## 3.2 MULTI-STEPS INFRARED MACRO-FINGERPRINTING OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

### 3.2.1 One-dimensional Infrared Spectral Analysis

Botanical materials could be rapidly monitored by one-dimensional infrared (1D-IR) spectroscopy and many researchers have used this technique as an initial step in the identification and quality control of medicinal herbs (Lu *et al.*, 2008). Figure 3.2.1.1 shows the comparison of 1D-IR spectra obtained for the leaves of *P. rotundifolia* and *P. praetermissa* collected from the Pasoh Forest Reserve (Negeri Sembilan), Labis Forest Reserve (Johor), Ampang Forest Reserve (Selangor) and Bukit Lagong (Selangor). Based on the spectra as shown in Figure 3.2.1.1a, similar absorption peaks were observed for *P. rotundifolia* and *P. praetermissa*. Generally, all the spectra have rather similar IR absorption peaks at 3396 $cm^{-1}$, 2922 $cm^{-1}$, 1718 $cm^{-1}$, 1691 $cm^{-1}$, 1622 $cm^{-1}$, 1443 $cm^{-1}$, 1370 $cm^{-1}$, 1313 $cm^{-1}$, 1229 $cm^{-1}$, 1149 $cm^{-1}$, 1099 $cm^{-1}$, 1027 $cm^{-1}$ and 912 $cm^{-1}$. The peak at 1313 $cm^{-1}$ showed more or less the same intensity in samples collected from all the locations (Figure 3.2.1.1b). However, some peaks in the range of 1900-850 $cm^{-1}$ showed slight variation in shape or intensity. The absorption peak at 1531 $cm^{-1}$ was only obvious in the spectra of *P. praetermissa* collected from Bukit Lagong. However, this sample did not show the peak at 1405 $cm^{-1}$ that was present in all other samples. Apart from that, the broad peak at 1370 $cm^{-1}$ was not found in the spectra of *P. praetermissa* collected from the Pasoh Forest Reserve. In this way, samples of *P. praetermissa* from the three locations can be distinguished from each other by studying their peak positions at 1531 $cm^{-1}$, 1405 $cm^{-1}$ and 1370 $cm^{-1}$. Conversely, the spectra of *P. rotundifolia* collected from the Pasoh Forest Reserve, Takar Melor and Sungai Batang exhibited similar absorption pattern. The IR absorption bands of different functional groups are tabulated in Table 3.2.1.1.

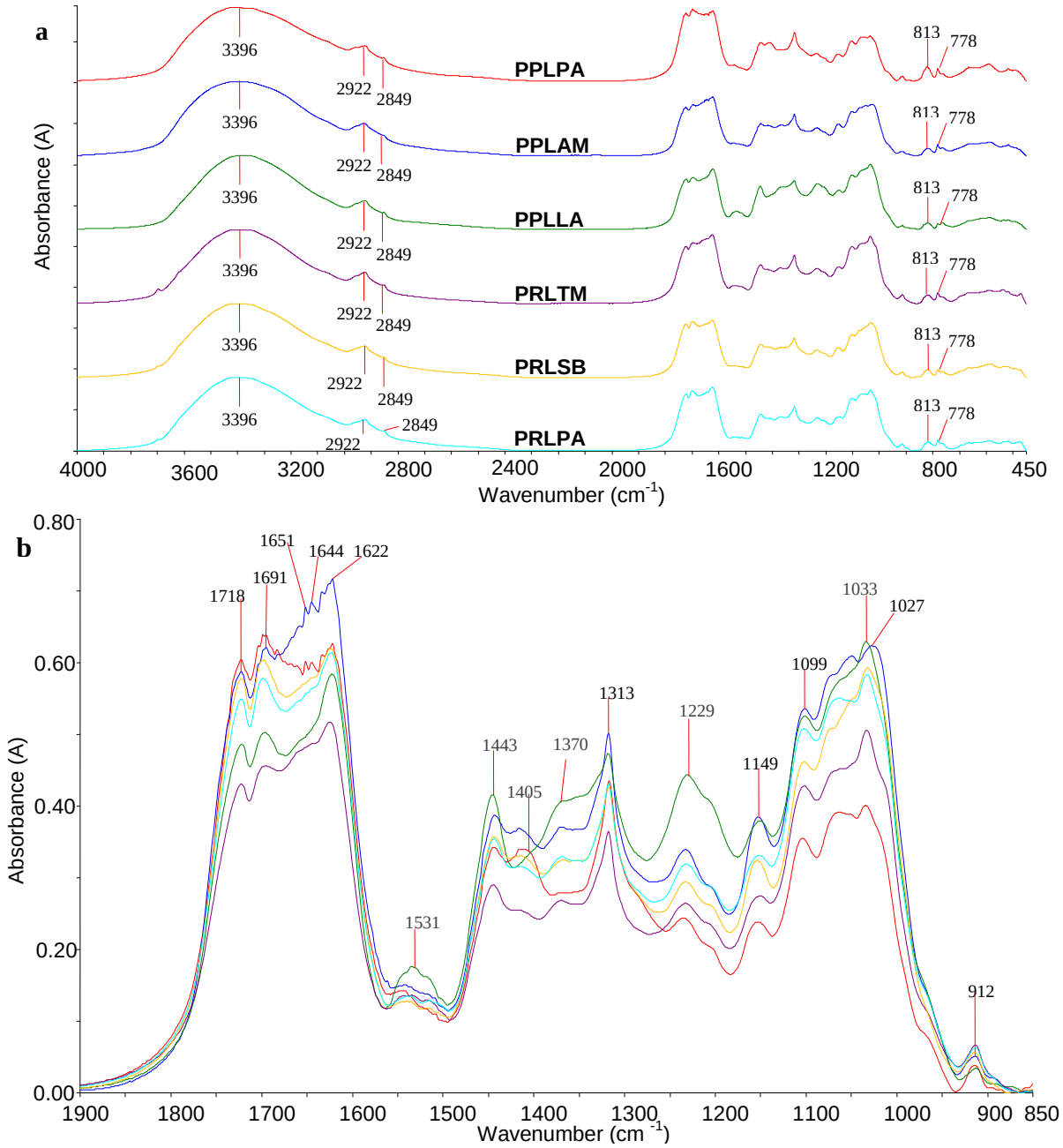



**Figure 3.2.1.1: a)** ID-IR spectra (4000-450 $cm^{-1}$) and **b)** Expanded spectra (1900-850 $cm^{-1}$) for *P. praetermissa* and *P. rotundifolia* collected from different localities.

**Table 3.2.1.1:** IR Spectral data of *P. rotundifolia* and *P. praeterm*issa.

| Wavenumber ($cm^{-1}$) | Base group and Vibration mode | Main attribution | References |
|---|---|---|---|
| 3396 | ν(O-H) | hydroxyl | Smith, 1998 |
| 2922 | $\nu_{as}$(C-H) | methylene | Smith, 1998 |
| 2849 | $\nu_{s}$(C-H) | methylene | Smith, 1998 |
| 1718, 1691 | ν(C=O) | ester, carbonyl, ketone | Noda, 2004 |
| 1622 | ν(C=C) | aromatic benzene ring | Lai *et al.*, 2009 |
| 1443 | ν(C-H) | methylene | Wu *et al.*, 2008a |
| 1313 | ν(C-C-O) | aromatic ester | Smith, 1998 |
| 1370 | δ(C-H) | methyl | Wu *et al.*, 2008a |
| 1229 | ν(C-O) | phenolic hydroxyl | Wu *et al.*, 2008a |
| 1149 | δ(C-H) | methyl, phenyl | Wu *et al.*, 2008a |
| 1099 | ν(C-O) | ester | Smith, 1998 |
| 1027 | ν(O-C-C) | ester | Smith, 1998 |
| 912 | γ(C-H) | end methylene | Wu *et al.*, 2008a |
| 813, 778 | γ(C-H) | benzene ring | Smith, 1998 |

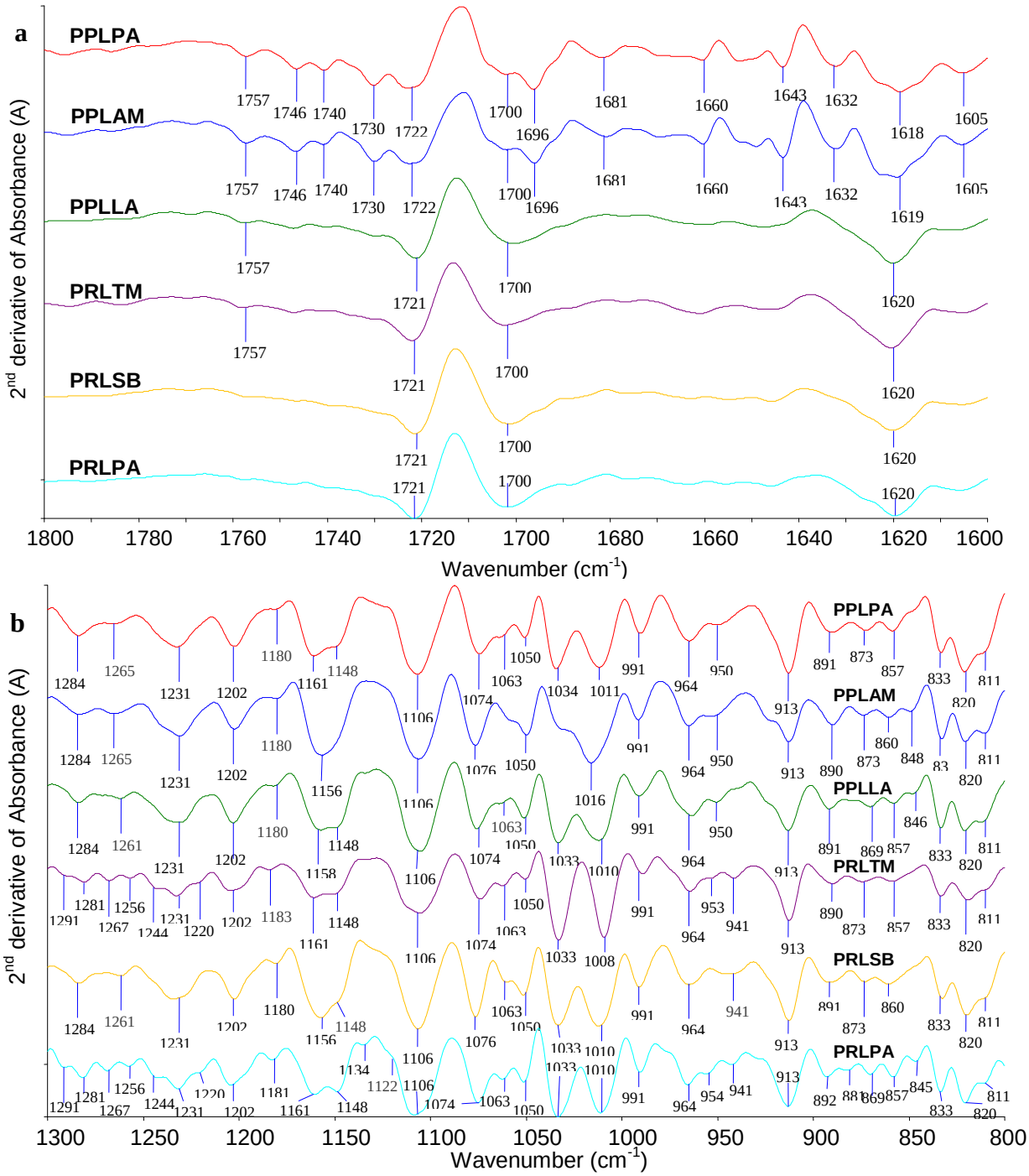
Note: ν, stretching or vibration; δ, in plane deformation; γ, out-of-plane deformation; $_{s}$, symmetrical; $_{as}$, asymmetrical.

### 3.2.2 Second Derivative Infrared Spectral Analysis

Second derivative infrared (IR) spectroscopy, the second step of multi-steps IR macro-fingerprinting could enhance the spectral resolution by amplifying tiny differences in the IR spectrum (Liu *et al.*, 2006a; Huang *et al.*, 2008; Wu *et al.*, 2008a). Some overlapped absorption peaks can be resolved by second derivative spectral analysis. Figure 3.2.2.1 shows the second derivative IR spectra of six representative samples of *P. rotundifolia* and *P. praetermissa* collected from the Pasoh Forest Reserve, Labis Forest Reserve, Ampang Forest Reserve and Bukit Lagong. More dissimilarities in term of absorption bands were observed among samples of *P. praetermissa* in the region of 1800-1600 $cm^{-1}$ (Figure 3.2.2.1a). Samples from the Pasoh and Ampang Forest Reserves showed characteristic peaks at 1746 $cm^{-1}$, 1740 $cm^{-1}$, 1730 $cm^{-1}$, 1696 $cm^{-1}$, 1681 $cm^{-1}$, 1660 $cm^{-1}$, 1643$cm^{-1}$ and 1632 $cm^{-1}$ but they were not obvious in samples from Bukit Lagong. The absence of these peaks could be used to discriminate samples of Bukit Lagong from that of the Pasoh and Ampang Forest Reserves. In the region of 1300-800 $cm^{-1}$, samples of the Pasoh Forest Reserve and Bukit Lagong demonstrated two strong absorption peaks at ~1034 $cm^{-1}$ and 1011 $cm^{-1}$, while samples of the Ampang Forest Reserve only showed one strong peak at 1016 $cm^{-1}$. In addition, the spectra of samples from the Pasoh Forest Reserve and Bukit Lagong exhibited two broad peaks at ~1161 $cm^{-1}$ and 1148 $cm^{-1}$ but that of the Ampang Forest Reserve has a sharp absorption peak at 1156 $cm^{-1}$ instead. The spectral features described above can be used to differentiate samples of the Pasoh Forest Reserve from that of the Ampang Forest Reserve.

On the other hand, samples of *P. rotundifolia* demonstrated some differences only in the region of 1300-800 $cm^{-1}$. The samples of Takar Melor and Pasoh Forest Reserve were characterised by the presence of absorption peaks at 1291 $cm^{-1}$, 1281 $cm^{-1}$, 1267 $cm^{-1}$, 1256 $cm^{-1}$, 1244 $cm^{-1}$, 1231 $cm^{-1}$ and 1220 $cm^{-1}$ while samples of Sungai Batang were

showing peaks at 1284 $cm^{-1}$, 1261 $cm^{-1}$, 1231 $cm^{-1}$ and 1202 $cm^{-1}$ (Figure 3.2.2.1b). These spectral features could be used for differentiation among the samples of *P. rotundifolia*.




Legend:

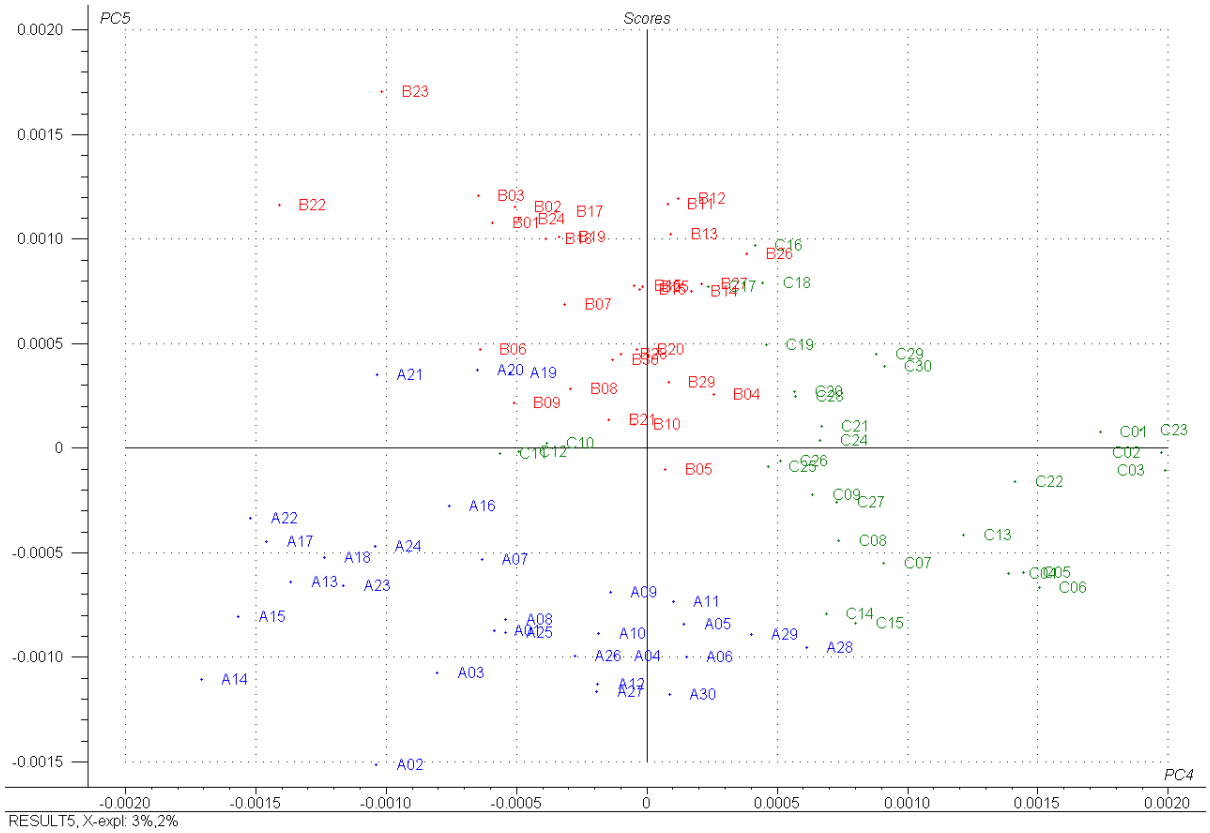
| | |
|---|---|
| **PRLPA** | *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan |
| **PRLSB** | *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor |
| **PRLTM** | *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor |
| **PPLLA** | *P. praetermissa* from Bukit Lagong, Selangor |
| **PPLAM** | *P. praetermissa* from Ampang Forest Reserve, Selangor |
| **PPLPA** | *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan |

**Figure 3.2.2.1:** Representative second derivative spectra for *P. rotundifolia* and *P. praetermissa* collected from different localities in the region of **a**) 1800-1600 $cm^{-1}$ and **b**) 1300-800 $cm^{-1}$.

### 3.2.3 Principal Component Analysis (PCA) of Infrared Spectra

Principal component analysis (PCA) is one of the major techniques used for chemical pattern recognition in exploratory data analysis (EDA). This technique is able to give a visual idea for the relationship between samples (Brereton, 2003; 2007). PCA is a useful tool in multivariate statistics analysis that can relate chemical function using a mathematical transformation (James *et al.*, 2005). In this study, the ten individual each in triplicate with a total of thirty spectra from each locality were statistically analyzed using PCA. All the spectra were preprocessed by multivariant scattering correction (MSC), baseline correction and second derivative using Savitzky-Golay with 13 points smoothing. After optimization, the spectral range of 2000-450 $cm^{-1}$ was utilised for analysis within the same species from different localities while the whole spectral range (4000-450 $cm^{-1}$) was used for comparing *P. rotundifolia* and *P. praetermissa*. Figures 3.2.3.1 and 3.2.3.2 show the PC scores plot of samples from different localities. In the *P. rotundifolia* scores plot, overlapping data was presented for samples collected from Sungai Batang (B) and Takar Melor (C) since the two samples were originated from the same forest, (Labis Forest Reserve) but from different plots (Figure 3.2.3.1). In the PC scores plot of *P. praetermissa*, samples from the Pasoh Forest Reserve, Ampang Forest Reserve and Bukit Lagong were able to separate from each other in three groups. Figure 3.2.3.3 displays the PC scores plot of ten individuals of *P. rotundifolia* and *P. praetermissa* from each location and Figure 3.2.3.4 shows the 3D scores plot. The 3D scores plot is able to give more visualize results using three principal components which enhances the separation of the overlapping groups. The first three PCs, PC1 (32 %), PC2 (27 %) and PC3 (7 %) explained 66 % of the total variance in the PC scores plot. Certain pattern is illustrated among the samples of *P. rotundifolia* (red) and *P. praetermissa* (blue) as shown in Figure 3.2.3.4a. The samples of *P. rotundifolia* (A-C) showed overlapping results among location A and B (Figure

3.2.3.4b). The result suggested the similarity of chemical characteristic in *P. rotundifolia* from Pasoh Forest Reserve (A) and Sungai Batang (B) and Takar Melor (C). On the other hand, samples of *P. praetermissa* (D-F) from Bukit Lagong (D), the Ampang Forest Reserve (E) and Pasoh Forest Reserve (F) were presented in three clusters (Figure 3.2.3.4b). Consequently, the identification and discrimination of *P. rotundifolia* and *P. praetermissa* could be realised by principal components analysis. A certain degree of variations among the samples could also be derived using PCA which could further provide characterisation among the samples.




Legend:

| Location | ID | Individual | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **PRLPA** | A | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |
| **PRLSB** | B | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |
| **PRLTM** | C | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |

**PRLPA:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **PRLSB:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **PRLTM:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve,Johor;

**Figure 3.2.3.1:** PCA scores plot of *P. rotundifolia* collected from different localities.


Legend:

| Location | ID | Individual | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **PPLLA** | **D** | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |
| **PPLAM** | **E** | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |
| **PPLPA** | **F** | 1-3 | 4-6 | 7-9 | 10-12 | 13-15 | 16-18 | 19-21 | 22-24 | 25-27 | 28-30 |

**PPLLA:** *P. praetermissa* from Bukit Lagong, Selangor; **PPLAM:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **PPLPA:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan.

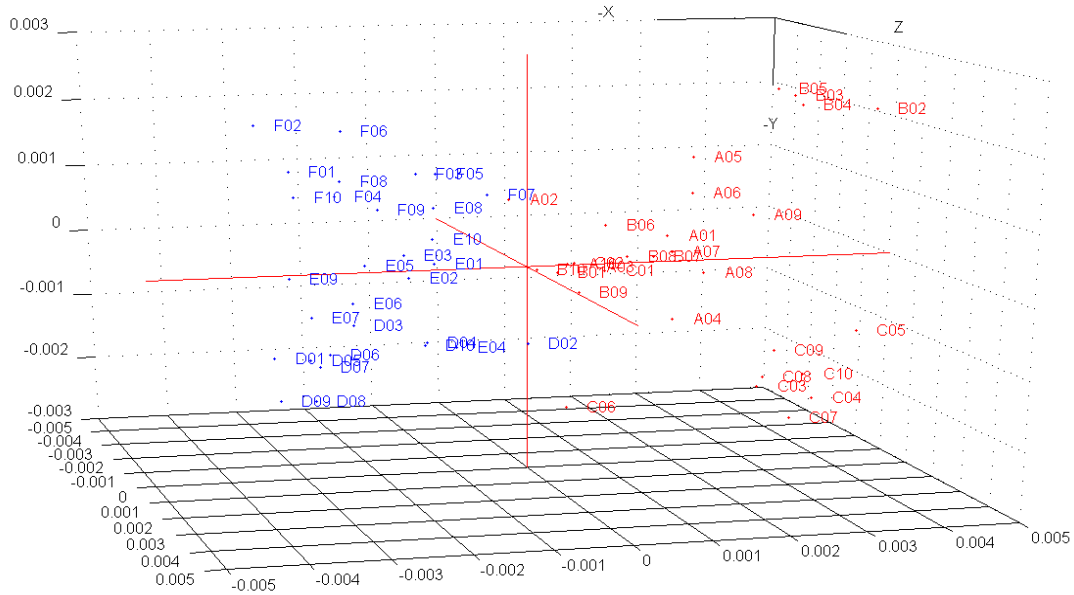
**Figure 3.2.3.2:** PCA scores plot of *P. praetermissa* collected from different localities.


Legend:

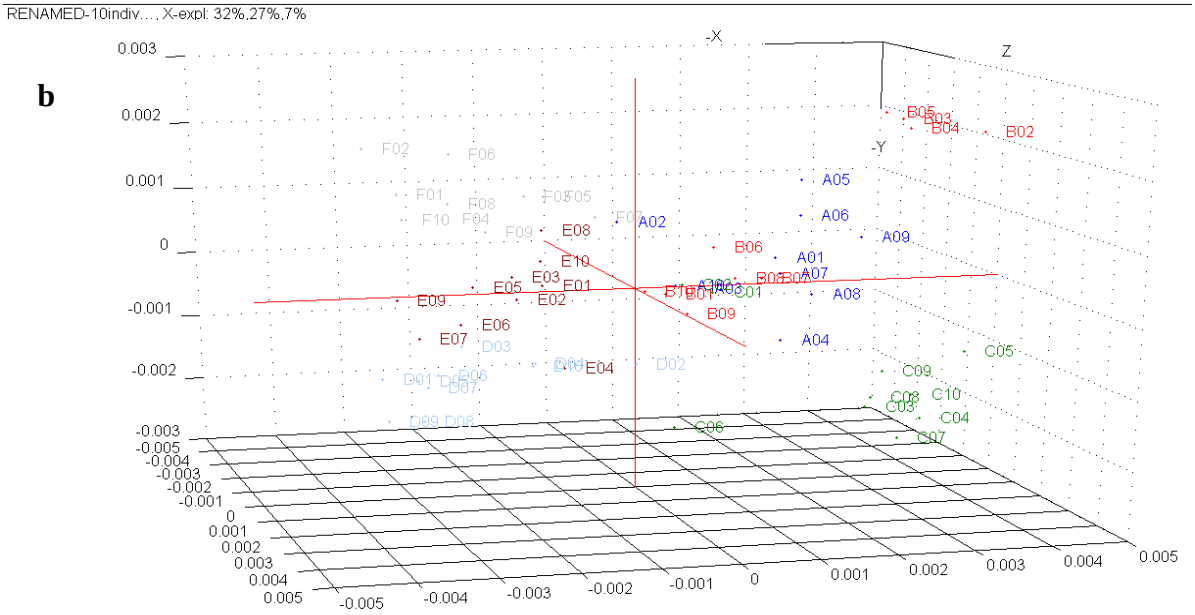
| | | |
|---|---|---|
| **A** | **PRLPA** | *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan |
| **B** | **PRLSB** | *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor |
| **C** | **PRLTM** | *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor |
| **D** | **PPLLA** | *P. praetermissa* from Bukit Lagong, Selangor |
| **E** | **PPLAM** | *P. praetermissa* from Ampang Forest Reserve, Selangor |
| **F** | **PPLPA** | *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan |

**Figure 3.2.3.3:** PCA scores plot of *P. rotundifolia* and *P. praetermissa* collected from each location.

**a**




**b**




X:PC1, Y:PC2, Z:PC3

**Figure 3.2.3.4:** PCA scores plot of **a**) *P. rotundifolia* (red) and *P. praetermissa* (blue) from **b**) different localities (**A:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **B:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **C:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor; **D:** *P. praetermissa* from Bukit Lagong, Selangor; **E:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **F:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan).

### 3.2.4 Two-dimensional Correlation Infrared Spectral Analysis

Two-dimensional (2D) correlation infrared (IR) spectroscopic technique can be applied to different types of samples stimulated by various perturbation methods such as temperature, composition, chemical reactions, optical and biological processes with a number of analytical tools (Noda, 2010). The 2D-correlation IR analysis is the study of spectrum through the vibration with external perturbation which is able to enhance the resolution of the spectra. For instance, the 2D-correlation IR spectrum based on thermal perturbation reveals molecular vibrating behavior of relative group of a molecule during the temperature perturbation (Li *et al.*, 2004; Liu *et al.*, 2008). The 2D-correlation synchronous plot is attained from forward Fourier transform of a dynamic spectrum at spectral variable and conjugate of the other Fourier transform of a dynamic spectrum and calculated over the interval of thermal perturbation (Noda, 2004). 2D-correlation IR spectral analysis with thermal perturbation has been utilised for identification and classification of traditional medicine. It serves as a distinct fingerprint for the complex mixture of chemical components in herbs.

In order to enhance the spectral resolution, we carried out the synchronous 2D-IR spectroscopy under thermal perturbation from 50-120 ˚C. All the spectra were subjected to baseline automatic correction and the lowest points of the smooth range of 2500-2000 $cm^{-1}$ of all spectra were set to be zero. The synchronous 2D-IR spectroscopic analysis further differentiated *P. rotundifolia* and *P. praetermissa* from the Labis Forest Reserve, Pasoh Forest Reserve, Ampang Forest Reserve and Bukit Lagong. Figure 3.2.4.1 presents the synchronous plots, auto peaks and the mesh plots of *P. rotundifolia* and *P. praetermissa* in the range of 1500-1000 $cm^{-1}$. All the samples of *P. rotundifolia* exhibited a cross peak at (1492 $cm^{-1}$, 1181 $cm^{-1}$) in the synchronous contour plot. This cross peak however was not shown obviously in samples of *P. praetermissa*. Therefore, it could be used to discriminate

the two *Phyllagathis* species. The samples of *P. rotundifolia* from Takar Melor, Sungai Batang and Pasoh Forest Reserve also showed similarity in their synchronous contour plots and mesh plots due to similar chemical characteristic. On the other hand, a relatively stronger positive cross peak at (1141 $cm^{-1}$, 1091 $cm^{-1}$) was noted for *P. praetermissa* from the Pasoh Forest Reserve while in the Ampang Forest Reserve, a weaker positive cross peak was observed at (1455 $cm^{-1}$, 1422 $cm^{-1}$) as compared to the other localities. There were six obvious auto peaks at 1090 $cm^{-1}$ (peak 1), 1140 $cm^{-1}$ (peak 2), 1190 $cm^{-1}$ (peak 3), 1311 $cm^{-1}$ (peak 4), 1420 $cm^{-1}$ (peak 5) and 1450 $cm^{-1}$ (peak 6) in all the samples. However, their signal intensities were different and their relative intensities are tabulated in Table 3.2.4.1. In general, samples of *P. rotundifolia* presented low values of relative intensity value for auto peaks at 1450 $cm^{-1}$, 1420 $cm^{-1}$ and 1311 $cm^{-1}$. In the case of *P. praetermissa*, the auto peaks at 1420 $cm^{-1}$ and 1311 $cm^{-1}$ in Bukit Lagong sample have lower intensities but at 1450 $cm^{-1}$, a higher intensity was observed as compared to samples of the other two locations. The peak at 1420 $cm^{-1}$ showed the highest value of relative intensity in samples of the Pasoh Forest Reserve. These differing features can be used to discriminate the three samples effectively. Therefore, the 2D-correlation IR analysis with thermal perturbation can be used as a fingerprint approach for the characterisation of *P. rotundifolia* and *P. praetermissa*.

**Table 3.2.4.1:** The relative values of intensities for the auto peaks of *P. rotundifolia* from Pasoh Forest Reserve (PRLPA), Sungai Batang (PRLSB), Takar Melor (PRLTM) and *P. praetermissa* from Bukit Lagong (PPLLA), Ampang Forest Reserve (PPLAM), Pasoh Forest Reserve (PPLPA) in the range of 1500-1000 $cm^{-1}$.

| | | *P. rotundifolia* | | | *P. praetermissa* | | |
|---|---|---|---|---|---|---|---|
| Peak number | Wavenumber ($cm^{-1}$) | PRLPA | PRLSB | PRLTM | PPLLA | PPLAM | PPLPA |
| 1 | 1090 | 0.416 | 0.362 | 0.358 | 0.438 | 0.472 | 0.688 |
| 2 | 1140 | 0.641 | 0.621 | 0.438 | 0.541 | 0.536 | 0.632 |
| 3 | 1190* | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| 4 | 1311 | 0.665 | 0.356 | 0.365 | 0.578 | 0.900 | 0.877 |
| 5 | 1420 | 0.271 | 0.477 | 0.257 | 0.199 | 0.344 | 0.741 |
| 6 | 1450 | 0.229 | 0.204 | 0.316 | 0.774 | 0.399 | 0.368 |

*The relative intensity of peak at 1190 $cm^{-1}$ is set as 1.

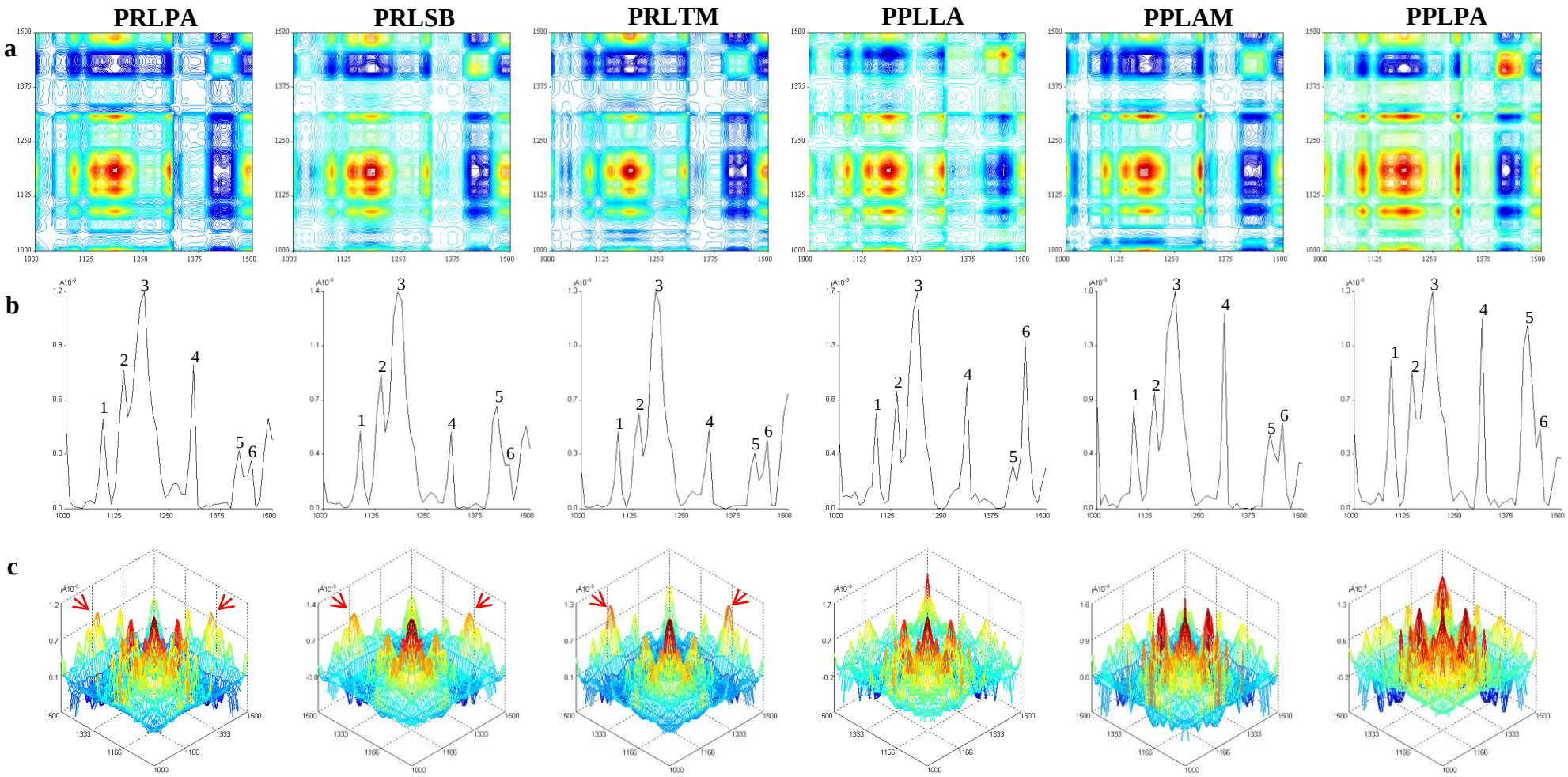



**Figure 3.2.4.1:** Synchronous 2D-IR contour plot **(a)**, synchronous 2D-IR auto peaks **(b)** and synchronous 2D-IR mesh plot **(c)** in the range of 1500-1000 $cm^{-1}$ for *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor (**PRLTM**), Sungai Batang, Labis Forest Reserve, Johor (**PRLSB**) Pasoh Forest Reserve (**PRLPA**), and *P. praetermissa* from Pasoh Forest Reserve (**PPLPA**), Ampang Forest Reserve (**PPLAM**), Bukit Lagong (**PPLLA**).

### 3.2.5 Multi-steps Infrared Macro-fingerprinting on Mixtures of *P. rotundifolia* and *P. praetermissa*

The IR analysis was performed on various mixtures of the dried and powdered leaves of *P. rotundifolia* (Pasoh Forest Reserve) and *P. praetermissa* (Bukit Lagong). In Figure 3.2.5.1, no apparent differences are observed in the 1D-IR spectra of the mixtures which varied from 100-0 % of *P. rotundifolia* (from top to bottom) and *vice versa* for *P. praetermissa*. However, when the data in the region of 1300-800 $cm^{-1}$ were derived to second derivatives spectra (Figure 3.2.5.1b), some changes in peak positions and intensities were observed in the range of 1300-1200 $cm^{-1}$ and 1150-1100 $cm^{-1}$ as shown in green and purple boxes. When the composition of *P. praetermissa* was increased, absorption peaks at 1291 $cm^{-1}$, 1281 $cm^{-1}$, 1267 $cm^{-1}$, 1256 $cm^{-1}$, 1244 $cm^{-1}$ and 1220 $cm^{-1}$ were substituted by absorption peaks at 1284 $cm^{-1}$ and 1261 $cm^{-1}$. These features are similar to that of *P. praetermissa* from Bukit Lagong. In addition, peaks at 1134 $cm^{-1}$ and 1122 $cm^{-1}$ were observed at lower intensities when the concentration of *P. praetermissa* was increased in the sample mixture.

Principal component analysis (PCA) is able to reduce the large data size without removing essential information in mathematical calculation. Subsequently, we applied PCA to look into the possibility of discrimination between *P. rotundifolia* and *P. praetermissa* in mixtures. The 1D-IR data were baseline corrected and transformed to second derivative data using Savitzky-Golay with 13 points smoothing. Multiplicative scatter correction (MSC) was performed to decrease the effect of dispersing and non homogeneous particles on spectrum. The spectral range of 2000-450 $cm^{-1}$ was chosen due to main contribution in IR characterisation. The sample mixtures consisting of *P. praetermissa* in *P. rotundifolia* from 0-100 % with interval of 5 % were categorised into four groups (0-25 %, 30-55 %, 60-75 % and 80-100 %). The PC scores plot reveals the trend with 50 % and 15 % of the

total variability being explained by the first two PCs. The first two PCs explained 65 % of the total variance and the variation could be due to differences in chemical composition for the two species. PCA scores plot was capable to classify the samples into four groups and denoted the relationships of samples by image (Figure 3.2.5.2). The closer a group, the more similar these samples within the groups were considered to be. This indicated that dissimilarity between the spectra of these two species were apparent. Thus, this approach can be used for rapid detection of the presence of mixtures in the raw material which is not easily revealed using traditional chemical analysis.

The 2D-correlation IR spectra for selected mixtures of *P. praetermissa* and *P. rotundifolia* are presented in the synchronous plot as shown in Figure 3.2.5.3. The cross peak at (1492 $cm^{-1}$, 1190 $cm^{-1}$) was obvious in sample with 100 % of *P. rotundifolia* but it became less clear in sample with 100 % of *P. praetermissa*, in relation to the auto peak at 1450 $cm^{-1}$ (peak 6). Extended 2D-correlation IR with thermal perturbation presented the intensity changes of auto peaks at 1420 $cm^{-1}$ (peak 5) and 1450 $cm^{-1}$ ( Figure 3.2.5.3). The synchronous mesh plot also showed intensity changes of cross peaks and auto peaks as indicated in the visual maps by the red arrows. In this way, the 2D-IR spectroscopy can also be used in the characterisation and correlation study between two different wavenumbers of genuine mixtures of herbs upon thermal perturbation. Hence, multi-steps IR macro-fingerprinting is useful in the herbal quality control of *P. rotundifolia*.

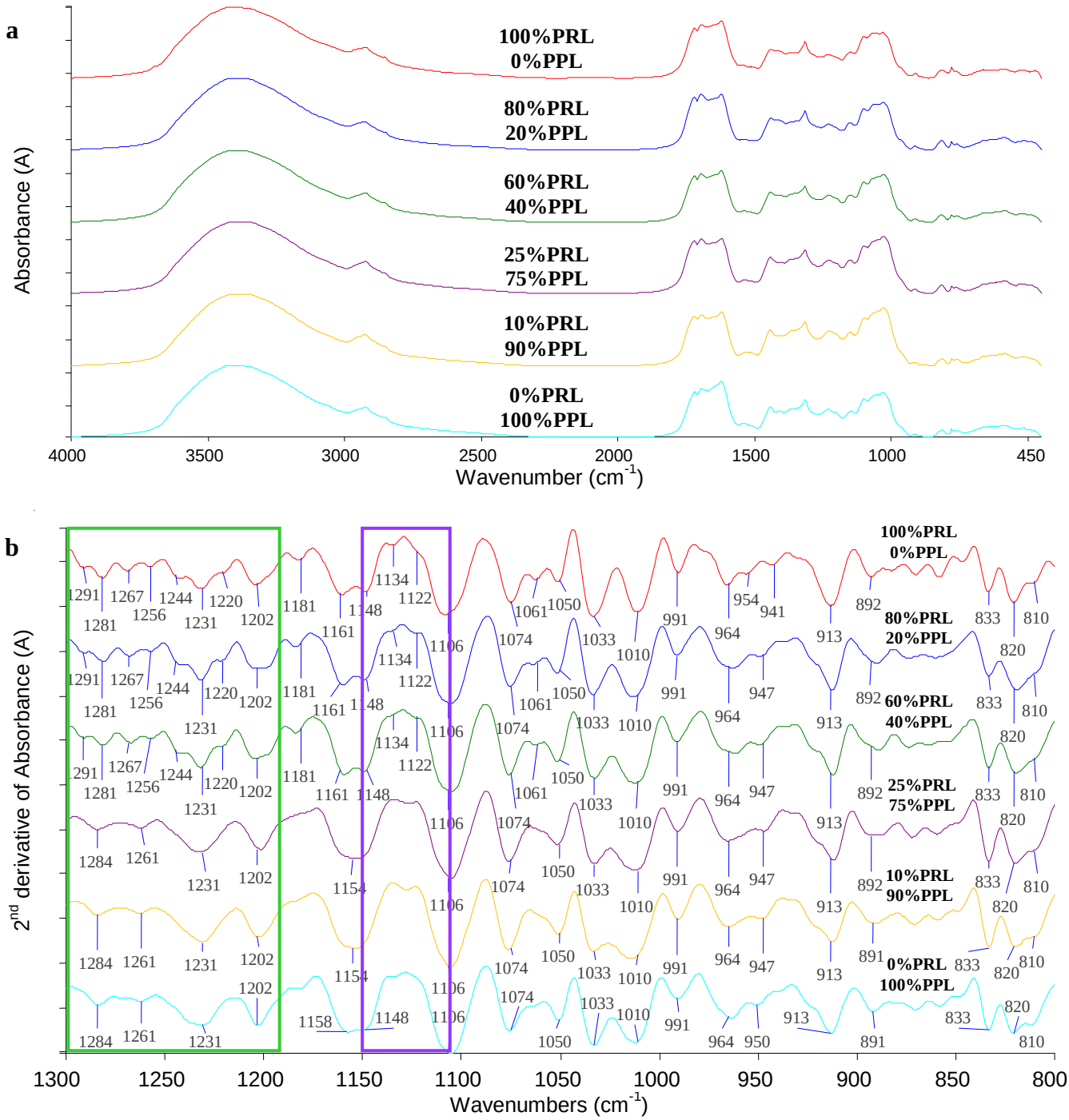



**Figure 3.2.5.1:** **a**) 1D-IR spectra (4000-450 $cm^{-1}$) and **b**) second derivative spectra (1300-800 $cm^{-1}$) for the mixture of *P. rotundifolia* (**PRL**) from Pasoh Forest Reserve and *P. praetermissa* (**PPL**) from Bukit Lagong.

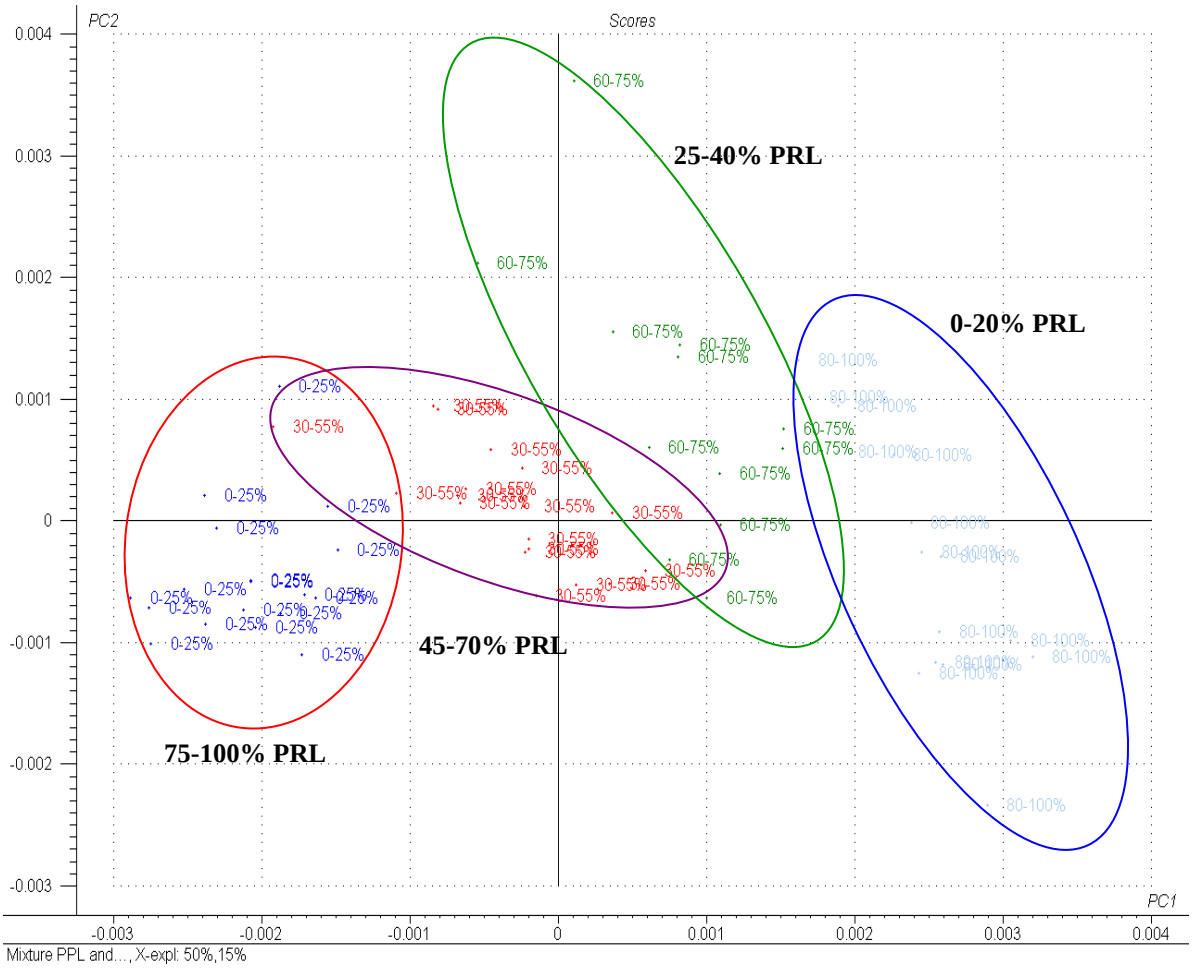



**Figure 3.2.5.2:** PCA scores plot obtained from 2000-450 $cm^{-1}$ IR spectra. The percentage indicated the adulteration of *P. praetermissa* (**PPL**) from Bukit Lagong in *P. rotundifolia* (**PRL**) from Pasoh Forest Reserve.

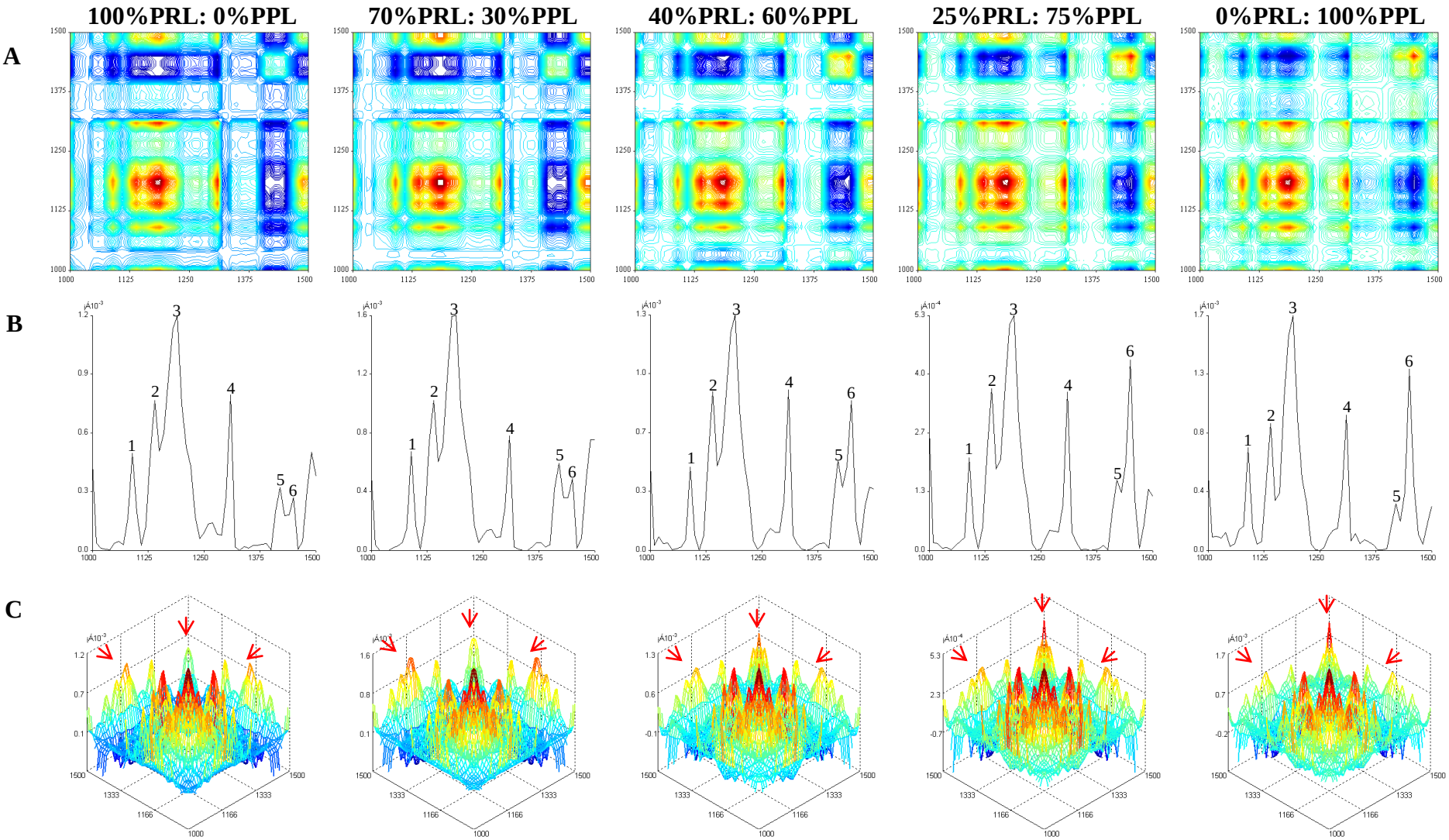


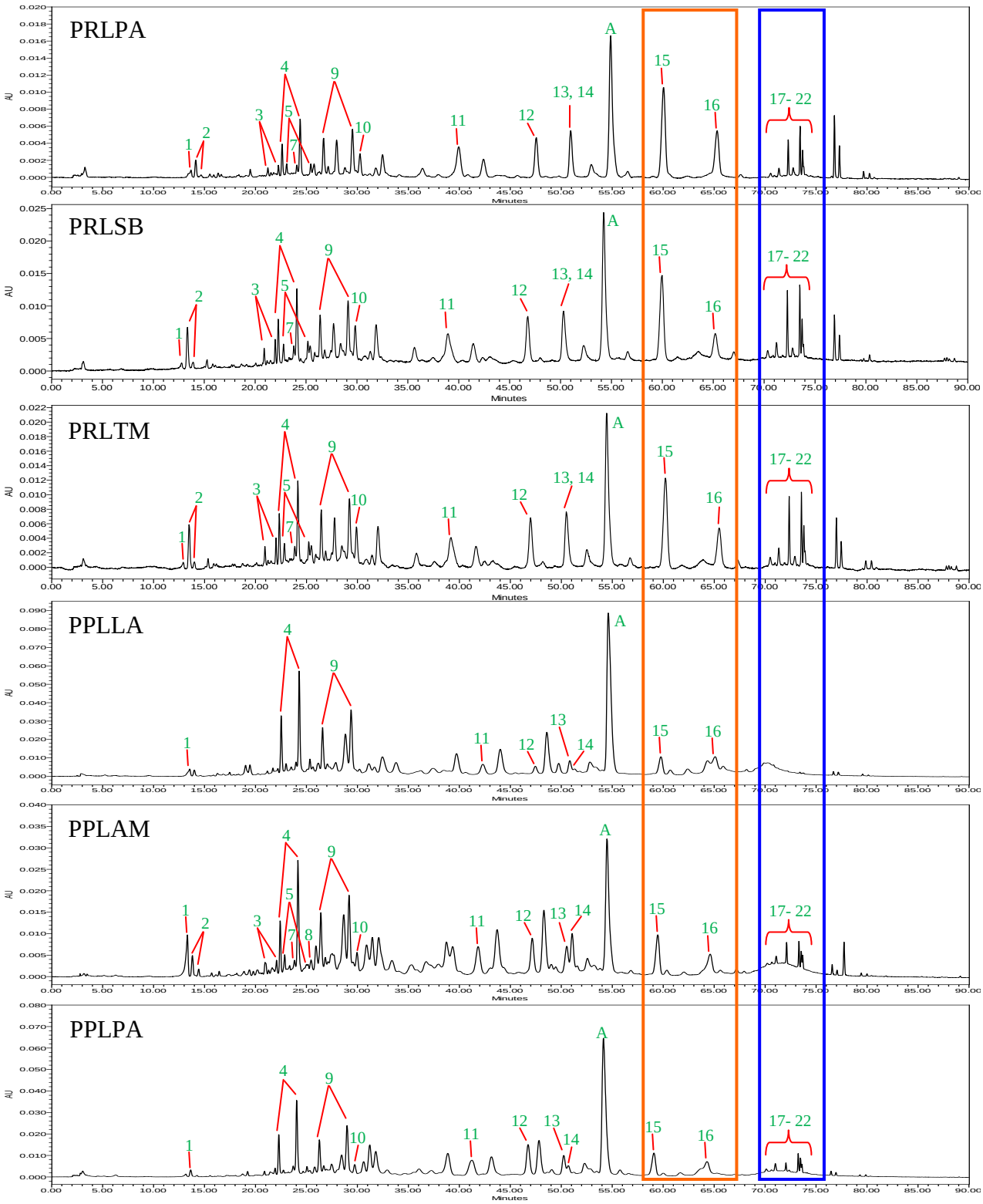

**Figure 3.2.5.3:** Synchronous 2D-IR contour plot **(A)**, synchronous 2D-IR auto peaks **(B)** and synchronous 2D-IR mesh plot **(C)** in the range of 1500-1000 $cm^{-1}$ for the mixture of *P. rotundifolia* from Pasoh Forest Reserve (**PRLPA**) and *P. praetermissa* from Bukit Lagong (**PPLLA**).

## 3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FINGERPRINTS OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

### 3.3.1 HPLC Fingerprints of *P. rotundifolia* and *P. praetermissa*

The representative HPLC chromatograms of *P. rotundifolia* (PRL) and *P. praetermissa* (PPL) from the Pasoh Forest Reserve (PA), Sungai Batang (SB) and Takar Melor (TM) from Labis Forest Reserve, Bukit Lagong (LA) and Ampang Forest Reserve (AM) are presented in Figure 3.3.1.1. The peak identities (**1-22**) were determined by spiking of the isolated compounds in these crude extracts. Generally, the HPLC fingerprints for *P. rotundifolia* (PRL) and *P. praetermissa* (PPL) exhibited similar profile. However, some levels of discrimination were found among the two species. As compared to *P. rotundifolia*, the HPLC chromatogram of *P. praetermissa* has lower peak intensities for the galloylated cyanogenic glucosides (peaks **17-22**) and these compounds were not present in *P. praetermissa* of Bukit Lagong (PPLLA). Furthermore, compounds 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (peak **15**) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (peak **16**) which represented the two major peaks in both species were shown in relatively lower peak area in *P. praetermissa* as compared to *P. rotundifolia*. Several compounds such as 6-*O*-galloyl-D-glucose (peak **2**), 3,6-di-*O*-galloyl-D-glucose (peak **3**), 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (peaks **4**), 3,4,6-tri-*O*-galloyl-D-glucose (peak **5**) and praecoxin B (peak **9**) were each observed as two peaks due to the formation of α and β-anomers of free C-1 glucose moiety. In addition, the two *Phyllagathis* species were found to contain another major compound labeled as peak **A** which was not stable in the purified form and in solution. It degraded to tetragalloyl-glucose and gallic acid methyl ester after a short period of time. Therefore, it was difficult to confirm the identity of peak **A**. All the HPLC chromatograms

were subjected to principal component analysis in order to obtain more visual discrimination for these two species. This will be discussed in the following section.



| Peaks | Retention Time (min) | $\lambda_{max}$ | Identification |
|---|---|---|---|
| **1** | 13.1 | 216, 271 | Gallic acid |
| **2** | 14.1, 15.4 | 216, 274 | 6-*O*-galloyl-D-glucose |
| **3** | 21.2, 22.3 | 216, 277 | 3, 6-di-*O*-galloyl-D-glucose |
| **4** | 22.3, 24.1 | 216, 263 | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose |
| **5** | 23.2, 25.6 | 216, 278 | 3,4,6-tri-*O*-galloyl-D-glucose |
| **6** | 23.5 | 216, 273 | Gallic acid methyl ester |
| **7** | 23.8 | 216, 279 | 1,2,3-tri-*O*-galloyl-β-D-glucose |
| **8** | 25.1 | 204, 272 | Casuarinin |
| **9** | 26.3, 29.0 | 216, 273 | Praecoxin B |
| **10** | 29.8 | 218, 279 | 1,4,6-tri-*O*-galloyl-β-D-glucose |
| **11** | 38.6 | 218, 279 | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose |
| **12** | 46.4 | 218, 280 | Pterocarinin C |
| **13** | 50.9 | 216, 274 | Prunasin 6'-*O*-gallate |
| **14** | 49.6 | 218, 281 | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose |
| **15** | 61.9 | 250, 365 | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside |
| **16** | 66.7 | 247, 367 | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside |
| **17** | 70.7 | 216, 277 | Prunasin 3',6'-di-*O*-gallate |
| **18** | 70.8 | 216, 275 | Prunasin 4',6'-di-*O*-gallate |
| **19** | 71.5 | 218, 275 | Prunasin 2',6'-di-*O*-gallate |
| **20** | 72.3 | 218, 278 | Prunasin 2',3',6'-tri-*O*-gallate |
| **21** | 73.7 | 216, 278 | Prunasin 3',4',6'-tri-*O*-gallate |
| **22** | 73.7 | 218, 279 | Prunasin 2',3',4',6'-tetra-*O*-gallate |

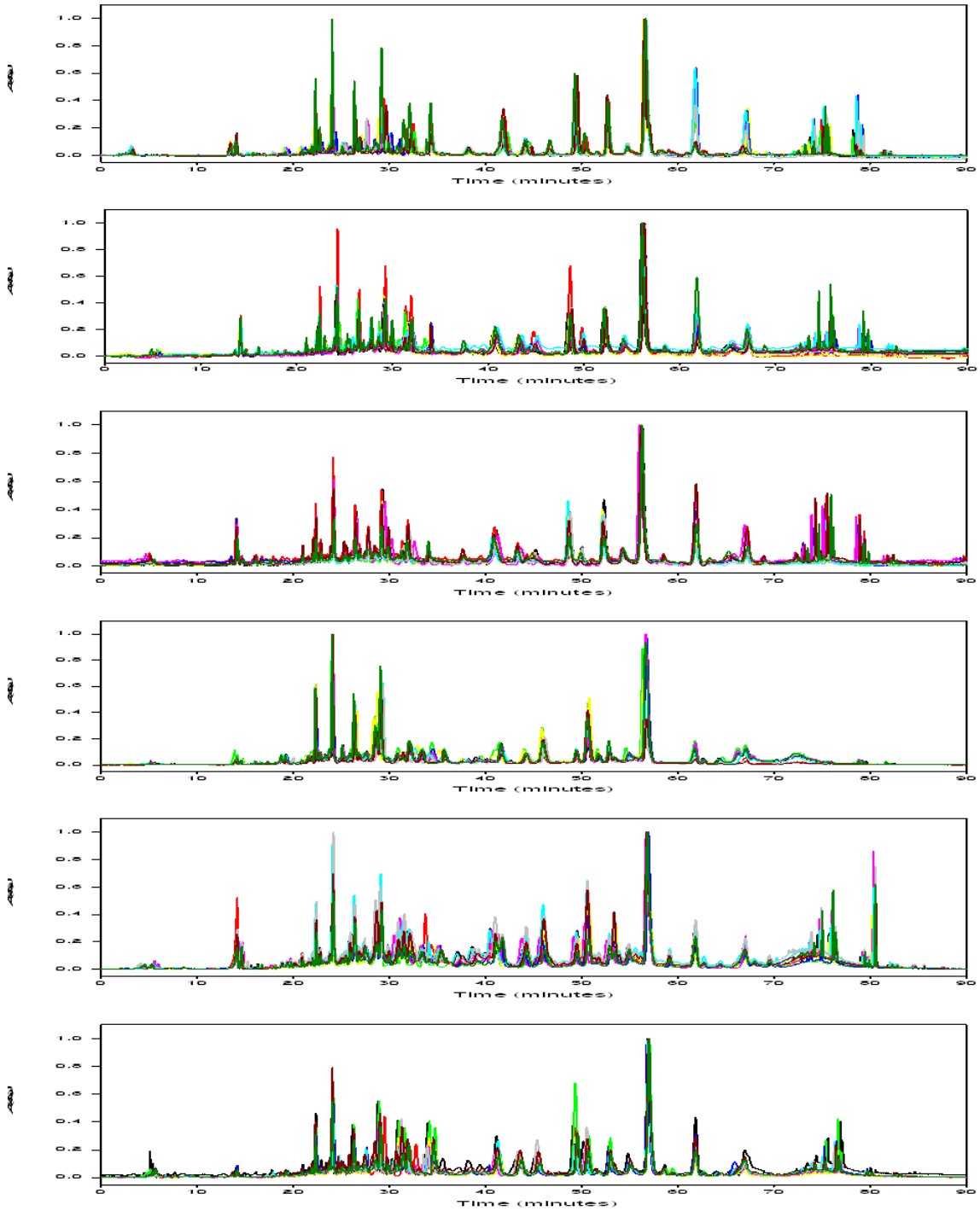
(**PRLPA:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **PRLSB:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **PRLTM:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor; **PPLLA:** *P. praetermissa* from Bukit Lagong, Selangor; **PPLAM:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **PPLPA:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan).

**Figure 3.3.1.1:** HPLC profiles from the leaves of *P. rotundifolia* and *P. praetermissa* from different localities.

### 3.3.2 Principal Component Analysis (PCA) of HPLC Chromatograms

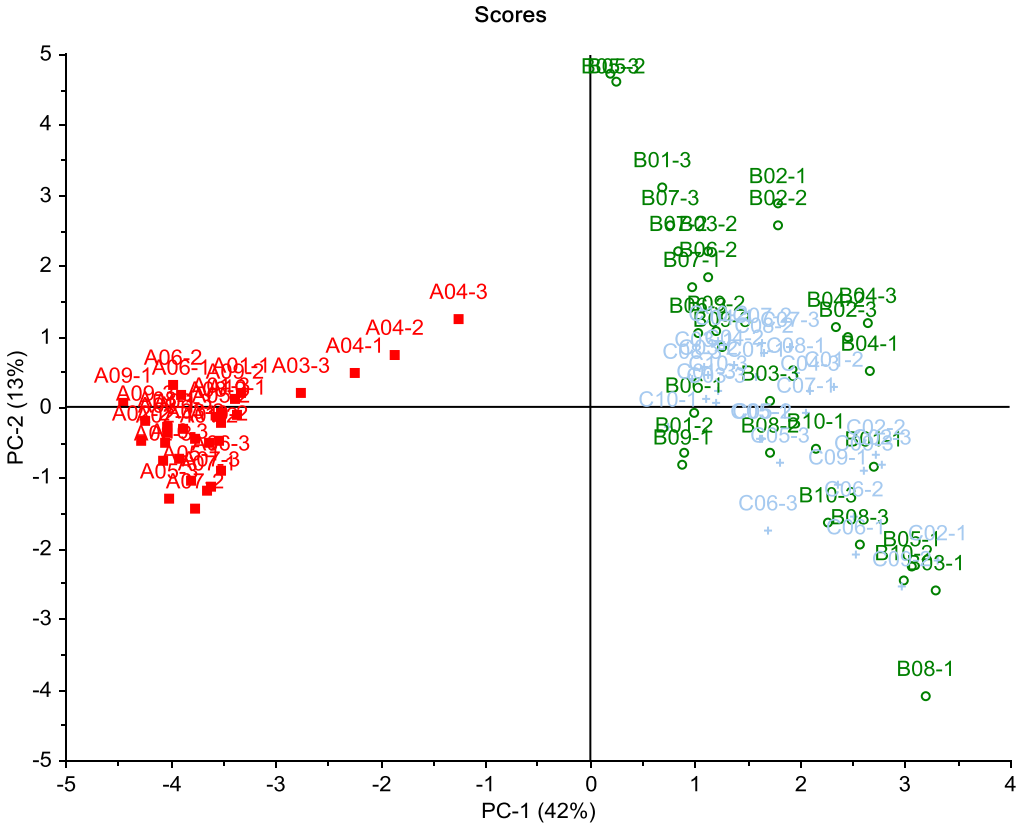
Principal component analysis (PCA) is unsupervised pattern recognition and generally it is applied to view the trends of data more clearly without losing any essential information (Brereton, 2003). A total of 180 HPLC chromatograms were obtained for *P. rotundifolia* and *P. praetermissa*. The alignment of chromatograms was essential due to instability of instrument performance as well as column ageing. Before performing the alignment of chromatograms along the time axis, a reference signal from a representative chromatogram was selected. These chromatographic profiles were then peak shifts corrected to ensure that the data were treated properly. The aligned HPLC chromatograms are illustrated in Figure 3.3.2.1. The results could be viewed as scores plot which described the PCs of the data set. From the scores plot, the grouping of the chromatographic profiles was easy to visualise. The PC scores plot for *P. rotundifolia* and *P. praetermissa* are presented in Figures 3.3.2.2 and 3.3.2.3, respectively. Two distinct groups were found in the *P. rotundifolia* scores plot, one group in Sungai Batang (B) and Takar Melor (C) and another group in the Pasoh Forest Reserve (A) as presented in Figure 3.3.2.2. The first two PCs revealed 42 % and 13 % of the total variability and 55 % of the total variance were being explained. These results indicated that the chromatographic profiles of samples from Sungai Batang and Takar Melor were rather alike. This could be explained by the similarity of environmental factors since both places were located in the Labis Forest Reserve. In the PC scores plot of *P. praetermissa*, three clusters consisting of Bukit Lagong (D), Ampang Forest Reserve (E) and Pasoh Forest Reserve (F) are illustrated in Figure 3.3.2.3 and first two PCs have explained 38 % of the total variance. The *P. praetermissa* from Bukit Lagong was differentiated from the group of Ampang Forest Reserve and Pasoh Forest Reserve groups. This result corresponded to the chromatographic profile of *P. praetermissa* collected from Bukit Lagong (Figure 3.3.1.1) where the samples did not show the presence

of galloylated cyanogenic glucosides as compared to other localities. Several samples of *P. praetermissa* from the Ampang Forest Reserve and Pasoh Forest Reserve were seen overlapped with each other that could be likely due to the similarity of chemical profiles exhibited among the individuals. Figure 3.3.2.4 shows the PC scores plot of *P. rotundifolia* and *P. praetermissa*. The first two PCs explained 48 % of the total variance where the PC1 explained 33 % and PC2 explained 15 % of the total variability. The samples of *P. praetermissa* from Bukit Lagong (D), Ampang Forest Reserve (E) and Pasoh Forest Reserve (F) were grouped by the yellow circle and the remaining cluster was consisted of *P. rotundifolia*. The results also showed that samples of *P. rotundifolia* from Sungai Batang (B) and Takar Melor (C) were clearly separated from that of Pasoh Forest Reserve (A) which indicated that the chemical profiles of *P. rotundifolia* from Labis Forest Reserve were different with that of the Pasoh Forest Reserve.




(**PRLPA:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **PRLSB:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **PRLTM:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor; **PPLLA:** *P. praetermissa* from Bukit Lagong, Selangor; **PPLAM:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **PPLPA:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan).

**Figure 3.3.2.1:** HPLC chromatogram of ten individuals for *P. rotundifolia* and *P. praetermissa* from different localities after alignment.

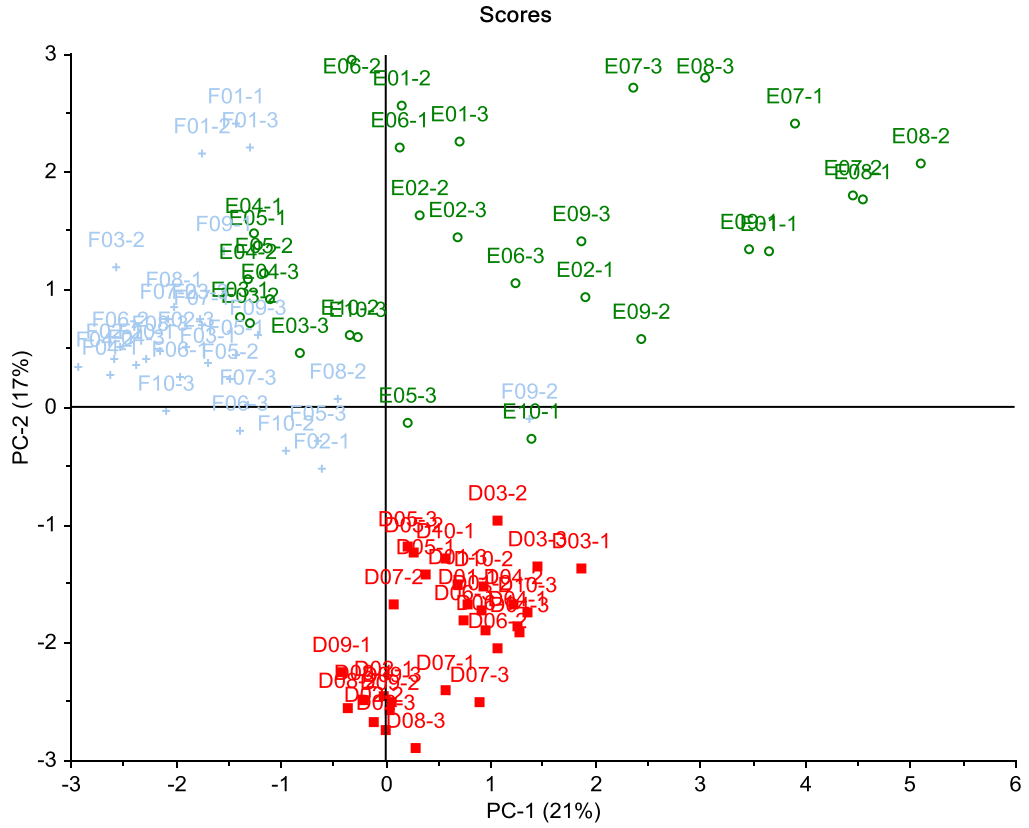



Legend:

| Location | ID | Individual | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 01- | 02- | 03- | 04- | 05- | 06- | 07- | 08- | 09- | 10- |
| **PRLPA** | A | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |
| **PRLSB** | B | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |
| **PRLTM** | C | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |

**PRLPA:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **PRLSB:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **PRLTM:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor;

**Figure 3.3.2.2:** PCA scores plot of *P. rotundifolia* chromatographic profiles from different localities.

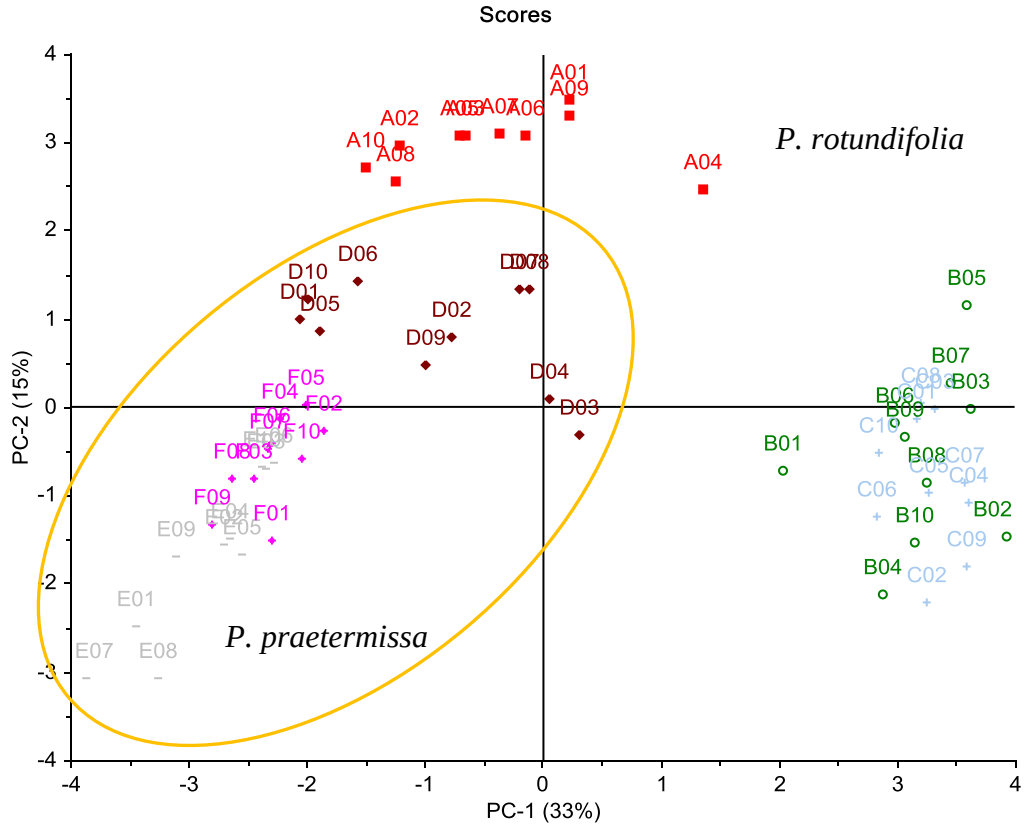



Legend:

| Location | ID | Individual | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 01- | 02- | 03- | 04- | 05- | 06- | 07- | 08- | 09- | 10- |
| **PPLLA** | D | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |
| **PPLAM** | E | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |
| **PPLPA** | F | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |

**PPLLA:** *P. praetermissa* from Bukit Lagong, Selangor; **PPLAM:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **PPLPA:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan.

**Figure 3.3.2.3:** PCA scores plot of *P. praetermissa* chromatographic profiles from different localities.




Legend:

| | | |
|---|---|---|
| **A** | **PRLPA** | *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan |
| **B** | **PRLSB** | *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor |
| **C** | **PRLTM** | *P. rotundifolia* from Takar Melor, Labis Forest Reserve, Johor |
| **D** | **PPLLA** | *P. praetermissa* from Bukit Lagong, Selangor |
| **E** | **PPLAM** | *P. praetermissa* from Ampang Forest Reserve, Selangor |
| **F** | **PPLPA** | *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan |

**Figure 3.3.2.4:** PCA scores plot of *P. rotundifolia* and *P. praetermissa* chromatographic profiles from each location.

## 3.4 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) FINGERPRINTS OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

### 3.4.1 LC-MS Fingerprints of *P. rotundifolia* and *P. praetermissa*

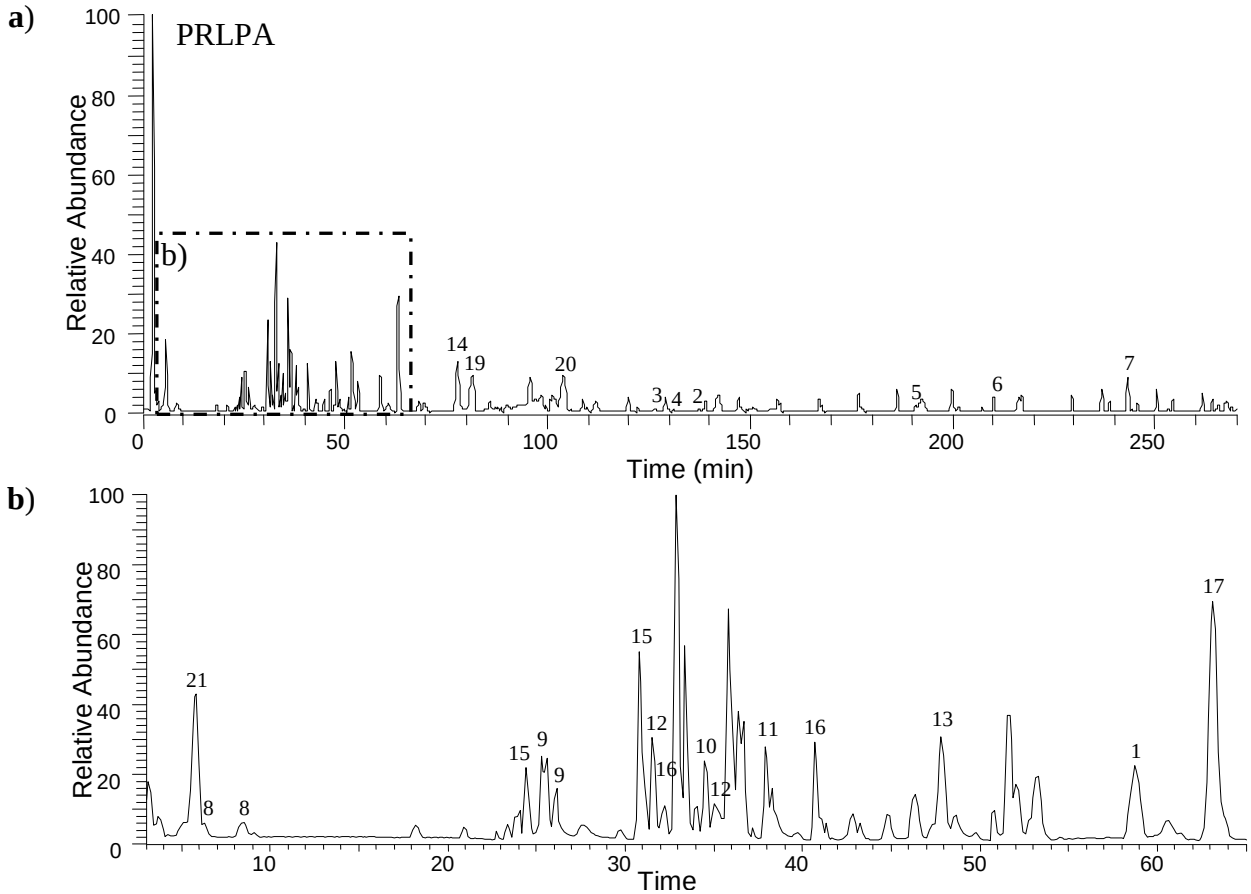
The ESI-$MS^n$ characterisation of the compounds (**1-21**) isolated from the leaves of *P. rotundifolia* and *P. praetermissa* has been described in the previous section (sections 3.1 and Table 3.1.2). These ESI-$MS^n$ data are applicable in the chemical fingerprinting of *P. rotundifolia* and *P. praetermissa* and facilitated the identification of galloylated cyanogenic glucosides, hydrolysable tannins and ellagic acid derivatives without going through timely isolation and purification steps. The LC-MS is a hyphenated technique that shows the advantage of providing tentative compound identification when the reference compounds are not accessible (Seeram *et al.*, 2006; Mullen *et al.*, 2003). Hence, it permits the identification of these compounds in complex samples that provide complete fingerprinting of plant materials.

The LC-MS analysis was performed over *m/z* in the range of 100-1000 after 60 min sonication of the plant materials in methanol. The LC-MS analysis was carried out in the negative ionisation mode since it provided superior data for the majority of compounds isolated from *P. rotundifolia* and *P. praetermissa*. In order to determine the correct molecular weight, the high resolution zoom scan analysis or isotopic patterns was used to enhance the isotope resolution of the compounds.

The representative chromatographic profile of *P. rotundifolia* from the Pasoh Forest Reserve (Figure 3.4.1.1), Sungai batang, Labis Forest Reserve (Figure 3.4.1.2) and Takar Melor, Labis Forest Reserve (Figure 3.4.1.3) and *P. praetermissa* from Bukit Lagong (Figure 3.4.1.4), Ampang Forest Reserve (Figure 3.4.1.5) and Pasoh Forest Reserve (Figure 3.4.1.6) are illustrated in Figure 3.4.1.7. The galloylated cyanogenic glucosides (**1-7**) were

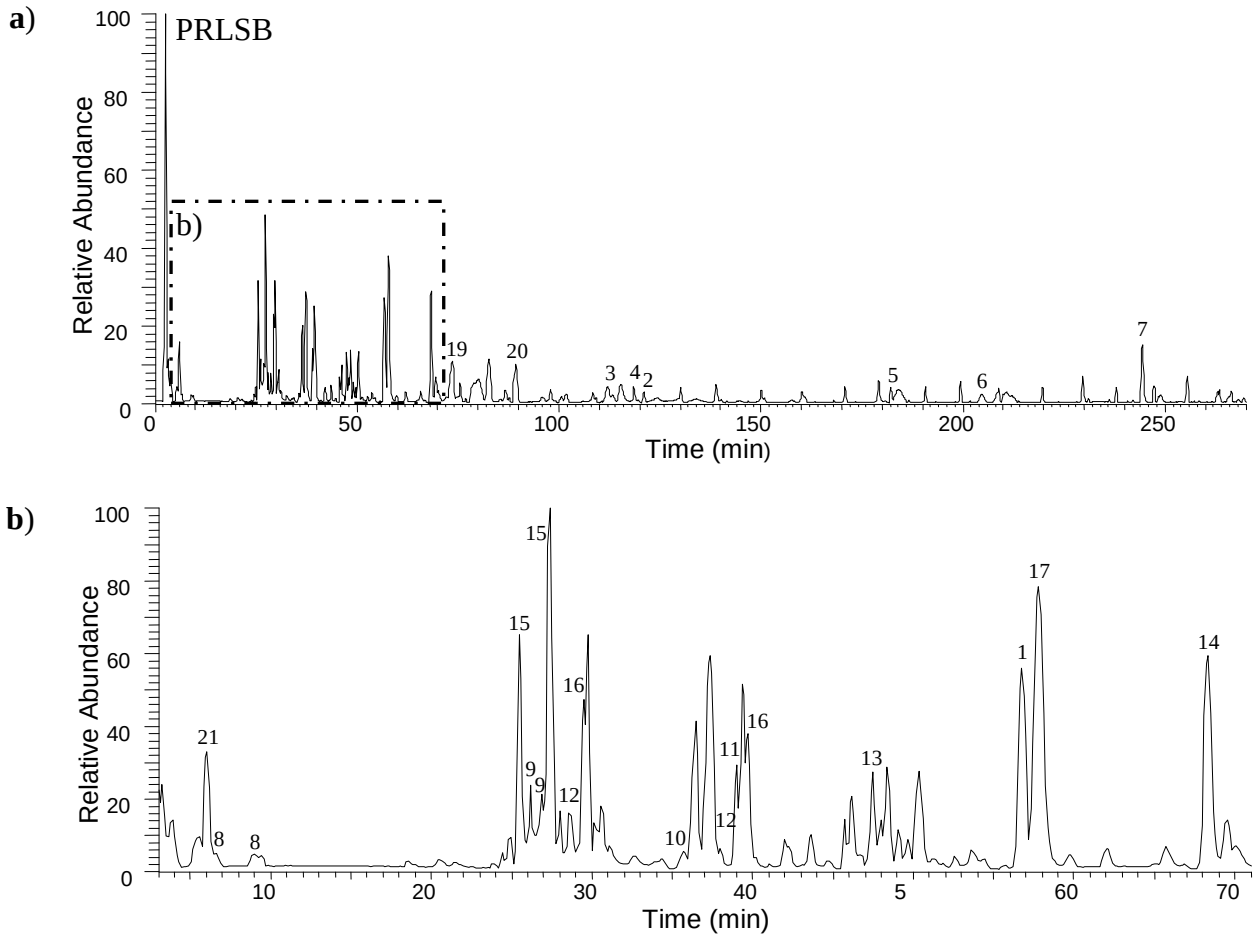
found in all samples of *P. rotundifolia*. However, they were present in relatively low abundance in *P. praetermissa* (Figure 3.4.1.7) and no noticeable presence of them was noted in *P. praetermissa* from Bukit Lagong. Some chemical constituents such as compounds **8**, **9**, **12**, **15** and **16** exhibited as two isomers due to the anomeric mixture of α and β-form of the chemical constituents. In addition, some samples showed the presence of peak **A** and peak **B** which exhibited as the isomers of monogalloyl-glucose and trigalloyl-glucose, respectively. Peak **A** gave $[M–H]^-$ ion at *m/z* 331 and the $MS^2$ spectrum of ion at *m/z* 271 yielded two major fragment ions at *m/z* 211 and 169. Similar fragmentation pattern was also observed for peak **B** which corresponded to compound **10**, **11**, and **12** representing the isomers of trigalloyl-glucose (Table 3.1.2). All the peaks in the chromatographic profiles are labelled and determined accordingly with their tandem mass data as tabulated in Table 3.1.2.

In short, the fragmentation schemes and tandem mass data of galloylated cyanogenic glucosides, gallotannins, ellagitannins and ellagic acid derivatives provide detailed $MS^n$ information which could facilitate rapid monitoring of seasonal variation, maturation process or growth stages and quality evaluation of *P. rotundifolia* and *P. praetermissa*.



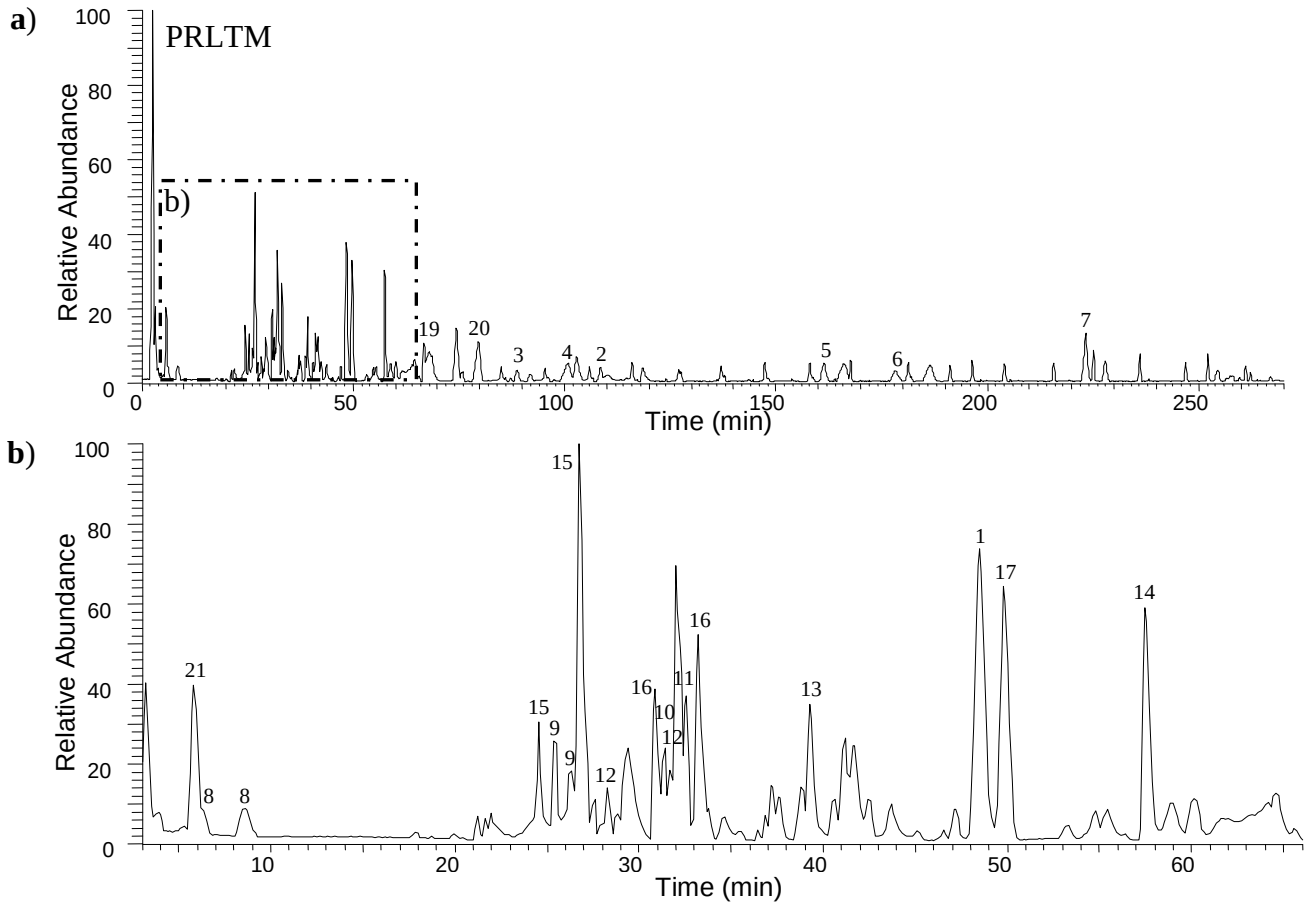

| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 58.74 | **11** | 1,4,6-tri-*O*-galloyl-β-D-glucose | 37.91 |
| **2** | Prunasin 2',6'-di-*O*-gallate | 137.38 | **12** | 3,4,6-tri-*O*-galloyl-D-glucose | 31.27, 35.33 |
| **3** | Prunasin 3',6'-di-*O*-gallate | 126.62 | **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 47.82 |
| **4** | Prunasin 4',6'-di-*O*-gallate | 131.35 | **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 77.77 |
| **5** | Prunasin 2',3',6'-tri-*O*-gallate | 190.95 | **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 24.46, 30.78 |
| **6** | Prunasin 3',4',6'-tri-*O*-gallate | 212.40 | **16** | Praecoxin B | 33.00, 40.70 |
| **7** | Prunasin 2',3',4',6'-tetra-*O*-gallate | 243.29 | **17** | Pterocarinin C | 63.16 |
| **8** | 6-*O*-galloyl-D-glucose | 6.28, 8.52 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 81.52 |
| **9** | 3, 6-di-*O*-galloyl-D-glucose | 25.26, 26.15 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 103.94 |
| **10** | 1,2,3-tri-*O*-galloyl-β-D-glucose | 34.50 | | | |

**Figure 3.4.1.1:** LC-MS chromatographic profile of **a**) *P. rotundifolia* (Pasoh Forest Reserve) and **b**) expanded chromatogram.



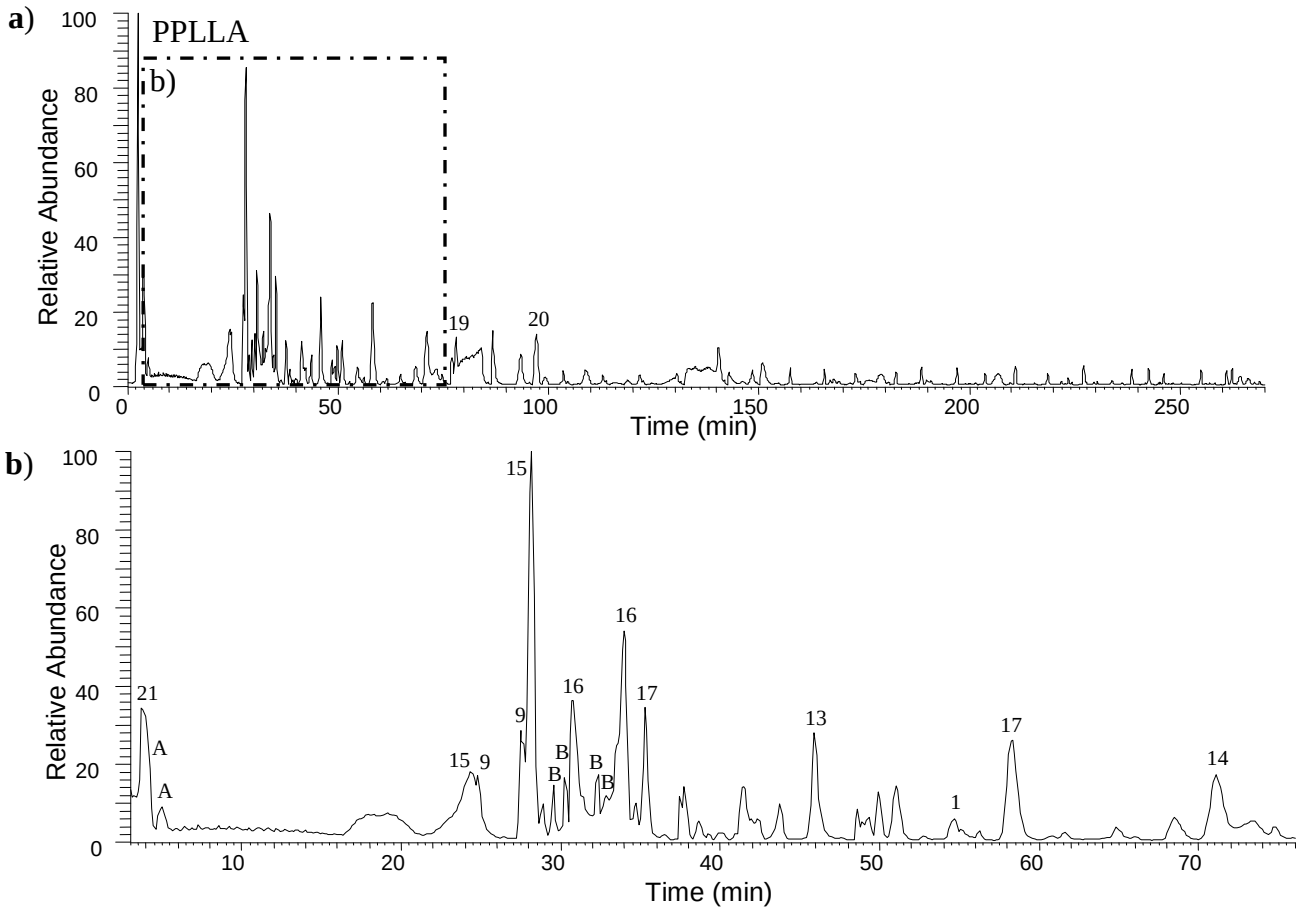

| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 56.75 | **11** | 1,4,6-tri-*O*-galloyl-β-D-glucose | 38.99 |
| **2** | Prunasin 2',6'-di-*O*-gallate | 121.01 | **12** | 3,4,6-tri-*O*-galloyl-D-glucose | 28.57, 37.84 |
| **3** | Prunasin 3',6'-di-*O*-gallate | 111.97 | **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 47.46 |
| **4** | Prunasin 4',6'-di-*O*-gallate | 118.37 | **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 68.35 |
| **5** | Prunasin 2',3',6'-tri-*O*-gallate | 182.11 | **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 25.45, 27.38 |
| **6** | Prunasin 3',4',6'-tri-*O*-gallate | 204.84 | **16** | Praecoxin B | 29.46, 39.71 |
| **7** | Prunasin 2',3',4',6'-tetra-*O*-gallate | 244.42 | **17** | Pterocarinin C | 57.80 |
| **8** | 6-*O*-galloyl-D-glucose | 6.60, 8.99 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 73.57 |
| **9** | 3, 6-di-*O*-galloyl-D-glucose | 26.20, 26.84 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 89.26 |
| **10** | 1,2,3-tri-*O*-galloyl-β-D-glucose | 35.68 | **21** | Gallic acid | 6.04 |

**Figure 3.4.1.2:** LC-MS chromatographic profile of **a**) *P. rotundifolia* (Sungai Batang, Labis Forest Reserve) and **b**) expanded chromatogram.



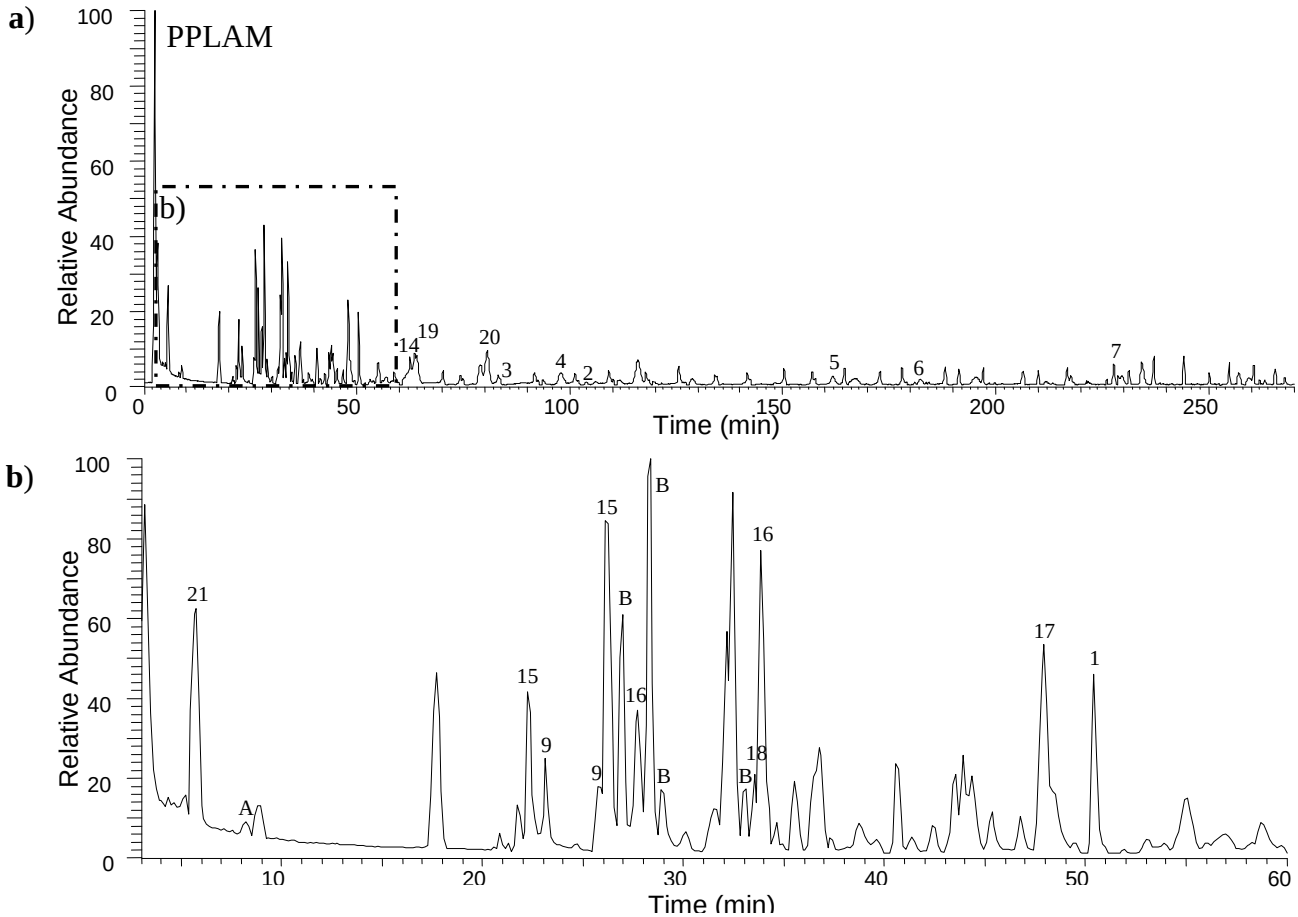

| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 48.47 | **11** | 1,4,6-tri-*O*-galloyl-β-D-glucose | 32.58 |
| **2** | Prunasin 2',6'-di-*O*-gallate | 108.60 | **12** | 3,4,6-tri-*O*-galloyl-D-glucose | 28.29, 31.66 |
| **3** | Prunasin 3',6'-di-*O*-gallate | 87.26 | **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 39.28 |
| **4** | Prunasin 4',6'-di-*O*-gallate | 100.89 | **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 57.45 |
| **5** | Prunasin 2',3',6'-tri-*O*-gallate | 161.33 | **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 24.56, 26.73 |
| **6** | Prunasin 3',4',6'-tri-*O*-gallate | 178.25 | **16** | Praecoxin B | 30.90, 33.41 |
| **7** | Prunasin 2',3',4',6'-tetra-*O*-gallate | 223.30 | **17** | Pterocarinin C | 49.82 |
| **8** | 6-*O*-galloyl-D-glucose | 6.23, 8.58 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 66.77 |
| **9** | 3, 6-di-*O*-galloyl-D-glucose | 25.36, 26.33 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 79.67 |
| **10** | 1,2,3-tri-*O*-galloyl-β-D-glucose | 31.40 | **21** | Gallic acid | 5.82 |

**Figure 3.4.1.3:** LC-MS chromatographic profile of **a**) *P. rotundifolia* (Takar Melor, Labis Forest Reserve) and **b**) expanded chromatogram.



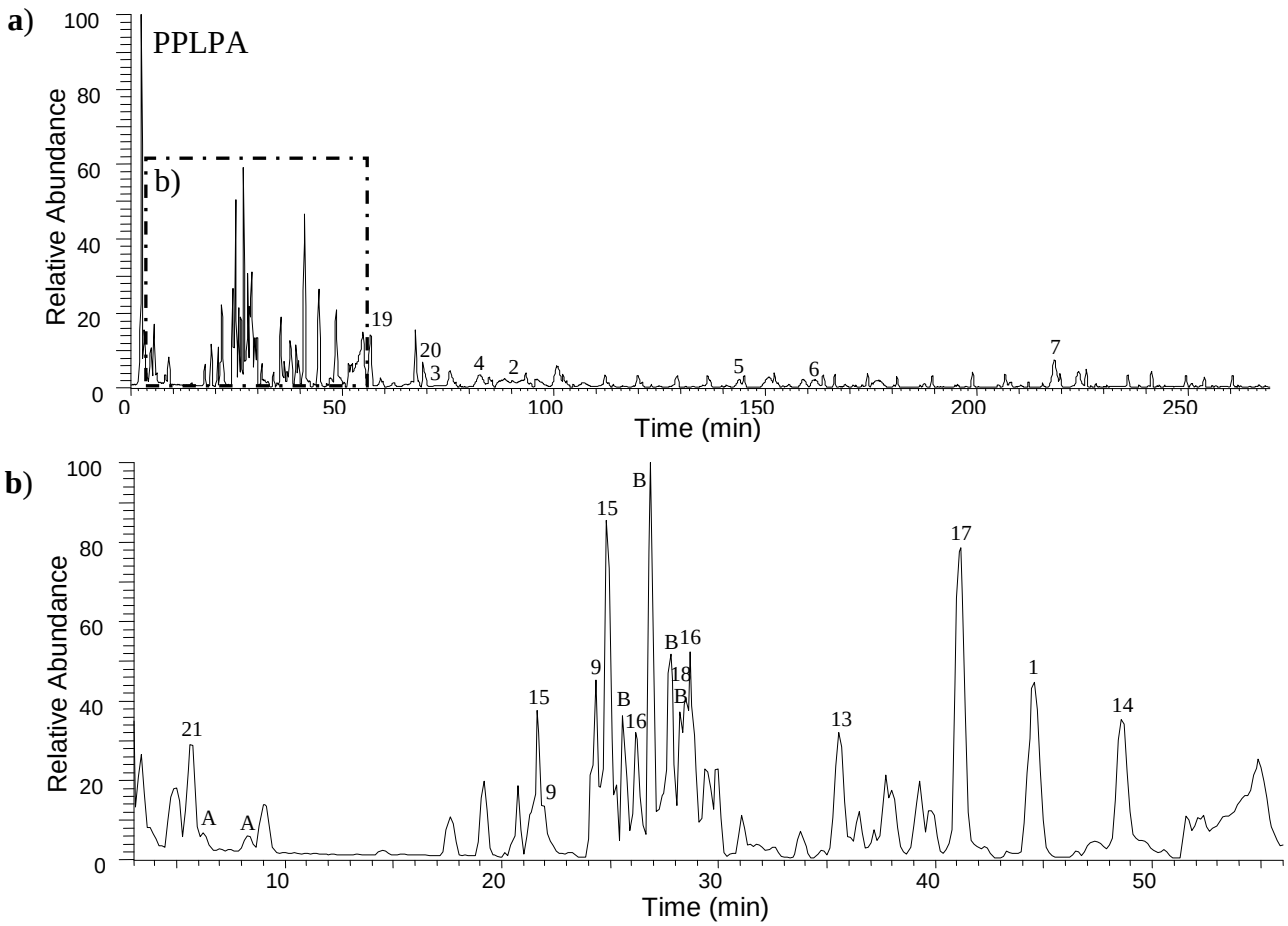

| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 54.78 | **18** | Casuarinin | 35.27 |
| **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 45.91 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 78.15 |
| **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 71.06 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 97.24 |
| **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 24.33, 28.11 | **21** | Gallic acid | 3.65 |
| **16** | Praecoxin B | 30.71, 33.95 | **A** | Monogalloyl-glucose | 3.74, 4.36 |
| **17** | Pterocarinin C | 58.22 | **B** | Trigalloyl-glucose | 28.87,30.25, 32.34, 33.02 |

**Figure 3.4.1.4:** LC-MS chromatographic profile of **a**) *P. praetermissa* (Bukit Lagong) and **b**) expanded chromatogram.



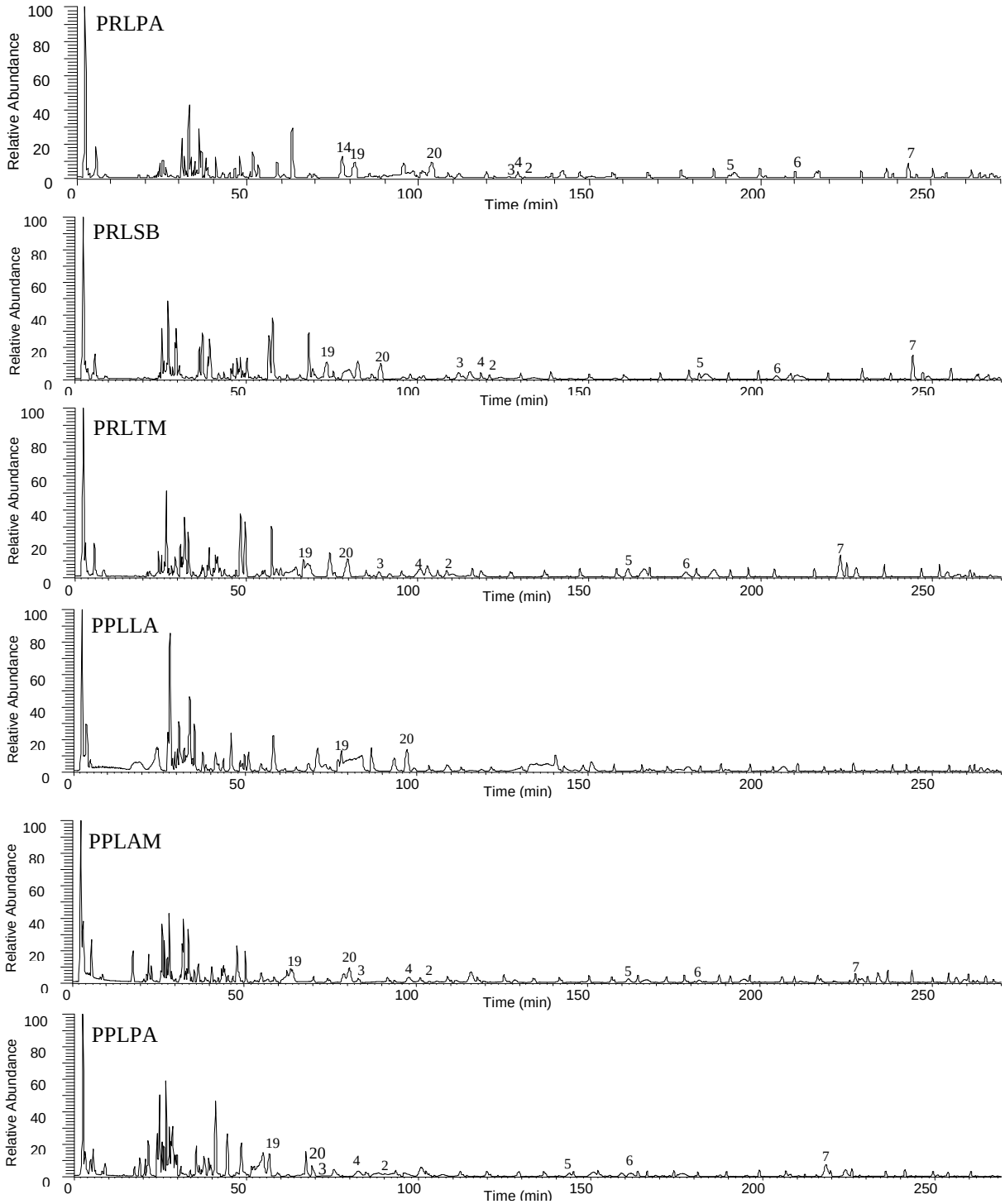

| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 50.43 | **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 22.26, 26.13 |
| **2** | Prunasin 2',6'-di-*O*-gallate | 106.01 | **16** | Praecoxin B | 27.74, 34.11 |
| **3** | Prunasin 3',6'-di-*O*-gallate | 84.39 | **17** | Pterocarinin C | 47.95 |
| **4** | Prunasin 4',6'-di-*O*-gallate | 97.90 | **18** | Casuarinin | 33.85 |
| **5** | Prunasin 2',3',6'-tri-*O*-gallate | 161.88 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 63.52 |
| **6** | Prunasin 3',4',6'-tri-*O*-gallate | 182.33 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 80.64 |
| **7** | Prunasin 2',3',4',6'-tetra-*O*-gallate | 227.79 | **21** | Gallic acid | 5.73 |
| **9** | 3, 6-di-*O*-galloyl-D-glucose | 23.13, 25.72 | **A** | Monogalloyl-glucose | 6.14, 8.25 |
| **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 40.56 | **B** | Trigalloyl-glucose | 27.22,28.59, 29.14, 33.52 |
| **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 62.54 | | | |

**Figure 3.4.1.5:** LC-MS chromatographic profile of **a**) *P. praetermissa* (Ampang Forest Reserve) and **b**) expanded chromatogram.




| No. | Compound | Retention Time (min) | No. | Compound | Retention Time (min) |
|---|---|---|---|---|---|
| **1** | Prunasin 6'-*O*-gallate | 44.56 | **15** | 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose | 21.63, 24.80 |
| **2** | Prunasin 2',6'-di-*O*-gallate | 90.37 | **16** | Praecoxin B | 26.16, 28.86 |
| **3** | Prunasin 3',6'-di-*O*-gallate | 71.33 | **17** | Pterocarinin C | 41.07 |
| **4** | Prunasin 4',6'-di-*O*-gallate | 82.69 | **18** | Casuarinin | 28.68 |
| **5** | Prunasin 2',3',6'-tri-*O*-gallate | 143.72 | **19** | 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside | 56.86 |
| **6** | Prunasin 3',4',6'-tri-*O*-gallate | 161.69 | **20** | 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside | 69.07 |
| **7** | Prunasin 2',3',4',6'-tetra-*O*-gallate | 218.26 | **21** | Gallic acid | 5.72 |
| **9** | 3, 6-di-*O*-galloyl-D-glucose | 21.72, 24.10 | **A** | Monogalloyl-glucose | 6.23, 8.26 |
| **13** | 1,2,3,6-tetra-*O*-galloyl-β-D-glucose | 35.54 | **B** | Trigalloyl-glucose | 25.71, 26.86, 27.25, 28.32 |
| **14** | 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose | 48.58 | | | |

**Figure 3.4.1.6:** LC-MS chromatographic profile of **a**) *P. praetermissa* (Pasoh Forest Reserve) and **b**) expanded chromatogram.




(**PRLPA:** *P. rotundifolia* from Pasoh Forest Reserve, Negeri Sembilan; **PRLSB:** *P. rotundifolia* from Sungai Batang, Labis Forest Reserve, Johor; **PRLTM:** *P. rotundifolia* from Takar Melor, Labis Forest Reserve,Johor; **PPLLA:** *P. praetermissa* from Bukit Lagong, Selangor; **PPLAM:** *P. praetermissa* from Ampang Forest Reserve, Selangor; **PPLPA:** *P. praetermissa* from Pasoh Forest Reserve, Negeri Sembilan).

**Figure 3.4.1.7:** LC-MS chromatographic profiles of *P. rotundifolia* and *P. praetermissa* from different localities.