was same as in S. PT content was gradually decreased during the storage period. It could be concluded that the adsorption process was not factor which affected to the PT content.

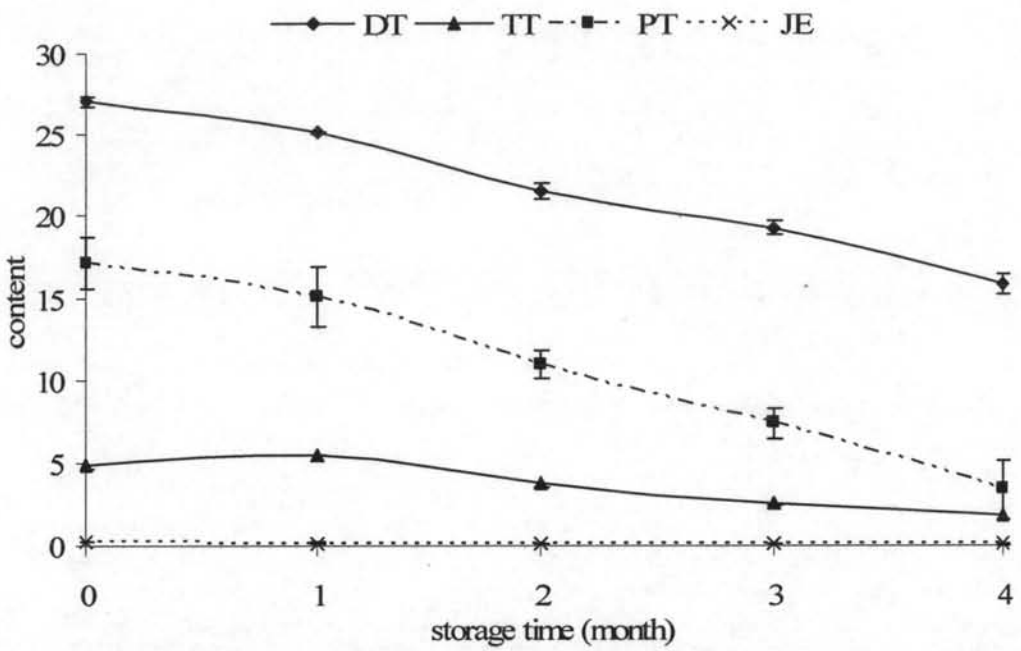



**Figure 17** The antigen contents of combined formulation (C) during storage periods at 2-8 °C.

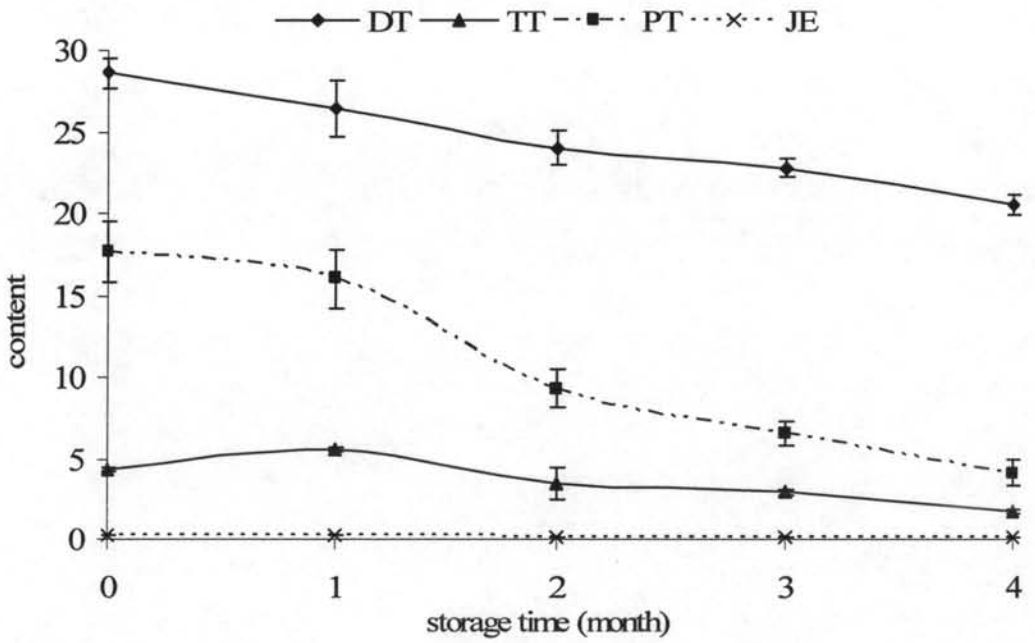


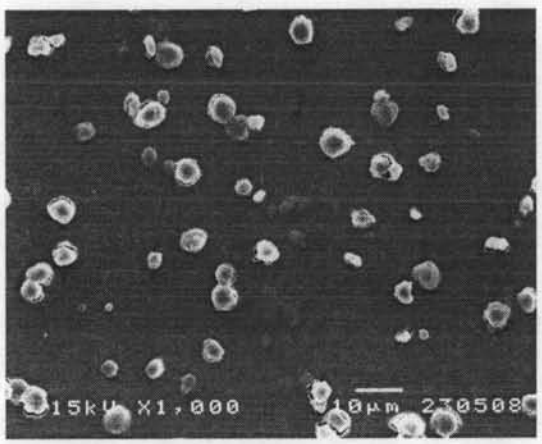

**Figure 18** The antigen contents of combined formulation (S) during storage periods at 2-8 °C.

As seen in Figure 17-18, the antigen content of JE was very low. C and S showed the similar pattern of JE content which they were constant or slightly decreased throughout storage period. If compared with other antigens, JE content of C and S were not changed or slightly decreased from initial to 4 months. It could not observe the change of antigen content from these figures. Therefore, the antigen content of DT and JE were affected by the adsorption process while TT and PT were not interfered from this factor.

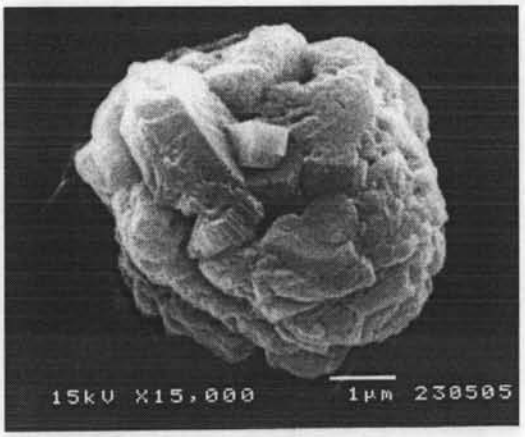
The production of adsorbed vaccine a lot of factors may influence the physicochemical process of the adsorption of antigens onto adsorbent particles. There is necessary to assess the quality of adsorbent and antigens, and to perform the adsorption process under optimal conditions. Taking into account that there are a lot of yet unknown or not understood immunological interaction in combined vaccines (Matheis et al., 2002) and impurities, such as amino acid, peptide and polysaccharides, reduce protein adsorption, probably by competing with antigen for adsorption site (Gupta et al., 1998) or may deform the structure of antigen, hence, the content of antigens were decreased during stability study.

**4.2 Scanning electron microscopy**

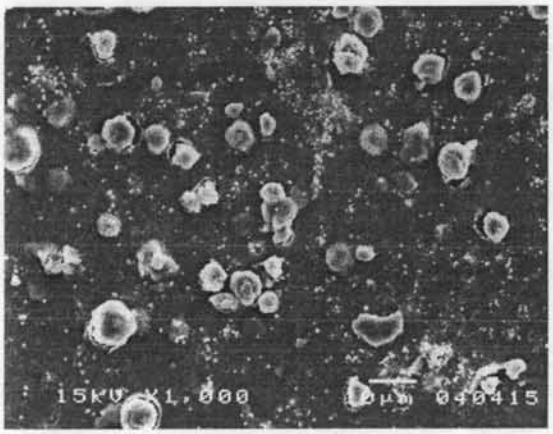
The morphology of AH, the preparations of C and S containing DT, TT, PT and JE at initial and after storage for 4 months at 2-8 °C by SEM are shown in Figure 19 and 20 respectively. AH was spherical complex particle (fig. 19B). The morphology of AH at 4-month storage (fig. 20B) was similar to the initial time. The appearance of C and S at initial (fig. 19D, 19F) showed some small fragments or some layers were attached on the surface of adjuvant. According to the study of Sripongsarn (2005) had shown that the photomicrographs of AH was spherical complex particle. The AH particles which were adsorbed with DT, TT and JE showed some small particles likely to be antigens attached on the adjuvant. As seen in Figure 20, the adsorbed AH particle of C and S after storage for 4 months were porous (fig. 20D, 20F). From stability result, each antigen contents were reduced after storage for 4 months, hence it showed that the porosities may be due to the reduction of antigens.



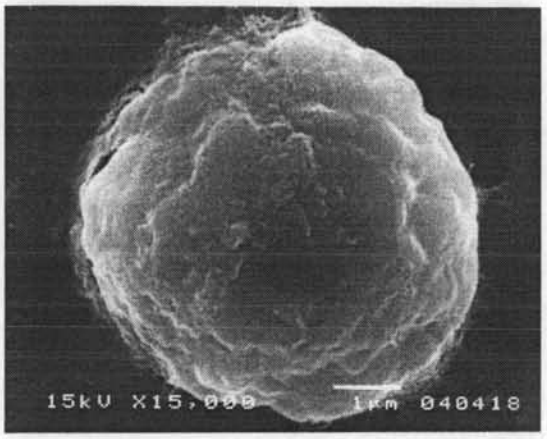

(A)



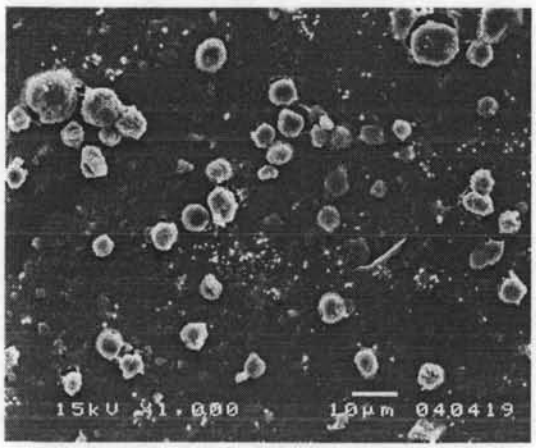

(B)



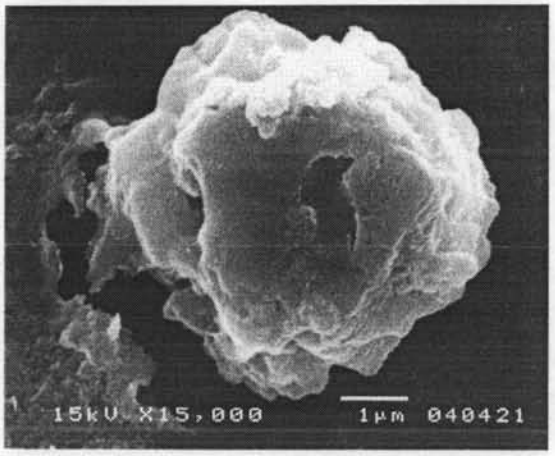

(C)




(D)

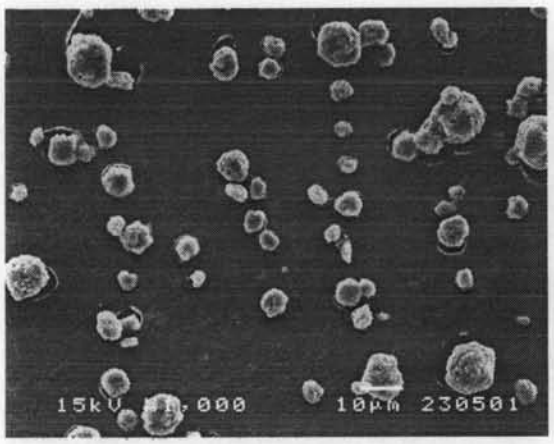



(E)

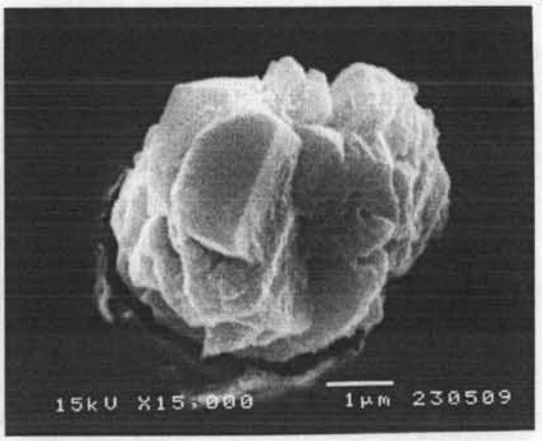



(F)

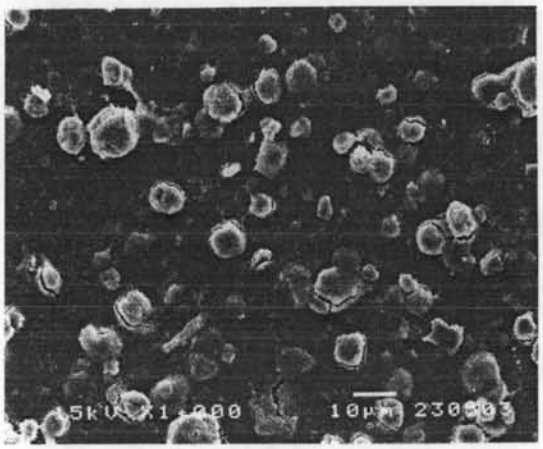
**Figure 19** SEM photomicrographs of (A) AH (1,000x), (B) AH (15,000x), (C) competitive adsorption (1,000x), (D) competitive adsorption (15,000x), (E) separate adsorption (1,000x) and (F) separate adsorption (15,000x): At initial time




(A)

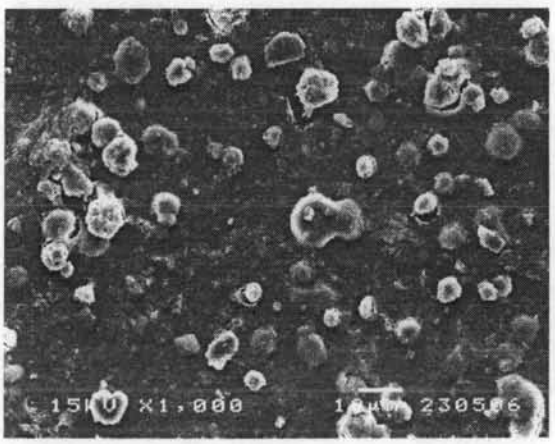



(B)

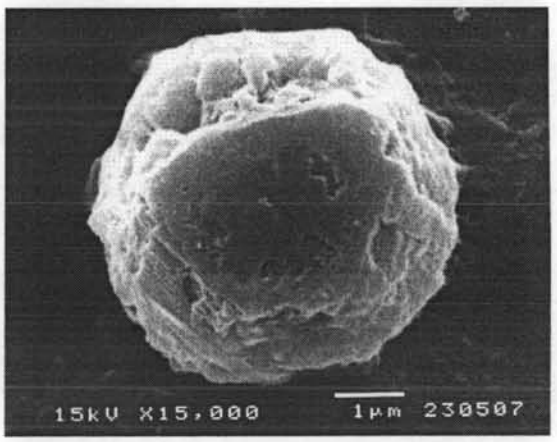


(C)


(D)



(E)




(F)

**Figure 20** SEM photomicrographs of (A) AH (1,000x), (B) AH (15,000x), (C) competitive adsorption (1,000x), (D) competitive adsorption (15,000x), (E) separate adsorption (1,000x) and (F) separate adsorption (15,000x): At 4-months storage time

### 4.3 Fourier Transform Infrared spectrometry

FT-IR analysis was used to ensure that no chemical interactions in the preparations. IR spectra of AH; C, t0 (at initial); S, t0; C, t4 (at 4-months storage) and S, t4 are shown in figure 21. Their spectra showed peaks at the wavenumbers of 1070.04, 1071.12, 1070.81, 1069.84, 1069.65 $cm^{-1}$, respectively which were O-H deformation region; 3097.65, 3090.68, 3091.74, 3088.36, 3087.86 $cm^{-1}$, respectively and 3411.95, 3409.93, 3410.96, 3399.60, 3398.81 $cm^{-1}$, respectively which were O-H stretching region. The results from O-H stretching were the broad peak between 3000-3500 $cm^{-1}$ which indicated the existence of structural hydroxyl environments and the shoulder peak around 3100 $cm^{-1}$.

These results were conformed to the studies of Shirodkar et al. (1990) and Lindblad (2004) that the principle peaks of AH were observed at wave numbers of 1070 in the O-H deformation region and a shoulder at 3100 $cm^{-1}$. The strong shoulder at 3100 $cm^{-1}$ is also unique for boehmite which is the mineralogical name of aluminium oxhydroxide.

No changes on absorption band position for the principle peaks were observed in the adsorbed adjuvant. This indicated no chemical changes of adsorbed AH due to the principle peaks were also represented. There were the minor differences between the absorption bands of C, t0; S, t0; C, t4; S, t4 with AH. C,t0; S,t0; C,t4; S,t4 showed the small peak at around 613, 729, 1533 $cm^{-1}$ and some tiny peak at around 2916-2973 $cm^{-1}$ but no these peaks at this position of AH. It could be concluded that the interaction between DT, TT, PT and JE with AH adjuvant by both competitive adsorption process and separate adsorption process may induce some chemical changes of AH structure which was not the important function for adsorption throughout the storage periods. In other word, the adsorption process and the storage time for 4 months had no effect on chemical structure of AH.

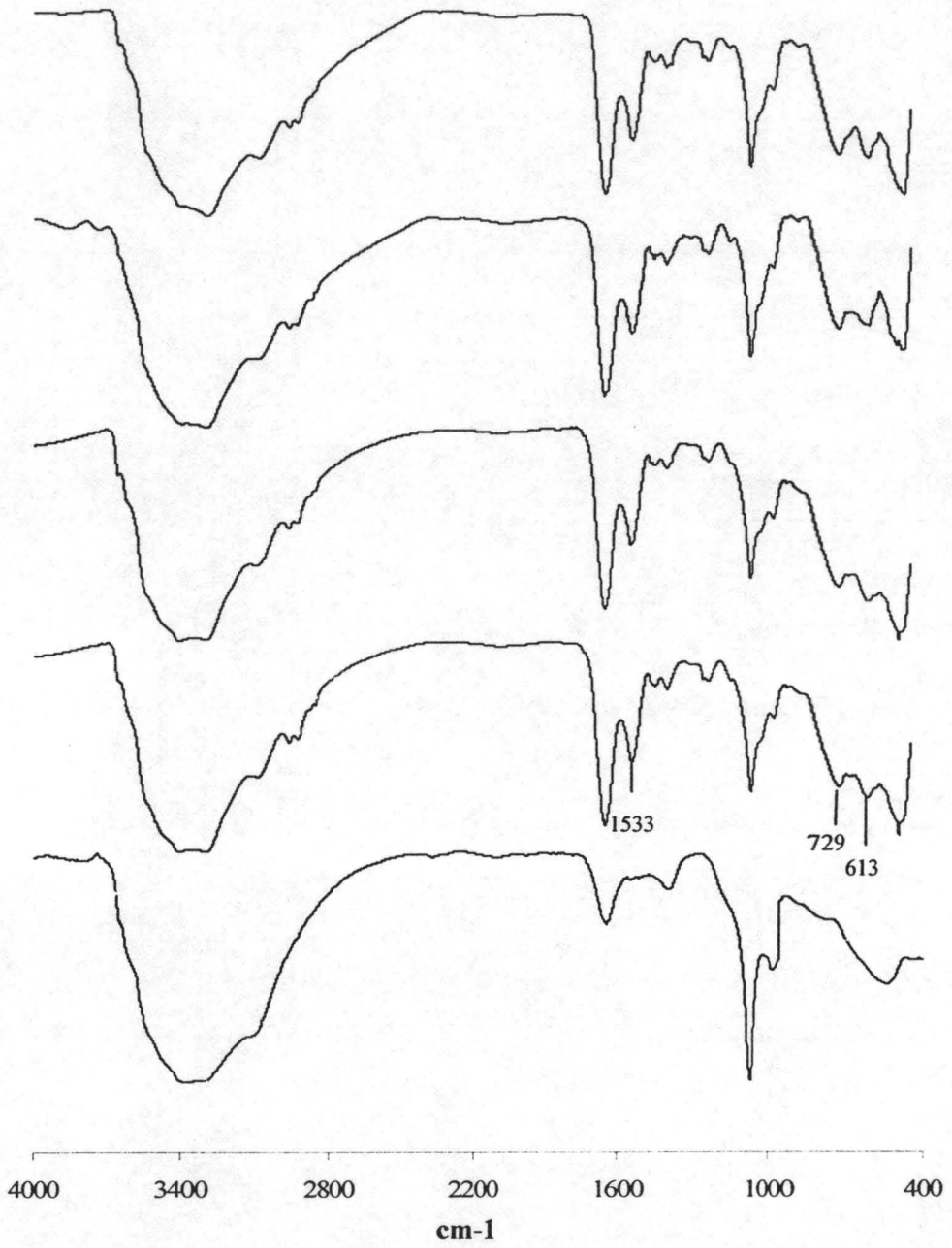



**Figure 21** FT-IR spectra of aluminium hydroxide and combined preparations by competitive adsorption process and separate adsorption process at initial (t0) and 4-month storage time (t4).

### 4.4 X-ray diffractrometry

The characterization of aluminium adjuvants is analyzed by X- ray diffractometry. The X-ray diffractograms of AH; C, t0; S, t0; C, t4 and S, t4 are shown in Figure 22.

The existing peaks were strong peaks at 4.72° which represented the peaks of all samples. It is obvious that AH exhibited crystalline state and there was no difference in position of diffraction band between AH with the two adsorption process preparations, competitive adsorption process and separate adsorption process, among storage for 4 months. In addition, both C and S had the reduction of the intensity of diffraction band from at initial. It may be due to the crystallinity of AH was reduced which supported by the porosity of AH from SEM after storage. The result could be concluded that no effect of adsorption processes and storage on the polymorphism of AH.

### 4.5 Particle size distribution

Particle sizes of adsorbed preparations were evaluated by laser diffractometry at initial and after storage 2-8 °C for 4 months in order to determine the physical stability of preparations. Table 15 and Figure 23 show the mean particle sizes of AH, C, S after 1-day production and 4-month storage, respectively.

At initial, the mean particle size of C was significantly larger than AH ($p<0.05$) and S was close to AH. According to the study of Sripongsarn (2005) had shown that the mean particle size of all adsorbed AH (DT-AH, TT-AH and JE-AH) were larger than AH. C exhibited the bigger than S. Due to AH was a complex fibrous particle (Sepelyak et al., 1984) which aggregated or de-aggregated during mixing. Because S had longer mixing time than C in adsorption process, so the AH structure of S was broken to smaller particle.

After storage for 4 months, the particle size of both C and S were significantly reduced from initial ($p<0.05$) and S was bigger than C. It might be assumed that among storage time, the structure of AH of C was broken more than S.

In conclusion, the results of particle sizes distribution which were measured by LD confirmed that the antigens were attached on the aluminium adjuvant. The particle sizes of adsorbed adjuvants which were smaller than unadsorbed adjuvant might be the result of structure conformation. The studies by Johnston et al. (2002) and Morefield et al. (2004) had shown that AH composed of very small primary particles. These particles formed irregularly shaped aggregates having diameters between 5 and 10 μm. They mixed AH adjuvant with labeled BSA and observed the label region with flow cytometry so the adjuvant aggregates underwent a de-aggregation and re-aggregation process during mixing. Therefore, the particle size of AH could be broken after the adsorption process.

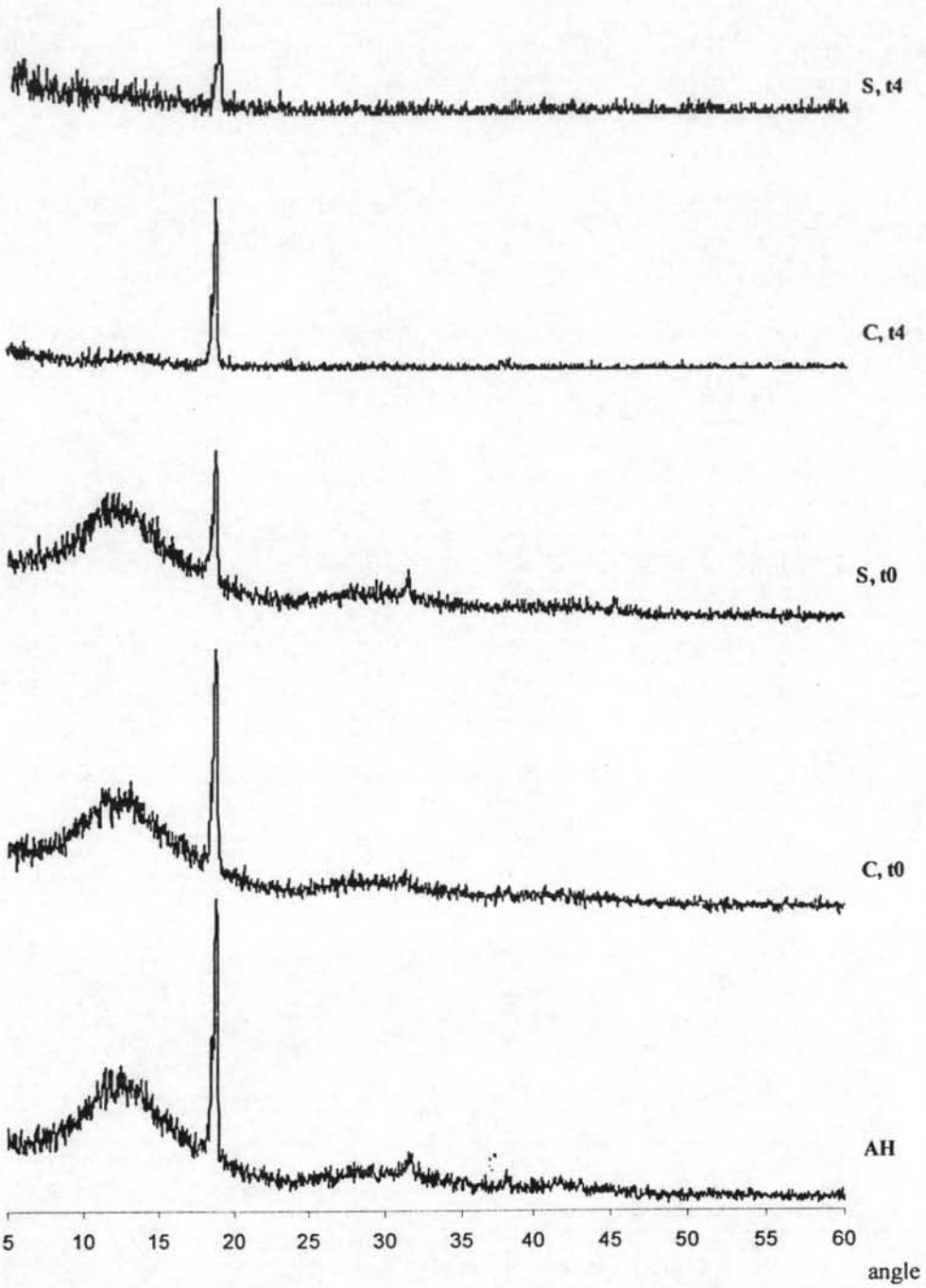



**Figure 22** X-ray diffractograms of aluminium hydroxide and combined preparations by competitive adsorption process and separate adsorption process at initial and 4-month storage time.

**Table 15** Particle sizes of AH and combined preparations at initial and after 4-months storage at 2-8 °C determined by laser diffractrometry.

| Formulation | Mean particle size (48) ± SD | | | |
|---|---|---|---|---|
| | Initial | | 4 months | |
| | D (v, 0.5) | uniformity | D (v, 0.5) | uniformity |
| AH | 9.40 ± 0.22 | 0.73 ± 0.03 | ND | ND |
| C | 10.95 ± 0.15 | 0.38 ±0.10 | 4.93 ± 0.19 | 0.59 ± 0.03 |
| S | 9.26 ± 0.07 | 0.70 ± 0.01 | 7.17 ± 0.07 | 0.33 ± 0.04 |

ND: not determined

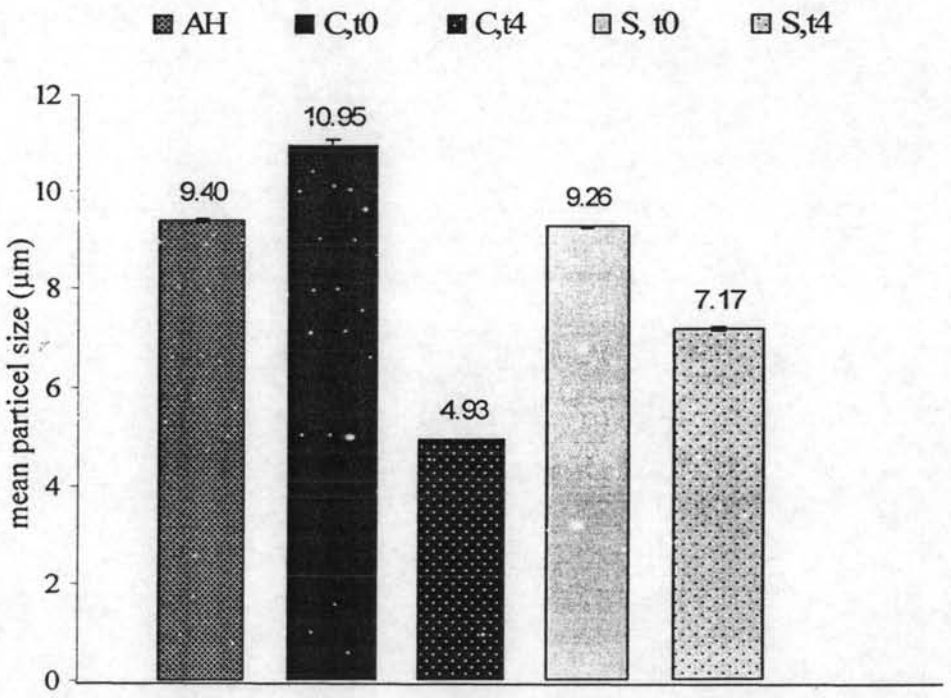



**Figure 23** Particle sizes of AH and combined preparations at initial (t0) and after 4 months storage (t4) at 2-8 °C determined by laser diffractrometry.

# CHAPTER V
# CONCLUSIONS

The results of this study were concluded as followed:

1. The percentage of aluminium content in aluminium hydroxide gel was $2.26 \pm 0.01$ %Al.

2. The results from the adsorptive capacity of single antigen on 1.67%w/v AH adjuvant indicated that the adsorptive capacity of DT and TT were 0.46 and 0.6 mg/mg Al, respectively. The adsorptive capacity of JE could not be determined because the adsorption pattern was not linear; however, the maximal adsorption was at 0.43 mg/mgAl. Therefore for DTP-JE vaccine, the amount of 1.67%w/v AH for adsorption of each antigens were 0.33 mg for DT, 0.08 mg for TT and because the total amount of AH was not more than 0.85 mg Al/dose as per USFDA allowance, thus for JE was 0.44 mg of 1.67%w/v AH.

3. The adsorption of single antigen on adjuvant at various processing variables could be concluded that the optimal processing variables for adsorption of DT, TT and JE on AH was temperature 5 °C; mixing speed 400 rpm and mixing time 5 hr because at this condition had the optimal %adsorption of DT, TT and JE.

4. Stability studies of combined preparations showed that

   4.1 S, which was prepared by separate adsorption at 5 °C, 400 rpm and for 5 hours, was the better optimal formulation process than C for the combined preparation under storage condition at 2-8°C for 4 months. The adsorption process may affect to DT and JE content, but not on TT and PT.

   4.2 The morphology by SEM showed that adsorbed AH particle of C and S had the attachment of some small fragments or layers likely

to be antigens. After storage for 4 months, the porosities on surface were shown which may be due to the reduction of antigens.

4.3 The adsorbed preparations prepared by C and S method, showed no change in the chemical structure of AH throughout the storage period at 2-8°C for 4 months due to the presented principle peaks in IR spectra.

4.4 The X-ray diffractrograms of adsorbed AH adjuvant by C and S presented the principle diffraction band. The diffraction band of adsorbed AH by C and S were no difference in position but the intensity decreased after storage for 4 months at 2-8°C, which indicated that antigens adsorption had no effect on the structure of AH while the reduction of crystallinity occurred.

4.5 The particle size of C was larger than that of AH while that of S was close to AH. After storage for 4 months at 2-8°C, the particle size of both C and S were reduced from initial.