CHARPTER IV

RESULTS

1. Establishment of Callus and Cell Cultures of *A. salviifolium*

1.1 Establishment of Callus Cultures

The young leaf explants of *A. salviifolium* were induced for callus formation using MS, B5 and WPM media supplemented with various kinds and concentrations of plant growth regurators, both auxins and cytokinins (see Materials and Methods). It seemed that no significant difference effects on callus initiation among the three basal media and the addition of various plant growth regulators. Most media studied could induce callus formation. However, based on callus appearance, it was found that WPM medium containing 0.3 mg/l BA and 0.3 mg/l 2,4-D could induce callus formation better than other media. The established callus culture of *A. salviifolium* appeared to be light-brown in colour with fluffy tissue on the outside, but highly aggregated inside (Figure 6). The callus culture could be maintained by subculturing. However, within 15 passages, the cultures turned brown gradually and dark eventually.

1.2 Establishment of Cell Suspension Cultures

Since *A. salviifolium* callus cultures showed slowly growth and turned dark quite rapidly in the course of subculturing, attempts were made to established the cell suspension cultures of the plant. The callus cultures were first inoculated into the liquid medium (WPM added 0.3 mg/l BA, 0.3 mg/l 2,4-D) to increase cell proliferation. In this liquid media as suspension cultures, the cultured cells showed faster growth rate and looked more healthy (Figure 7).

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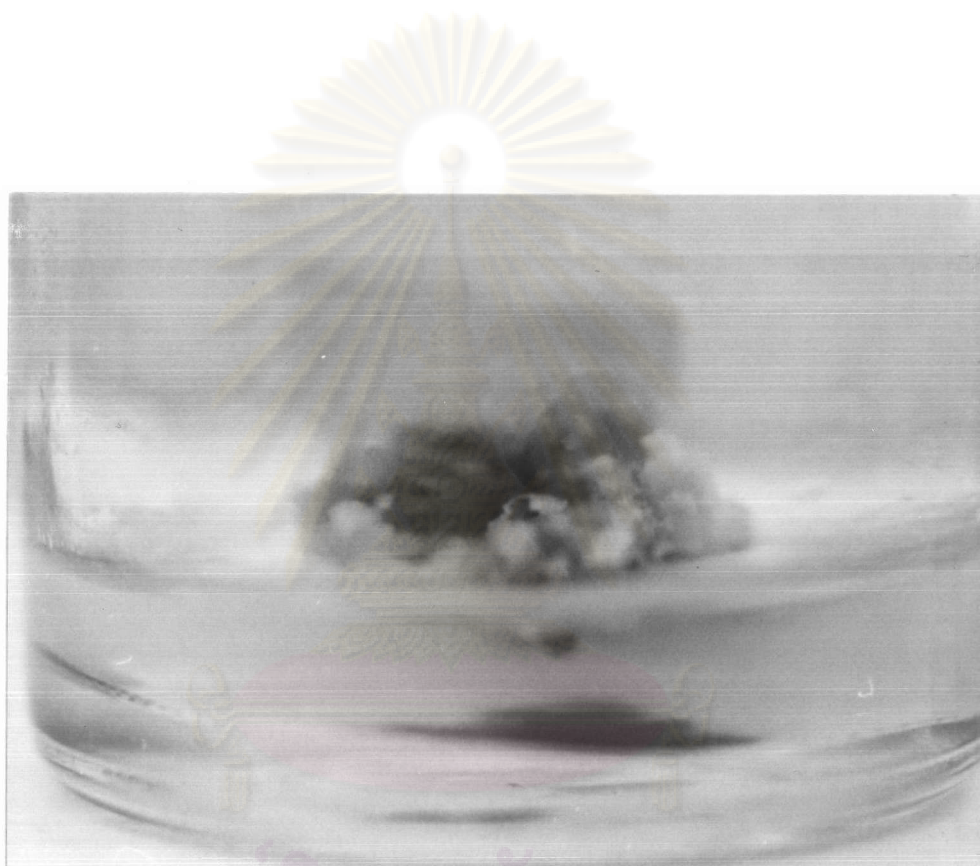

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Figure 6 The callus culture of *A. salviifolium* on WPM containing 0.3 mg/l BA and 0.3 mg/l 2,4-D

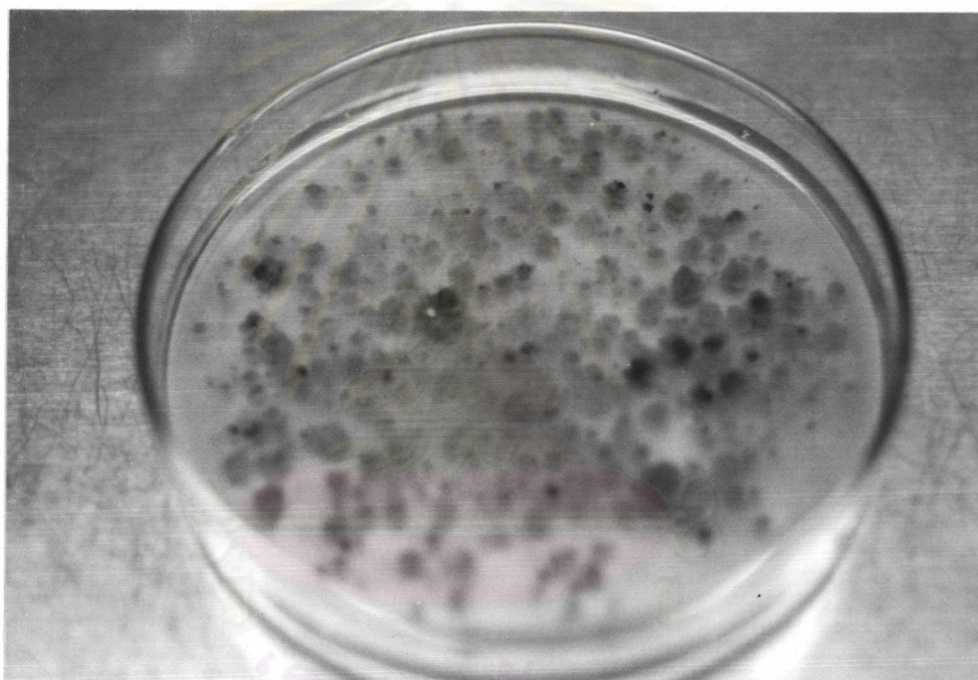

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Figure 7 The suspension culture of *A. salviifolium* in WPM containing 0.3 mg/l BA and 0.3 mg/l 2,4-D

In order to improve cell growth of the culture, effect of nitrogen levels were studied. This was carreid out by varying NH_4NO_3 concentrations (nitrogen source) to obtain total nitrogen concentrations as follows : 14.46, 28.92, 43.38, 57.84 and 72.30 mM. After being incubate for 1 week, the result showed that all cell suspensions had similar appearance : the medium and the cultured cells were light-brown in colour with some aggregates. Further incubation for 2-3 weeks also showed no difference of the culture. Therefore, it seemed that NH_4NO_3 had no significance on cell growth of *A. salviifolium.*

For studying the effect various potassium concentration, KNO_3 was varied to obtain the concentrations of 5.56 mM and 24.75 mM., the latter was as much as the potassium concentration in B5 medium. The cultured cells were also found to be no difference from each other and from the cells in various nitrogen concentration media. The growth rate was still slow.

It has been reported that the emetine-producing cell cultures of *Cephaelis ipecacuanha* are successfully maintained in the medium containing 1.0 mg/l 2,4-D and 4.0 mg/l NAA (Jha *et al.*, 1991). Therefore, WPM medium supplemented with the same growth regulators was tried. However, it was found that the medium could only reduce cell aggregation but could no increase cell fresh weight, On the other hand, the suspension cultures in WPM supplemented with 4.0 mg/l IAA, 4.0 mg/l NAA and 8.0 mg/l IBA made the cultured cells more aggregated and turned brown eventually.

1.3 Establishment of Root Cultures

The established suspension cultures maintained in WPM were subsequently subcultured in RM containing 2.0 mg/l GA_3, 1.0 mg/l BA and 0.1 mg/l Ki. It was found that some cell aggregates could initiate short roots (Figure 8 and 9) but their growth was very slow and the cultures turn brown eventually. Attempts were also

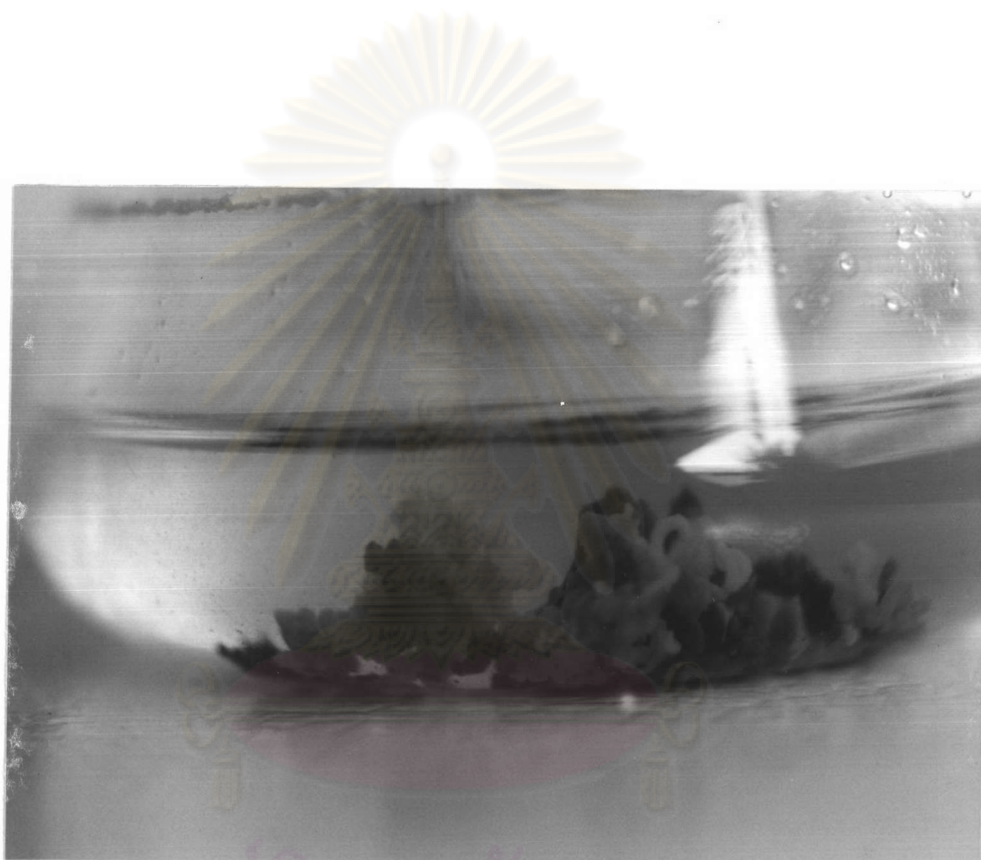

Figure 8 The root culture of *A. salviifolium* in RM containing 2.0 mg/l GA_3, 1.0 mg/l BA and 0.1 mg/l Ki

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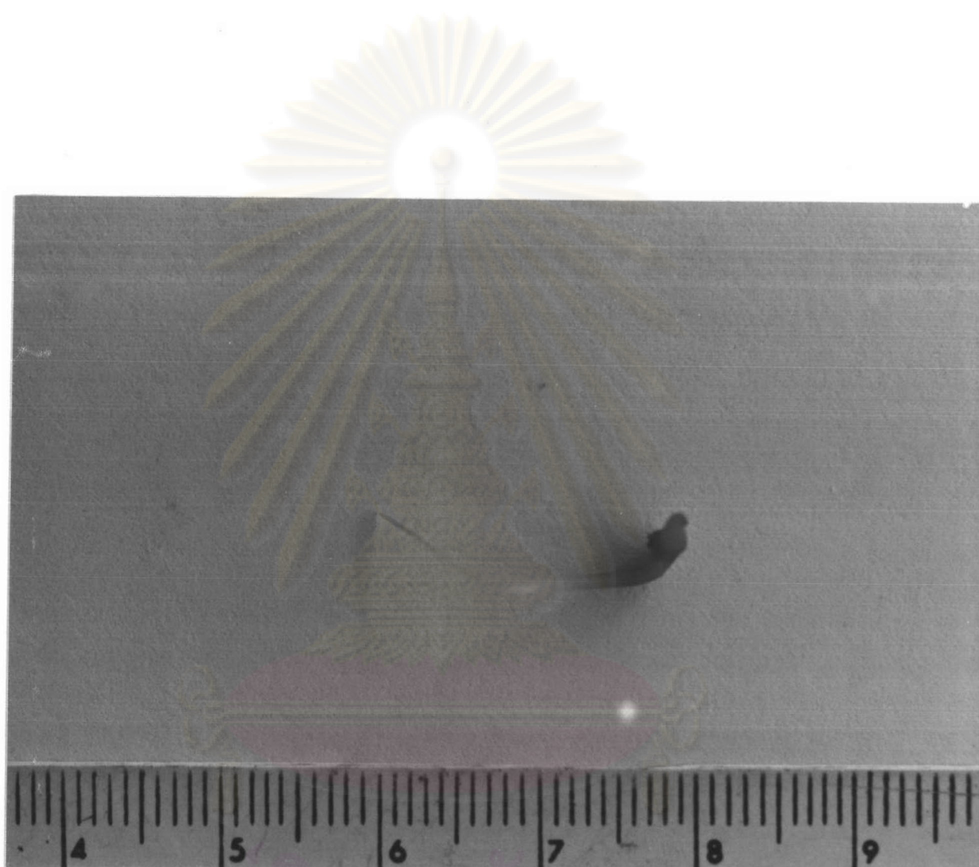

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Figure 9 The separated root from root culture of *A. salviifolium*

made by using rootlets of the plant root for initiation of root culture in the same RM. However, no growth was observed and the root died eventually.

2. Alkaloid Detections from *A. salviifolium*

2.1 Alkaloid Detection in the Leaves of *A. salviifolium*

There are many alkaloids accumulated in the leaves of *A. salviifolium* as observed by TLC (Figures 10 and 11). After acid-base separating method and purification, an alkaloid was observed. It has Rf value of 0.23 when using the solvent system of toluene:ethyl acetate:diethylamine = 7:2:1, detection: Dragendorff's reagent (Figure10) and Rf 0.72 with the solvent system of dichloromethane:methanol:ammonia = 90:9:1, detection: Dragendorff's reagent (Figure 11). Mass analysis of the compound, EI 70 V, shows m/z (% relative intensity) = 475 (base peak) (100.00), 304(12.44), 290(39.02), 262(97.31), 185(79.58), 171(69.31)(Figures 12 and 13). The fragmentation pattern and molecular mass indicated the alkaloid was alangimarckine.

2.2 Alkaloid Detection in the Roots of *A. salviifolium*

This was different from the detection of alkaloids in the leaves. All alkaloids in the roots were only extracted and spoted to TLC in order to compare with standards cephaeline and emetine in two solvent systems. Cephaeline has Rf 0.23 and Rf 0.61 , while emetine has Rf 0.38 and Rf 0.78 (Figures 8 and 9) respectively.

2.3 Alkaloid Detections in the Cell Cultures of *A. salviifolium*

Tissue cultures of *A. salviifolium* including callus cultures, suspension cultures and root cultures were also detected for the presence of alkaloids. The results, however, showed that no alkaloid was detected in all the tissue cultures (Figures 8 and 9).

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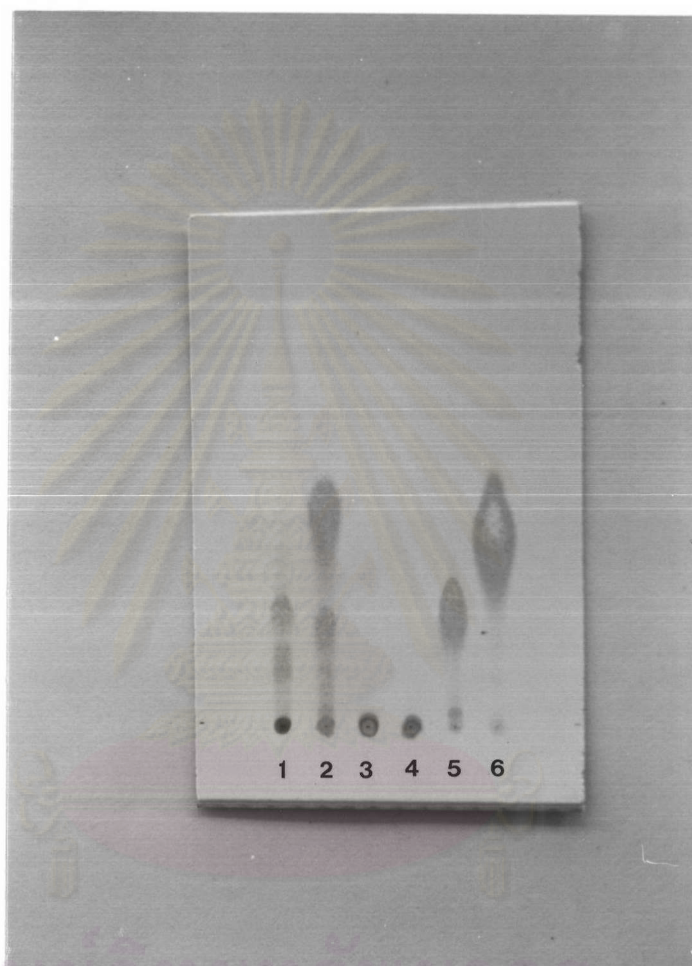

Figure 10 TLC pattern of the ethanolic extracts of 1) leaves, 2) roots 3) callus cells, 4) sespension cells, 5) standard cephaeline, and 6) standard emetine ; using the solvent system of toluene : ethyl acetate : diethylamine (7:2:1), detection : Dragendorff's reagent

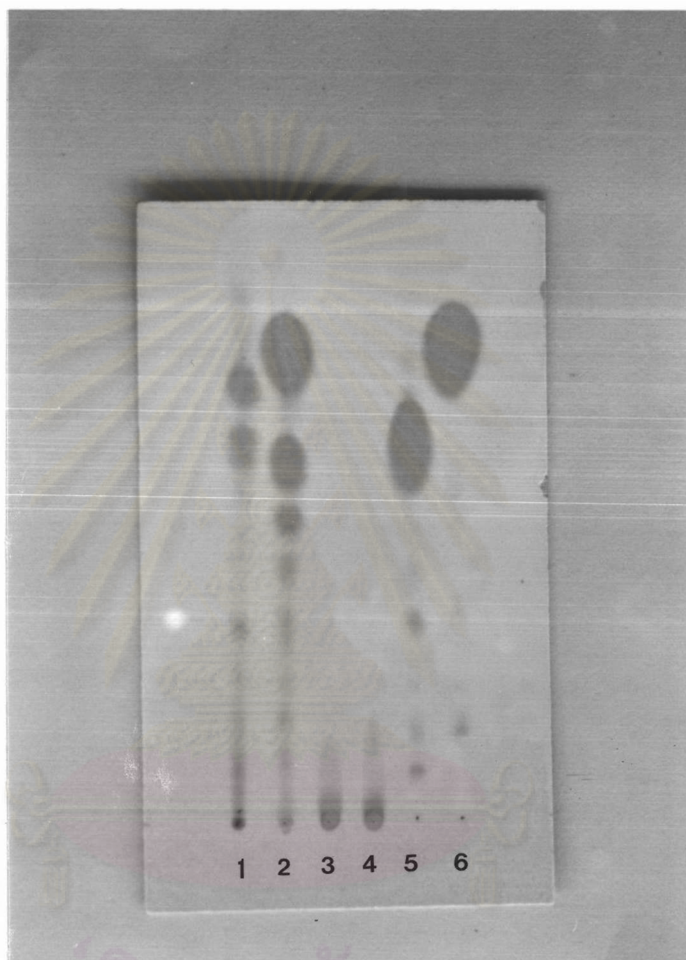

Figure 11 TLC pattern of the ethanolic extracts of 1) leaves, 2) roots 3) callus cells, 4) sespension cells, 5) standard cephaeline, and 6) standard emetine ; using the solvent system of dichloromethane : methanol : ammonia (90:9:1), detection : Dragendorff's reagent

Mass spectrum EI, 70 V

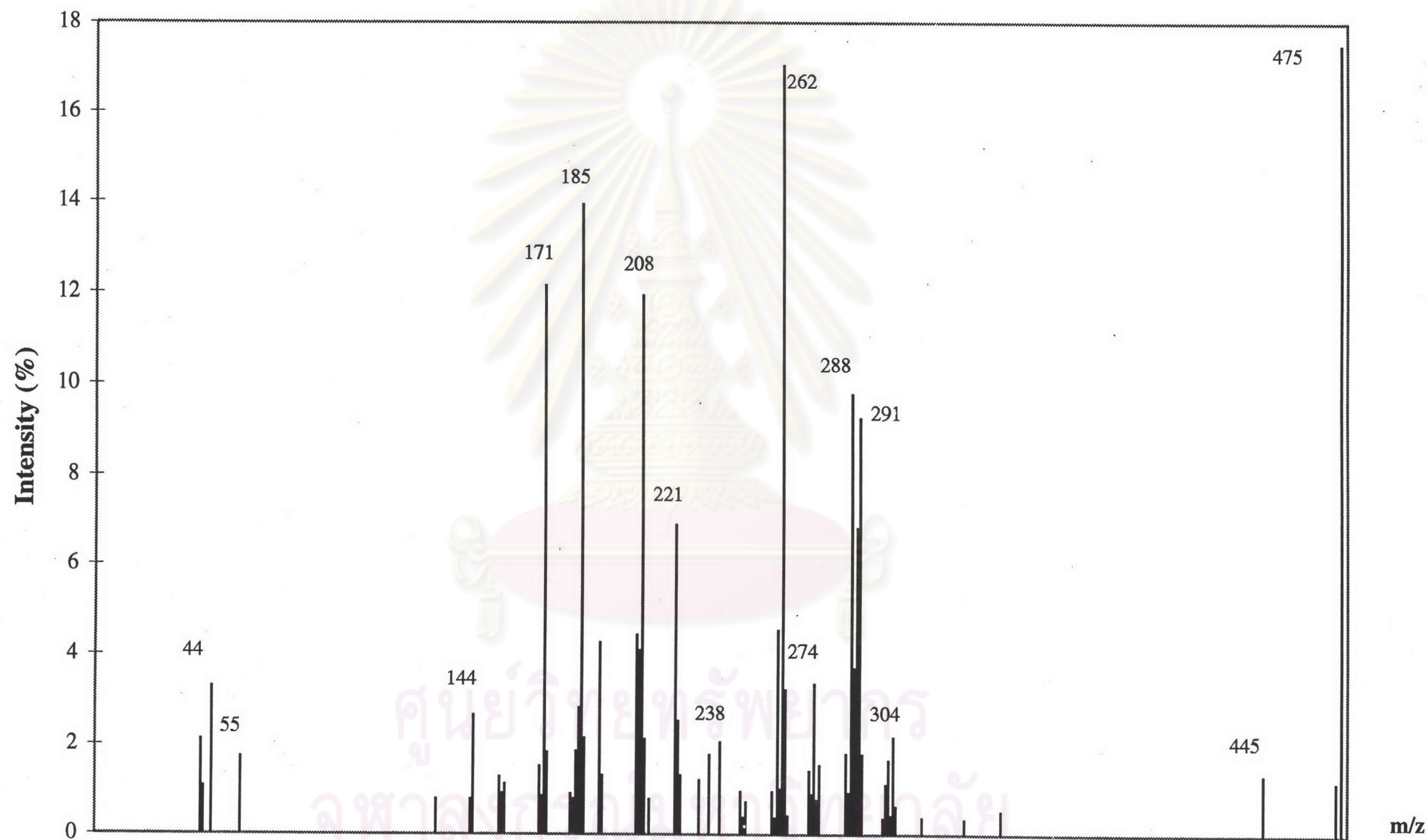

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Figure 12 Mass spectrum, EI 70 V, of alangimarckine

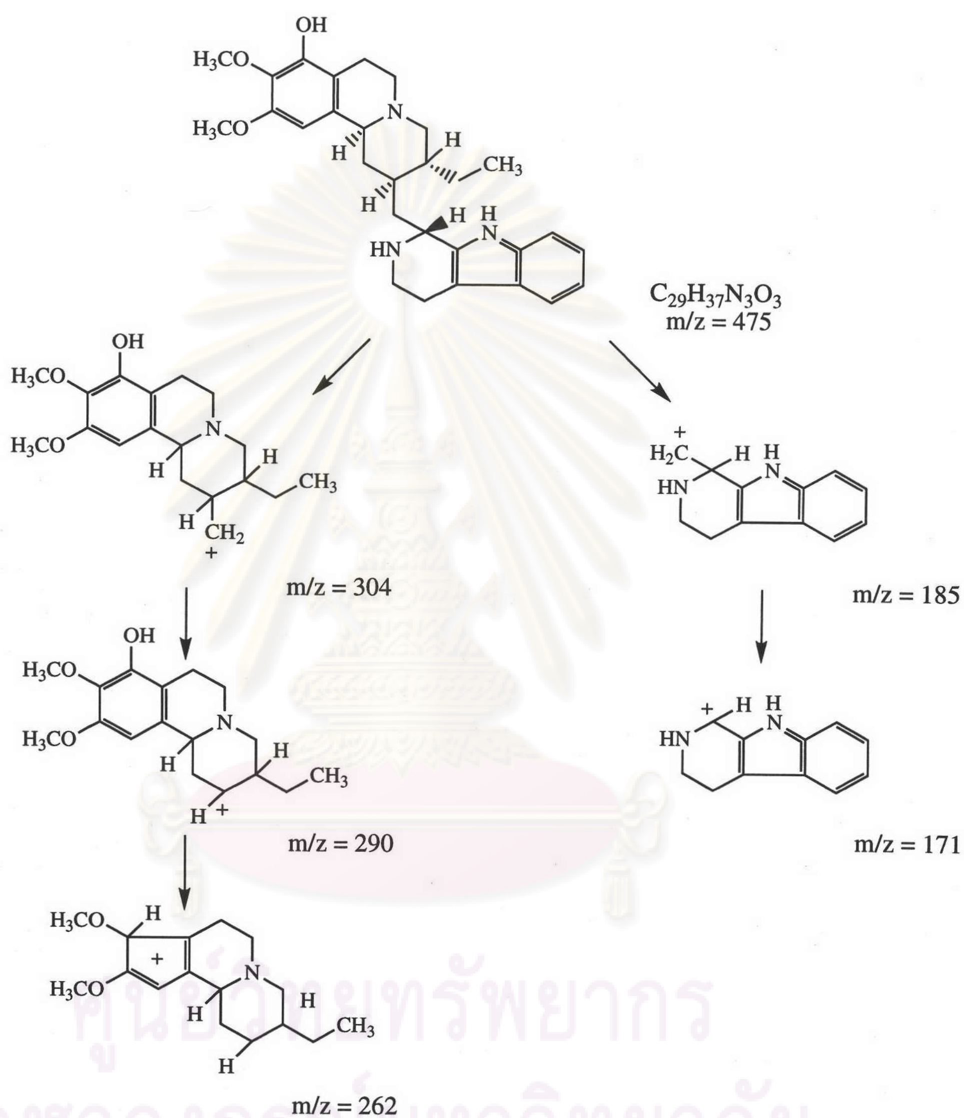

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Figure 13 Mass fragmentation pattern of alangimarckine

3. Enzyme Detection and Product Identification

The incubation of dopamine and secologanin with crude enzyme extracts of *A. salviifolium* at pH 7.5 resulted in a rapid appearance of the new product. The product was distinguished from secologanin by using wavelength 290 nm instead of 240 nm (Figures 14 and 15). Absolutely no transformation of the substrate occurred in the control (boiled). Time-course study of the enzyme activity resulted in the increase of the reaction product and simultaneously with the decrease of its substrate, dopamine (Figure 16). The highest accumulation appeared within 2 hr.

The UV-absorption spectra of the reaction product produced by TLC-densitometer clearly showed the combination of λ_{max} of both dopamine and secologanin (at 240 nm and 290 nm) and a detectable absorption similar to the spectrum of chemically synthesized deacetyl(iso)ipecoside (Figure 17).

However, the products obtained by large-scale (100-fold) incubation were identified as demethylalangiside and demethyisoalangiside by HPLC with retention times of 7.21 min and 15.16 min respectively (Figures 18 and 19). This was also confirmed by LC-MS, it was found that the enzymatic products showed their CI mass spectra with M+1 at 492 for both peaks. These were corresponded to the molecular mass 491 of demethylalangiside and demethylisoalangiside (Figures 20 and 21). These results, therefore, confirmed that the immediate products of dopamine and secologanin condensation were deacetylipecoside and deacetylisoipecoside which, by the process of purification, rapidly converted to demethylalangiside and demethylisoalangiside, respectively (Figure 22).

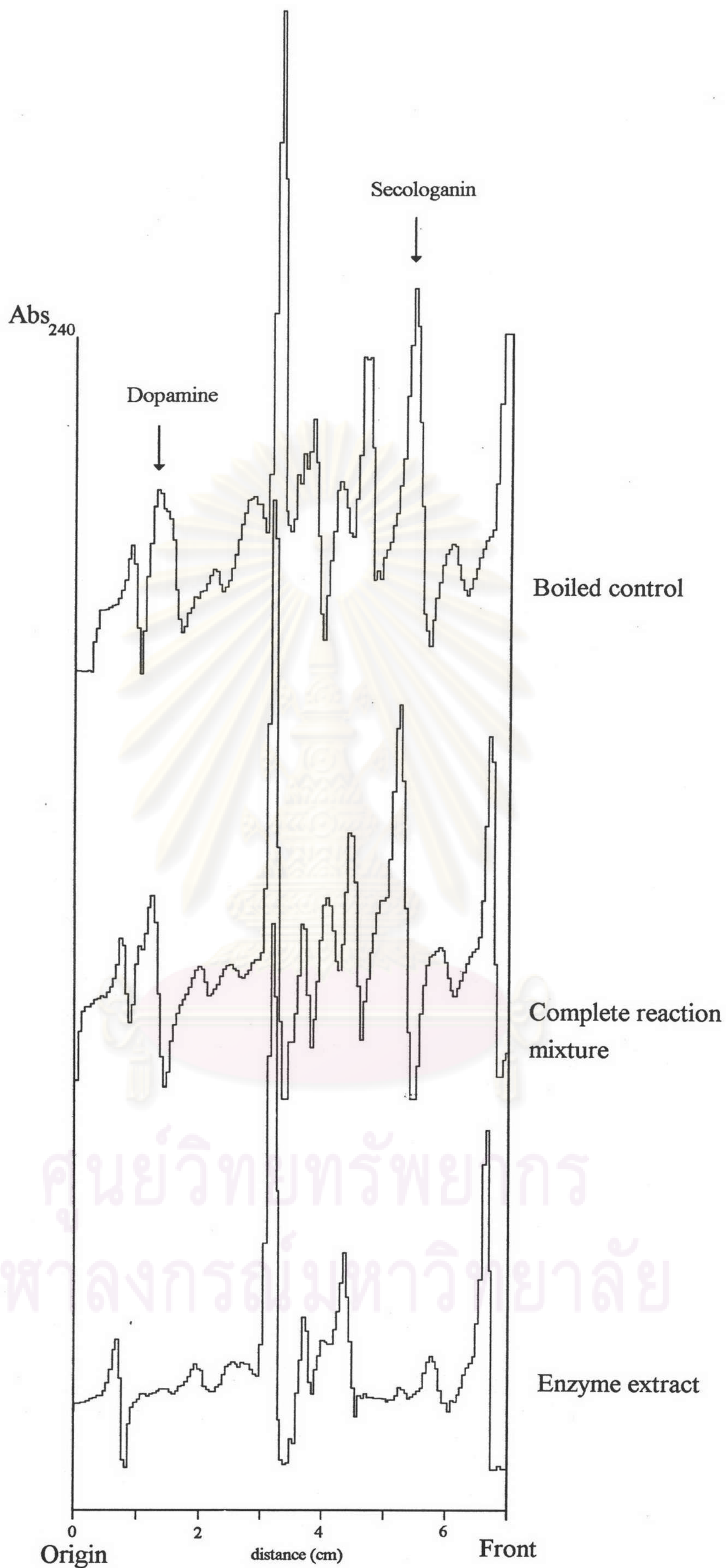

Figure 14 TLC-densitometric chromatograms (240 nm) of the control (boiled), complete reaction mixture and enzyme extract

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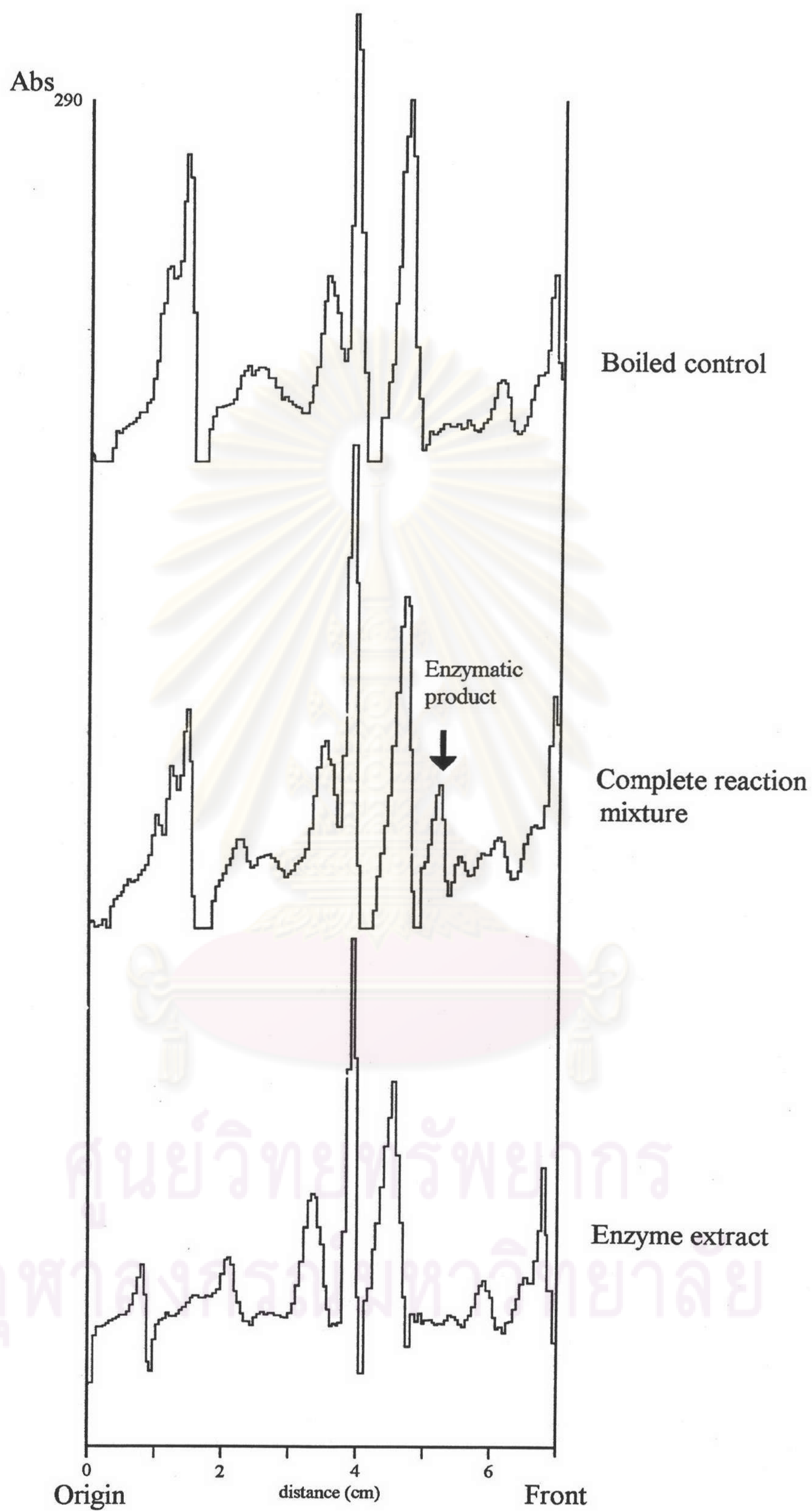

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Figure 15 TLC-densitometric chromatograms (290 nm) of the control (boiled), complete reaction mixture and enzyme extract

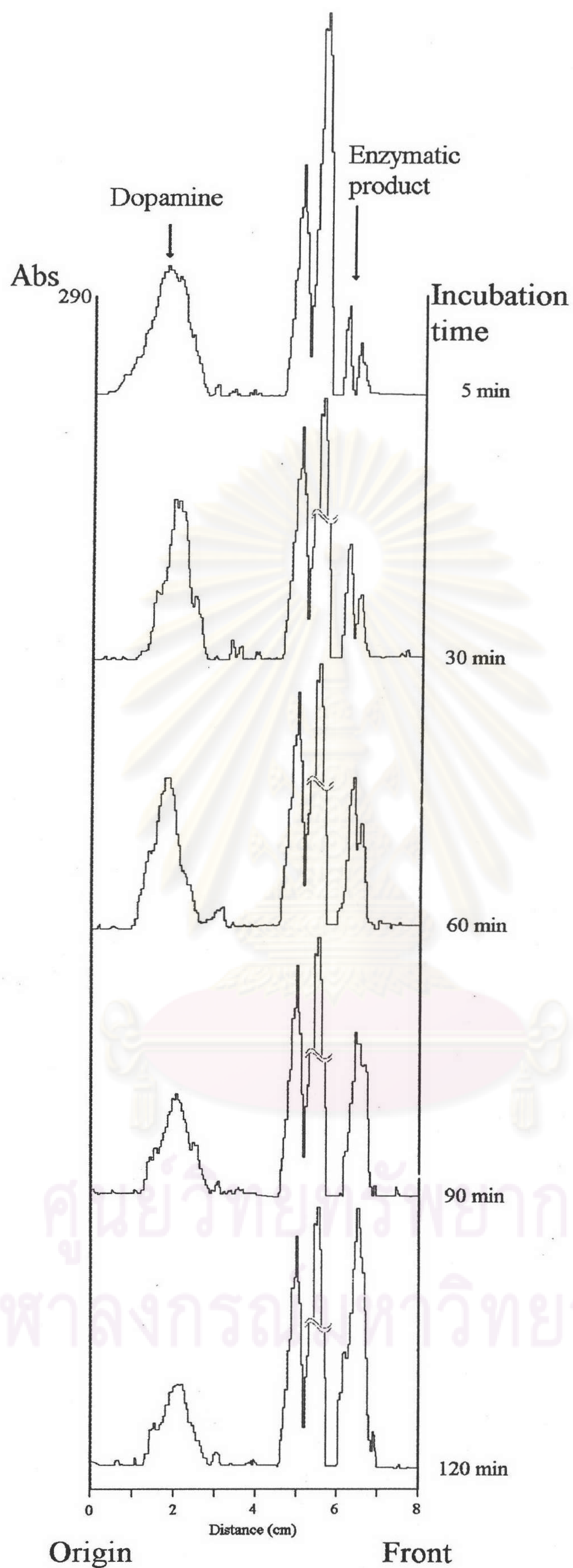

Figure 16 TLC-densitometric chromatograms of time-course studies in enzymatic activities at 5, 30, 60, 90 and 120 min

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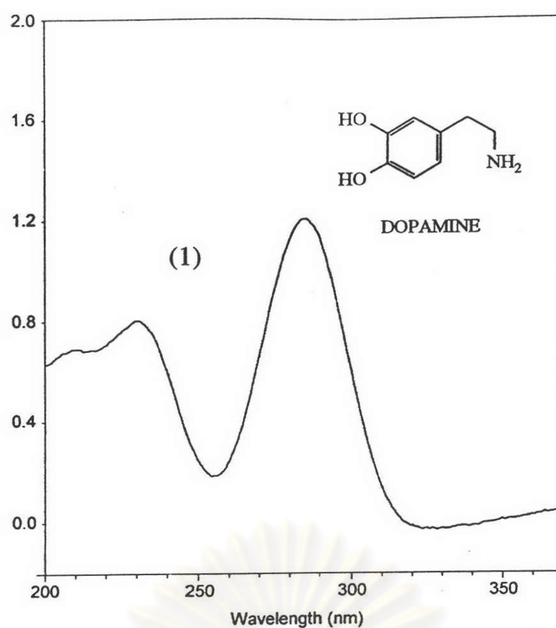

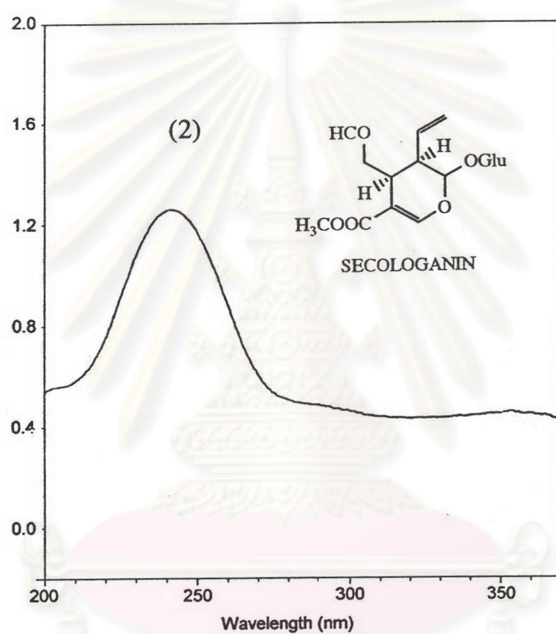

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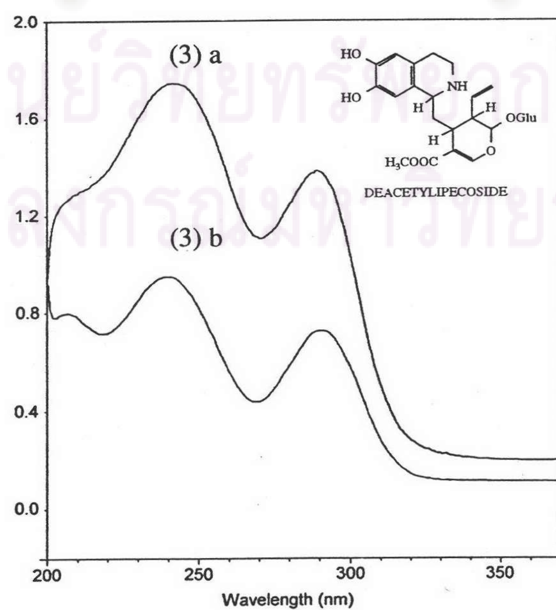
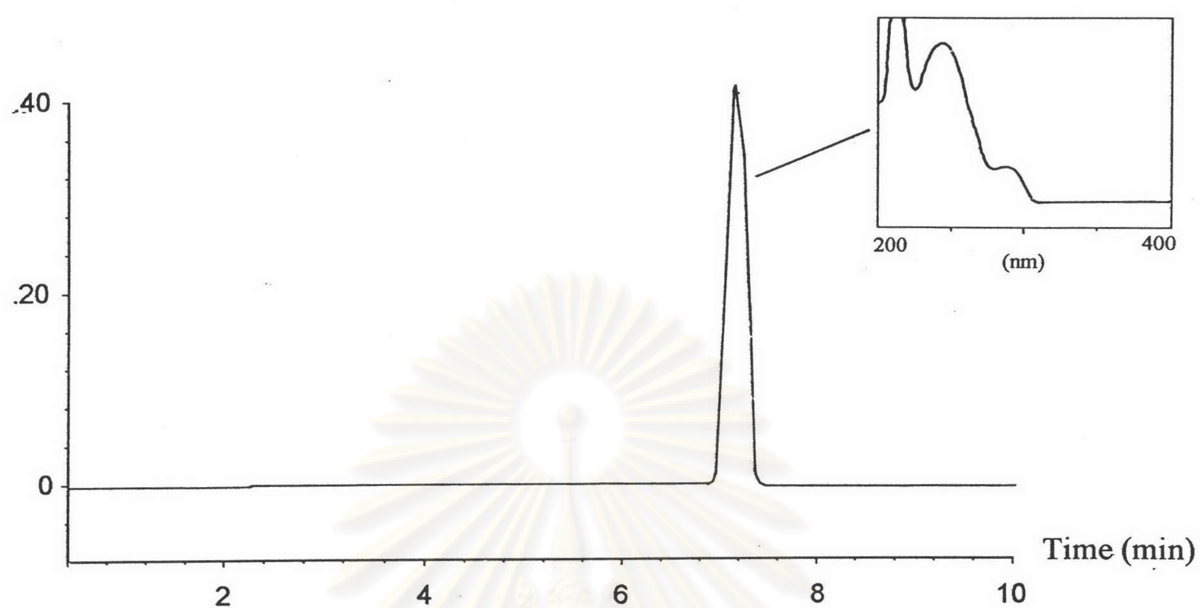
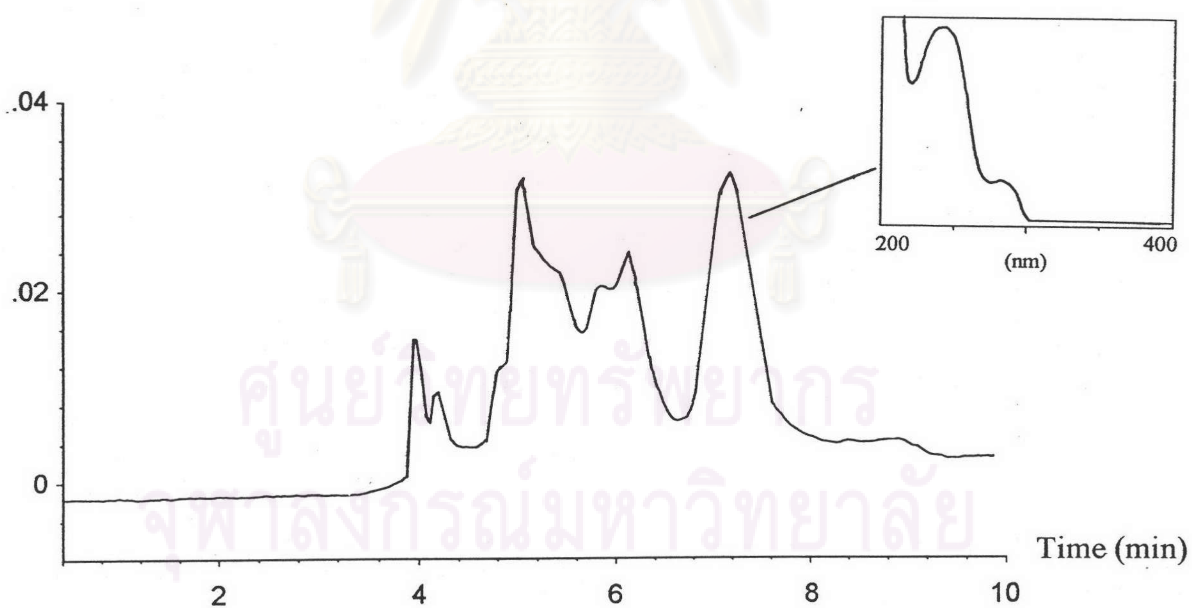


Figure 17 UV spectra of (1) dopamine, (2) secologanin, (3) a.deacetyl(iso)ipecoside, b.product from enzymatic reaction



(1)



(2)

Figure 18 HPLC chromatograms (photodiode array detector) of
(1) demethylalangiside (standard),
(2) enzymatic product

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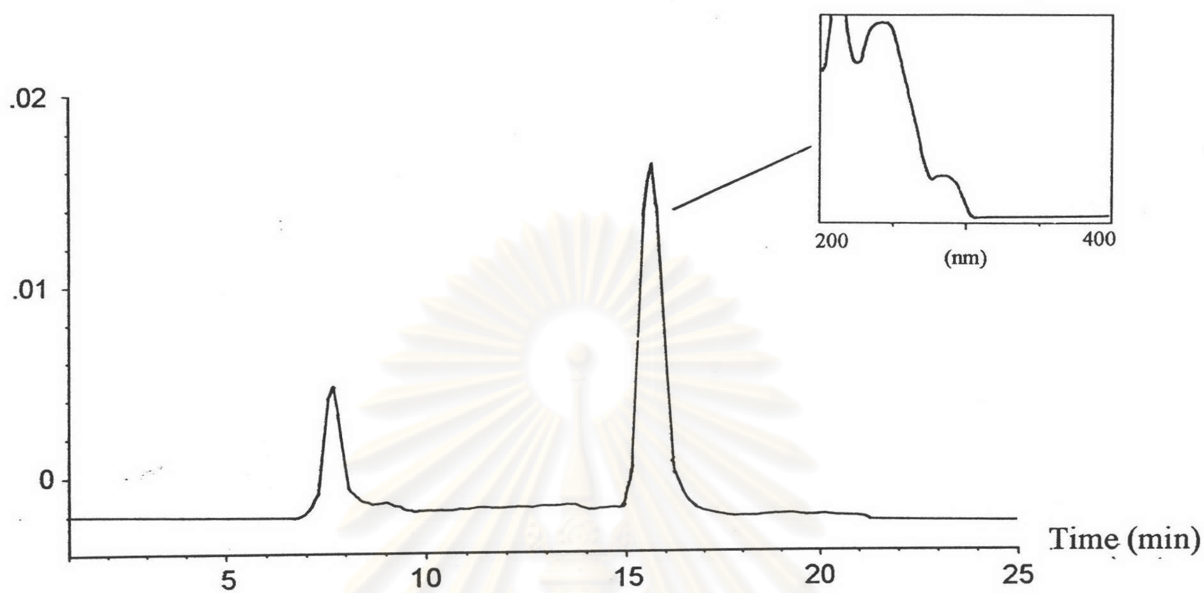

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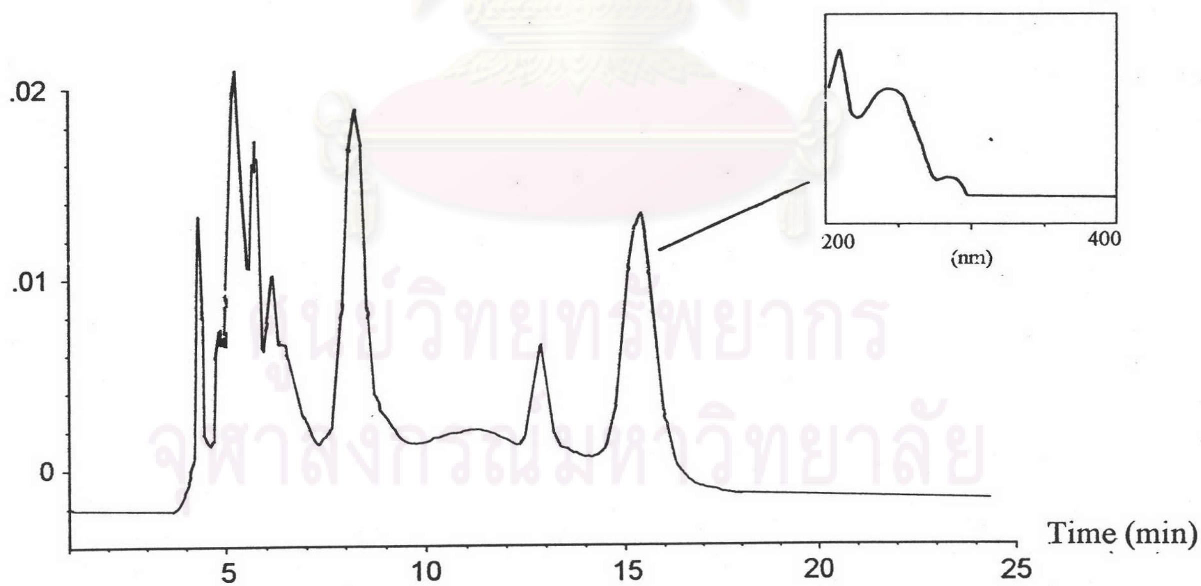

(2)

Figure 19 HPLC chromatograms (photodiode array detector) of
(1) demethylisoalangiside (standard),
(2) enzymatic product

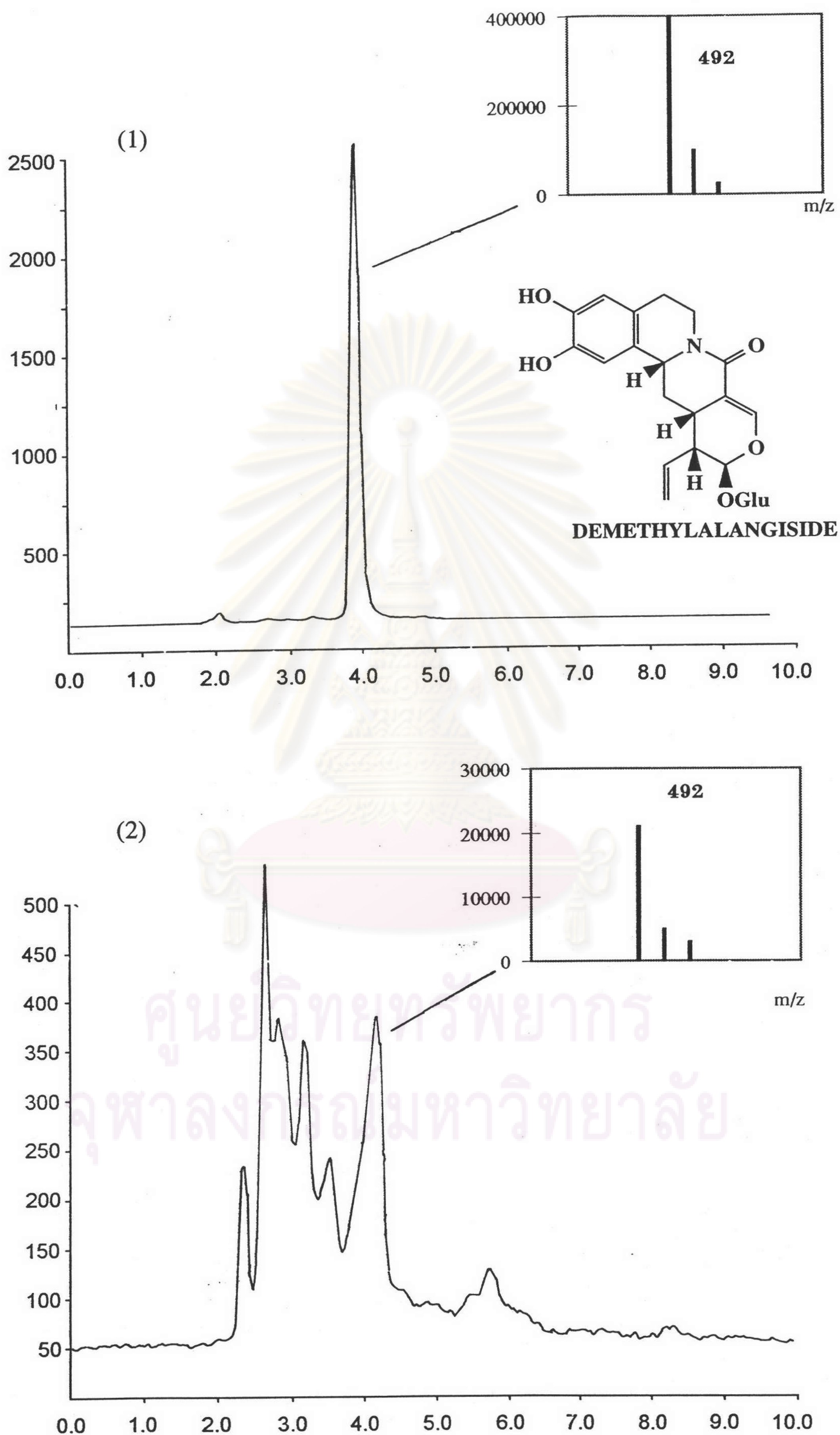

Figure 20 LC-MS chromatograms (UV detector) of
(1) demethylalangiside (standard),
(2) enzymatic product

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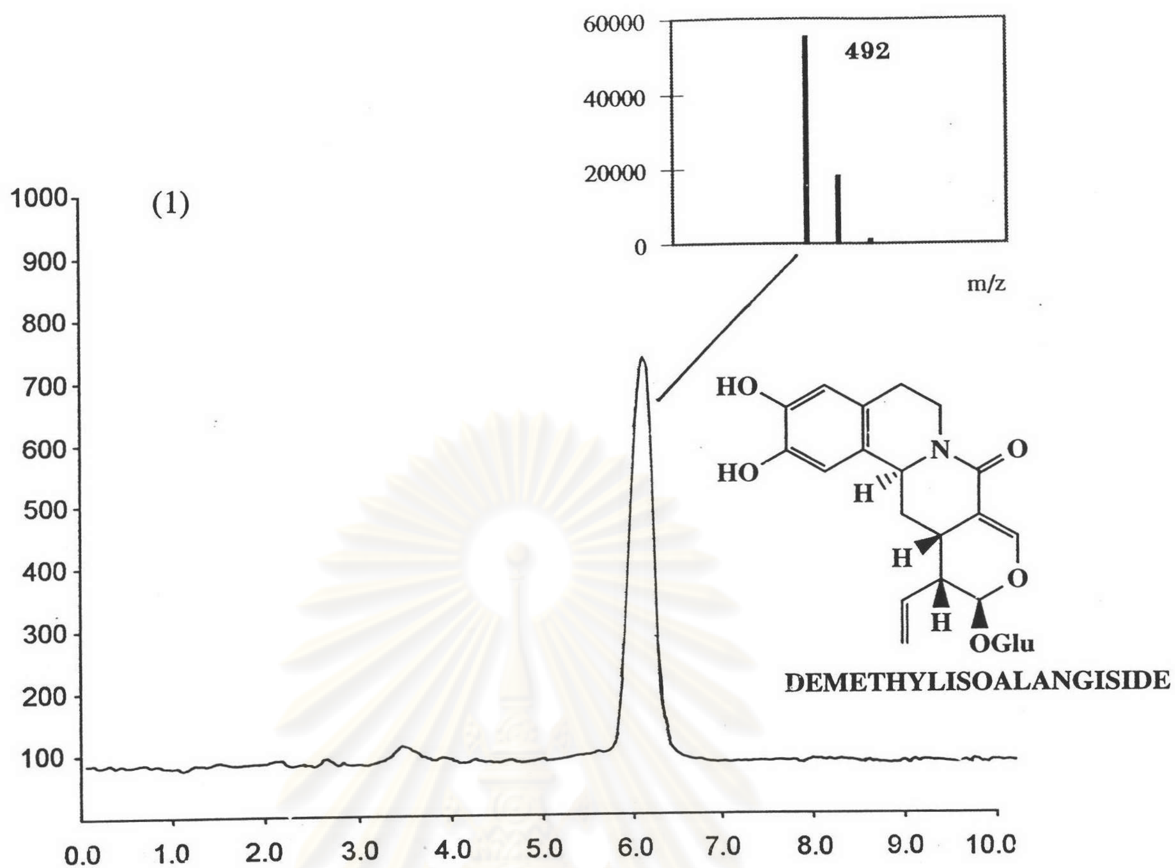

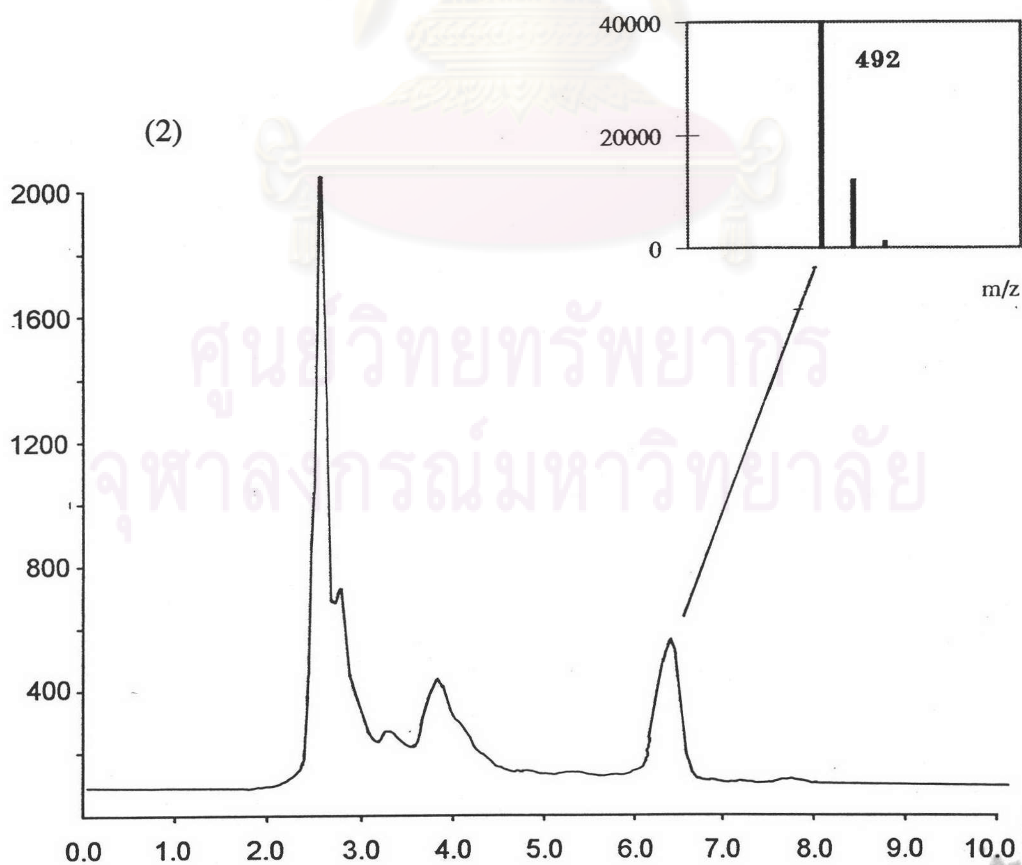

Figure 21 LC-MS chromatograms (UV detector) of
(1) demethylisoalangiside (standard),
(2) enzymatic product

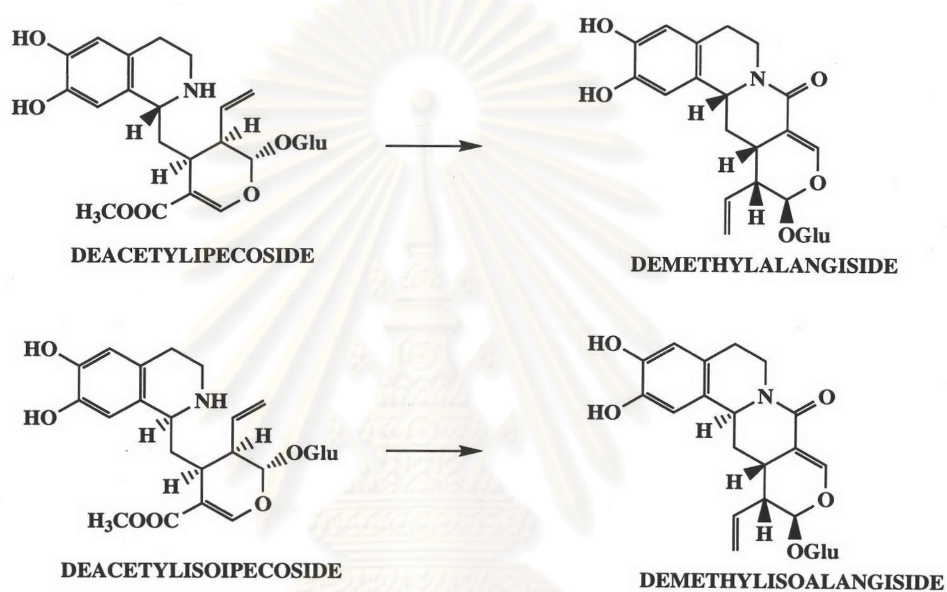

Figure 22 Deacetylipecoside and deacetylisoipecoside could be cyclised in a basic condition. This could take place also in a neutral condition while purification (A. Itoh, T.Tanahashi and N.Nagakura, J. Nat. Prod. in press)

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