CHAPTER IV

RESULTS

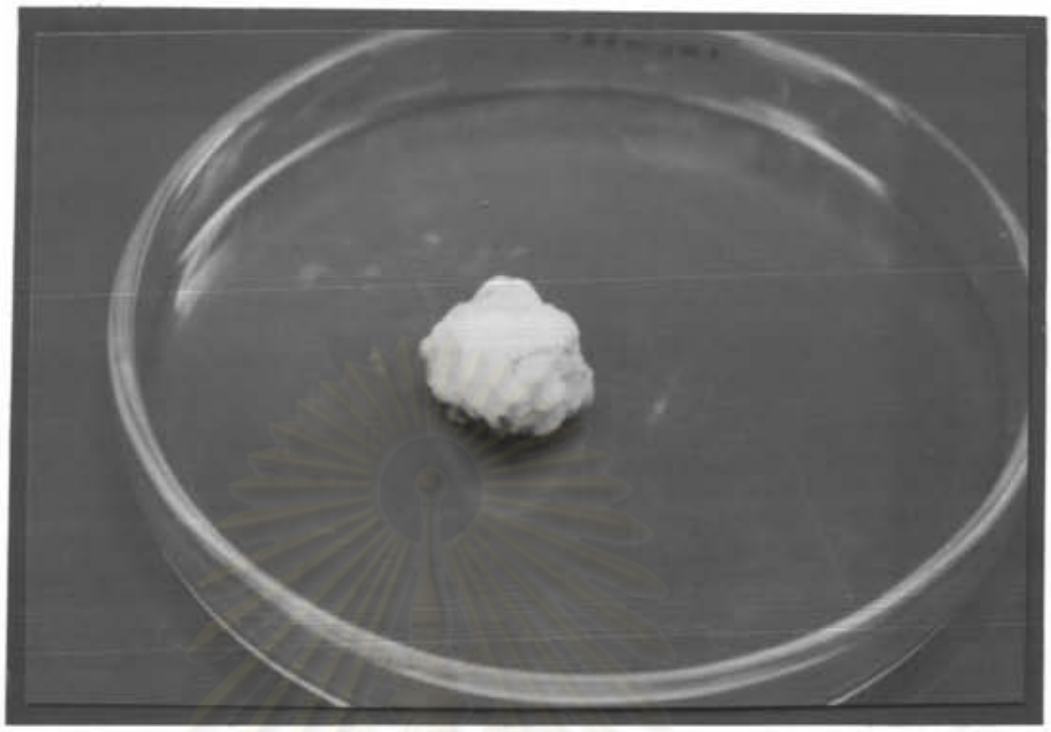
1. Establishment of callus cultures from the tubers of *Stephania pierrei*

In a preliminary experiment, the effect of basal media and culture conditions on callus induction from *S. pierrei* explants were studied. The explants of tubers were cultured on various media, including MS, B5, WPM, LS, NN, RT and White agar media. These media were all supplemented with 30 g/l sucrose, 1.0 mg/l 2,4-D and 0.1 mg/l kinetin at 25°C under 16 hrs of light. Under these conditions, the ability of each tuber explant from callus was very low, no callus induction was observed. Moreover, the explants turned brown and the basal agar media were all yellow after 2 weeks. From the literature, it has been reported that callus formation of*S.cepharantha* tuber was accomplished by incubating in the dark (Sugimoto, Sugimura and Yamada, 1988). We, therefore, reported the same experiment by culturing the explants on same basal agar media as above but incubated them in the dark at 26°C instead. After a month of incubation, callus formation on White medium was observed, while negative results were observed in other media. However, with the basal medium of White, the developed calli appeared to grow slowly. Therefore,the effect of type and concentration of growth regulators on callus formation were investigated further. By manipulation of various plant hormones, it was found that callus formation was induced successfully on White agar medium supplemented with the combination of 30 g/l sucrose, 0.1 mg/l kinetin, 0.2 mg/l BA, 1.0 mg/l NAA, 1.0 mg/l GA3 and 0.8% w/v agar. As shown in Figure 12, the calli were initiated at the top edge of tuber segments before enlarging to the bottom and formed as compact yellowish nodules after a month of incubation. The segment with calli were subcultured every 4 weeks on the same White medium and incubated in the dark. Under this condition, the callus appeared to grow rapidly, therefore, the size of segments were enlarged in about twofold in two months and the cali were fluffy in texture and turned yellowish white (Figure 13).

Although, the calli grew rapidly on the semisolid medium, no differentiation was observed. Furthermore, when the calli nodules were cut as the slit sheet and cultured on various agar media, including the same White medium, the developed

Figure 12 Callus cultures of *S. pierrei* on White medium.

a



b

Figure 13 Callus cultures. (a) 1 month (b) 3 months

callus turned brown, wilted and died eventually. With the medium containing gellan gum, however the callus enlarged rapidly, in the first 2 weeks and then turned brown and died in the next 4 weeks. Therefore, our attempt to establish stable callus cultures of *S. pierrei* was not successful.

2. Root regeneration from callus cultures.

After the callus tissue of *S. pierrei* were subcultured on White agar medium containing 30 g/l sucrose, 0.1 mg/l kinetin, 0.2 mg/l BA, 1.0 mg/l NAA and 1.0 mg/l GA_3 for 2 months, the differention of the root buds from the calli were observed. There were 4-6 buds per group. After the roots were 0.5 cm long, they were cut and transfered into White and Root media supplemented with the same growth regulators and rotated at 85 rpm on a rotary shaker in the dark. The excised roots under these conditions were appeared as texture with 2-3 nodules after a week of incubation and were then swallen and dispersed after next 2 weeks.

With no success in establishing the root cultures in liquid media, we went back to imporve our semisolid medium to support the growth of the callus cultures. In doing this, the callus cultures with shorted roots were transfered into RT medium containing 30 g/l sucrose, 0.1 mg/l kinetin, 0.2 mg/l BA, 0.1 mg/l NAA, 1.0 mg/l GA3 and 0.3% w/v gellan gum and incubated in the dark. After 4 weeks, it appeared that the roots could elongate to 3-5 cm in length (Fig. 14), they were harvest, and the callus cultures were maintained in the dark in the same medium. Under these conditions, the roots could reelongate and were harvested up to 4 times. After the forth harvest, the callus turned brown and died. Attempt to subculture the callus was not successful. The harvested roots were pooled and kept in -20°C refrigerator for detection of isoquinoline alkaloids and their content.

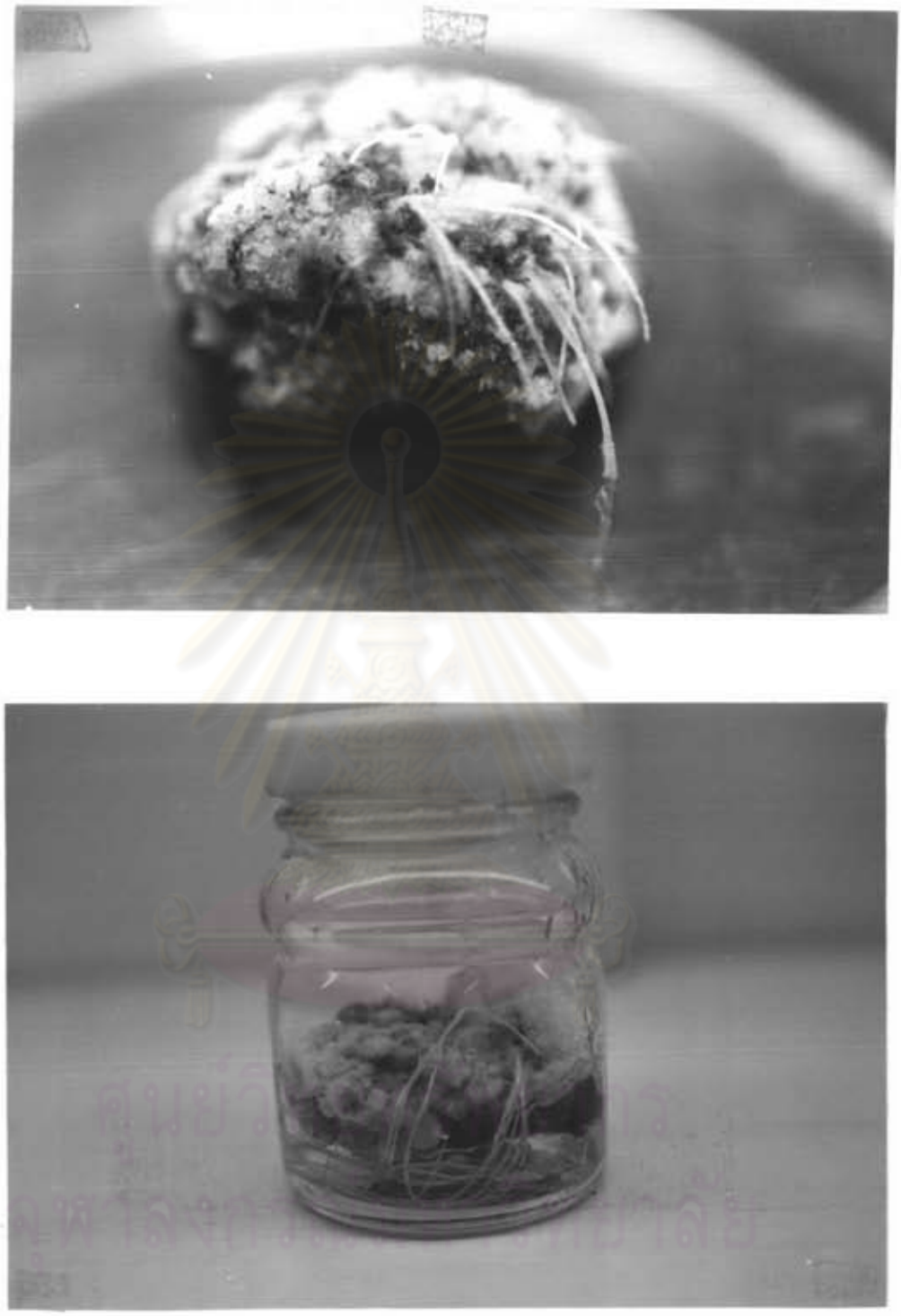


Figure 14 Root cultures on Root medium

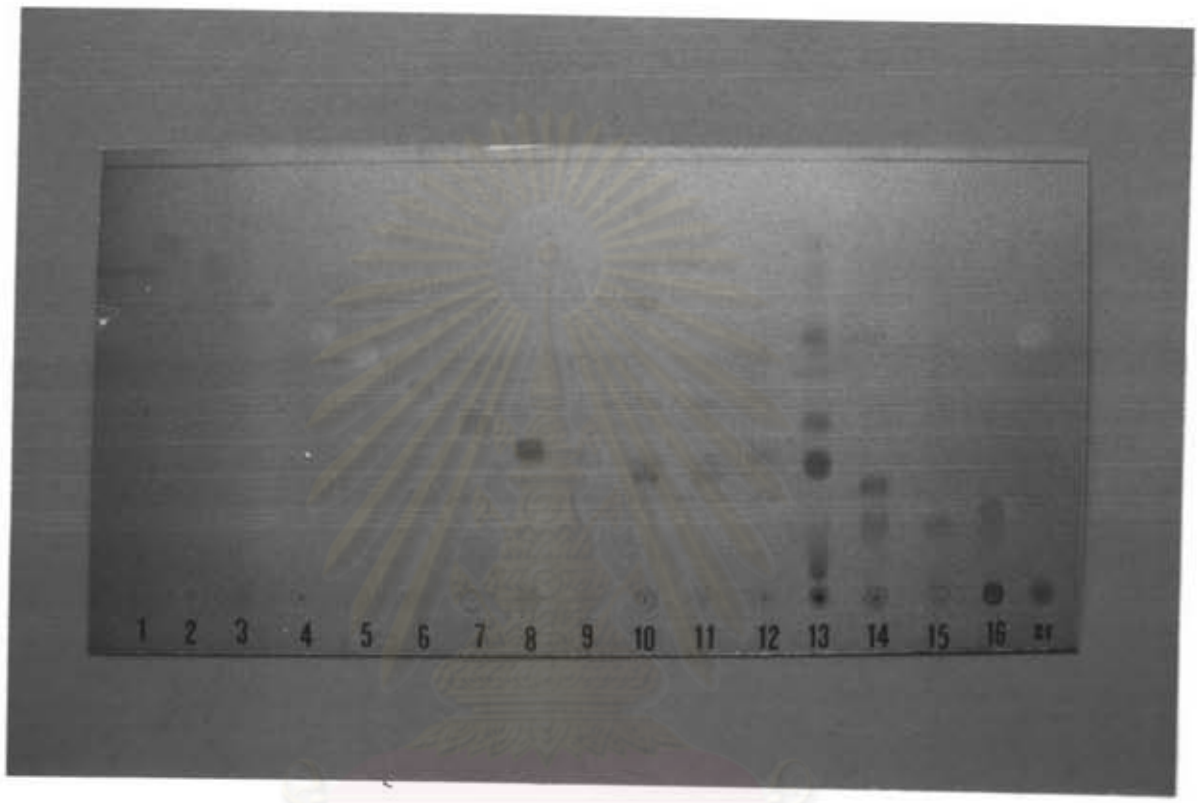


Figure 16 TLC patterns of authentic isoquinoline alkaloids
for names and Rf values of each see the Table 7
(next page)

Table 7 Rf values of authentic isoquinoline alkaloids in toluene : EtOAc:DEA (7:2:1)

Alkaloids	Rf values
1. Tetrahydropalmatine	0.81
2. Delavaine	0.76
3. Isolaureline	0.68
4. Dicentrine	0.60
5. Tetrahydrostephabine	0.53
6. Thaicanine	0.48
7. Codamine	0.40
8. Phanostenine	0.34
9. Corydalmine	0.31
10. Xylopine	0.28
11. Annonaine	0.28
12. Salutaridine	0.23
13. Nordicentrine	0.30
14. Cassythicine	0.16
15. Asimilobine	0.15
16. Reticuline	0.16

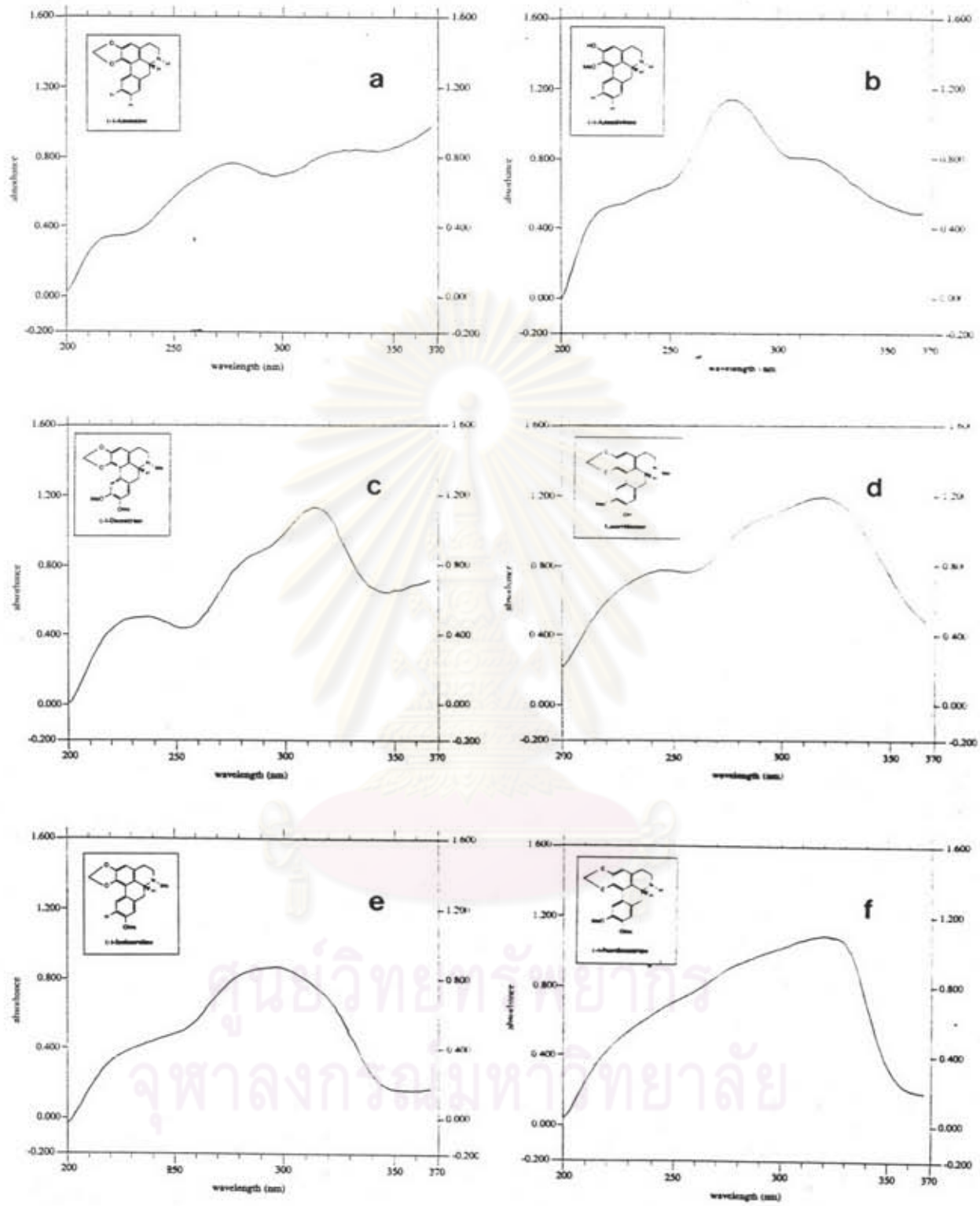


Figure 17 UV-absorption spectra of authentic isoquinoline alkaloids

a). (-)-Anonaine b). (-)-Asimilobine c). (-)-Dicentrine

d). Cassythicine e). Isolaureline f). (-)-Nordicentrine

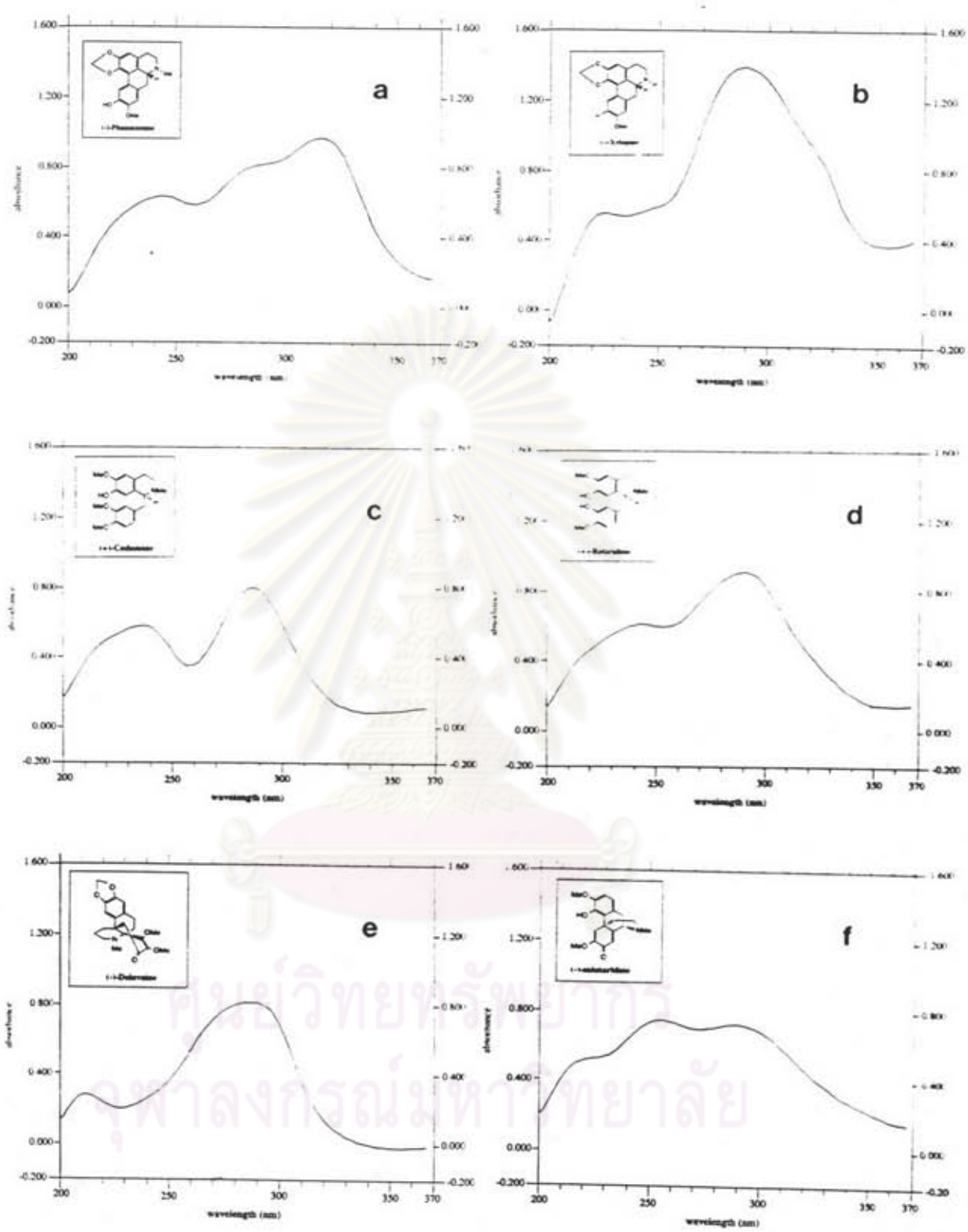


Figure 18 UV-absorption spectra of authentic isoquinoline alkaloids
a). (-) Phanostenine b). (-)-Xylopine c). (+)-Codamine
d). (+)-Reticuline e). (-)-Delavaine f). (-)-Salutaridine

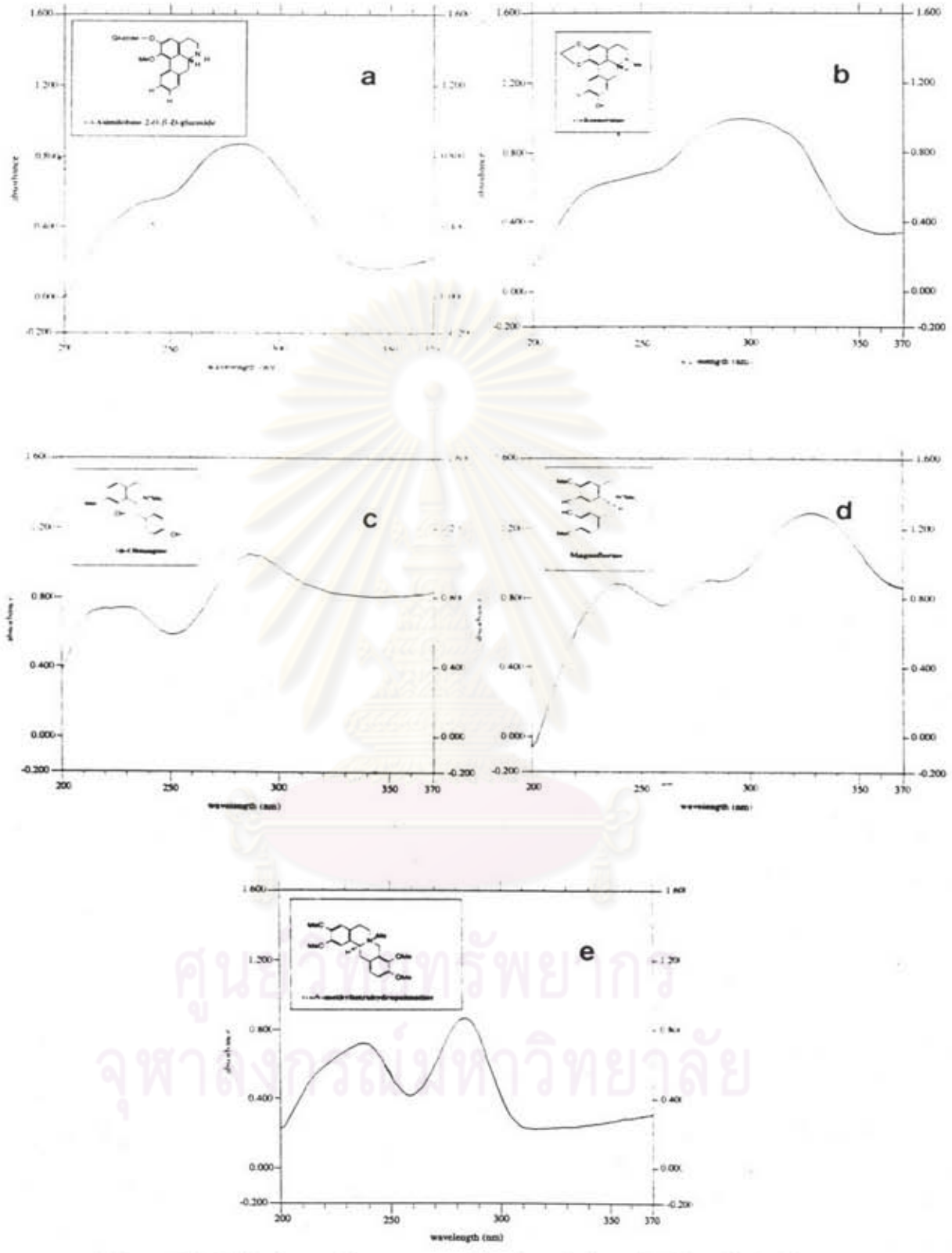


Figure 19 UV-absorption spectra of authentic isoquinoline alkaloids

a). (-)-Asimilobine-2-*O* -β-*D* -glucoside

b). (-)-Roemeroline c). (±)-Oblongine d). Magnoflorine

e). (-)-*N*-methyltetrahydropalmatine

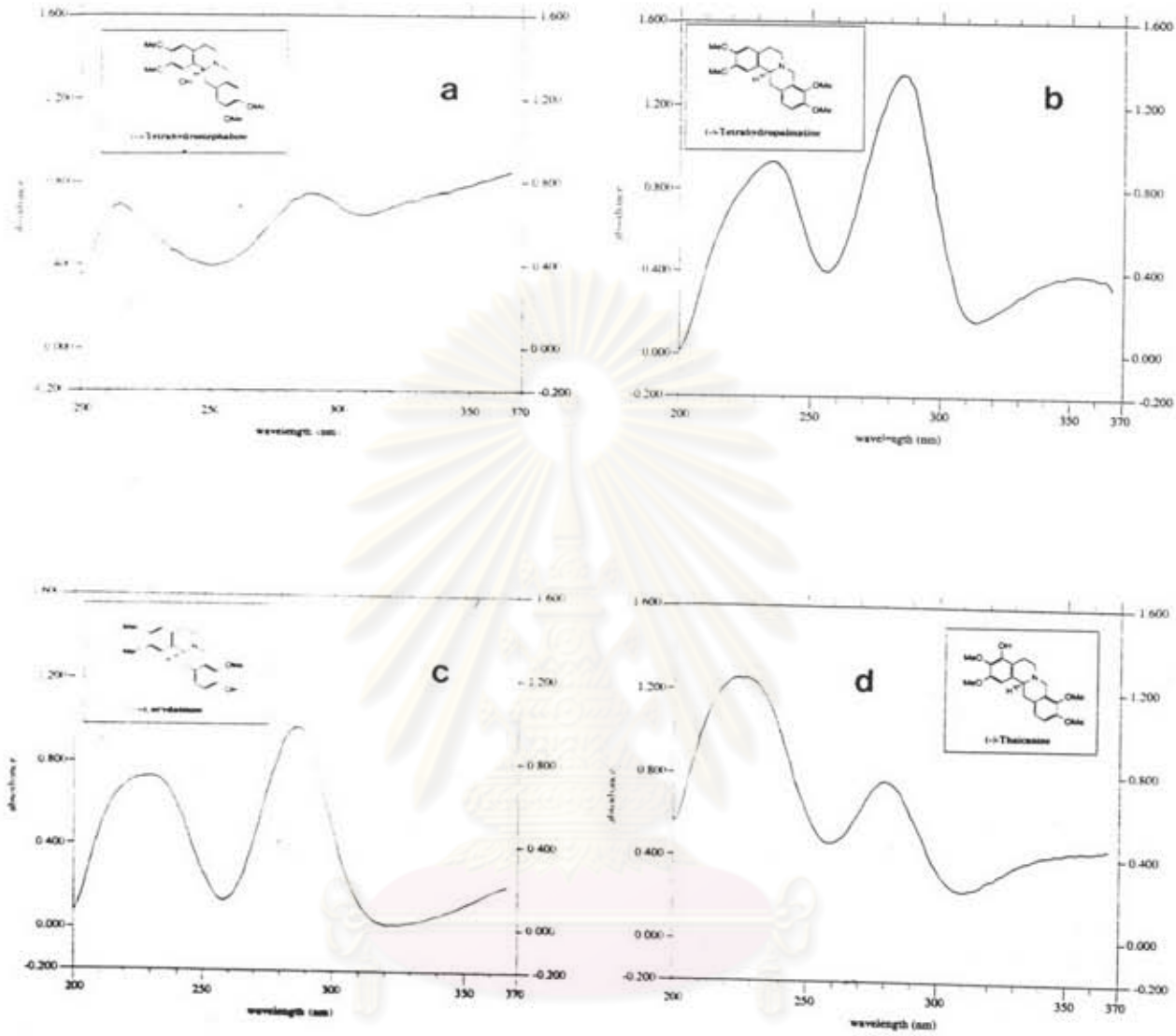


Figure 20 UV-absorption spectra of authentic isoquinoline alkaloids
a). (-)-Tetrahydrostephabine b). (-)-Tetrahydropalmatine
c). (-)-Corydalmine d). (-)-Thaicanine

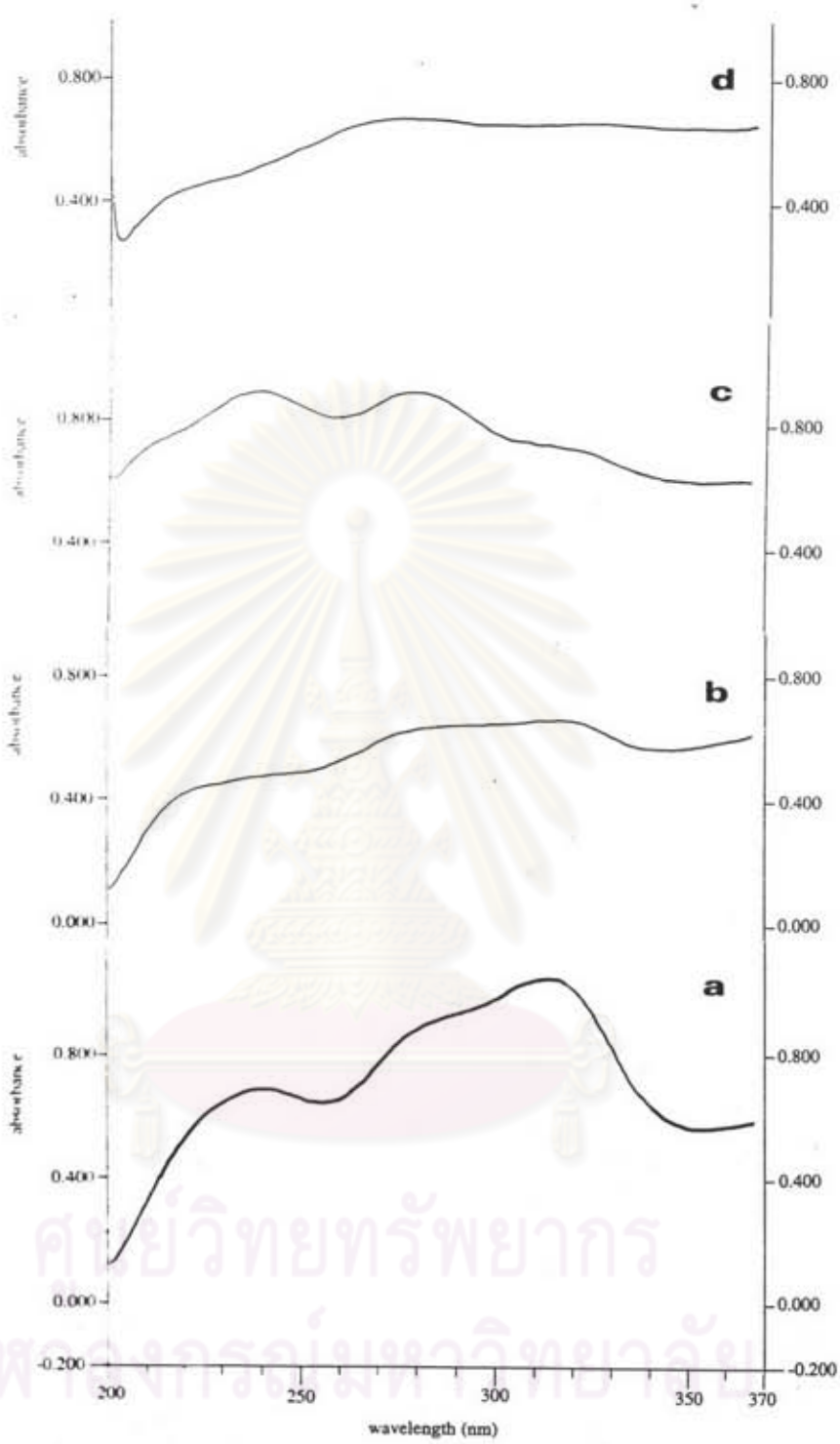


Figure 21 UV- absorption spectra of spots on TLC plate using EtOAc:MeOH (5:1)
(a) Rf = 0.34 (b) Rf =0.56 (c) Rf = 0.64 (d) Rf = 0.73

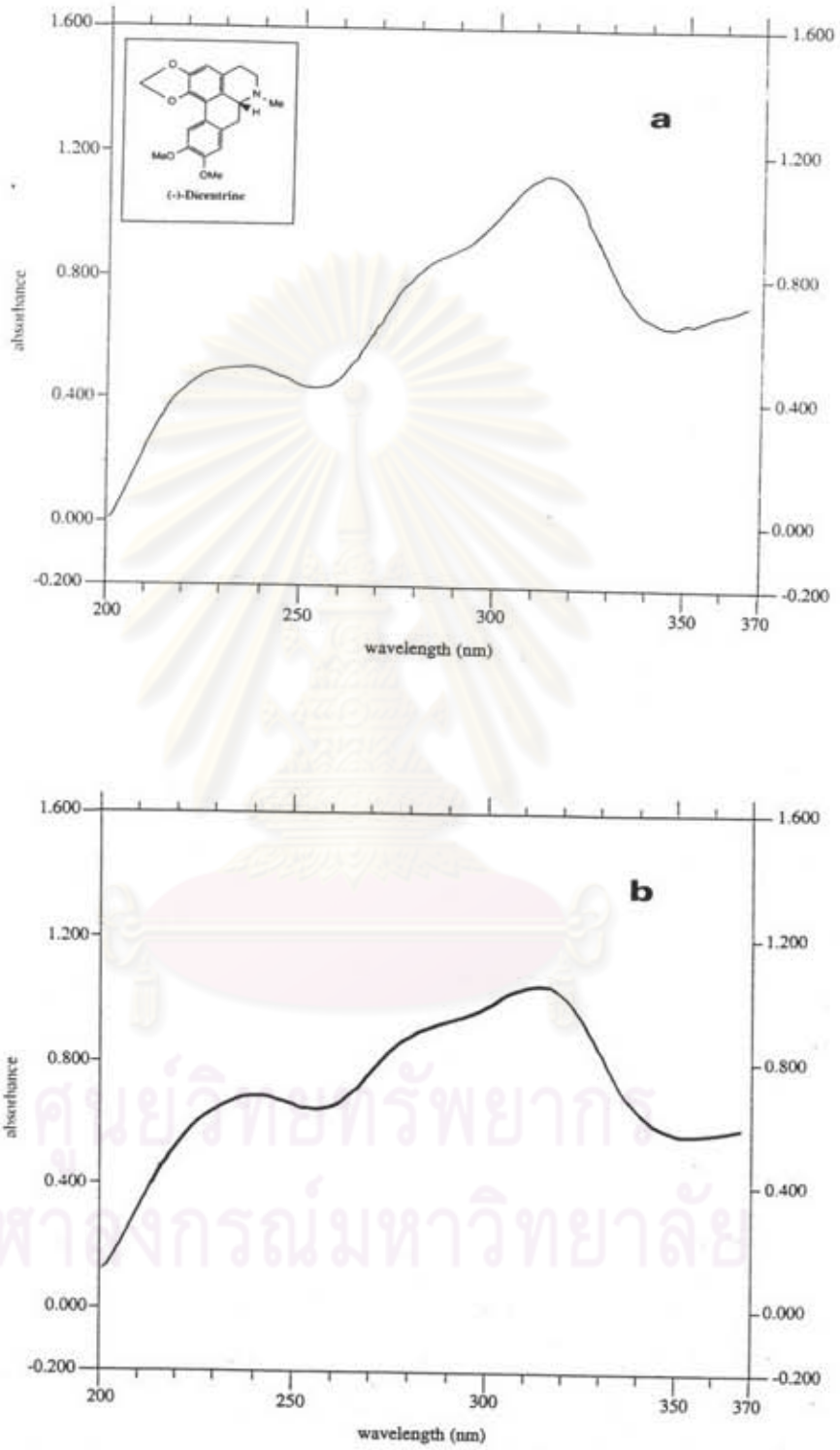


Figure 22 UV-absorption spectra of (a) authentic dicentrine and (b) spot at Rf= 0.34

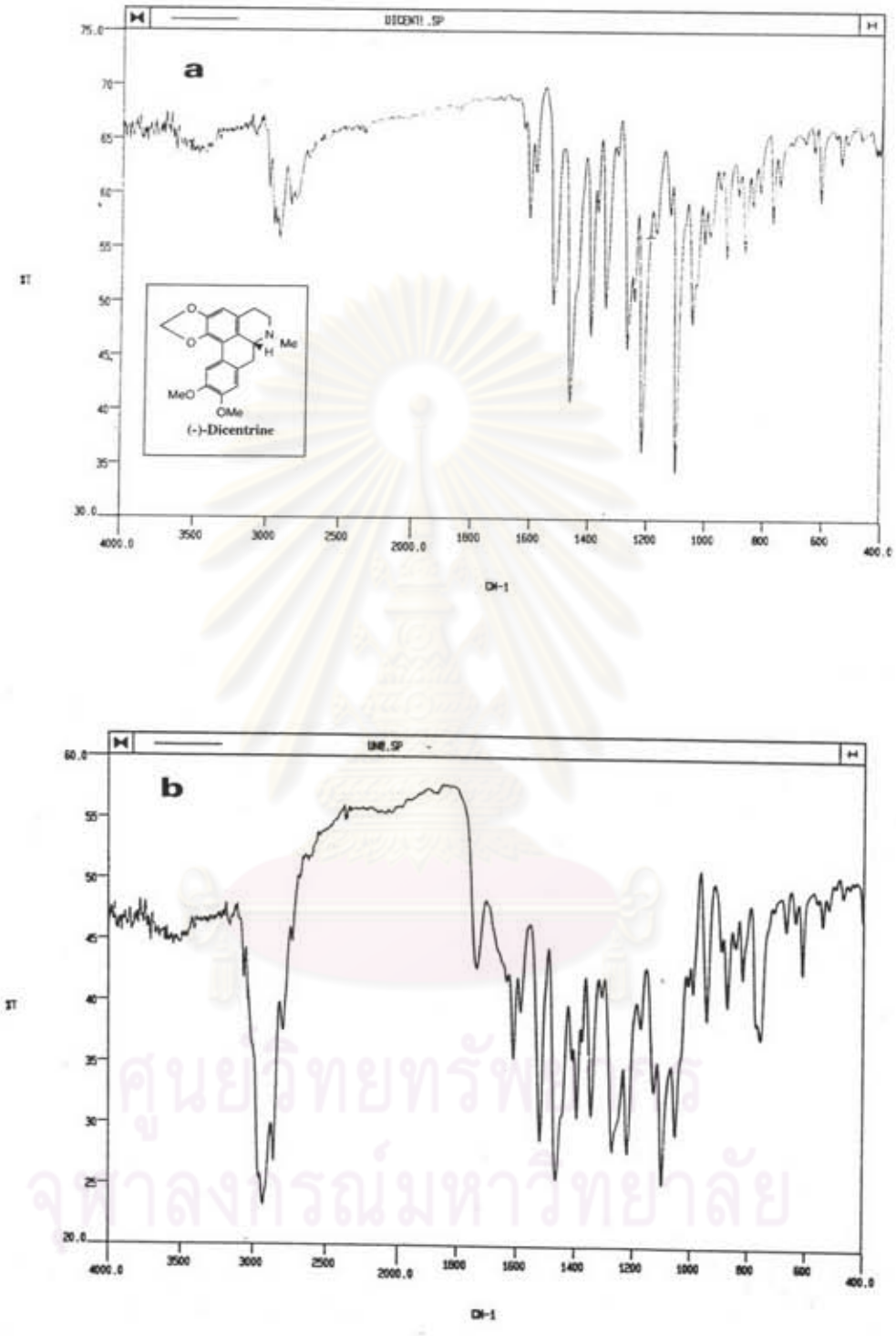


Figure 23 IR spectra of dicentrine (a) and purified sample from cultured roots (b).

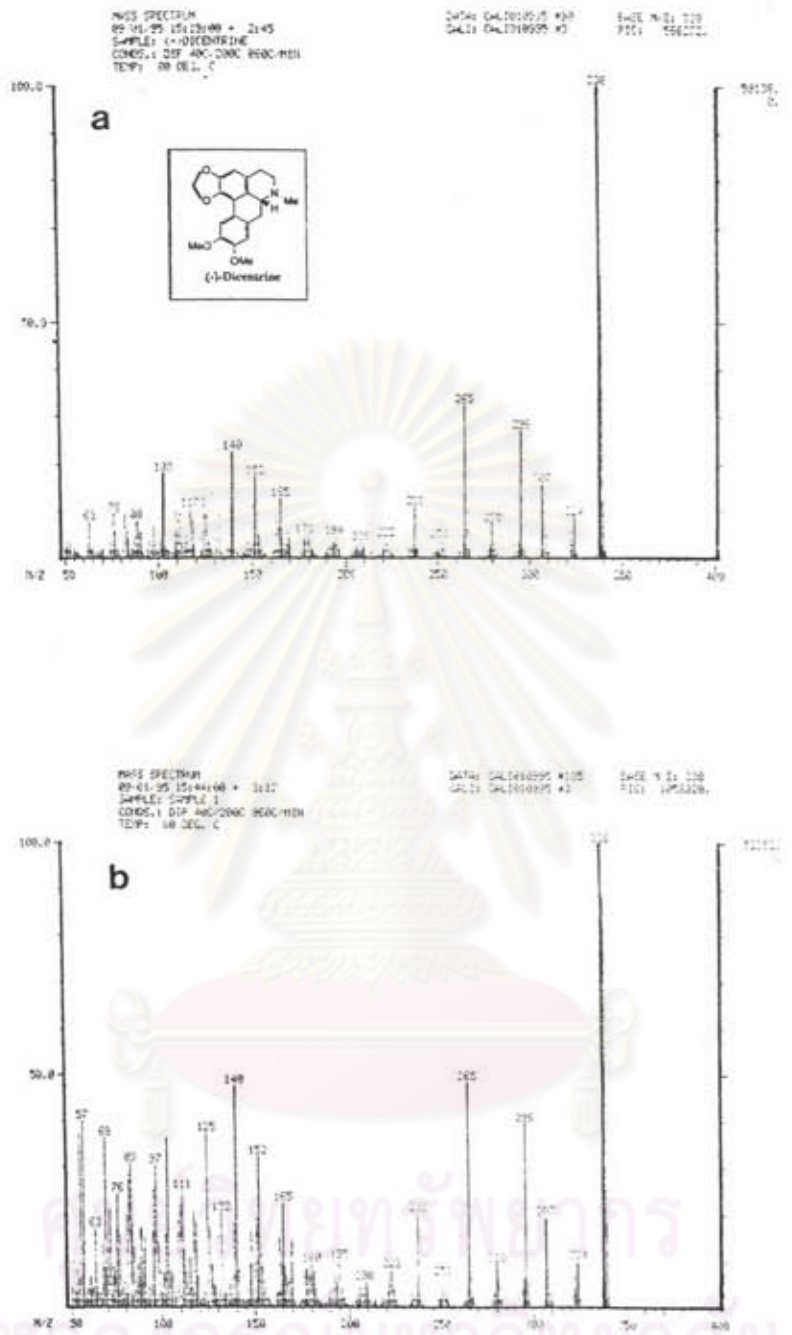


Figure 24 Mass spectra of dicentrine (a) and purified sample from cultured roots (b).

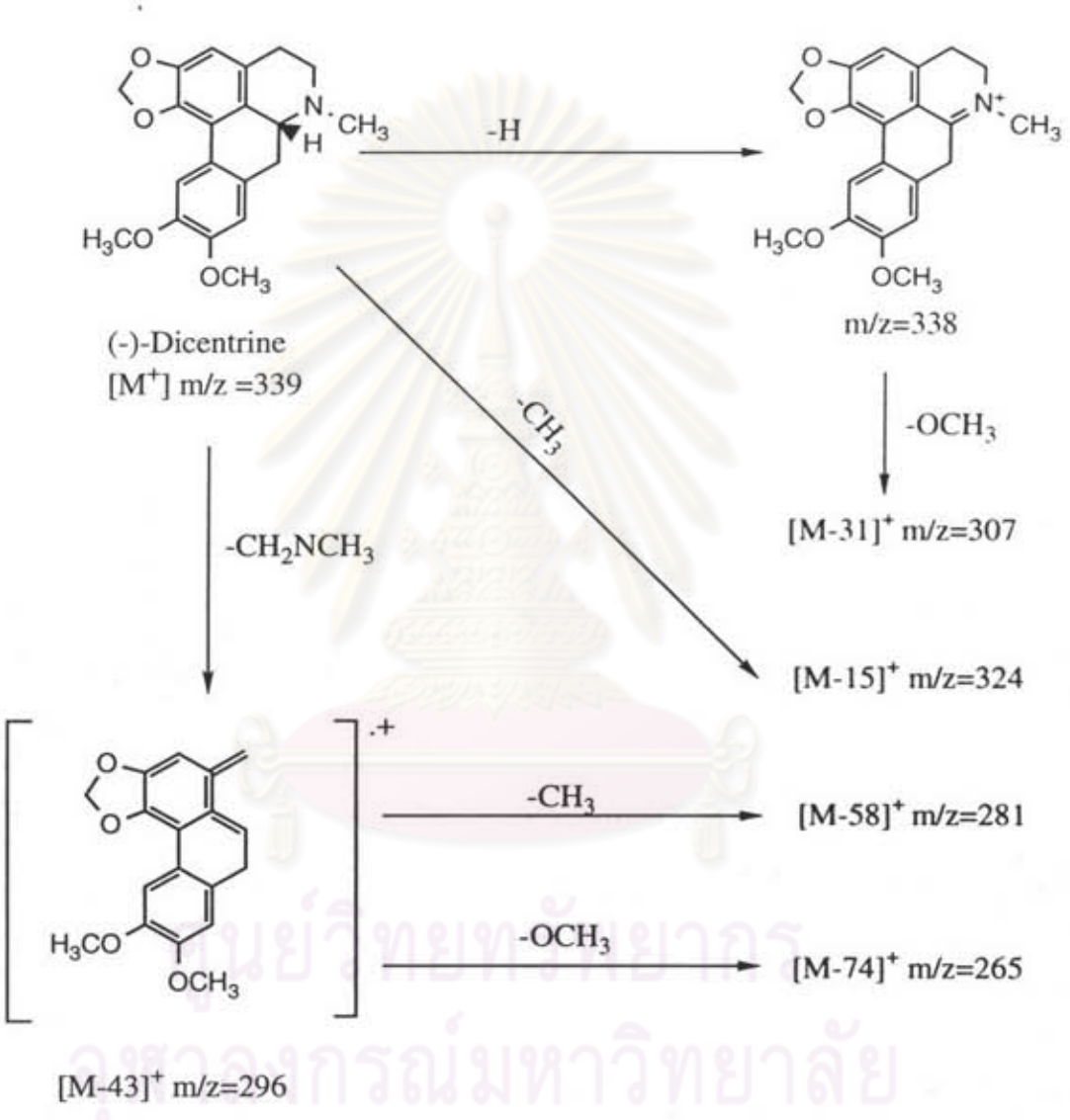


Figure 25 Mass fragmentation pattern of (-)-dicentrine.

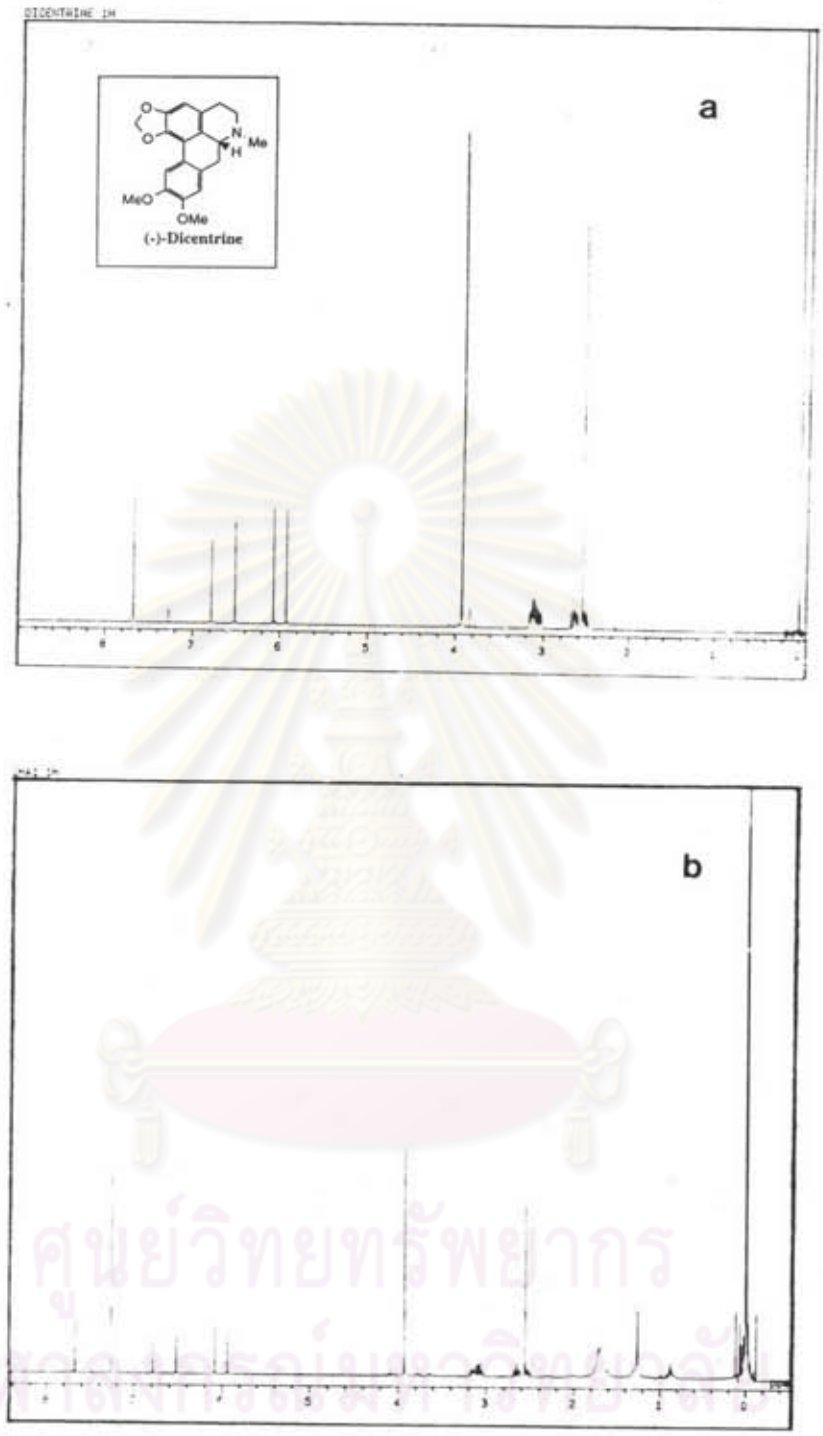


Figure 26 ^{1}H-nmr spectra of dicentrine (a) and purified sample from cultured roots (b)

4. Quantitative analysis of dicentrine from the root cultures and plant parts of *S. pierrei*

The TLC separation of dicentrine from other constituents using the solvent system of ethylacetate and methanol (5:1) allowed the compound be quantitated by the method of densitometry which generated a chromatogram. The area under the peak of dicentrine could be used for calculation of its content if a calibration curve of authentic dicentrine is available. In this study, the calibration curve of standard dicentrine was obtained by plotting the peak areas against dicentrine concentration (Figure 27). This graph showed linearity relationship between 0.25 to 2.50 µg for a 5 µl application volume of each dicentrine concentration which was equivalent to 0.05 to 0.5 mg dicentrine per ml. The regression analysis and the correlation coefficient was found to be 0.999936 and its linear slope was 0.52.

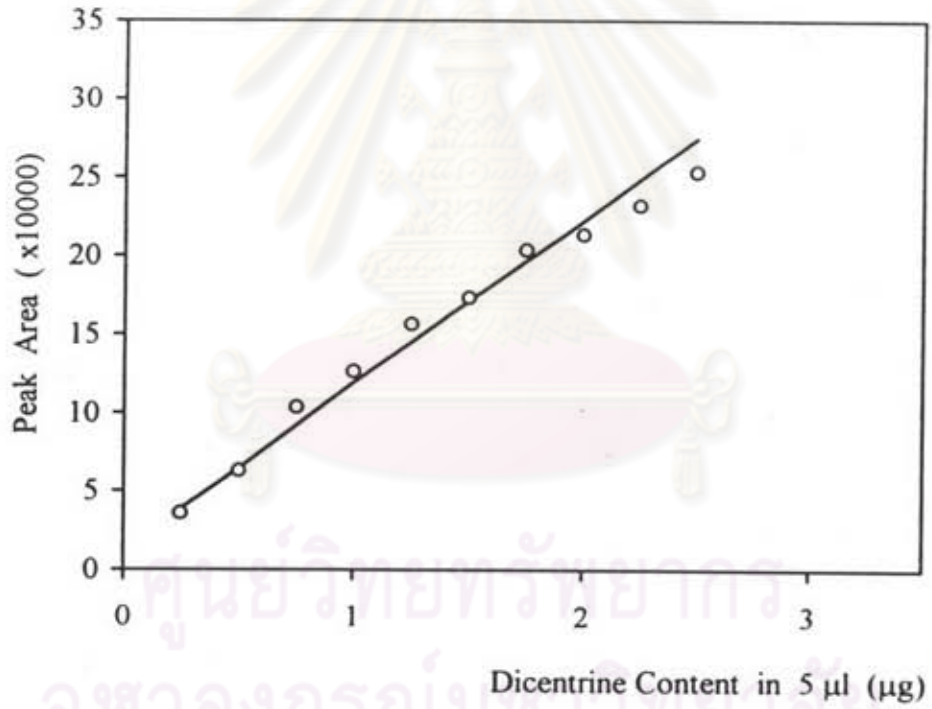


Figure 27 Standard curve of dicentrine

Based on this densitometric analysis, the TLC chromatograms of various crude extracts were produced (Fig. 28) and its content of dicentrine was obtained. It was found that the dicentrine content in the tubers was only 0.013% w/w dry weight and that in the leaves extract was not detected (Table 8). On the other hand, the root cultures accumulated dicentrine up to 0.82% w/w dry weight which appeared to be sixty-two times higher than its content found in the tubers.

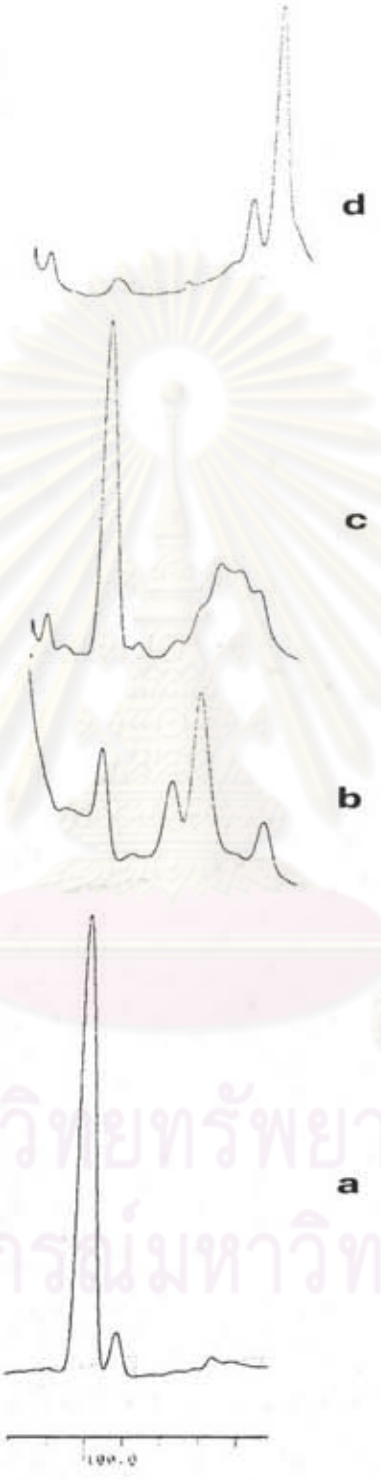


Figure 28 Chromatogram of plant parts and cultured roots extract
(a) authentic dicentrine (b) tuber extract
(c) cultured root extract (d) leaves extract

Table 8 Quantitative analysis of dicentrine in various plant parts and cultured roots

Plant parts	Dried Crude (g)	Total volume (ml)	Peak aera ($x10^4$)	Dicentrine in 5µl (µg)	Total Dicentrine (mg)	% w/w dry weight
Tuber	2.5	5	4.95	0.33	0.33	0.013
Leaves	2.5	5	-	-	-	-
Cultured roots	0.18	6	15.05	1.24	1.48	0.82