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APPENDICES

APPENDIX I

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC

Chromatography was the most useful technique for the separation of phytochemicals. The chromatographic analysis were distributed between two phases, one of which was a stationary one while the other moved. The separation occurred because, under an optimum set of condition, each component in the mixture would interacted with the two phases differently relative to the other components in the mixture. The methods including thin layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). TLC is basically and widely used chromatographic technique available for the analysis of plant constituents, especially for preparation purposes. It is very simple, no special apparatus needed. GC and HPLC are more convenience, efficiency and resolution technique than TLC. The mobile phase of TLC and HPLC are liquid while the mobile phase of GC is gaseous. TLC and HPLC are thus the main analytical techniques for analysis of non-volatile compounds whereas GC is a technique of choice for volatile compounds. GC was limited to secondary metabolites which had boiling point below 450 ^{o}C and which were stable at the temperature of the separation. Therefore, HPLC had the advantage over GC in that it was not necessary to prepare volatile derivatives.

Chromatography is described and measured in terms of four major concepts: capacity, efficiency, selectivity and resolution. The capacity and selectivity of the column are mostly variable controlled by the column manufacturer, whereas efficiency and resolution could be controlled, to some extent, by the chromatographer. Important parameters in chromatographic are the retention time of a solute (t_R) and an unretained compound (t_0), the corrected time of a solute ($t_{R'}$), the volume of solute (V_R), the volume of solvent or void volume (V_0), the peak width at the baseline (w) and halfway of the peak ($w_{0.5}$).

The capacity factor (k'_R) of a column is a direct measure of the strength of the interact of the sample with the packing material. It was mostly function of packing material but could be manipulated to a degree by varying the solvent strength.

$$k'_R = \frac{(t_R - t_0)}{t_0} = \frac{(V_R - V_0)}{V_0}$$

The selectivity of the chromatographic system is a measure of the difference in retention times (or volumes) between two given peaks and described how effectively a chromatographic system could separate two compounds.(Figure 2) Selectivity is controlled using mobile phase and temperature and defined in term of a, where

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{V_2 - V_0}{V_1 - V_0} = \frac{k'_2}{k'_1}$$

The resolution is a term used to describe the degree of separation between neighboring solute bands or peaks. It was affected by the selectivity (a), efficiency (N) and capacity (k') of column.

$$R_S = \frac{1}{4} \frac{\alpha - 1}{\alpha} (N^{1/2}) \frac{k'}{1 + k'}$$

The efficiency of column is a number that described peak broadening as a function of retention and it is defined in term of number of theoretical plates, N and N is defined in term of the retention time (t_r) of the solute and halfway of the peak. ($w_{0.5}$) The greater the number of theoretical plates, the more efficient the column is considered to be. The movement of a solute along the column is viewed as a stepwise transfer from on theoretical plates, the greater number of theoretical plate to the next. The thinner the theoretical plates, the greater the number that could be envisaged within a given length of column.

$$H = L / N$$

$$N = 5.54 (t_r / w_{0.5})^2$$

where L is the length of the column (millimeters). Thus, the smaller the height equivalent to a theoretical plate (HETP, or H), the greater is the efficiency of the column. In general, the H value is smaller for small stationary phase particle sizes, low mobile phase flow rates, less viscous mobile phases, higher separation temperature and smaller solute molecular sizes.

The most effective way to alter resolution is to change the selectivity or the capacity of the column. The effect of increasing the efficiency of the column by increasing the column length or flow-rate velocity is less significant, as resolution increase proportionally as the square root of the number theoretical plates. If increased resolution is required, a column with a higher capacity factor is the best choice. However, increasing the capacity factor would increase the analysis time, so a compromise must be reached between resolution and analysis time.

APPENDIX II

ISOFLAVONE HPLC FINGERPRINTS OF REFERENCE STANDARD AND *P. mirifica*

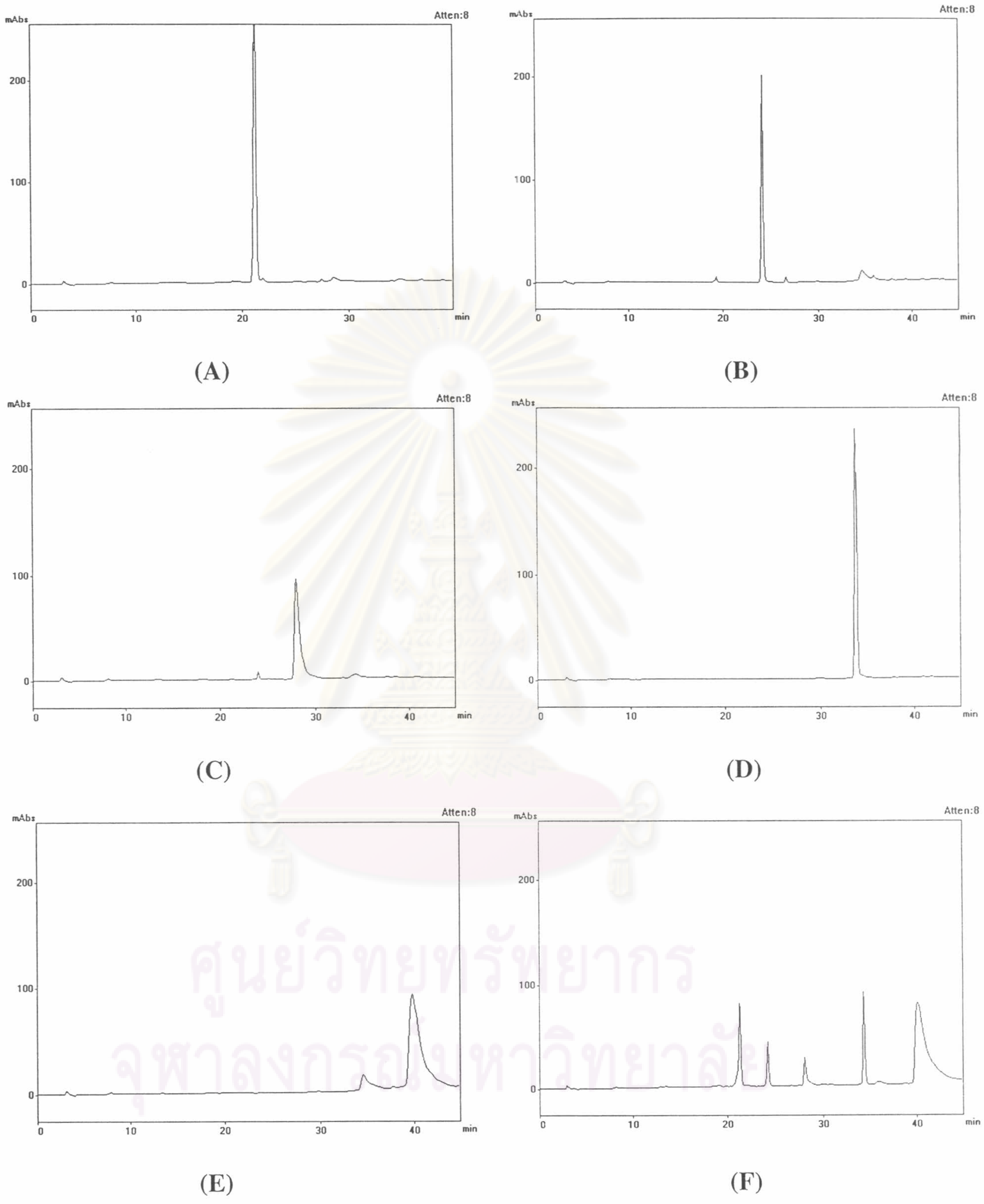
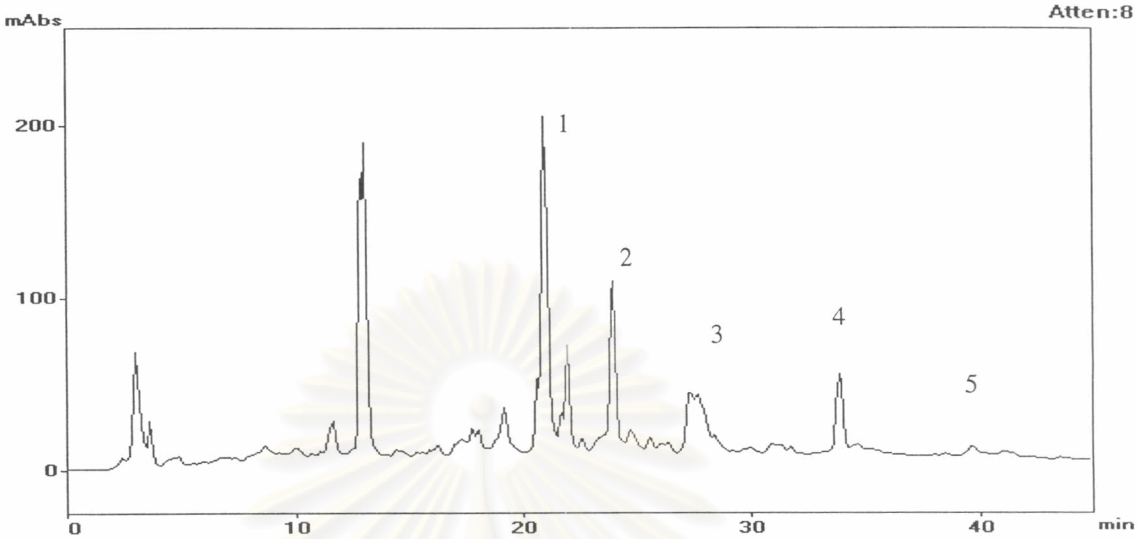
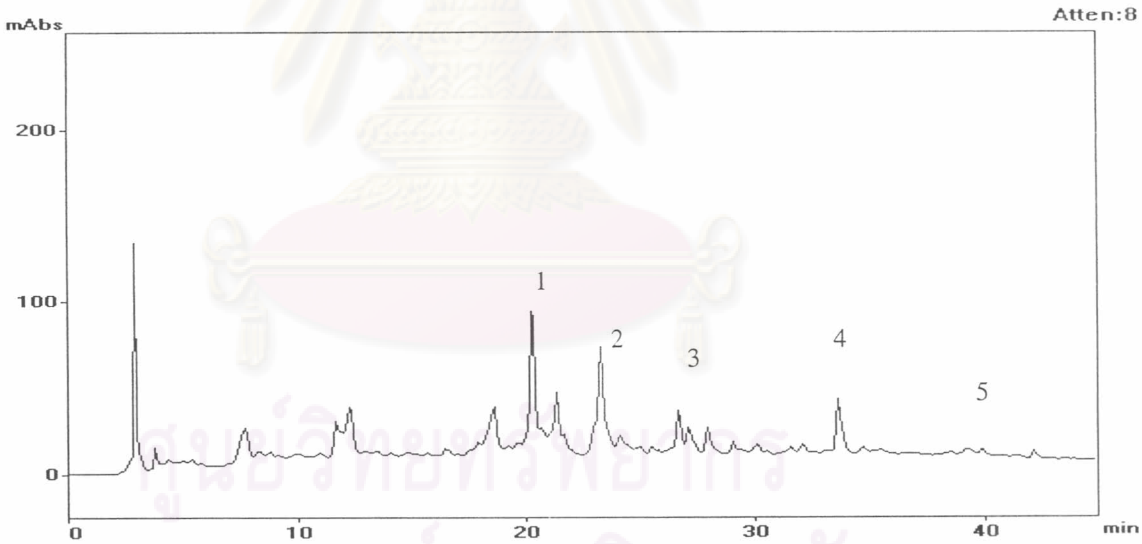


Figure 43 Isoflavone HPLC fingerprint of reference standard (A) Puerarin (B) Daidzin (C) Genistin (D) Daidzein (E) Genistein (F) mixed standard
1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein



(A)



(B)

Figure 44 Isoflavone HPLC fingerprint of wild *P. mirifica* clone Chiang Dao at wavelength (A) 254 and (B) 280 nm

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

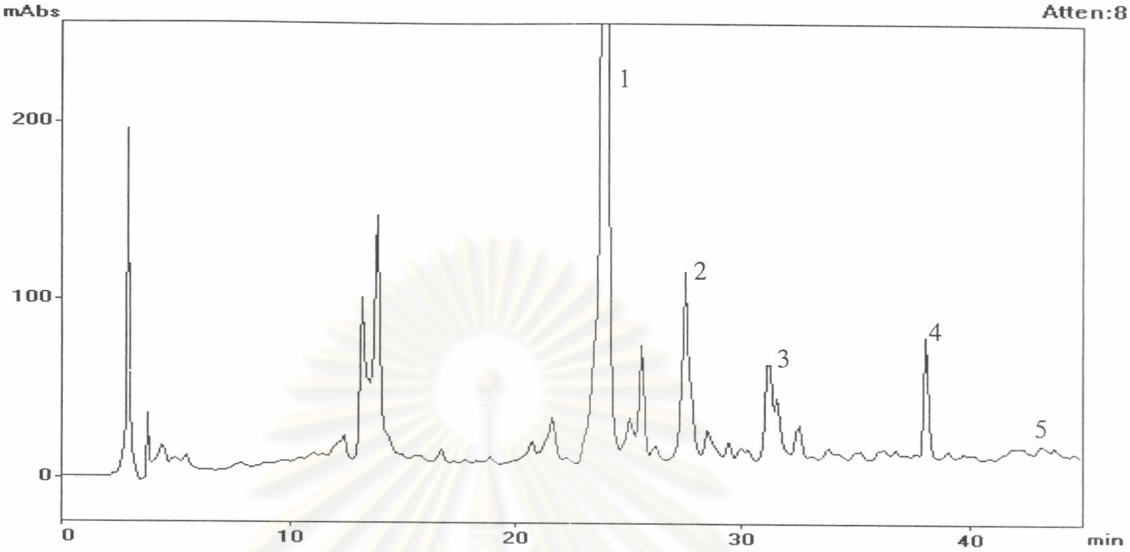


Figure 45 Isoflavone HPLC fingerprint of *P. mirifica* clone Chiang Dao spike with puerarin

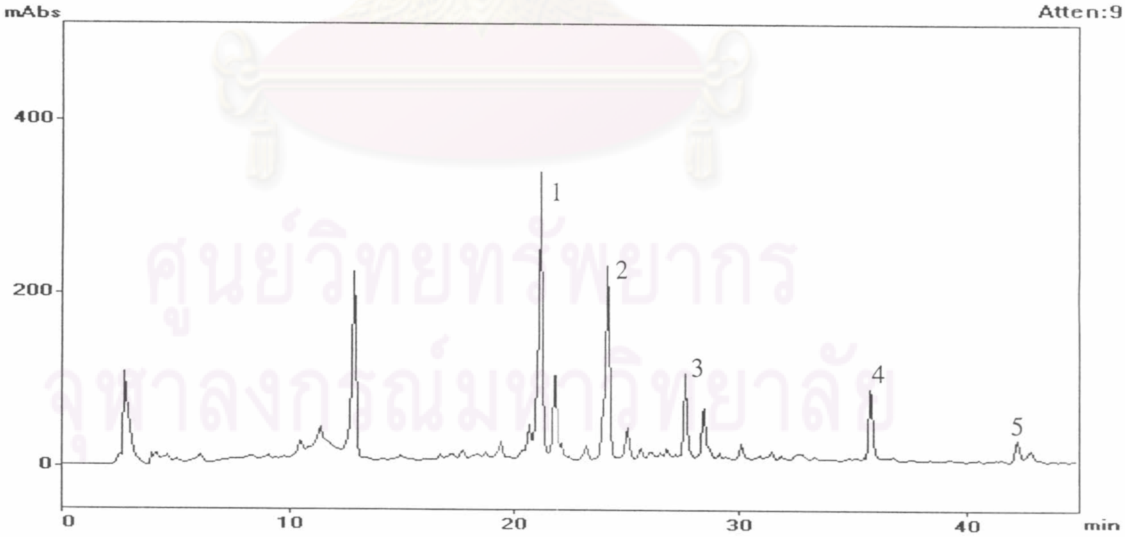


Figure 46 Isoflavone HPLC fingerprint of *P. mirifica* clone Chiang Dao spike with daidzin

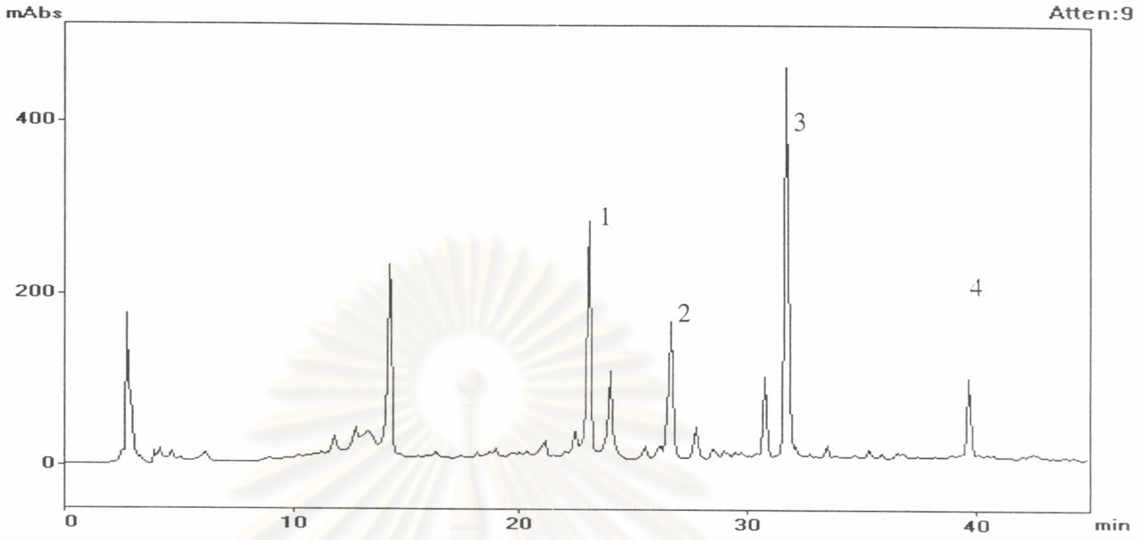


Figure 47 Isoflavone HPLC fingerprint of *P. mirifica* clone Chiang Dao spike with genistin

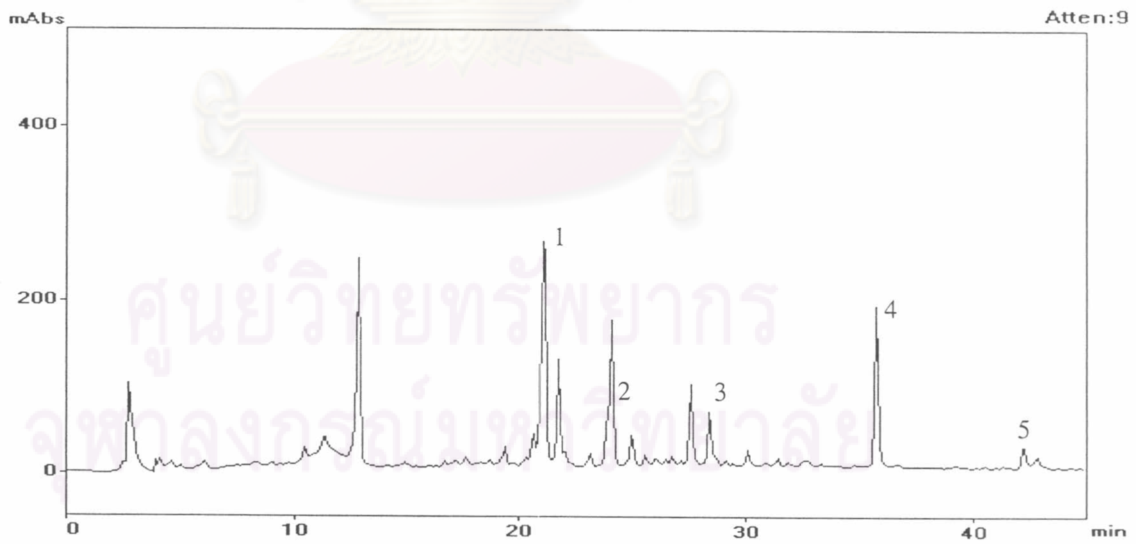


Figure 48 Isoflavone HPLC fingerprint of *P. mirifica* clone Chiang Dao spike with daidzein

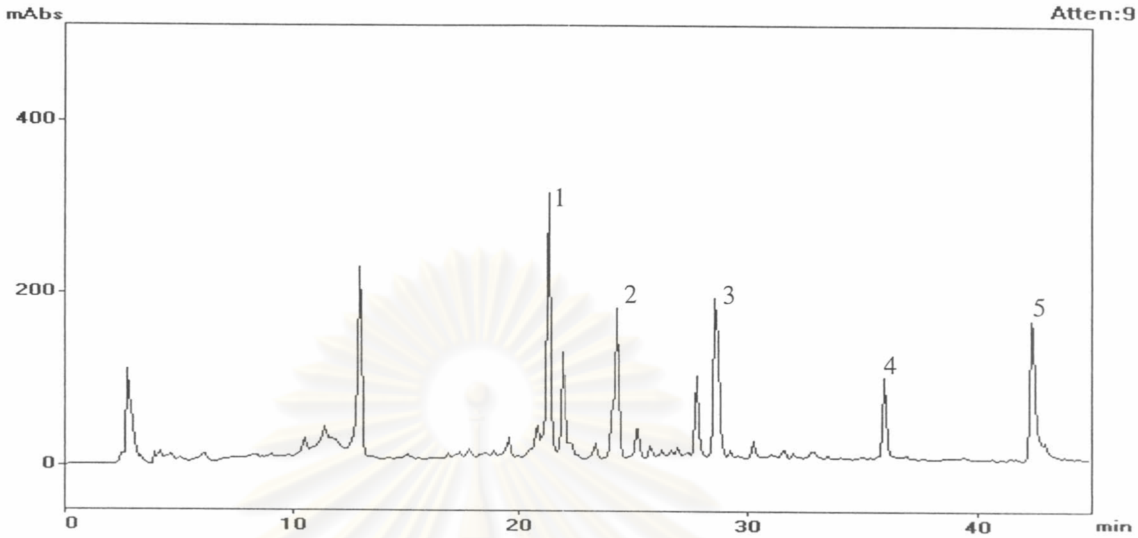


Figure 49 Isoflavone HPLC fingerprint of *P. mirifica* clone Chiang Dao spike with genistein

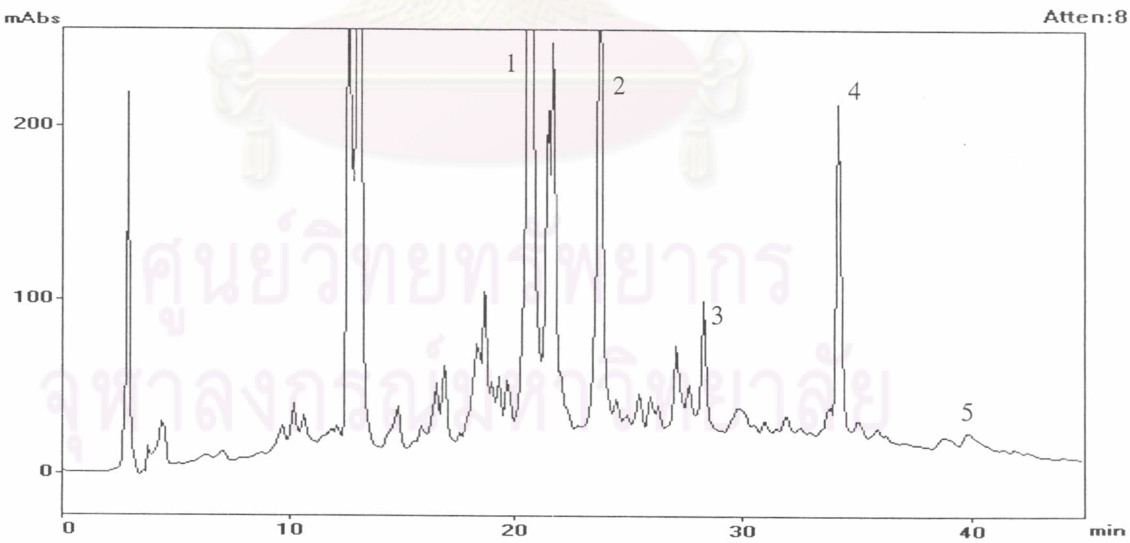


Figure 50 Isoflavone HPLC fingerprint of wild *P. mirifica* clone Chiang Dao, Spray Dry preparation

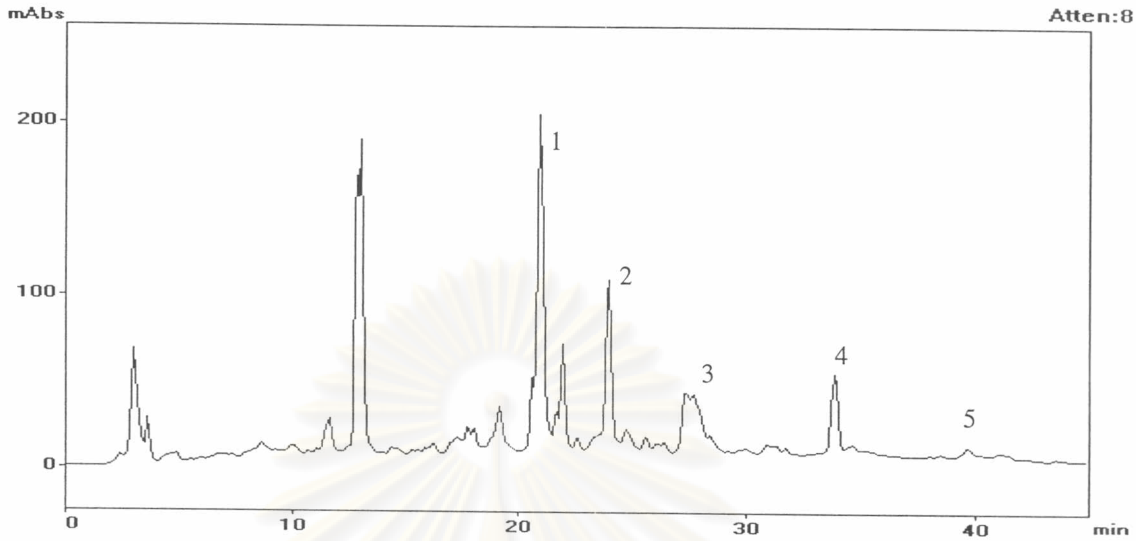


Figure 51 Isoflavone HPLC fingerprint of wild *P. mirifica* clone Chiang Dao extracted in methanol

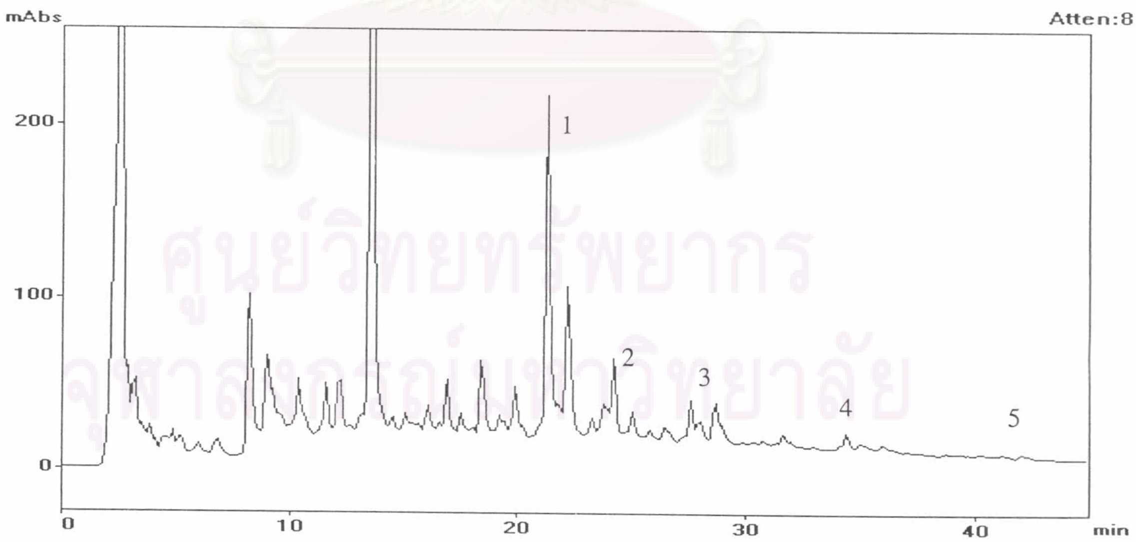


Figure 52 Isoflavone HPLC fingerprint of wild *P. mirifica* clone Chiang Dao extracted in water

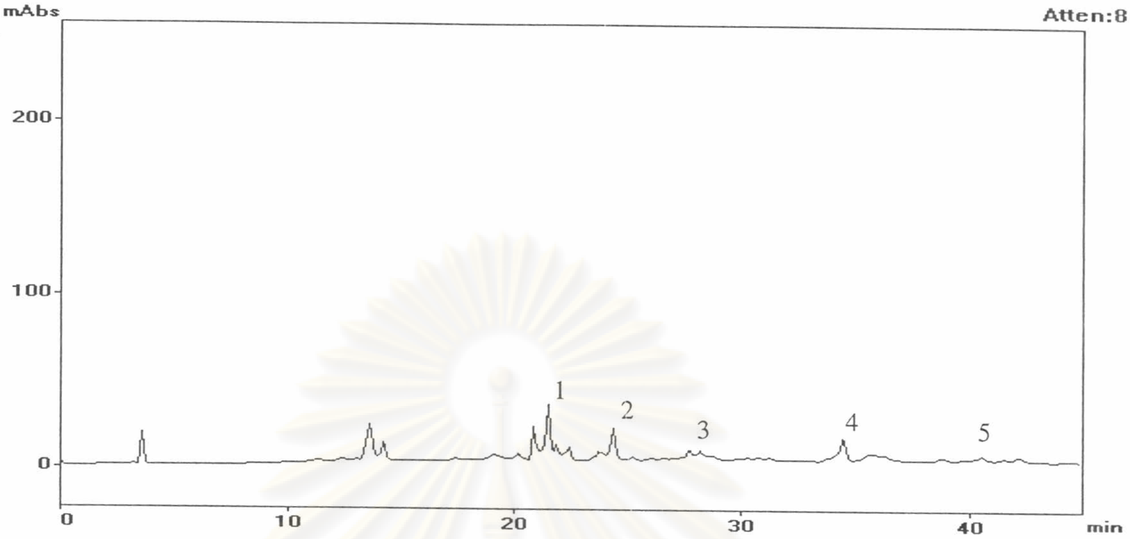


Figure 53 Isoflavone HPLC fingerprint of wild *P. mirifica* clone Chiang Dao extracted in ethanol

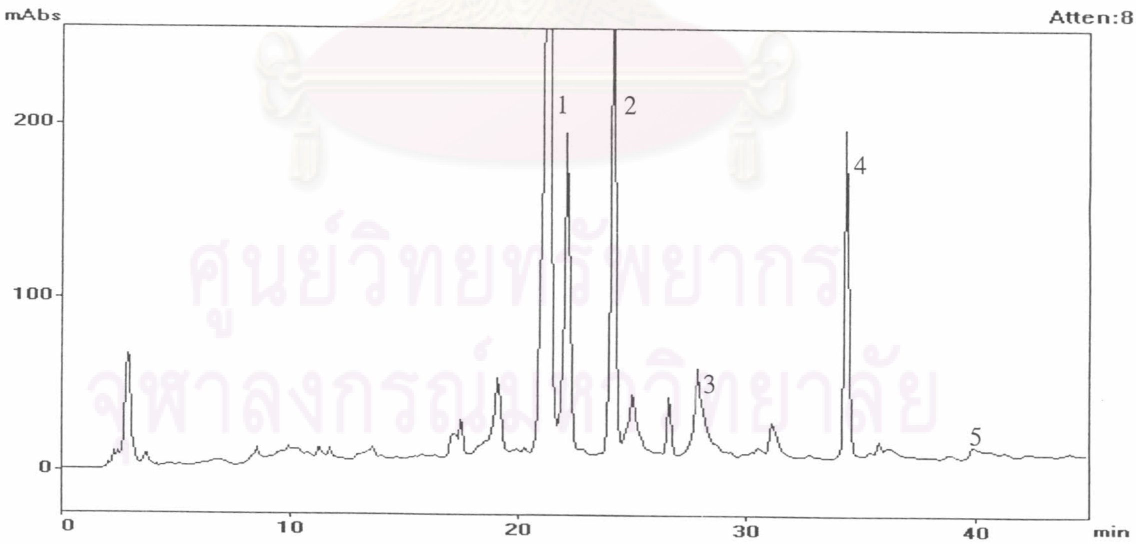


Figure 54 Isoflavone HPLC fingerprint of *P. lobata* collected from China

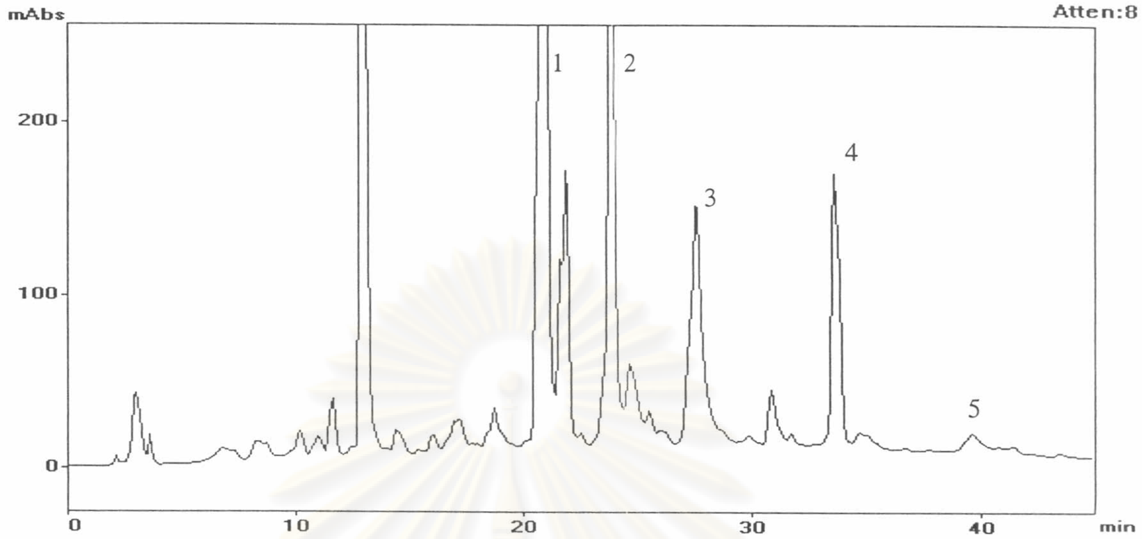


Figure 55 Isoflavone HPLC fingerprint of *P. mirifica* collected in Kanchanaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

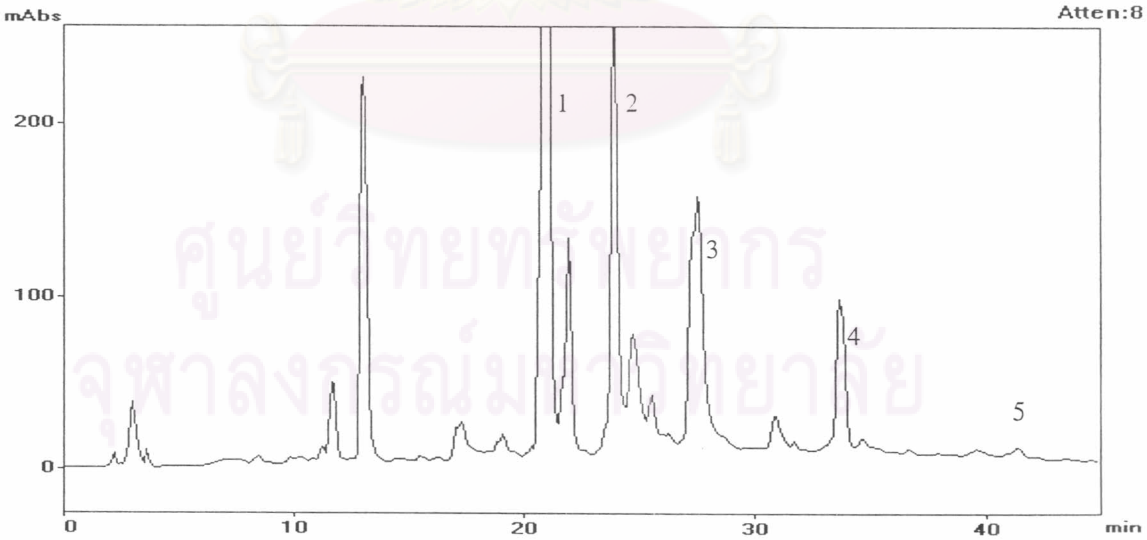


Figure 56 Isoflavone HPLC fingerprint of *P. mirifica* collected in Lamphun province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

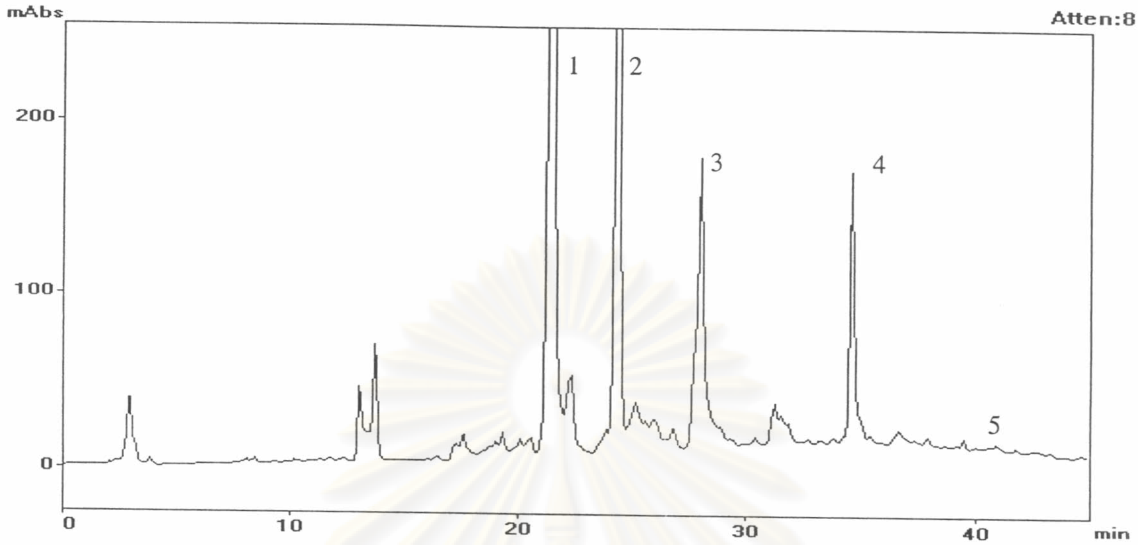


Figure 57 Isoflavone HPLC fingerprint of *P. mirifica* collected in Chiang Mai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

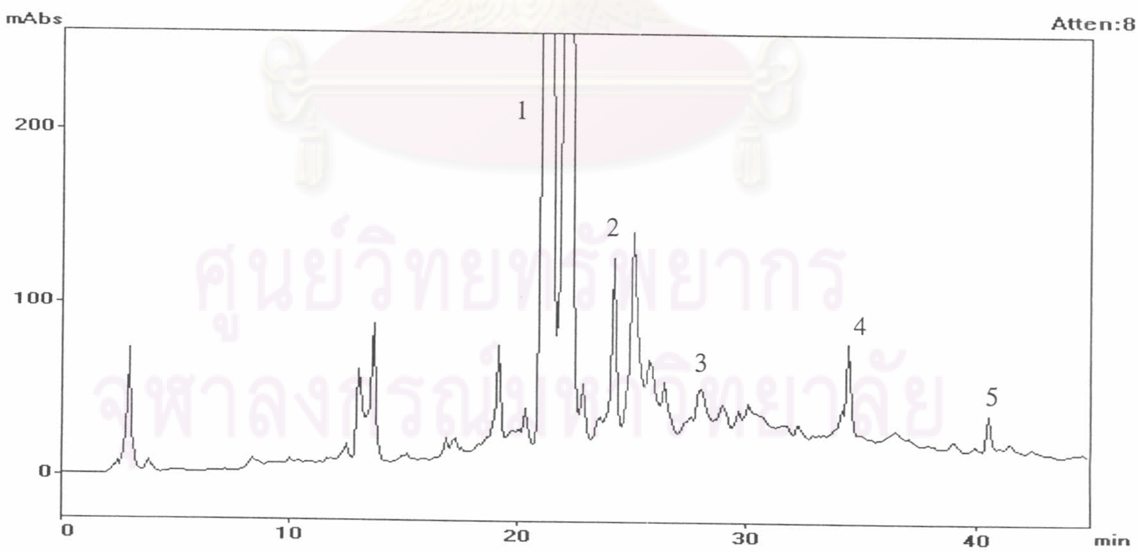


Figure 58 Isoflavone HPLC fingerprint of *P. mirifica* collected in Sakon Nakhon province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

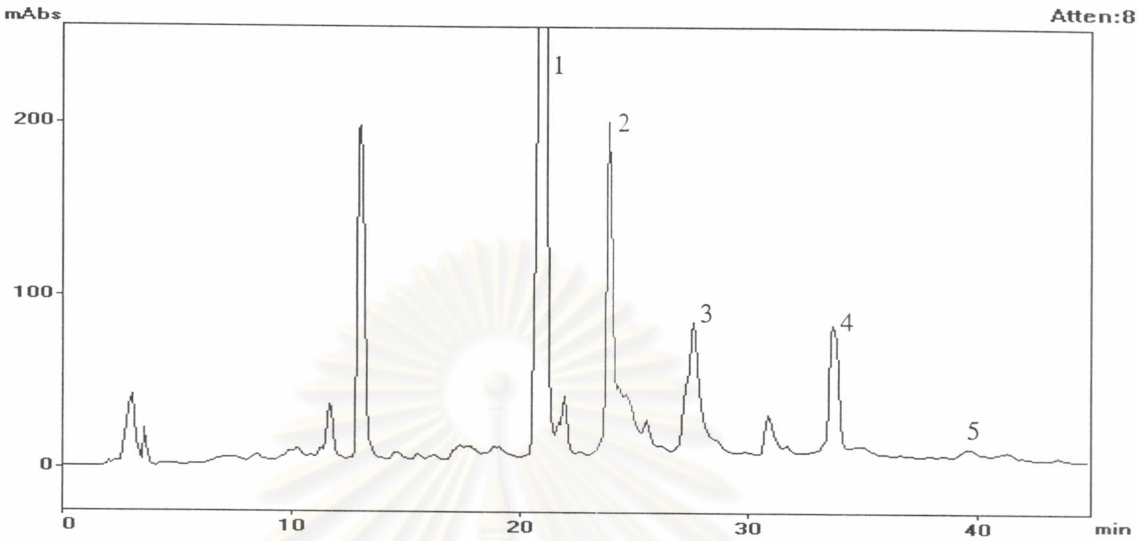


Figure 59 Isoflavone HPLC fingerprint of *P. mirifica* collected in Mae Hong Son province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

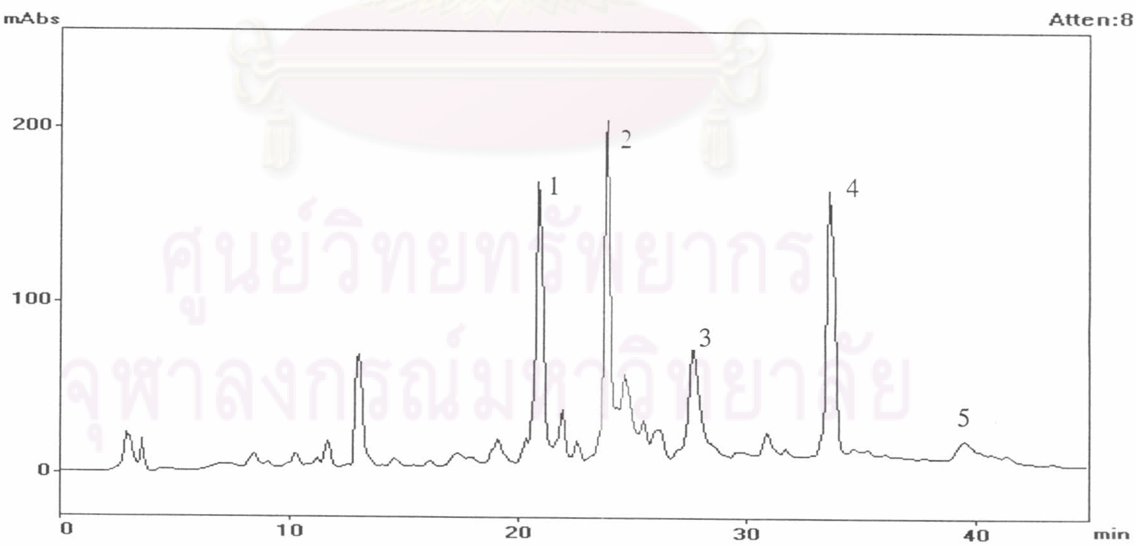


Figure 60 Isoflavone HPLC fingerprint of *P. mirifica* collected in Uthai Thani province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

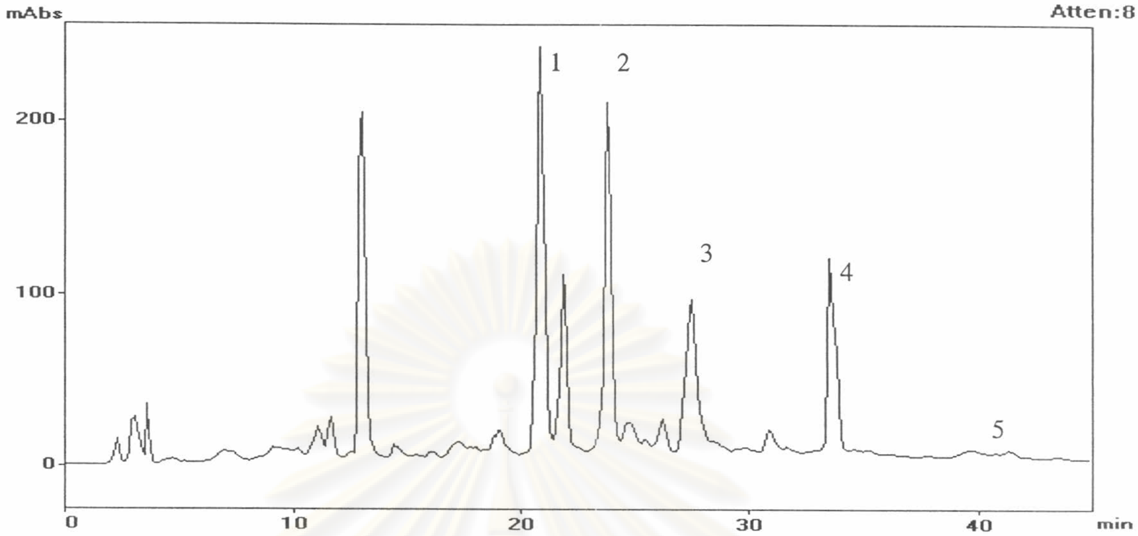


Figure 61 Isoflavone HPLC fingerprint of *P. mirifica* collected in Sukhothai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

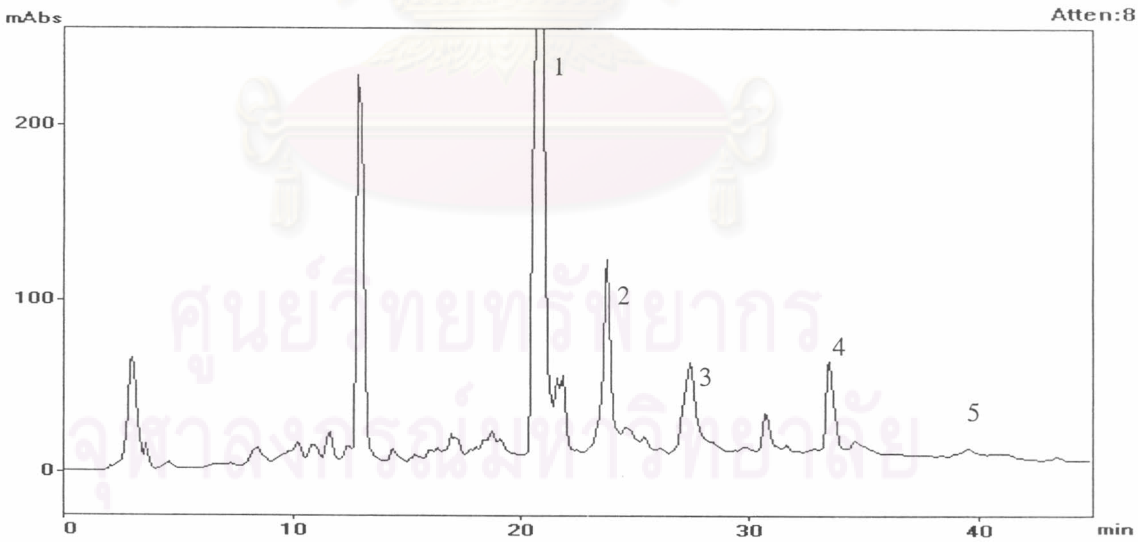


Figure 62 Isoflavone HPLC fingerprint of *P. mirifica* collected in Lampang province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

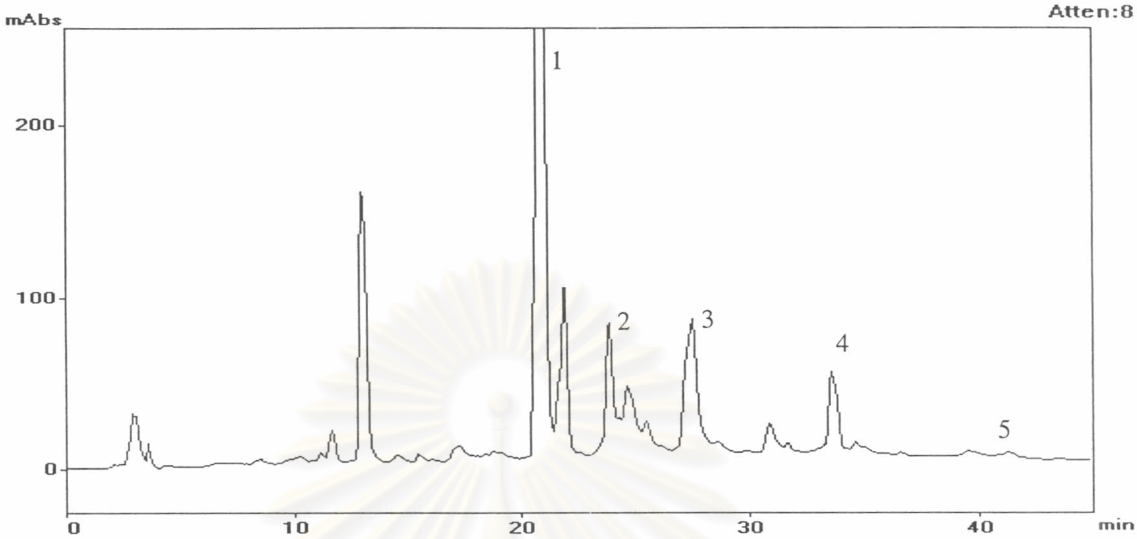


Figure 63 Isoflavone HPLC fingerprint of *P. mirifica* collected in Tak province
1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

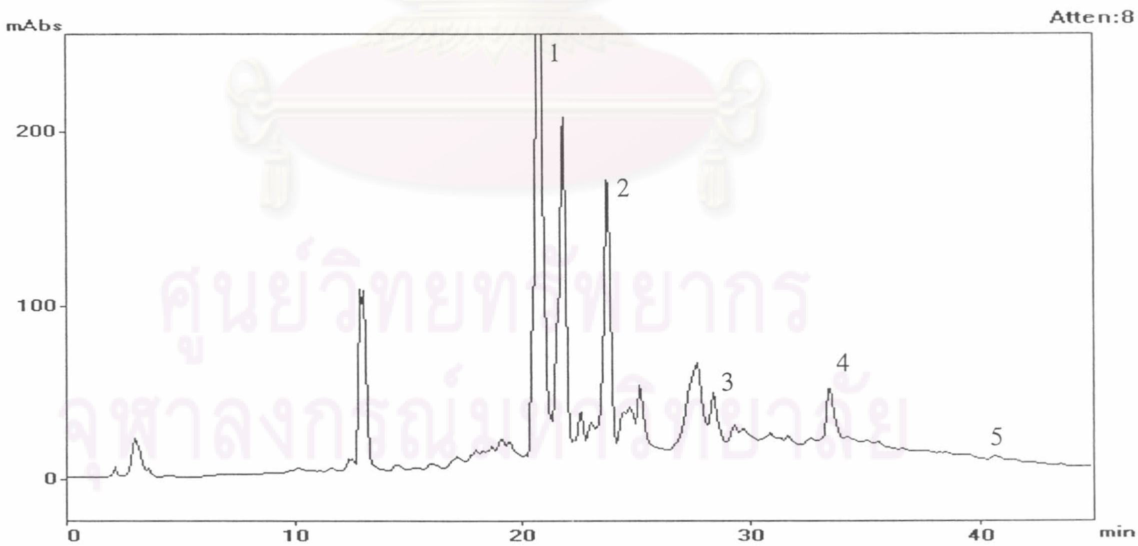


Figure 64 Isoflavone HPLC fingerprint of *P. mirifica* collected in Saraburi province
1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

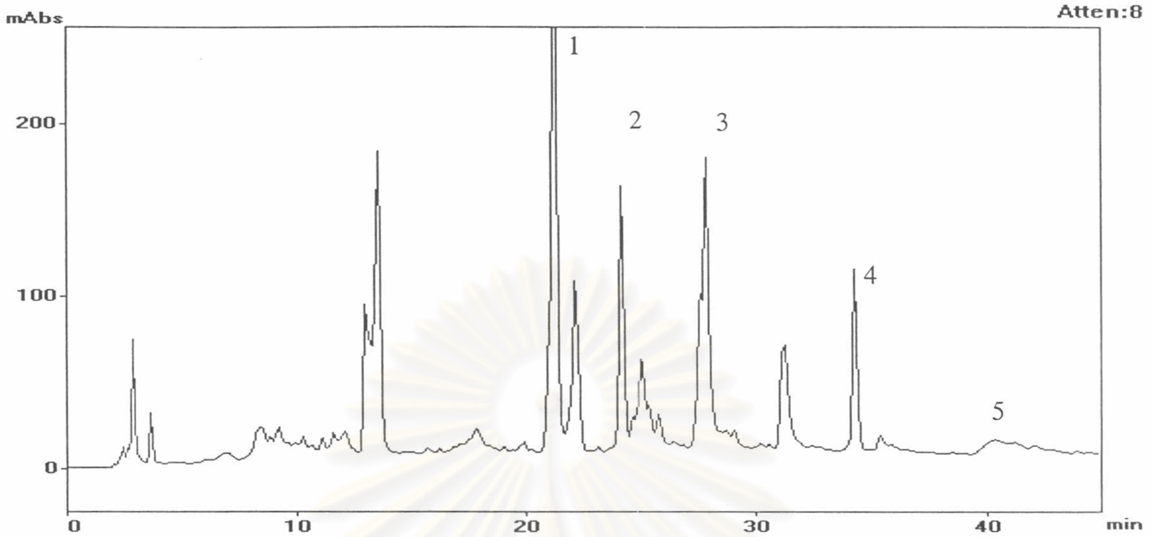


Figure 65 Isoflavone HPLC fingerprint of *P. mirifica* collected in Ratchaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

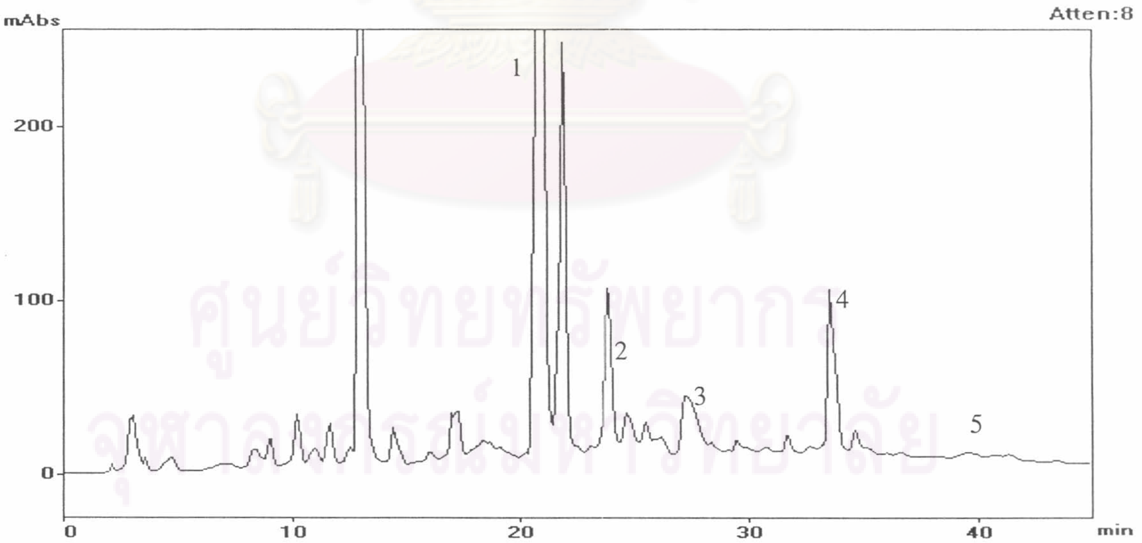


Figure 66 Isoflavone HPLC fingerprint of *P. mirifica* collected in Phitsanulok province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

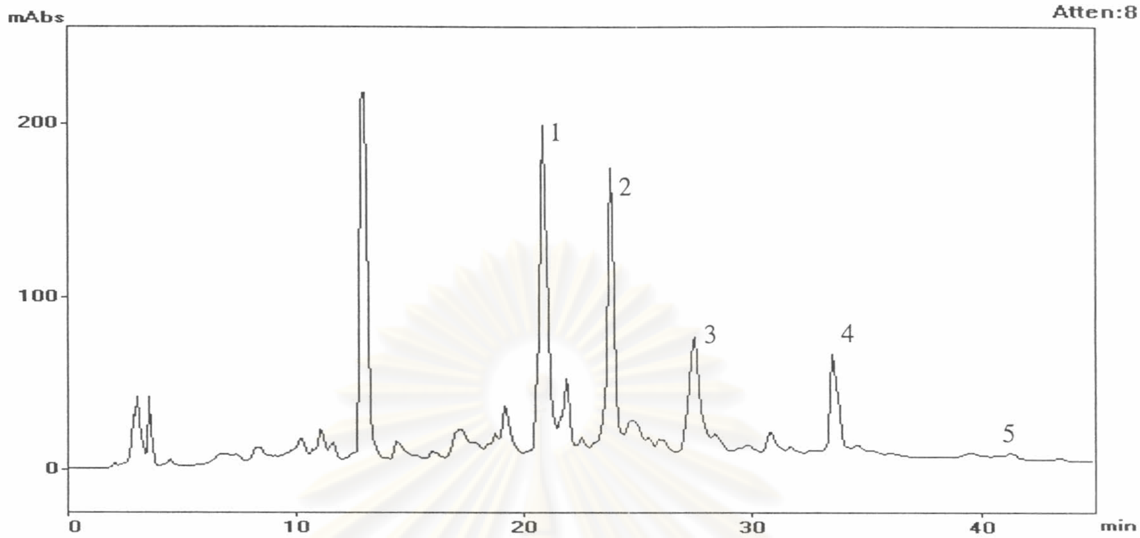


Figure 67 Isoflavone HPLC fingerprint of *P. mirifica* collected in Phetchaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

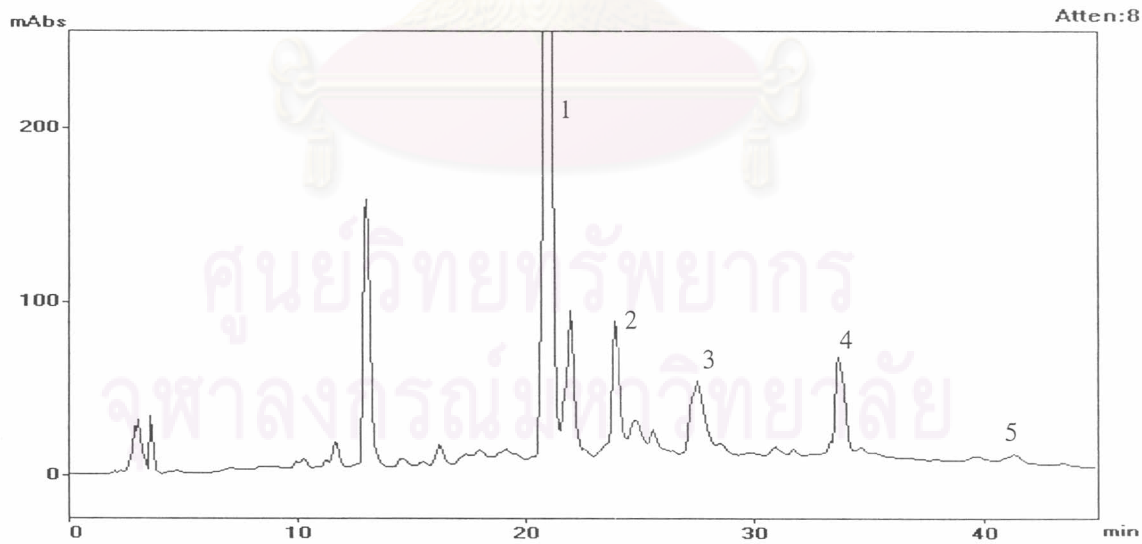


Figure 68 Isoflavone HPLC fingerprint of *P. mirifica* collected in Phrae province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

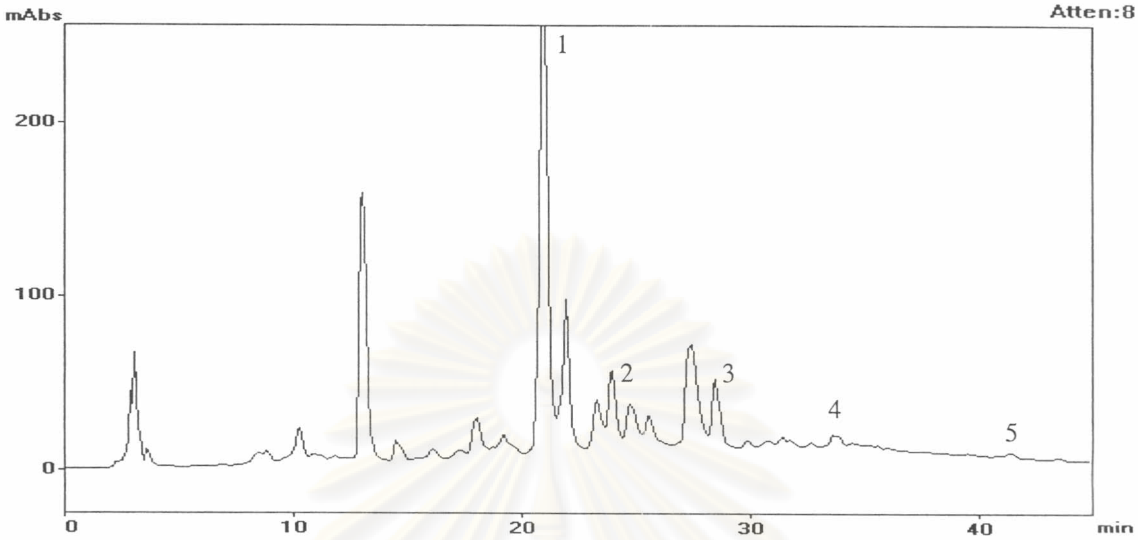


Figure 69 Isoflavone HPLC fingerprint of *P. mirifica* collected in Lop Buri province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

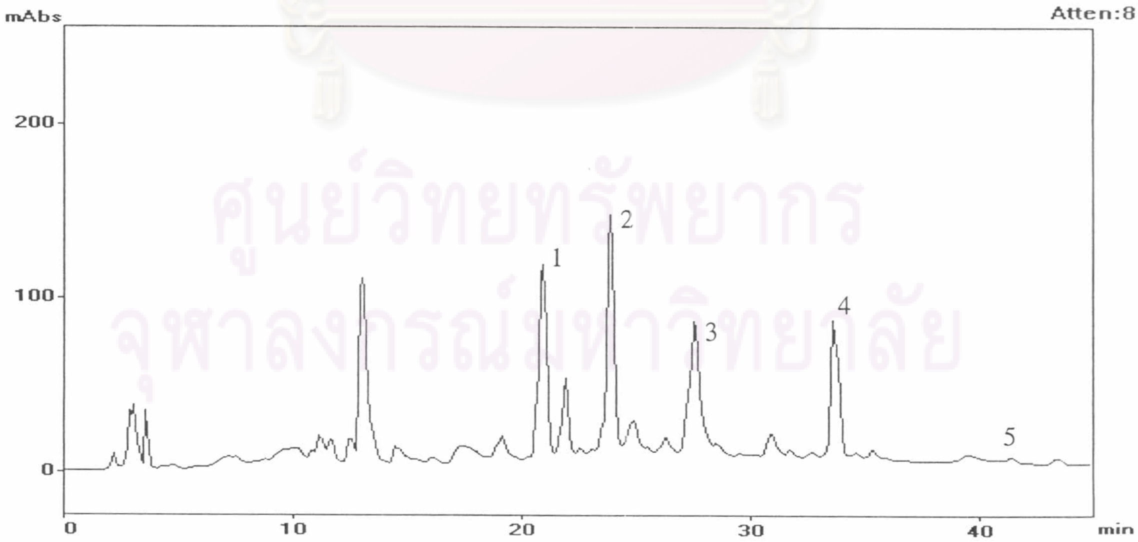


Figure 70 Isoflavone HPLC fingerprint of *P. mirifica* collected in Loei province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

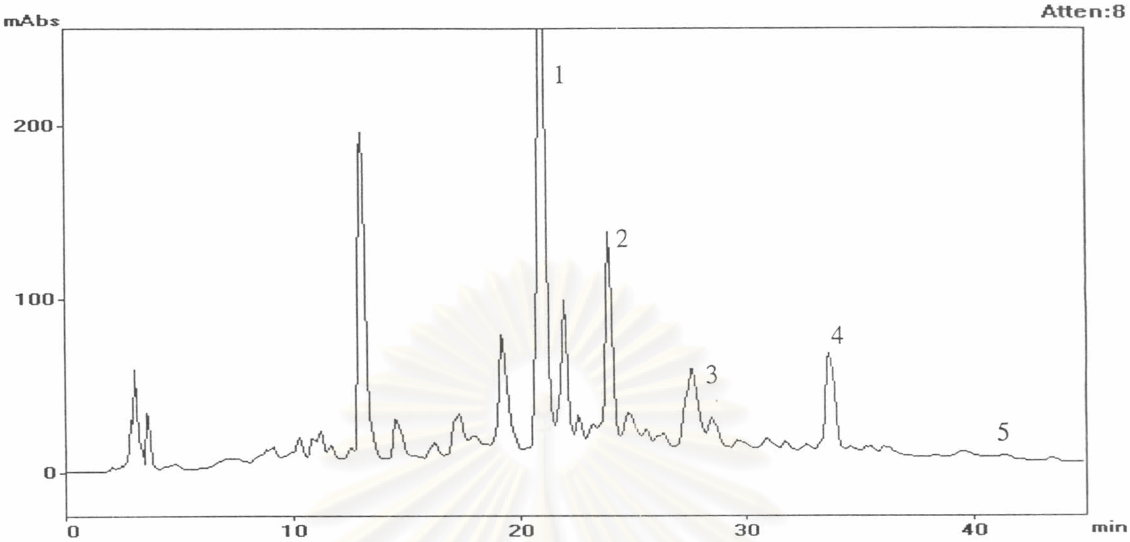


Figure 71 Isoflavone HPLC fingerprint of *P. mirifica* collected in Chaiyaphum province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

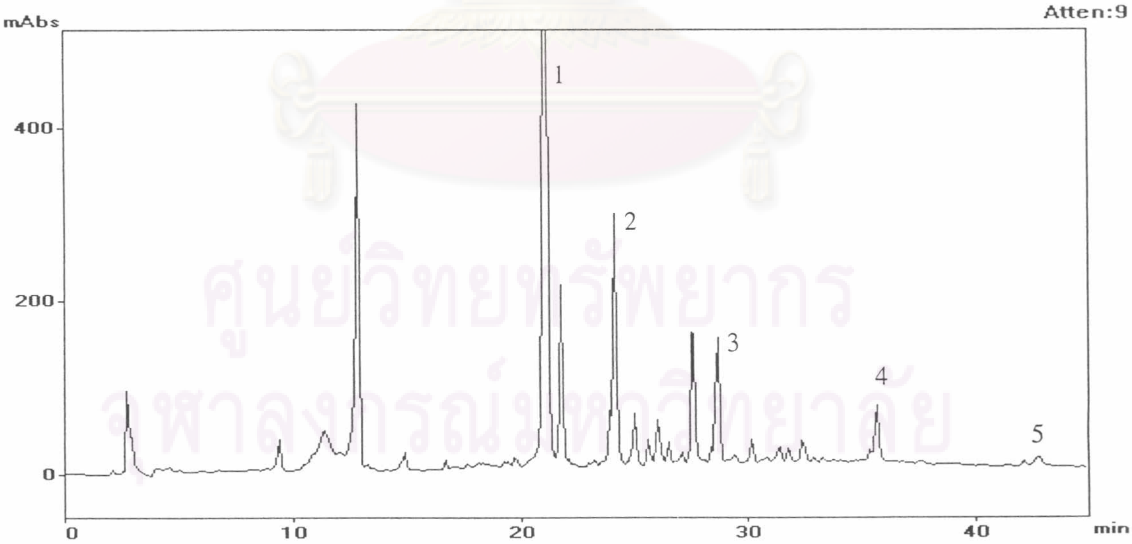


Figure 72 Isoflavone HPLC fingerprint of *P. mirifica* collected in Uttharadith province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

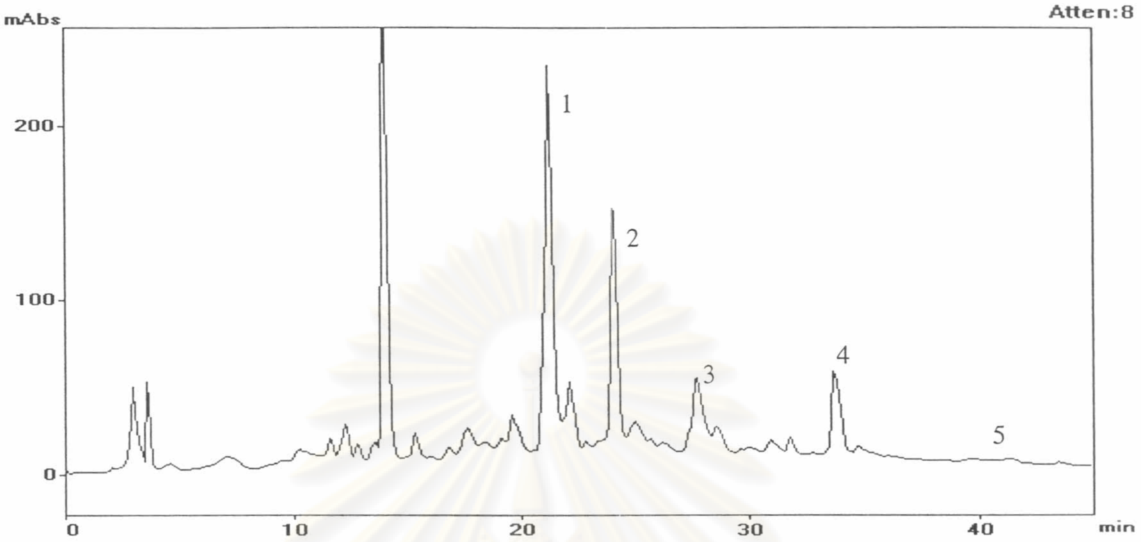


Figure 73 Isoflavone HPLC fingerprint of *P. mirifica* collected in Nakhon Sawan province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

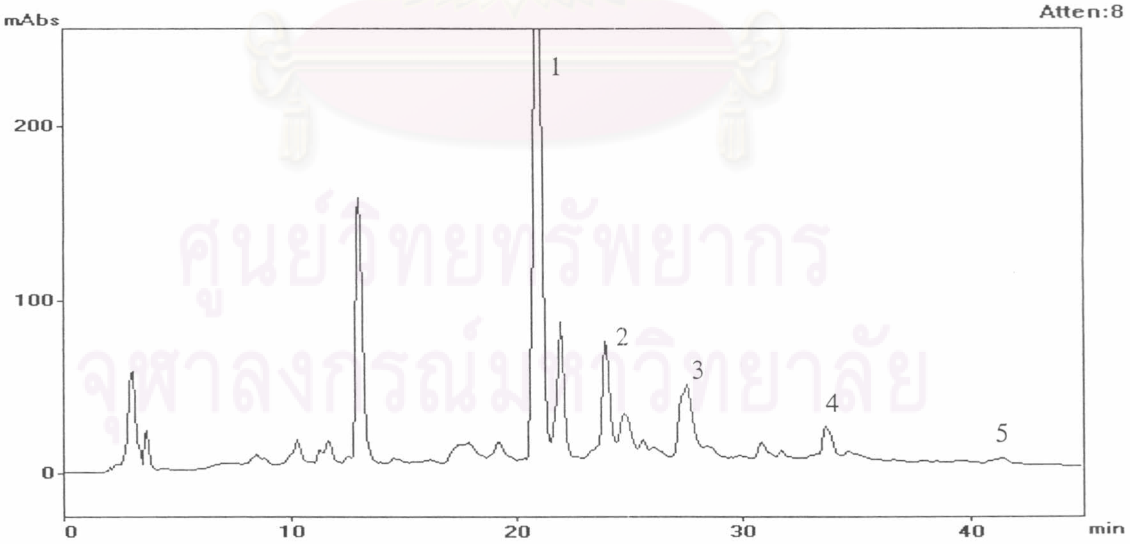


Figure 74 Isoflavone HPLC fingerprint of *P. mirifica* collected in Chiang Rai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

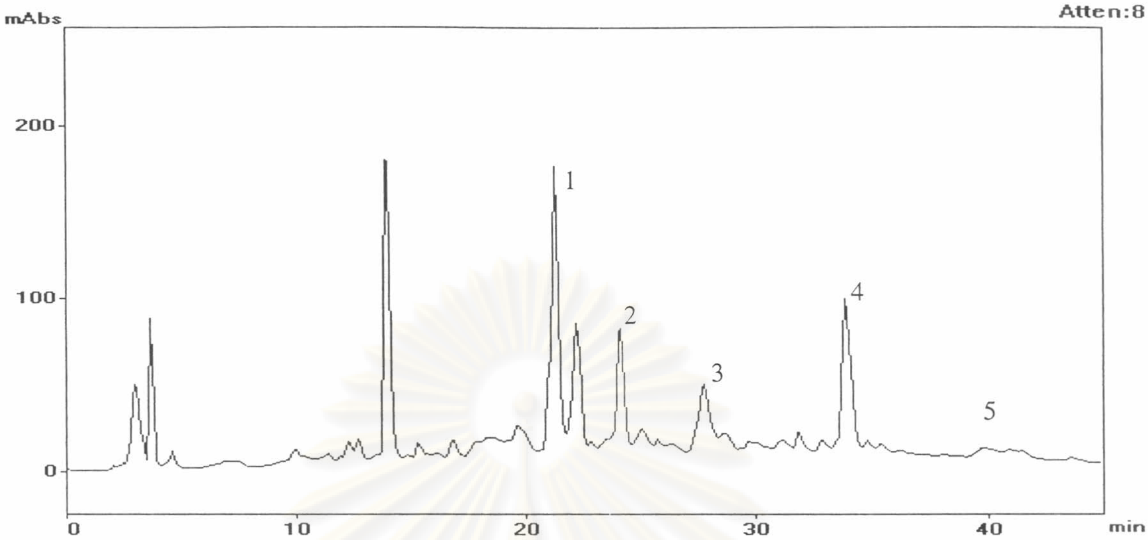


Figure 75 Isoflavone HPLC fingerprint of *P. mirifica* collected in Nong Bua - Lam Phu province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

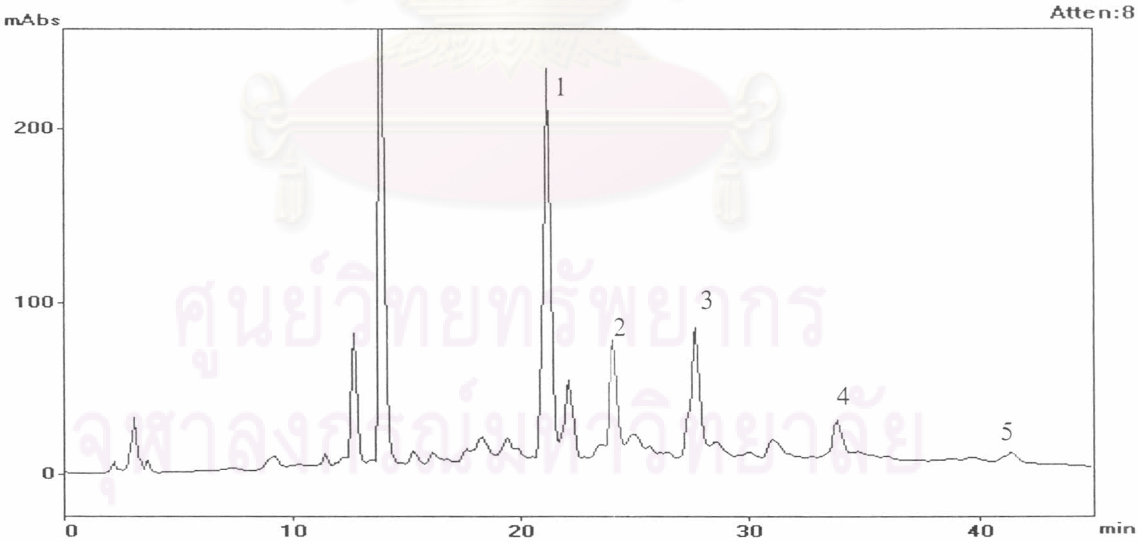


Figure 76 Isoflavone HPLC fingerprint of *P. mirifica* collected in Phayao province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

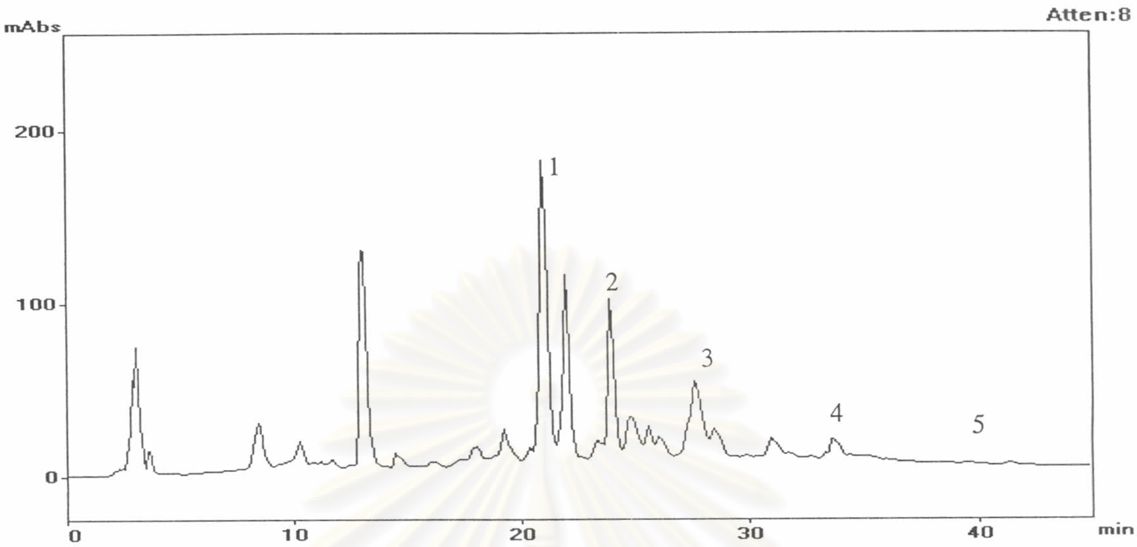


Figure 77 Isoflavone HPLC fingerprint of *P. mirifica* collected in Prachuab - Khiri Khan province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

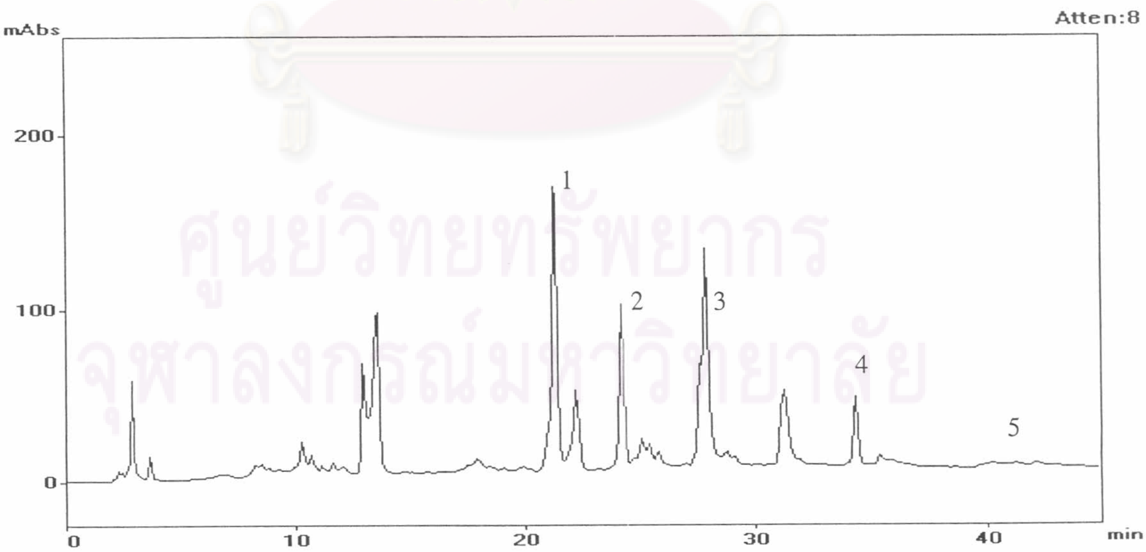


Figure 78 Isoflavone HPLC fingerprint of *P. mirifica* collected in Chumphon province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

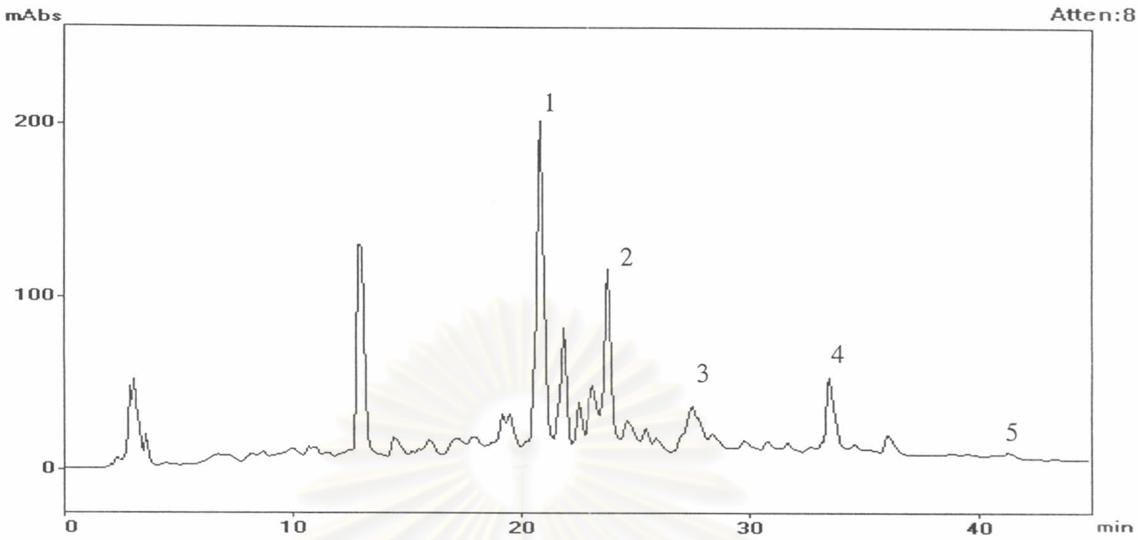


Figure 79 Isoflavone HPLC fingerprint of *P. mirifica* collected in Prachin Buri province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

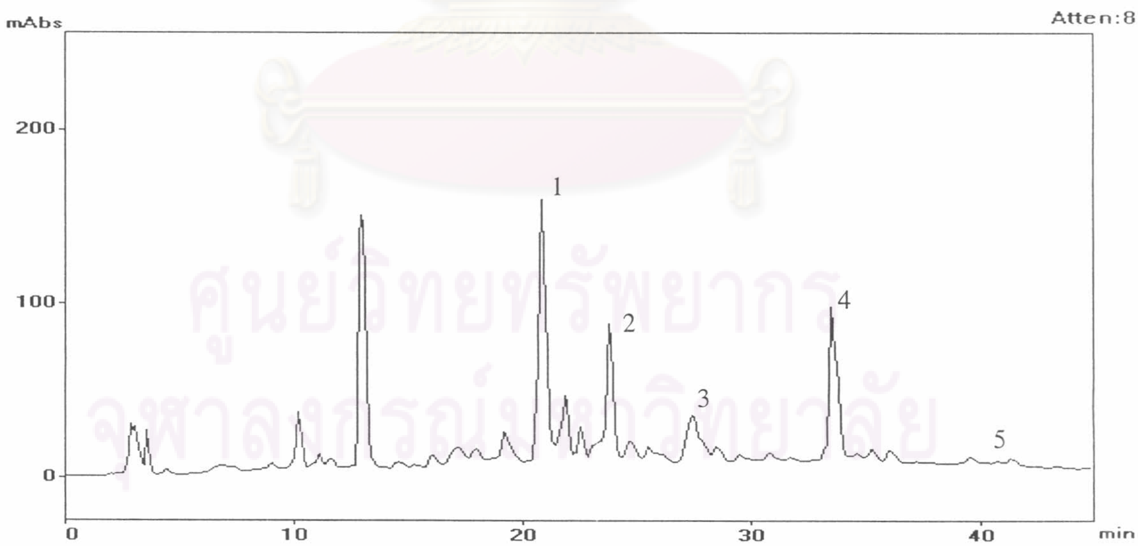


Figure 80 Isoflavone HPLC fingerprint of *P. mirifica* collected in Phetchabun province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

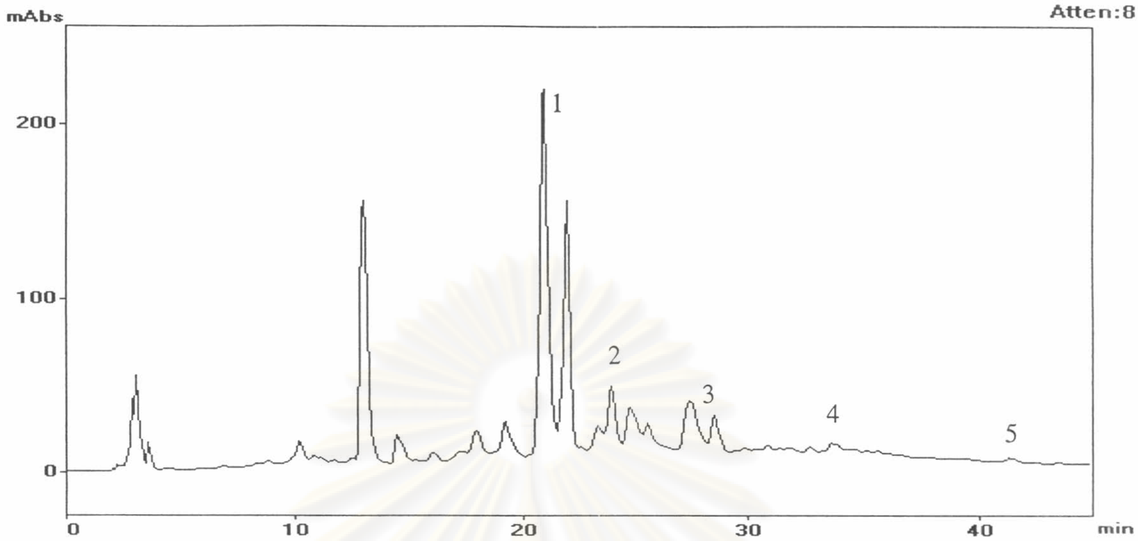


Figure 81 Isoflavone HPLC fingerprint of *P. mirifica* collected in Nakhon Ratchasima province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

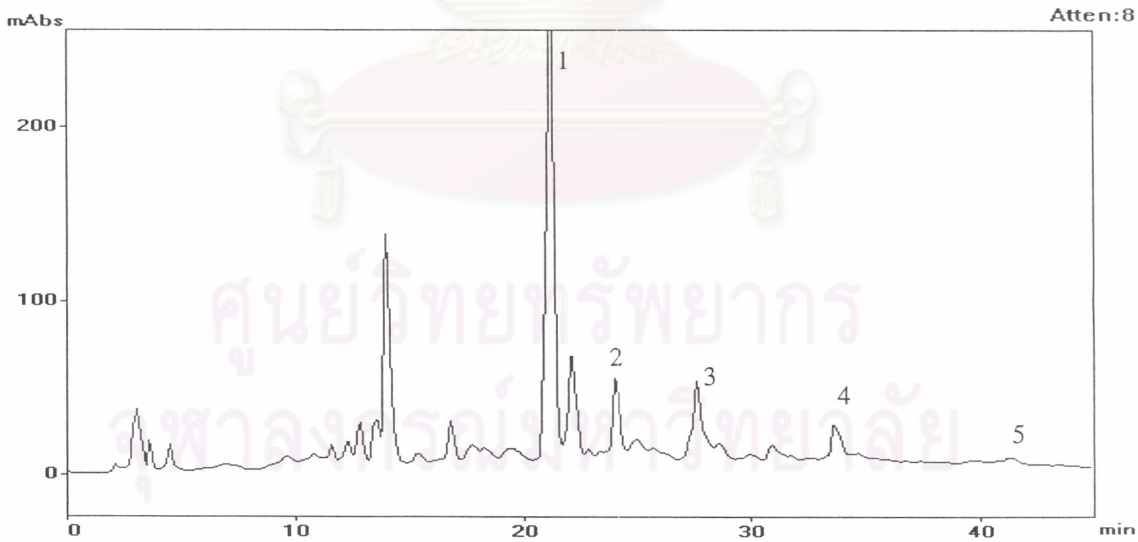


Figure 82 Isoflavone HPLC fingerprint of *P. mirifica* collected in Kamphaeng-Phet province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

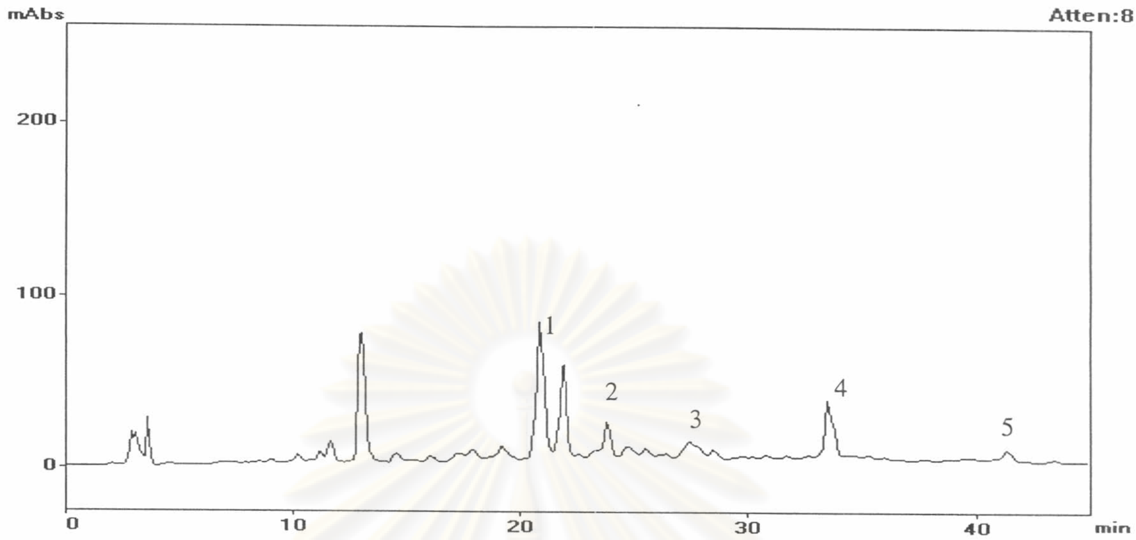


Figure 83 Isoflavone HPLC fingerprint of *P. mirifica* collected in Nan province
1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

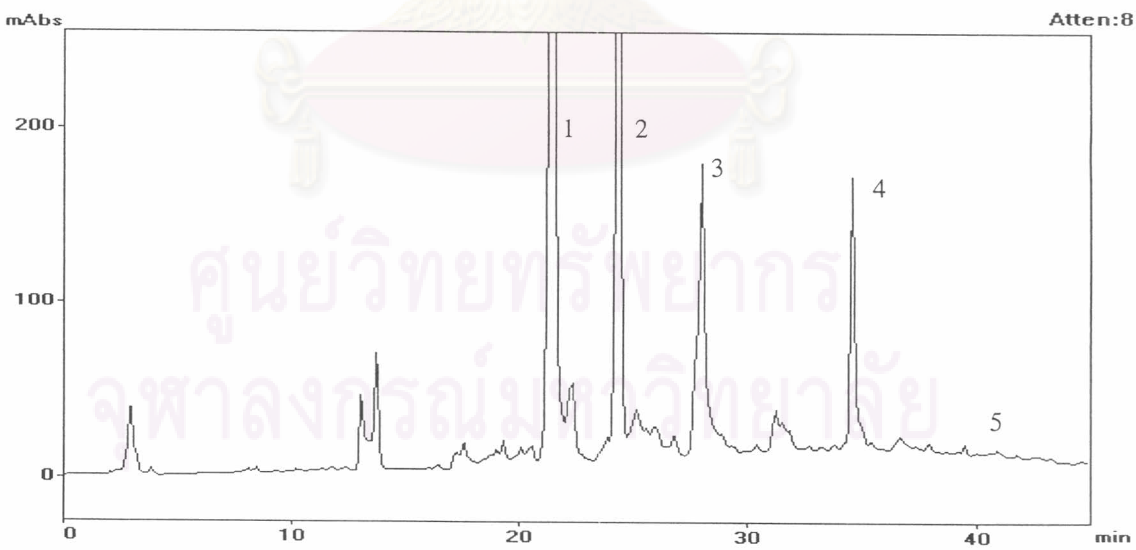


Figure 84 Isoflavone HPLC fingerprint of *P. mirifica* from Hod district in Chiang Mai province
1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

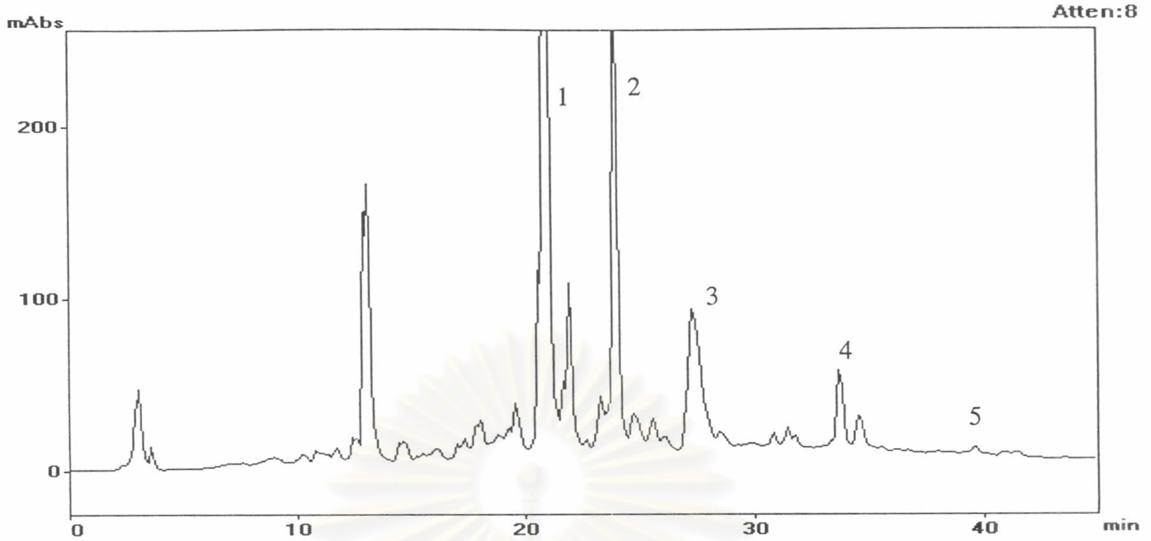


Figure 85 Isoflavone HPLC fingerprint of *P. mirifica* from Doi Tao district in Chiang Mai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

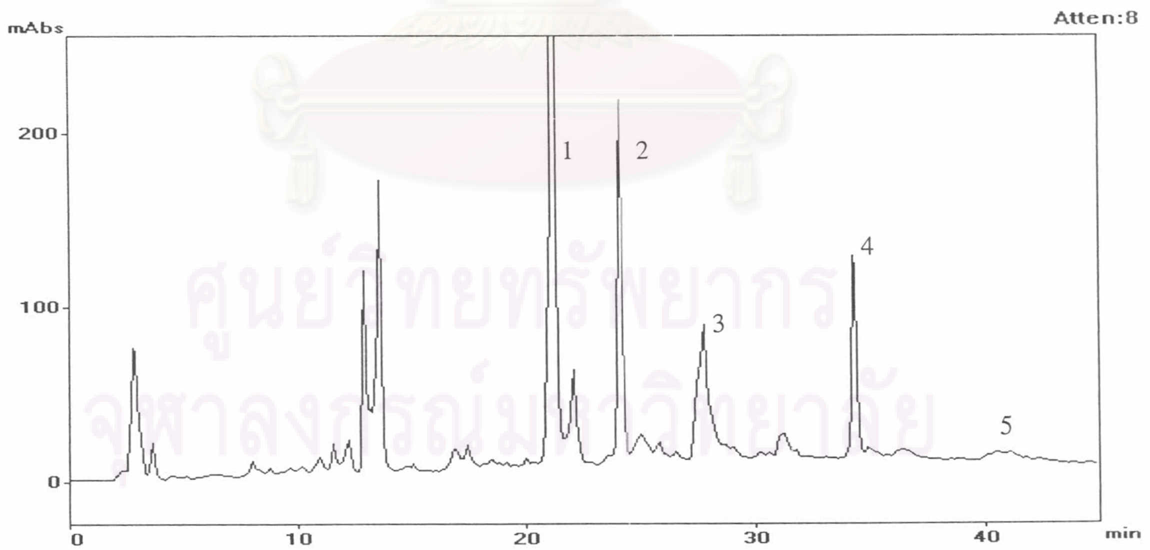


Figure 86 Isoflavone HPLC fingerprint of *P. mirifica* from Doi Saket district in Chiang Mai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

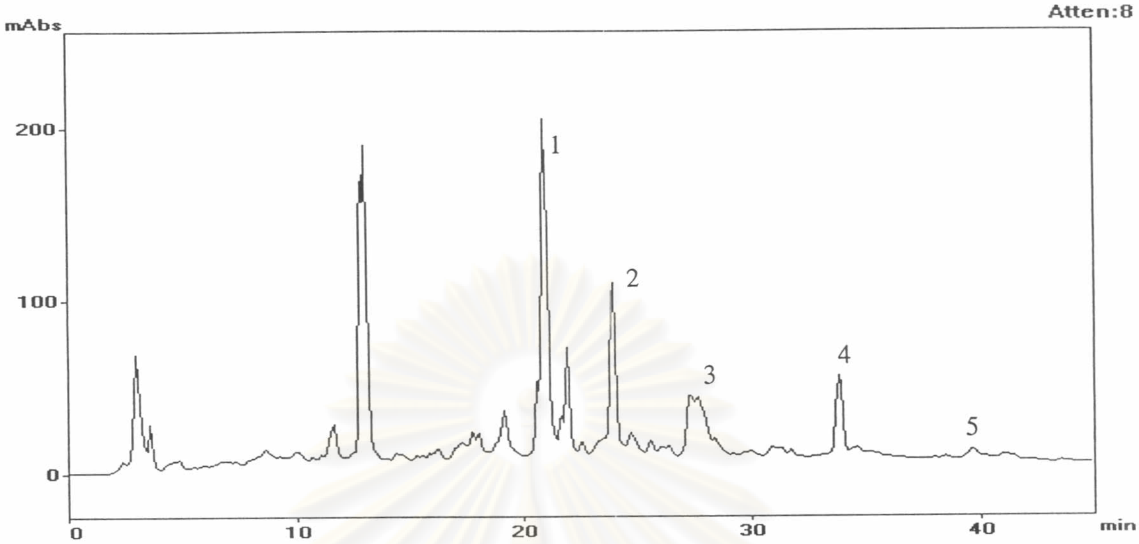


Figure 87 Isoflavone HPLC fingerprint of *P. mirifica* from Chiang Dao district in Chiang Mai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

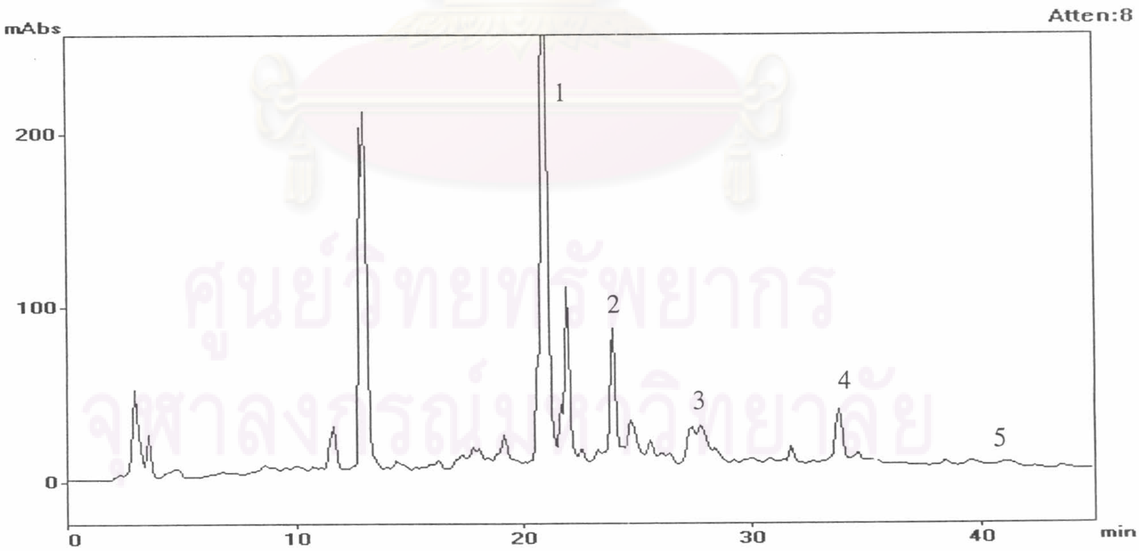


Figure 88 Isoflavone HPLC fingerprint of *P. mirifica* from Chaiprakarn district in Chiang Mai province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

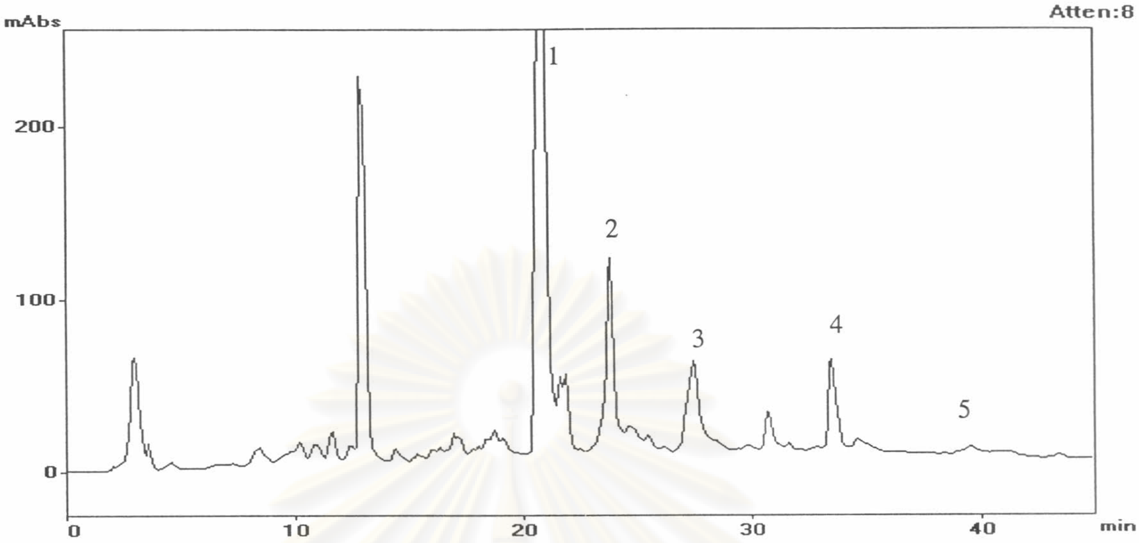


Figure 89 Isoflavone HPLC fingerprint of *P. mirifica* from Koh Ka district in Lampang province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

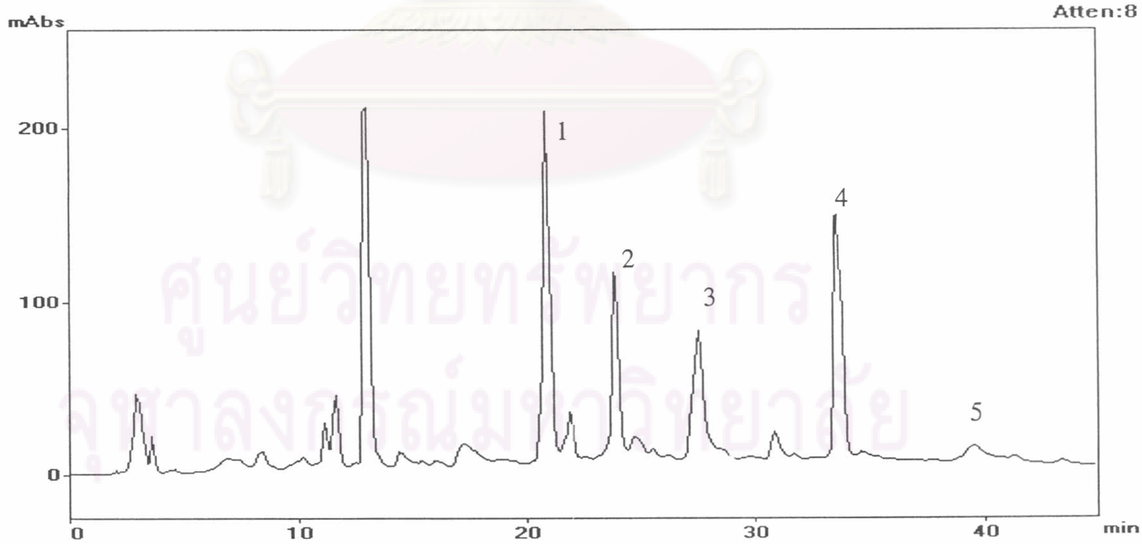


Figure 90 Isoflavone HPLC fingerprint of *P. mirifica* from Hang Chat district in Lampang province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

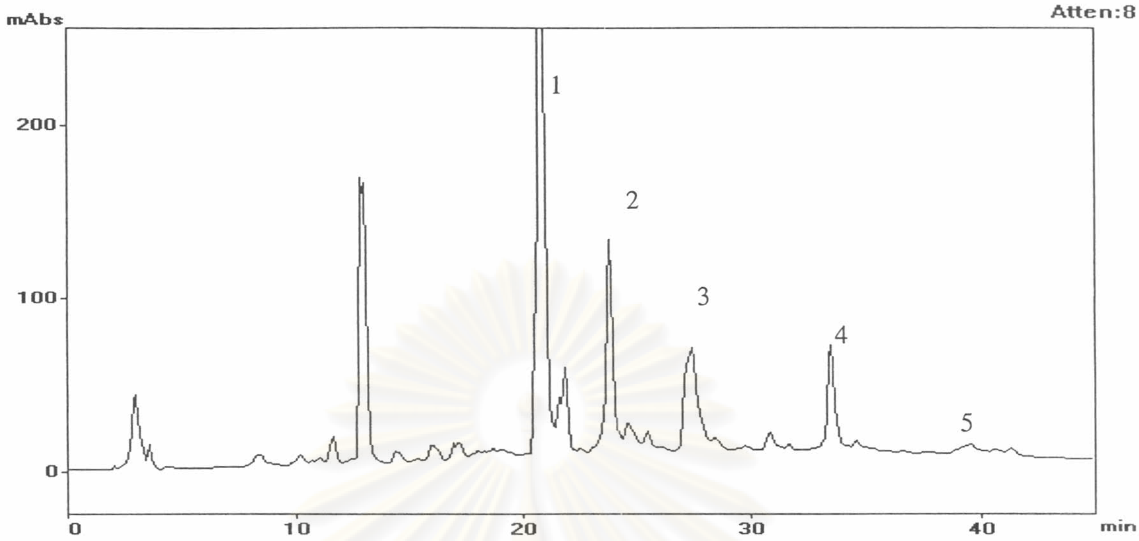


Figure 91 Isoflavone HPLC fingerprint of *P. mirifica* from Thern district in Lampang province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

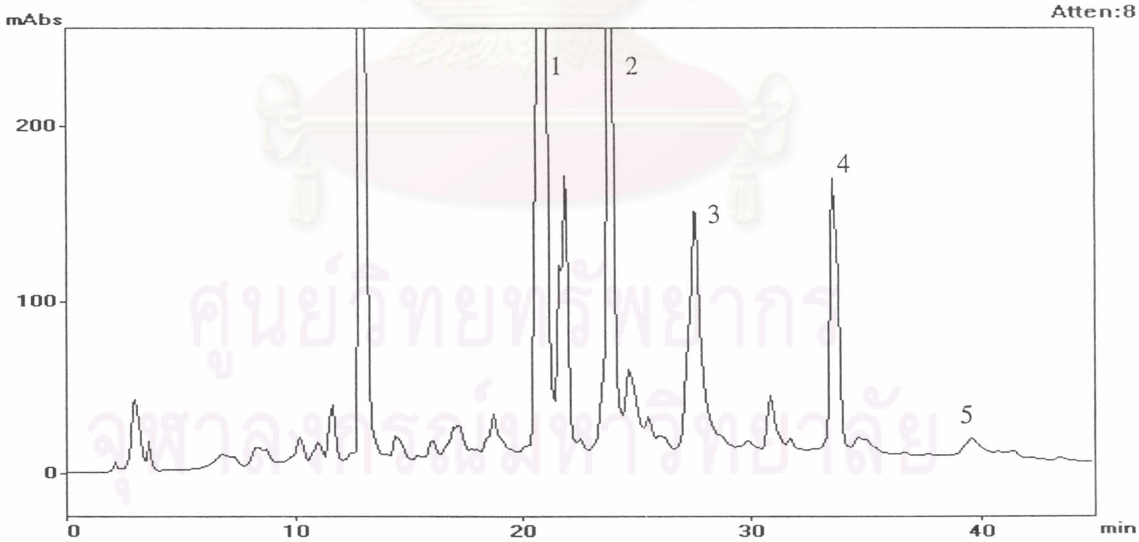


Figure 92 Isoflavone HPLC fingerprint of *P. mirifica* from Srisawat district in Kanchanaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

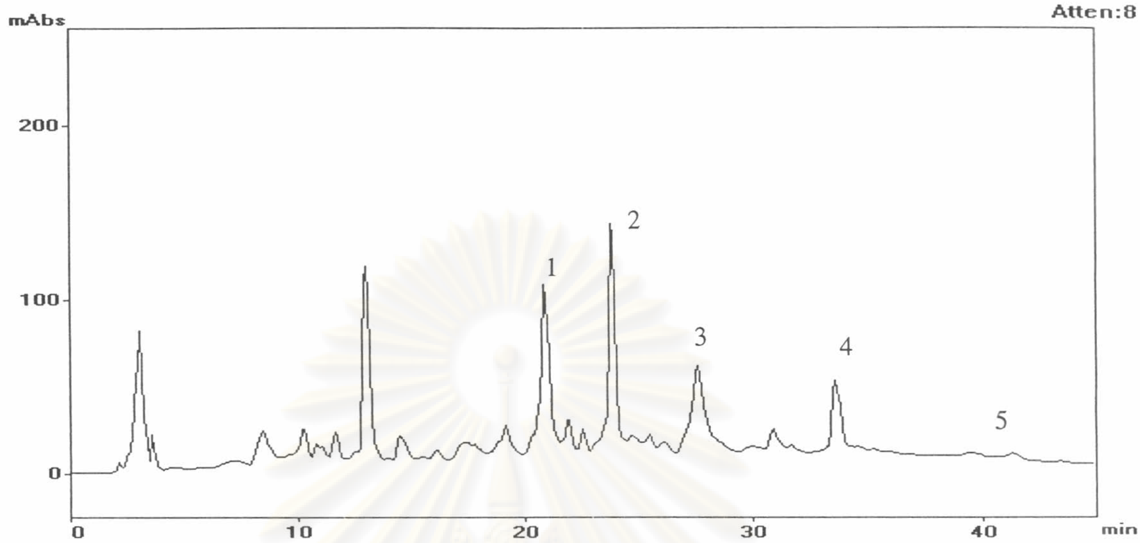


Figure 93 Isoflavone HPLC fingerprint of *P. mirifica* from Sai Yoke district in Kanchanaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

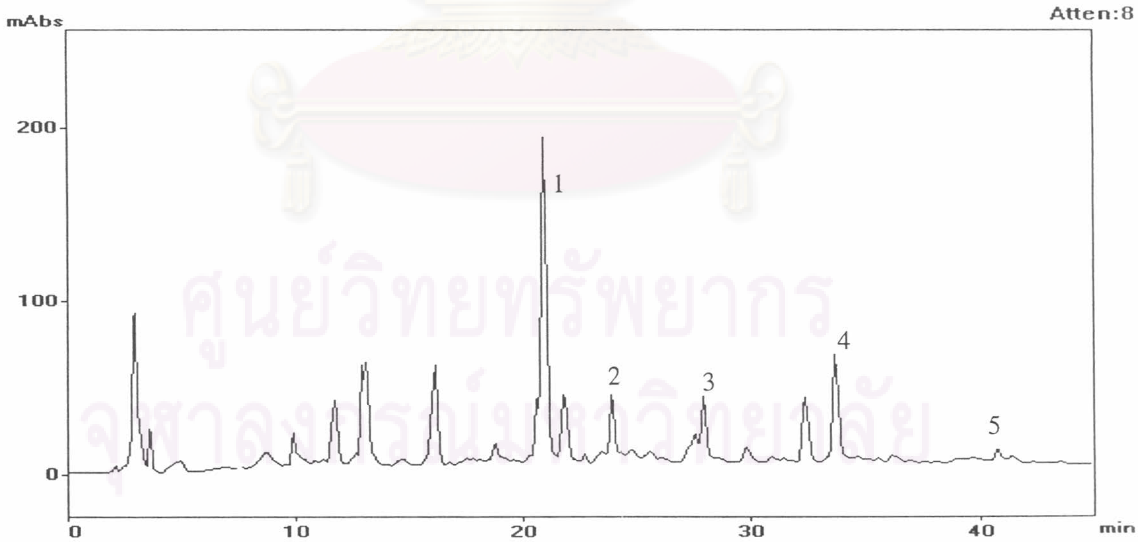


Figure 94 Isoflavone HPLC fingerprint of *P. mirifica* from Thongphaphum district in Kanchanaburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

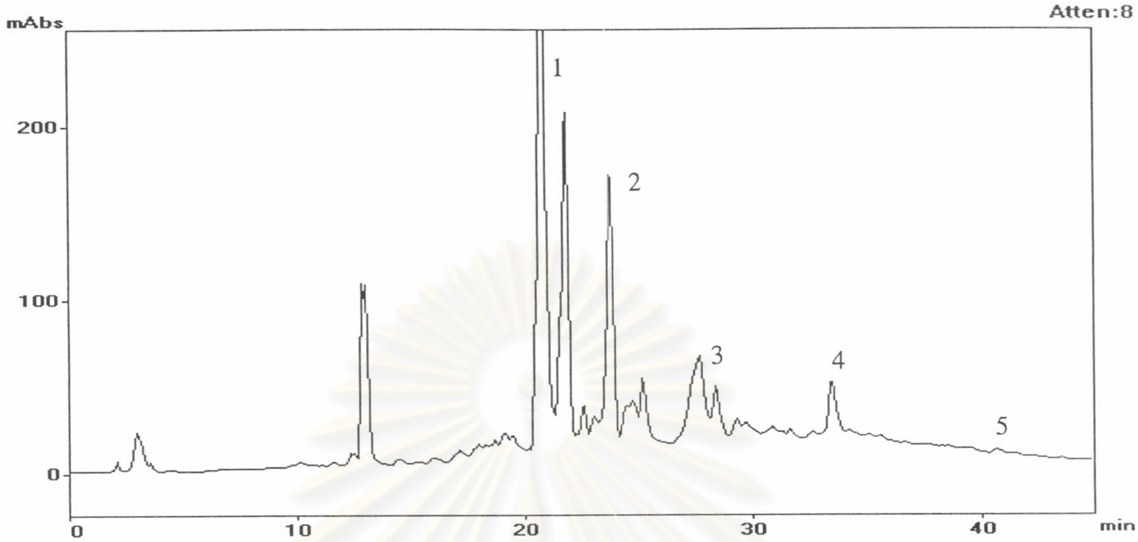


Figure 95 Isoflavone HPLC fingerprint of *P. mirifica* from Muak Lek district site I in Saraburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

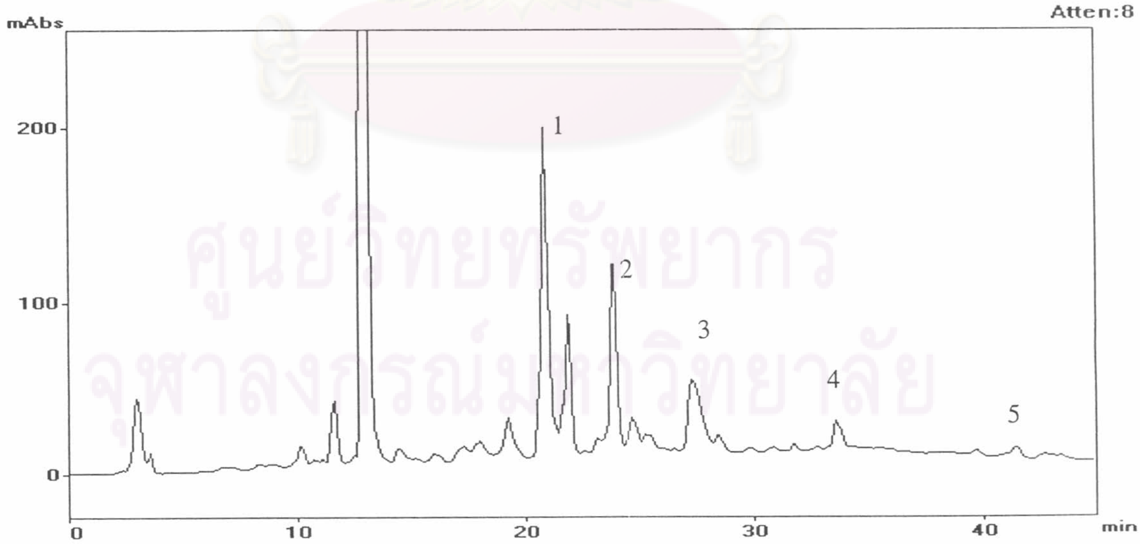


Figure 96 Isoflavone HPLC fingerprint of *P. mirifica* from Phra Putthabat district in Saraburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

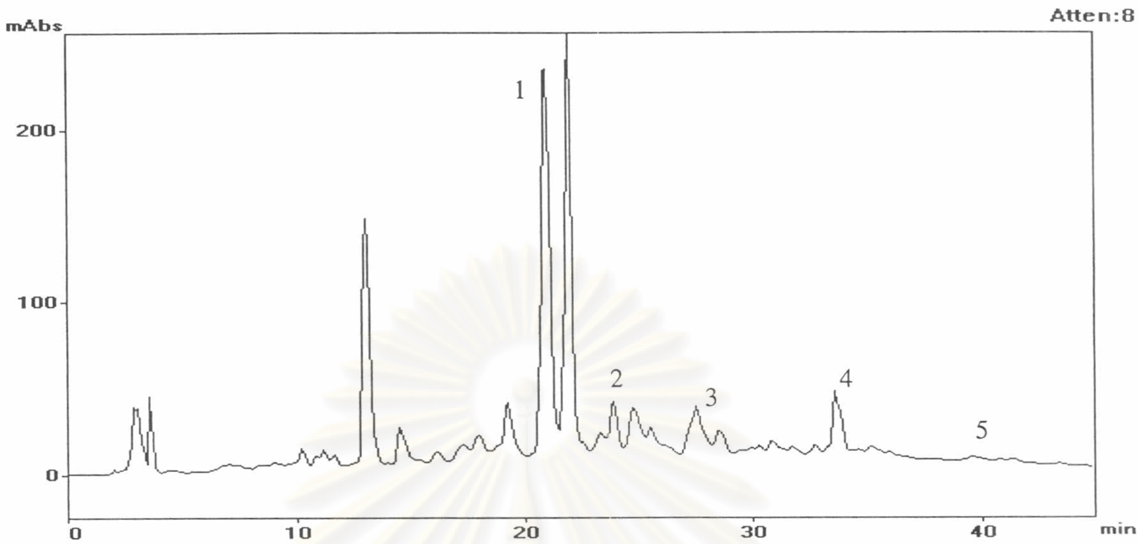


Figure 97 Isoflavone HPLC fingerprint of *P. mirifica* from Muak Lek district site II in Saraburi province

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

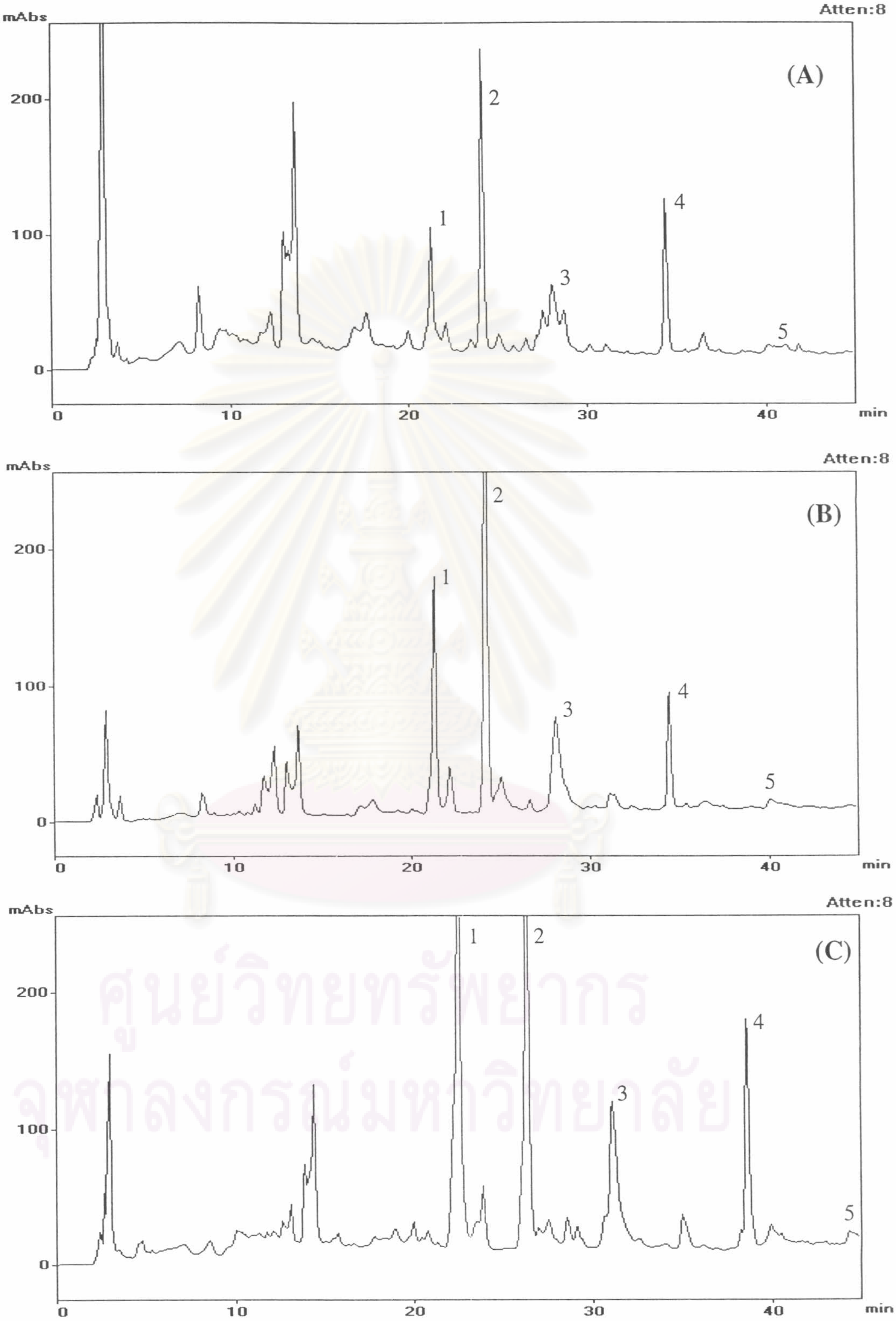
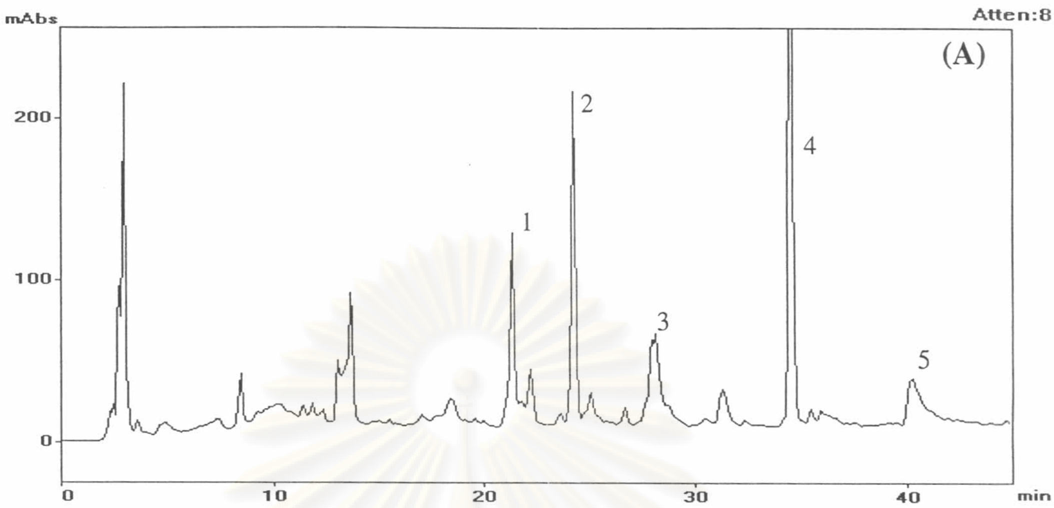


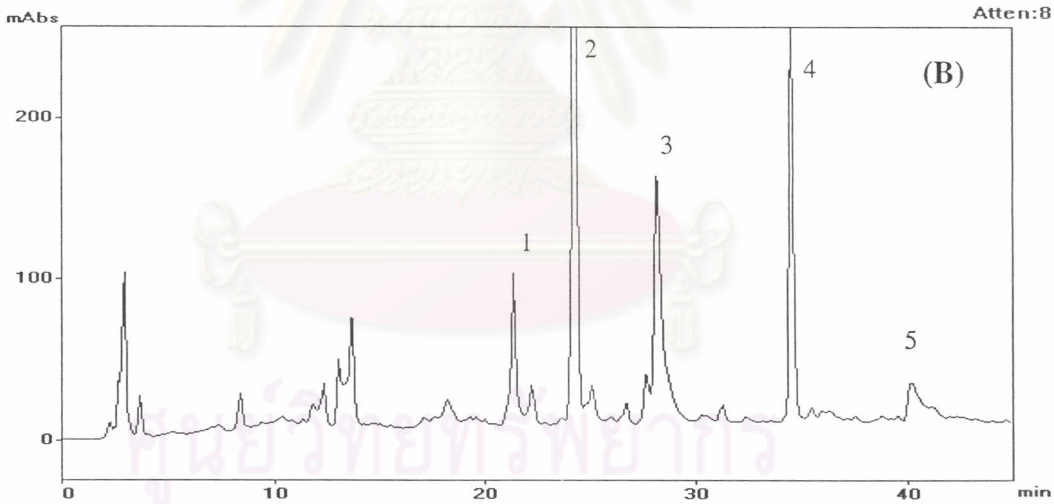
Figure 98 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao cultivated in different location

(A) Chaing Rai (B) Bangkok (C) Ratchaburi

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein



(A)



(B)

Figure 99 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Chaiprakarn cultivated in different location

(A) Chaing Rai (B) Ratchaburi

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

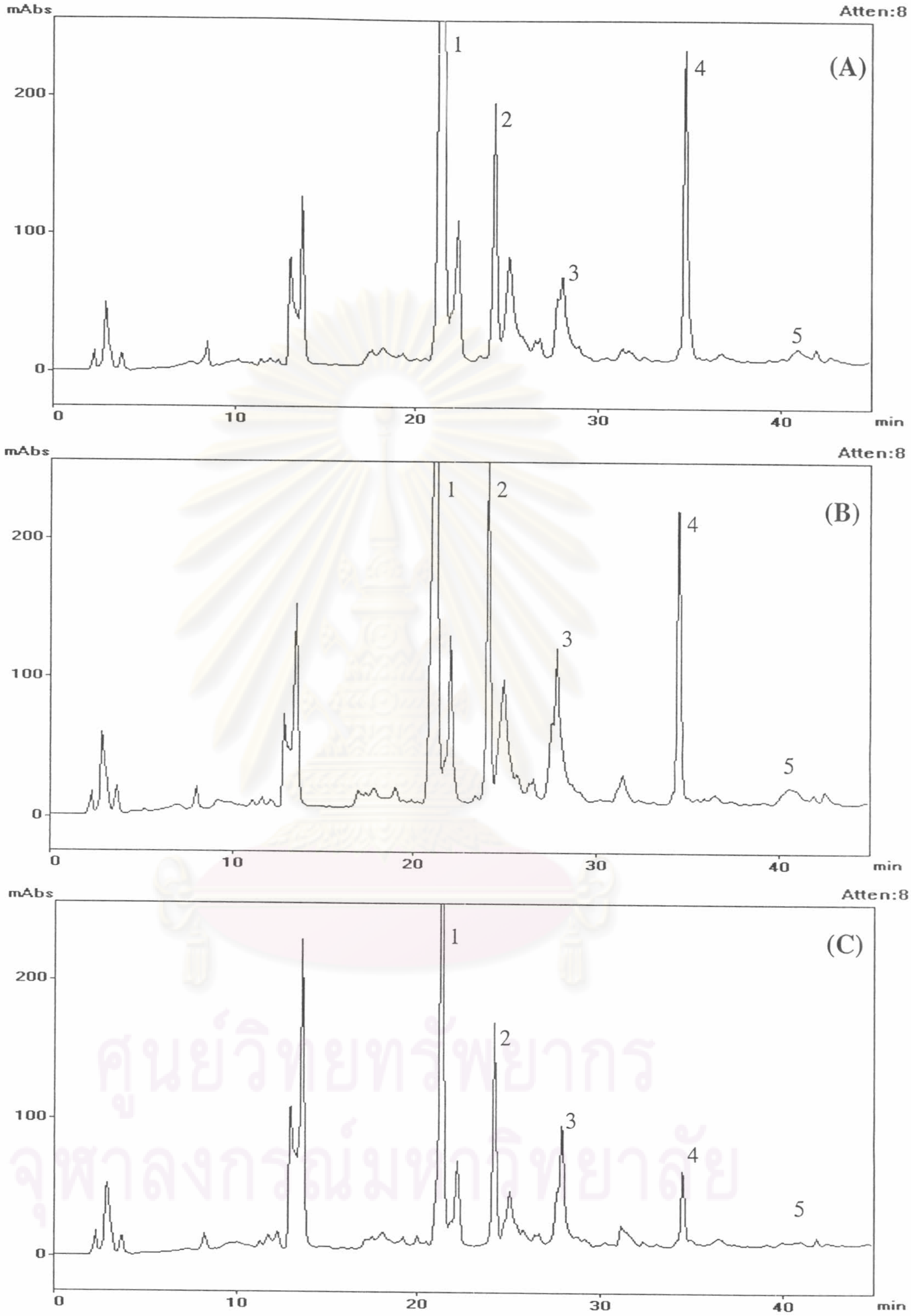


Figure 100 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao cultivated in different season

(A) Rainy (B) Winter (C) Summer

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

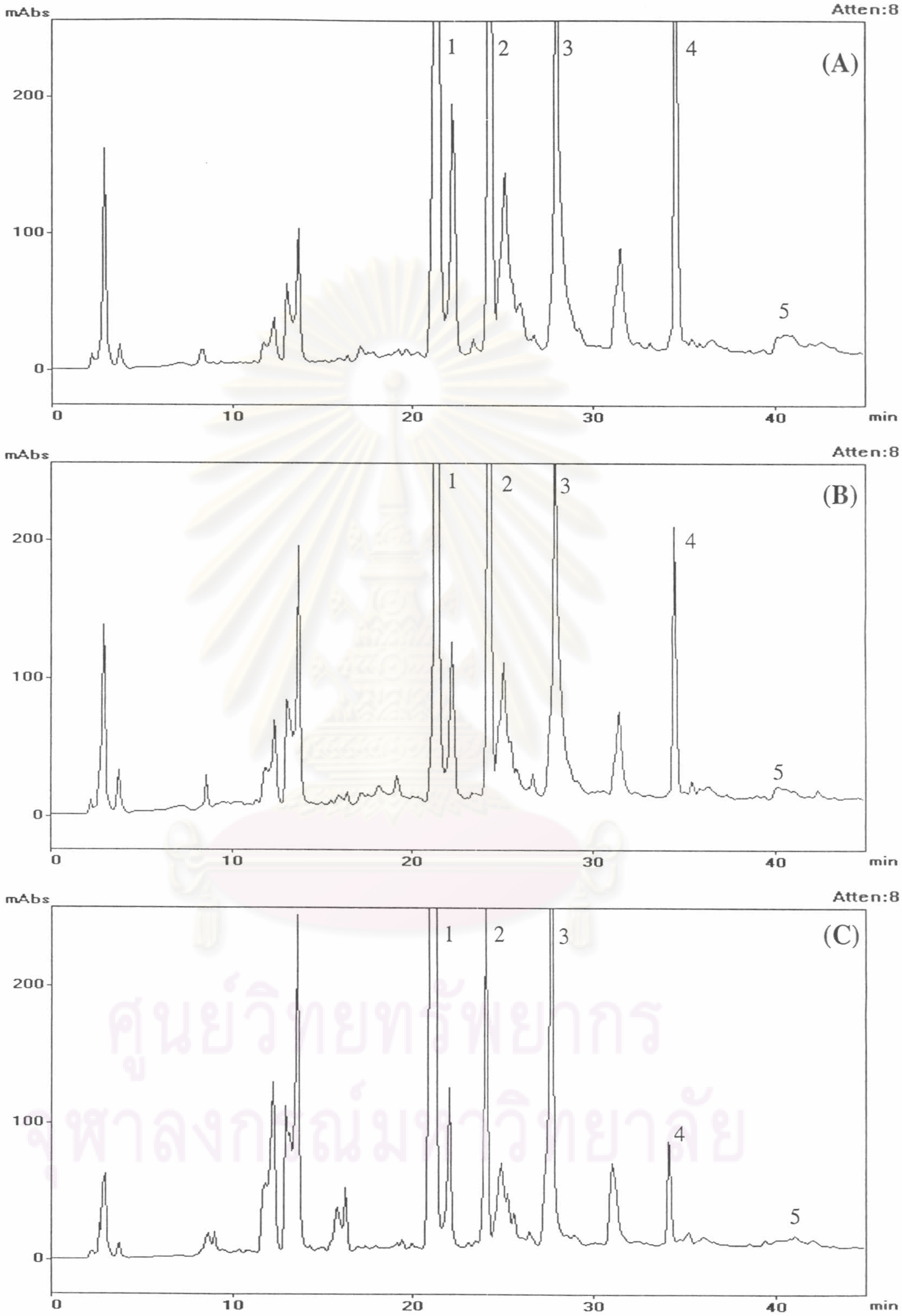


Figure 101 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Chaiprakarn cultivated in different season

(A) Rainy (B) Winter (C) Summer

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

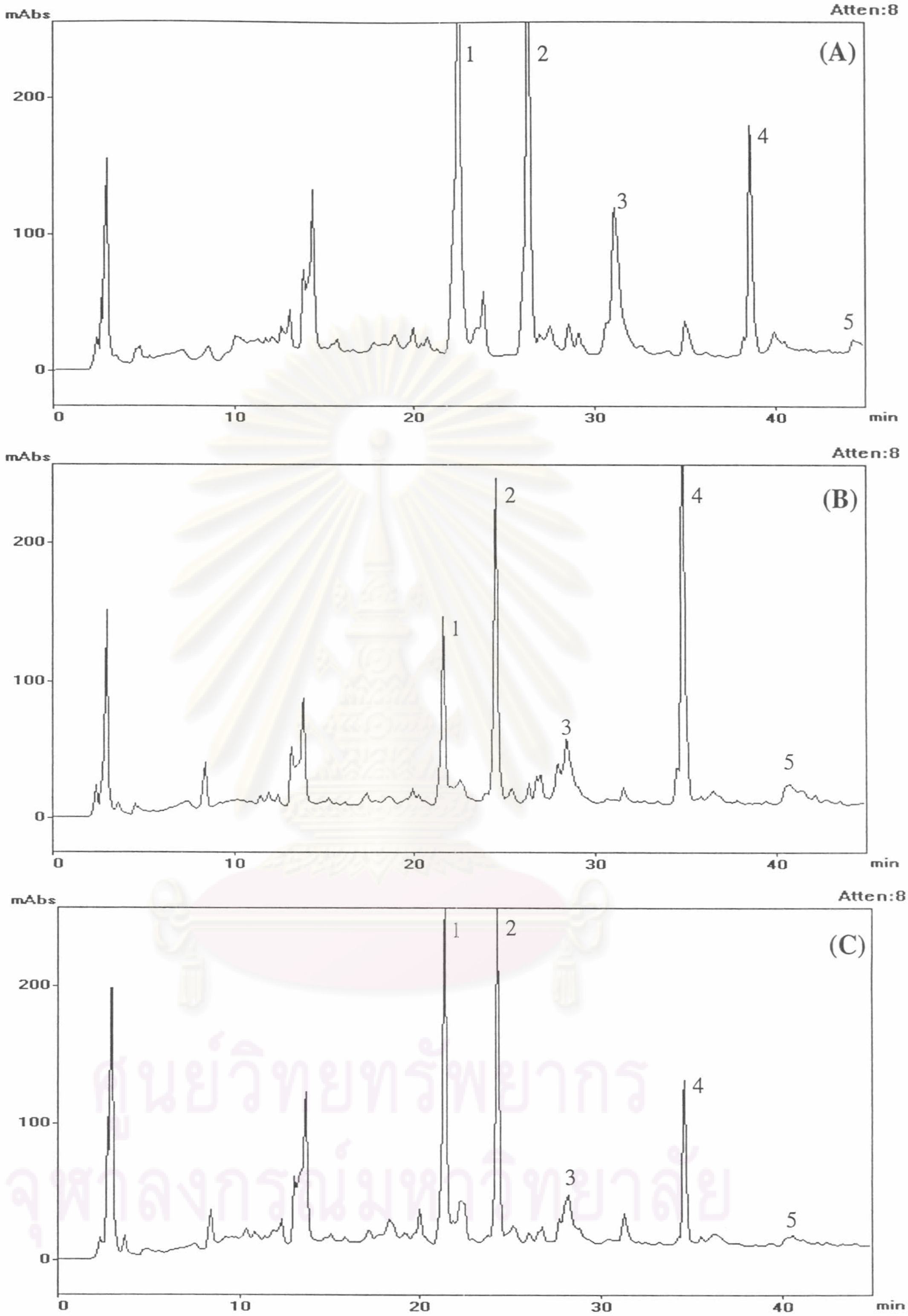


Figure 102 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao (F1) cultivated in Ratchaburi province

(A) sub-clone I (B) sub-clone II (C) sub-clone III

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

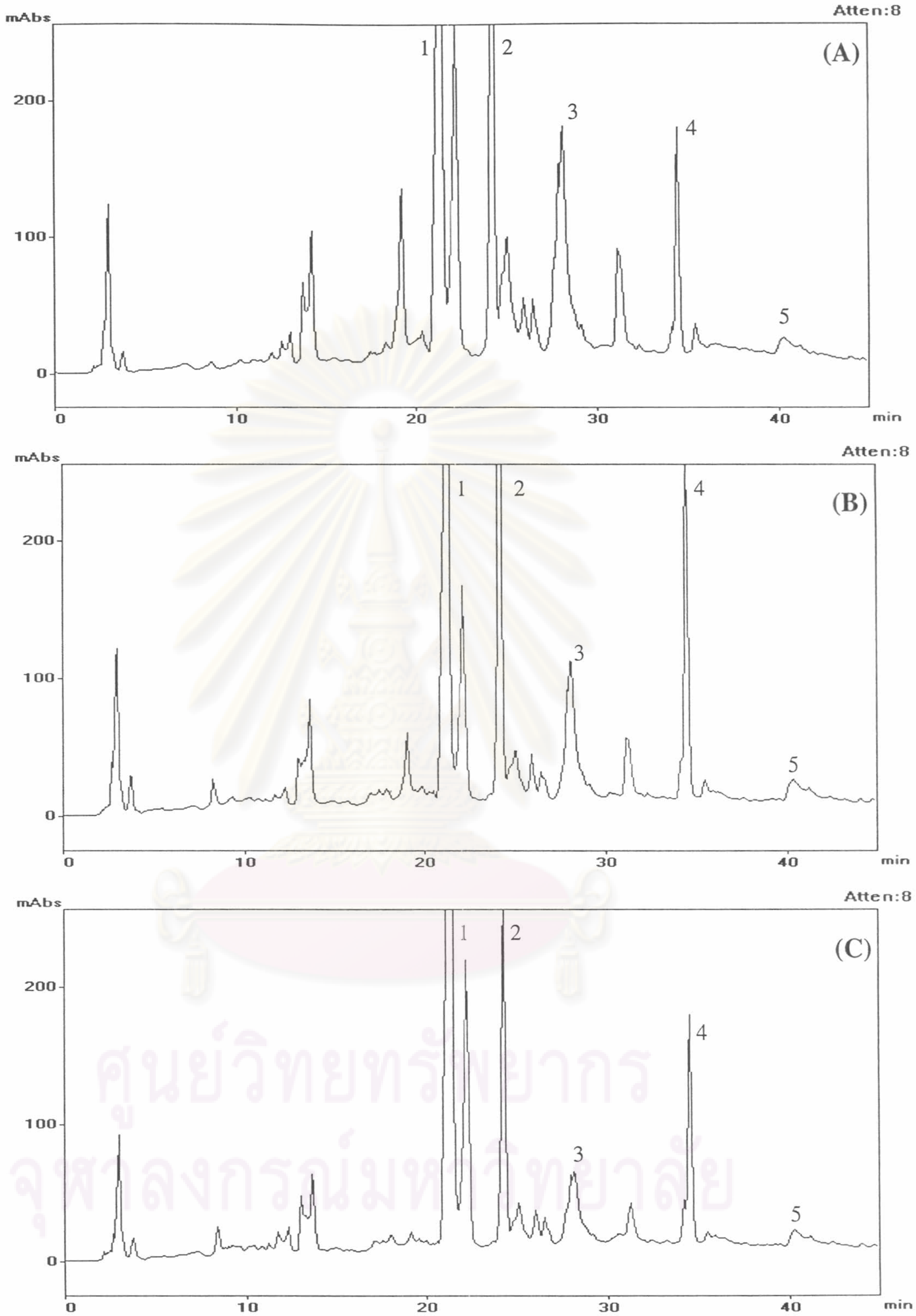


Figure 103 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Sai Yoke (F_1) cultivated in Ratchaburi province

(A) sub-clone I (B) sub-clone II (C) sub-clone III

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

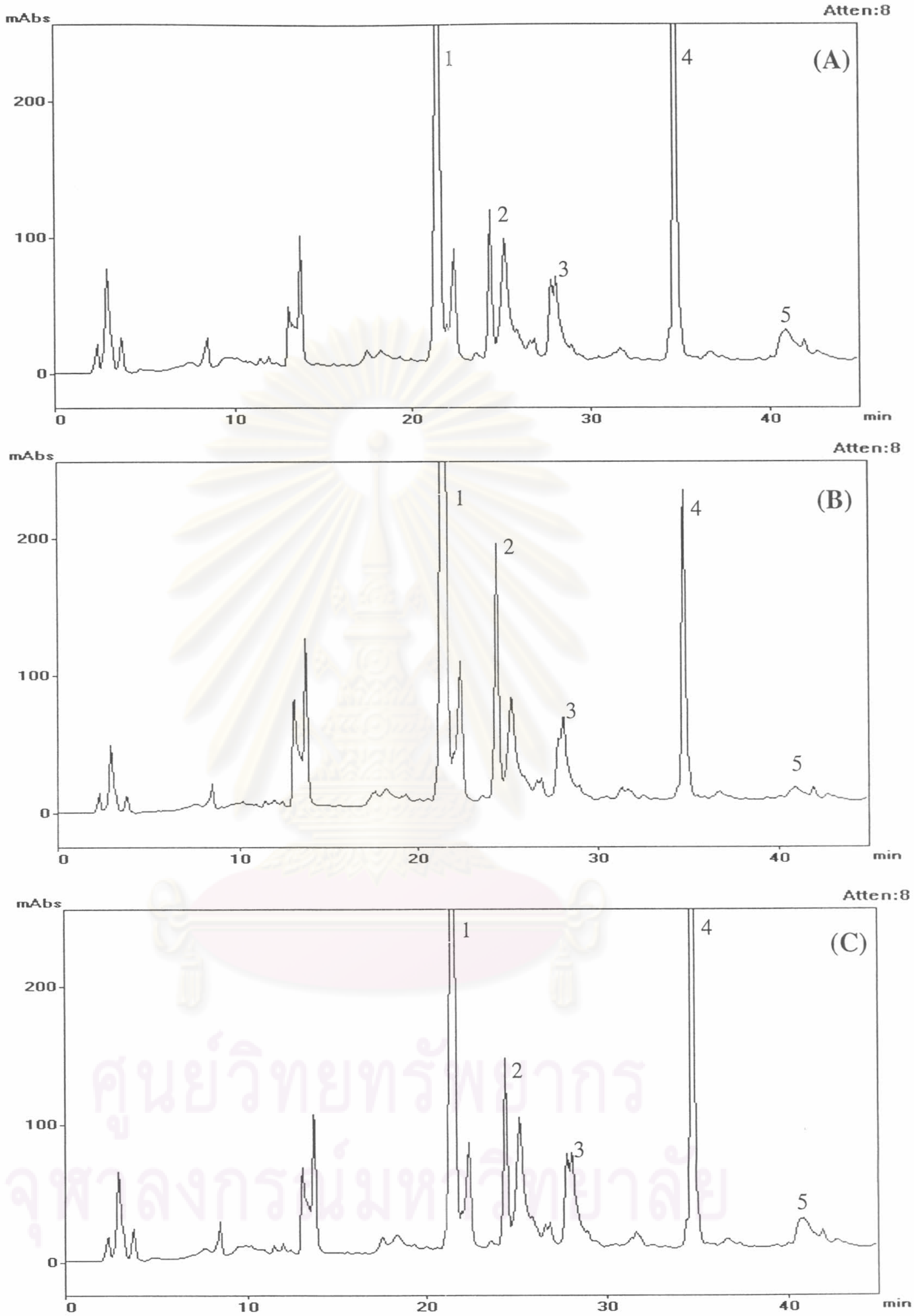


Figure 104 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao cultivated in the rainy season

(A) sub-clone I (B) sub-clone II (C) sub-clone III

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

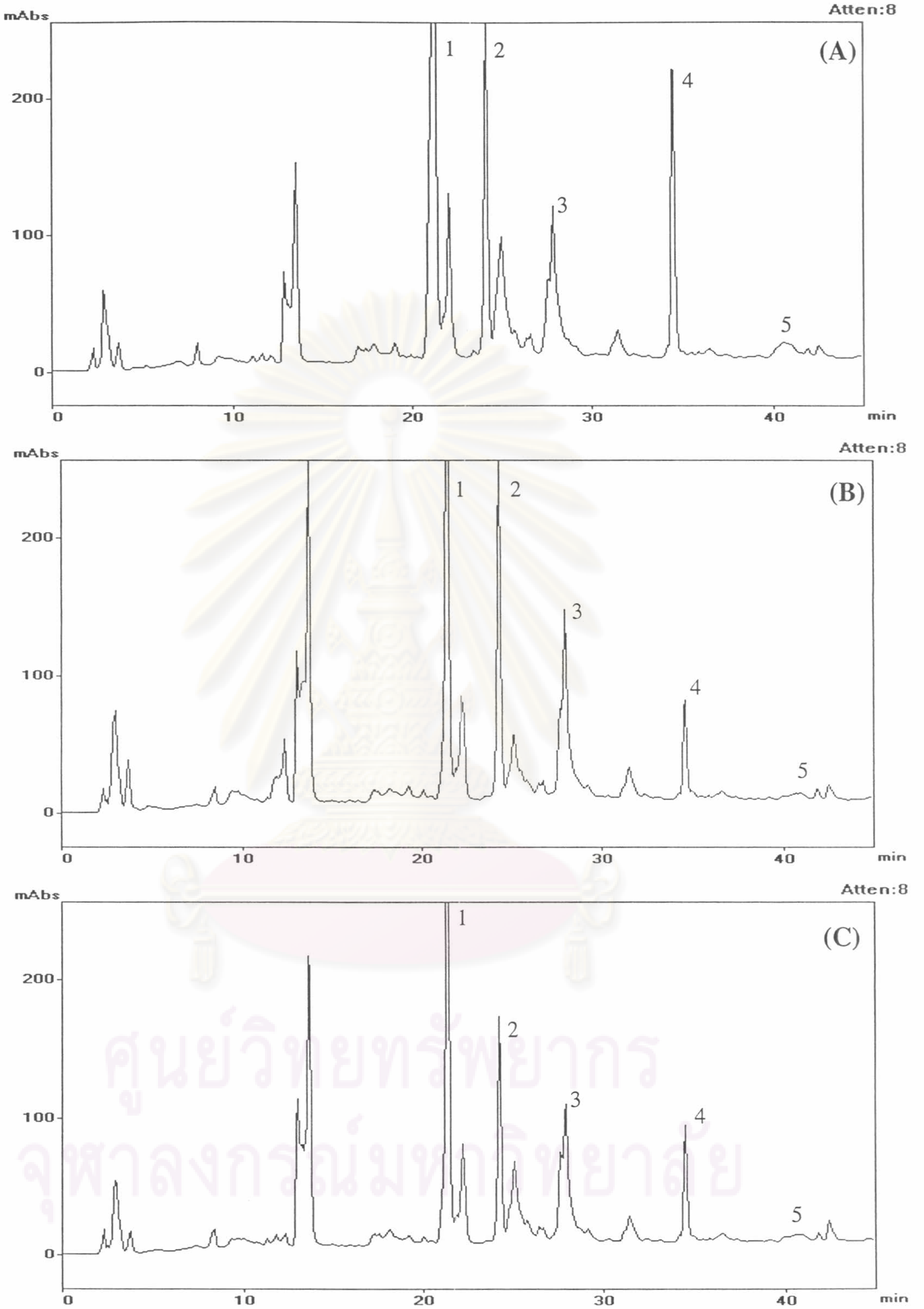


Figure 105 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao cultivated in winter

(A) sub-clone I (B) sub-clone II (C) sub-clone III

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

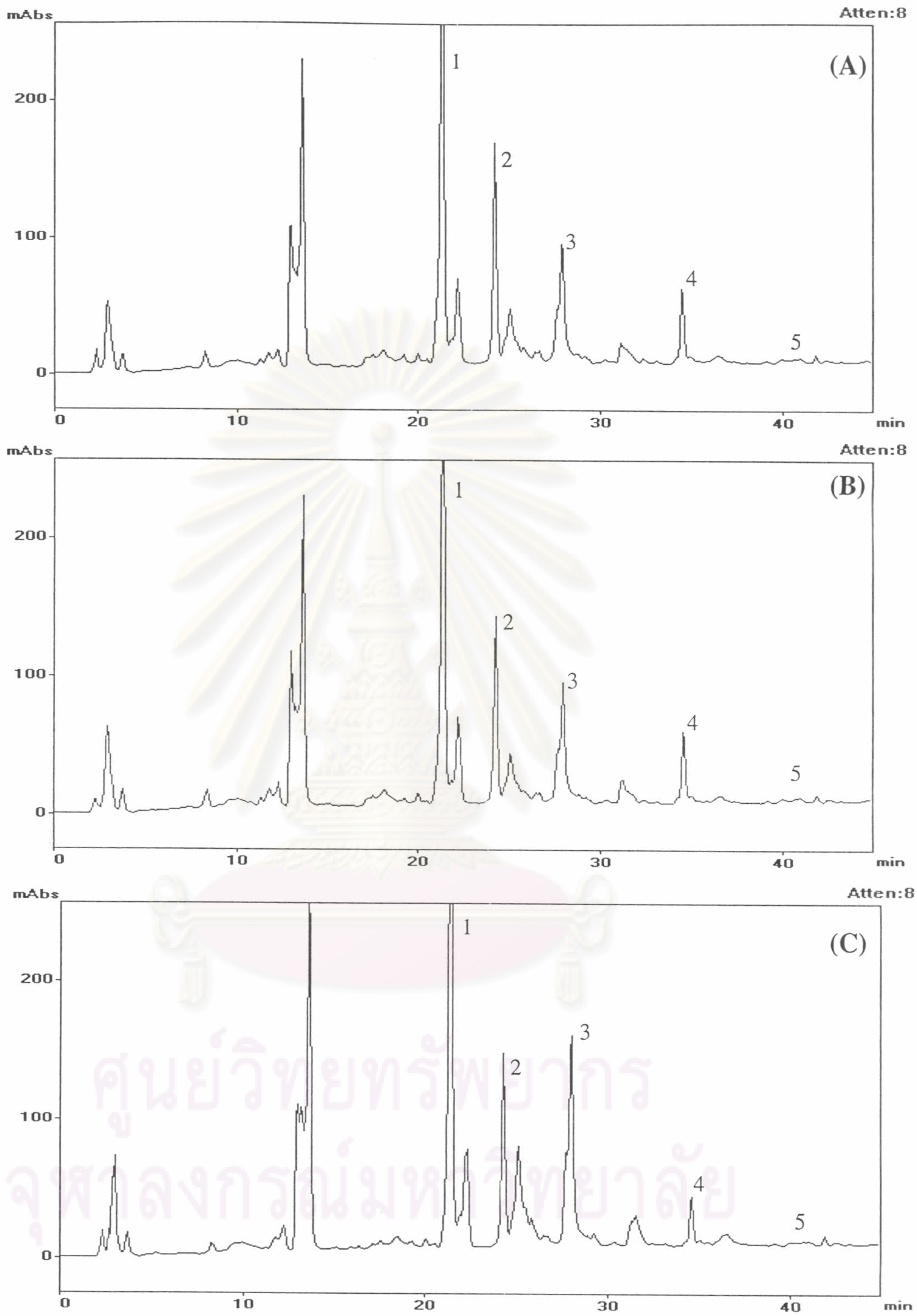


Figure 106 Isoflavone HPLC fingerprint of field grown *P. mirifica* clone Doi Tao cultivated in summer

(A) sub-clone I (B) sub-clone II (C) sub-clone III

1 = Puerarin 2= Daidzin 3 = Genistin 4 = Daidzein 5 = Genistein

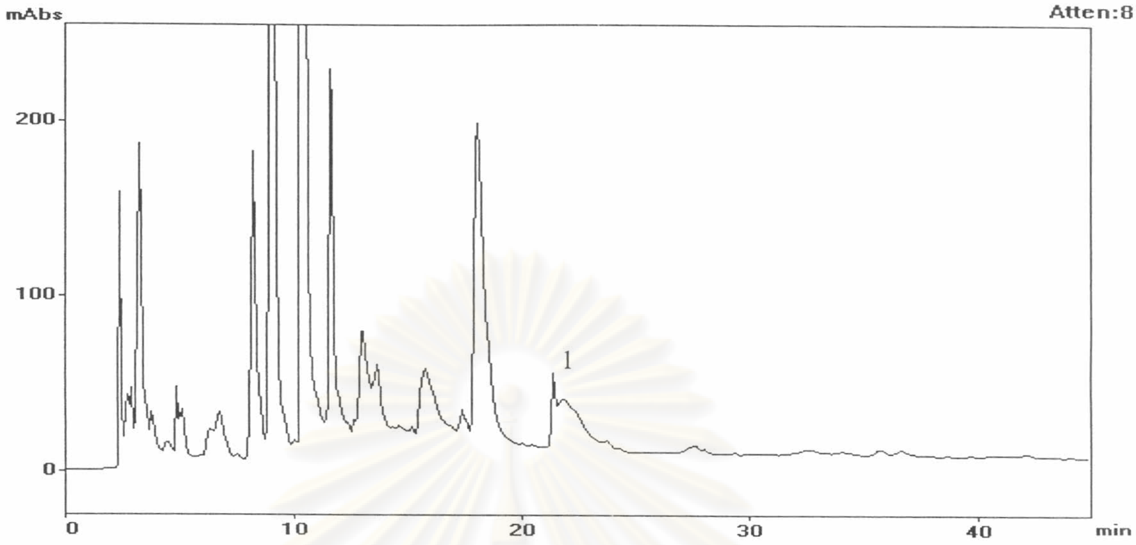


Figure 107 Isoflavone HPLC fingerprint of *P. mirifica* in chicken essence

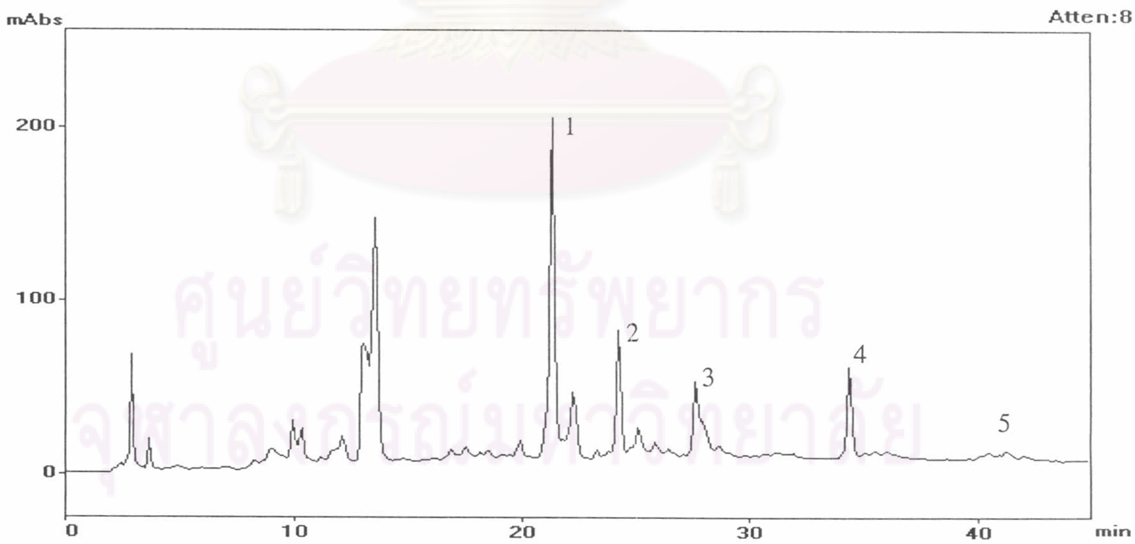


Figure 108 Isoflavone HPLC fingerprint of gamma-irradiated *P. mirifica*

APEENDIX III

HPLC FINGERPRINTS OF *B. superba*

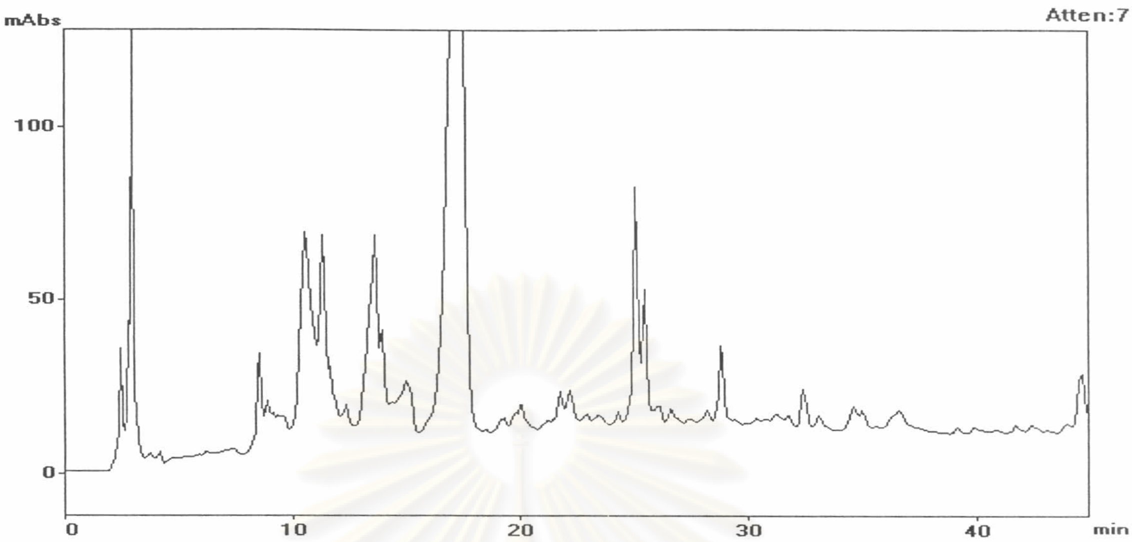


Figure 109 HPLC fingerprint of wild *B. superba* collected in Lampang province

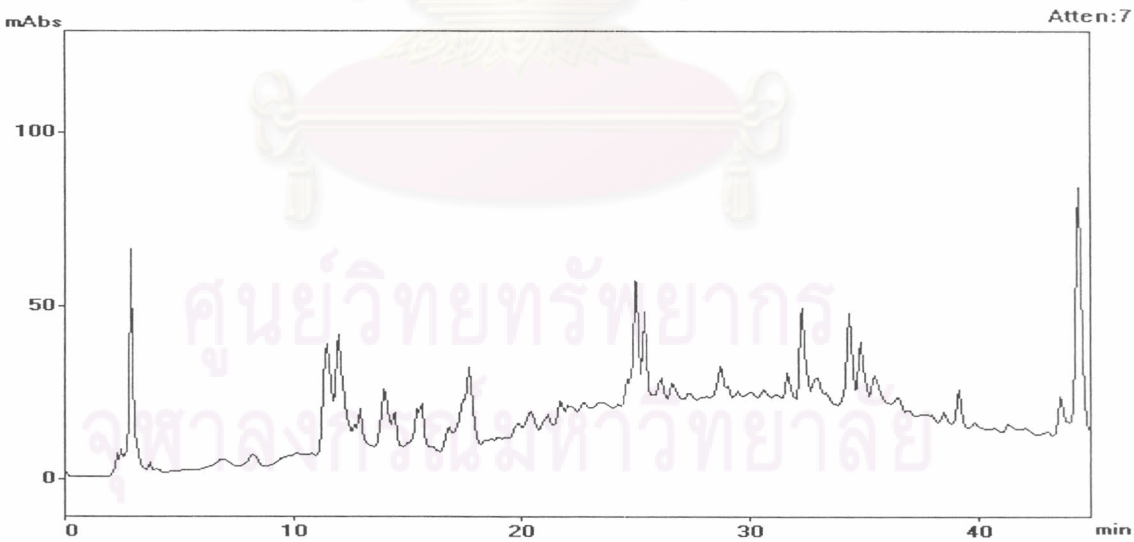


Figure 110 HPLC fingerprint of wild *B. superba* collected in Ratchaburi province

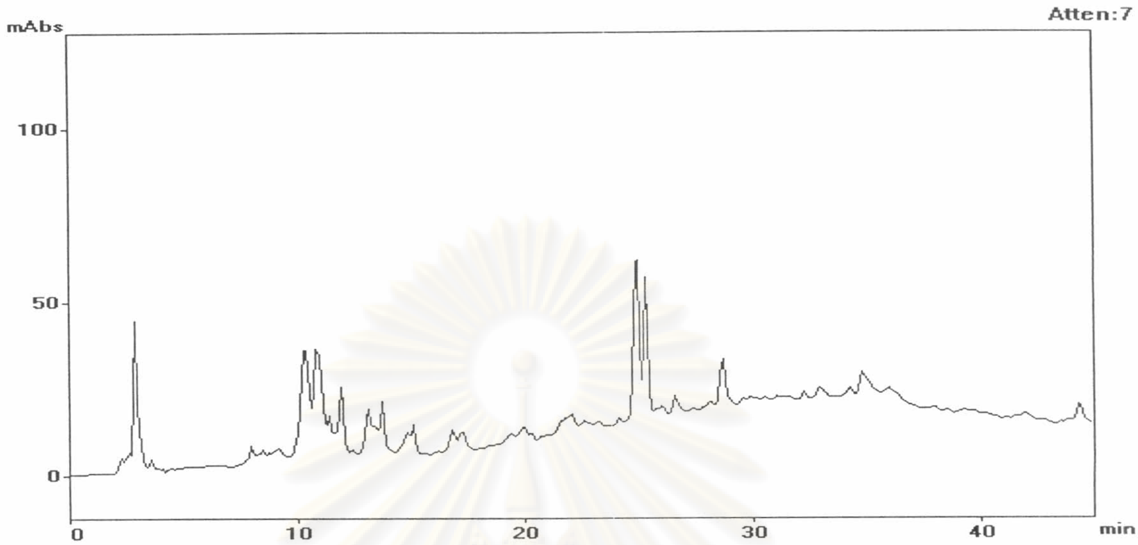


Figure 111 HPLC fingerprint of wild *B. superba* collected in Khon Kaen province

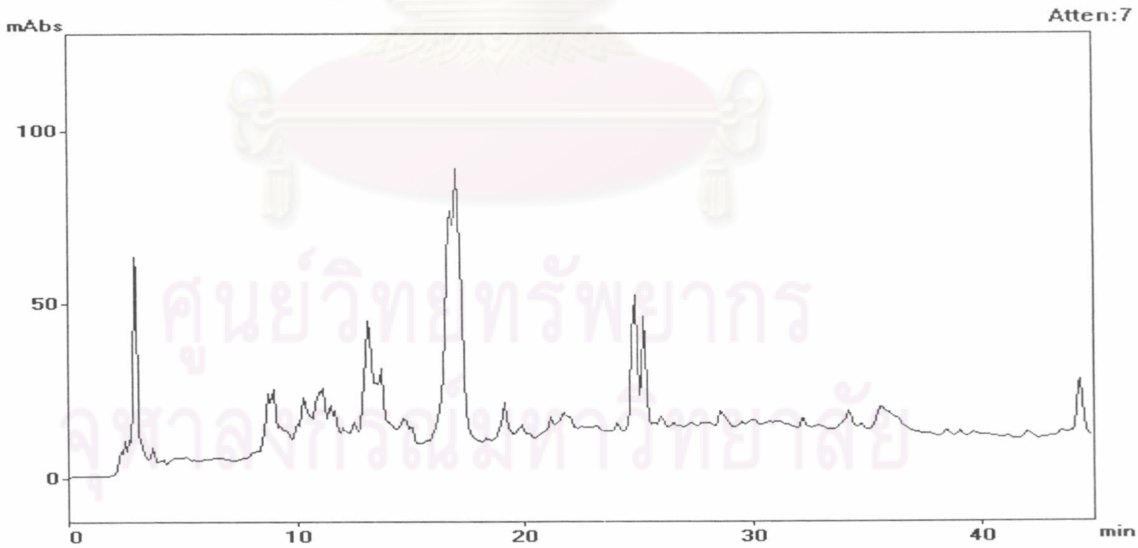


Figure 112 HPLC fingerprint of wild *B. superba* collected in Chantaburi province

APPENDIX IV

HPLC FINGERPRINTS OF *M. collettii*

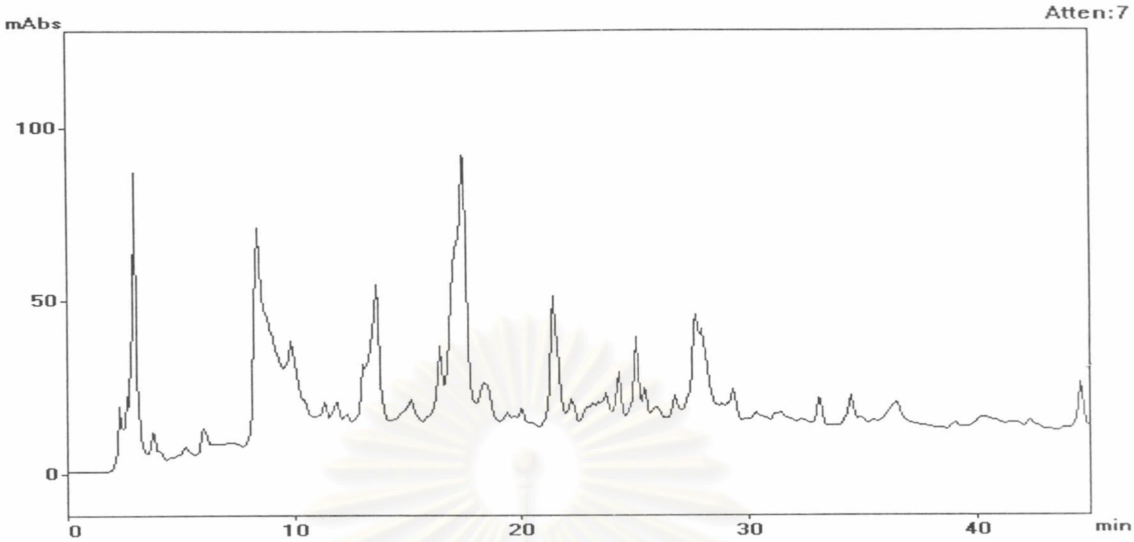


Figure 113 HPLC fingerprint of wild *M. collettii* collected in Chiang Rai province

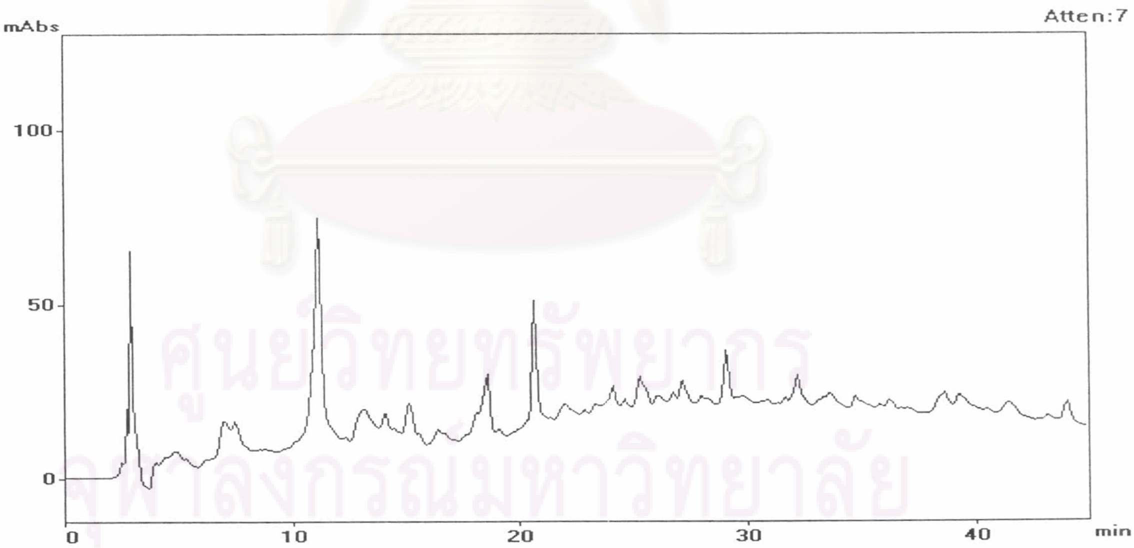


Figure 114 HPLC fingerprint of wild *M. collettii* collected in Lampang province

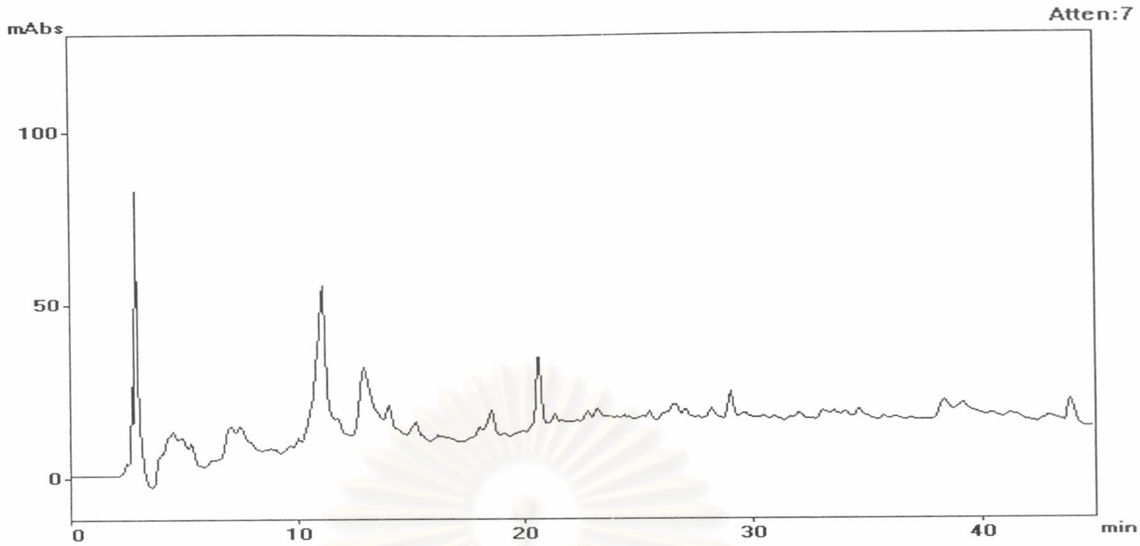


Figure 115 HPLC fingerprint of wild *M. collettii* collected in Kanchanaburi province

Biography

Ms. Subongkoch Subtang was born December 13, 1970 in Lamphang, Thailand. She was graduated with Bachelor degree of Science in Chemistry, Faculty of Science, Naraesuan University in 1992. She has enrolled in the Graduate School, Chulalongkorn University for Master Degree of Science in Biotechnology during 2000-2003.