

APPLICATION OF INFRARED SPECTROSCOPY FOR CLASSIFICATION OF THALASSEMIA AND HEMOGLOBIN E

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Abstract

Thalassemia and hemoglobinopathy are forms of hereditary anemia. They are caused by hemoglobin (Hb) disorders. There are many comprehensive and accurate diagnostic methods but they are complex, expensive, and labor-intensive and, in many countries, there are limited facilities to diagnose these diseases. Therefore, the application of the infrared (IR) spectroscopy technique for classification of thalassemias and hemoglobinopathy, especially hemoglobin E (HbE) which is common in Thailand, was studied because it is easy to use and has many advantages: it is reagent free, it uses a small amount of sample (2 μ l), and it is simple as it does not require more training. In this study, a total of 124 samples was obtained; 24 samples were normal hemoglobin and there were 4 groups with 20 samples of β^+ -thalassemia, β^0 -thalassemia, HbE, α^+ -thalassemia, and α^0 -thalassemia hemoglobin, respectively. The results of this study by IR spectroscopy (652 spectra) were analyzed between the normal hemoglobin and the hemoglobin of β^+ -thalassemia, β^0 -thalassemia, HbE, α^+ -thalassemia, and α^0 -thalassemia using principal component analysis and unsupervised hierarchical cluster analysis of the Unscrambler software. The results found that this technique could be used to classify between the normal hemoglobin and abnormal hemoglobin of all the thalassemias. Among these thalassemic groups, α^0 -thalassemia was the most differentiated from the normal group, followed by HbE, α^+ -thalassemia, β^+ -thalassemia, and β^0 -thalassemia, respectively.

Keywords: Thalassemia, hemoglobin E, infrared spectroscopy, β -thalassemia, α -thalassemia

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Introduction

Thalassemia and hemoglobinopathy are forms of hereditary anemia caused by hemoglobin (Hb) abnormality. Thalassemia is when the Hb decreases or does not synthesize, while hemoglobinopathy is when the Hb synthesizes but is deformed. These unusual conditions of red blood cells, which have a short lifespan, lead to anemia. In 2008, the World Health Organization reported the prevalence of the carriers of hemoglobin gene variants and affected conceptions. The prevalence of the population in carrying the significant variations, α^+ thalassemia, and any other variants were 5.2%, 20.7%, and 24%, respectively (Modell and Darlison, 2008a). The worldwide risk of thalassemia and hemoglobinopathy for having affected children with the hemoglobin disorders were around 1.1% and 2.7% per 1000 conceptions, respectively. However, the prevalence of the affected births with mortality under the age of 5 years was 3.4% (Modell and Darlison, 2008b). In Thailand, about 1% of the population have the disease (Ministry of Public Health, 2007; Wibulpolprasert, 2011) and the prevalence of α -thalassemia, β -thalassemia, and hemoglobin E (HbE) was 16-30%, 2-10%, and 8-54%, respectively (Ratanasiri, 2007). The only treatment for these diseases in most countries is regular blood transfusion and iron chelation that is recommended for patients with iron overload. Therefore, improvement in the diagnosis, prevention and management has been investigated in many countries for preventing and controlling these diseases (Fucharoen and Winichagoon, 2010).

Normal Hb consists of 2 α -globin chains and 2 non- α globin chains. Alpha globin chains are controlled by the gene cluster on chromosome 16 which synthesizes the ζ and α chains and consists of 141 amino acids. The other chains are controlled by the gene cluster on chromosome 11 which establishes the ϵ , γ , β , and δ chains and consists of 146 amino acids. Synthesis of the Hb chains depends on the patterns and the periods. During the gestation period, there are Hb Gower I, Hb Gower II, and Hb Portland, respectively. After birth, they switch to be HbF, HbA, and HbA2. Later, HbF decreases

and HbA increases until adulthood. It is found that HbA is the highest form of Hb in adults with approximately 95-97.5%, followed by HbA2 (2.5-3.5%), and HbF (less than 1%) (Nienhuis, 1986; Bain *et al.*, 2010).

Thalassemias are the result of autosomal recessive genes inherited from both the father and mother. They are an imbalance of the Hb resulting from decreasing some Hb chains and increasing the other Hb chains which causes the hypochromic red blood cells (RBCs). These hypochromic RBCs are destroyed rapidly, so the thalassemic patients show spleen and liver enlargements and anemia (Bank, 1978). Hemoglobinopathy is an abnormal structure of the Hb. It is caused by mutation of the gene which changes the type or amount of amino acid in the Hb polypeptides. For example, HbE is an unusual amino acid in the β -chain at position 26 that changes from glutamic acid to lysine (Scott *et al.*, 2013).

Diagnosis of thalassemia starts with the screening methods which include a one-tube osmotic fragility (OF) test, dichlorophenolindophenol (DCIP) precipitation test, and red blood cell indices [mean corpuscular volume (MCV) <80 femtoliter (fL) and mean corpuscular hemoglobin (MCH) <27 picogram (pg)]. Later, high performance liquid chromatography (HPLC) or electrophoresis will be used to separate the types of the Hb, and then molecular testing techniques are finally used to confirm the diagnosis (Fucharoen *et al.*, 2004; Bain, 2011). These techniques are more complex, costly, and require special instruments. From the research of Liu *et al.* (2003), a novel approach was found of infrared spectroscopy (IR) which was used to distinguish the normal Hb, β thalassemia, and HbH. The principle of this technique is using the chemical groups to vibrate the spectra insight into the binding of ligands (Liu *et al.*, 2003; Wan *et al.*, 2013).

Materials and Method

Samples

The 100 samples of thalassemic patients were obtained from the Health Promoting

Hospital in Nakhon Ratchasima and the Genome Molecule Laboratory Company confirmed the thalassemic types by DNA analysis. These samples were divided into 5, with 20 samples for each group, as α^0 -thalassemia, α^+ -thalassemia, β^0 -thalassemia, β^+ -thalassemia, and HbE. For the normal group, 24 samples were obtained from blood donors at the Regional Blood Center in Nakhon Ratchasima and also were confirmed by DNA analysis. All samples were reviewed and approved by the Ethics Committee for Researches Involving Human Subjects, based on the Declaration of Helsinki with project code EC-56-28 of Suranaree University of Technology.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a modern infrared spectroscopy. The Fourier transform is a mathematical process that converts the raw spectrum (or interferogram) to an actual spectrum. It is performed by 2 Fourier transformations: 1 by the interferometer and 1 by the computer. The interferometer originates the Fourier transform of the spectrum when a monochromatic source is regarded. This study used a Vertex 70 FTIR spectrometer (Bruker Corp., Billerica, MA, USA) coupled to an IR microscope (Hyperion 2000, Bruker Corp.) equipped with a KBr beamsplitter and the mercury cadmium telluride detector cooled with liquid nitrogen with a measurement range of $4000\text{--}200\text{ cm}^{-1}$ which is a mid-IR.

IR Measurement

Each specimen used 2 μL aliquot of the hemolysates which was evenly spread on an IR reflective slide (MIRR-IR Corner Frosted, Kevley Technologies, Chesterland, OH, USA) with a 3 millimetre diameter. The IR absorption spectra require an IR spectrometer coupled with an IR microscope and connected to the detector cooled with liquid nitrogen over the wavenumber range from 4000 cm^{-1} to 200 cm^{-1} . A spectral acquisition was made in reflection mode with a focal plane aperture size of $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ at 4 cm^{-1} spectral resolution with 64 co-added scans at $36\times$ objectives (Bruker Optics, Ettlingen, Germany); measurement of the background was 1 time every 5 times of the

specimen measurement and the temperature and the humidity were controlled to be lower than 27°C and 20%, respectively. All interferograms were co-added and converted to absorb spectra using the OPUS 7.2 software.

Spectra Manipulation

The recorded IR spectra with the Vertex 70 FTIR spectrometer were transformed with the OPUS 7.2 software. They were performed to the second derivative spectra and vector normalization over the regions $1800\text{--}1400\text{ cm}^{-1}$ and $3400\text{--}2800\text{ cm}^{-1}$. All spectra were qualitative selected with a weak absorbance (an absorbance of $1684\text{--}1656\text{ cm}^{-1}$ region <0.30 units) or a high absorbance (an absorbance of $1684\text{--}1656\text{ cm}^{-1}$ region >1.2 units) (Thumanu *et al.*, 2014). Then, these areas were integrated for clustering and classification trials.

Statistical Analysis

Various spectral features differed between the spectra of the Hb from the normal control and each Hb from 4 groups: the α^0 -, α^+ -, β^0 -, and β^+ -thalassemic patients, as well as among the individual spectra acquired for HbE. The next step, therefore, applied a separating analysis using the OPUS 7.2 software and the Unscrambler[®] X software package (CAMO Software AS, Oslo, Norway) to discriminate whether the individual IR spectra can be classified according to their clinical diagnosis. This method uses a statistical approach to classify spectra into groups according to subtle spectral differences. The outcome of discrimination can be visualized with a dendrogram in which the discriminant formation is plotted as a horizontal, interconnecting line located at the distance at which the discriminant is being formed.

Results and Discussion

The outcome of the measurement from IR spectroscopy was 652 spectra. It was divided into the normal, β^+ -thalassemia, β^0 -thalassemia, HbE, α^0 -thalassemia, and α^+ -thalassemia groups, which were 114, 166, 70, 105, 89, and 108 spectra, respectively (Figure 1(a)). Later, it was performed on the second derivative spectra using the

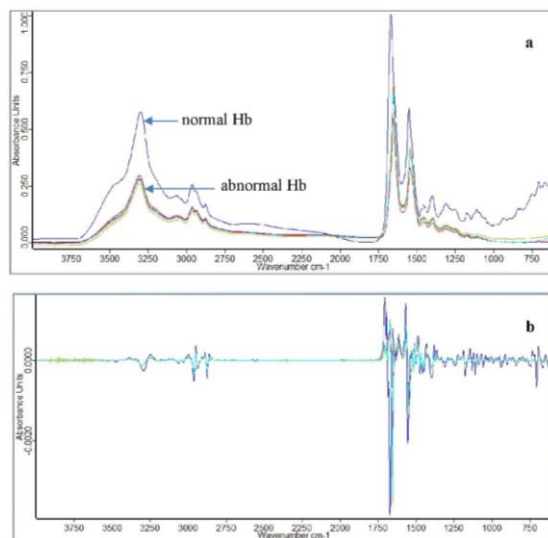


Figure 1. The average IR spectrum of Hb (a) and the second derivative spectrum (b) from normal 24 samples: 114 spectra, β^+ -thalassemia 20 samples: 166 spectra, β^0 -thalassemia 20 samples: 70 spectra, HbE 20 samples: 105 spectra, α^0 -thalassemia 20 samples: 89 spectra, and α^+ -thalassemia 20 samples: 108 spectra

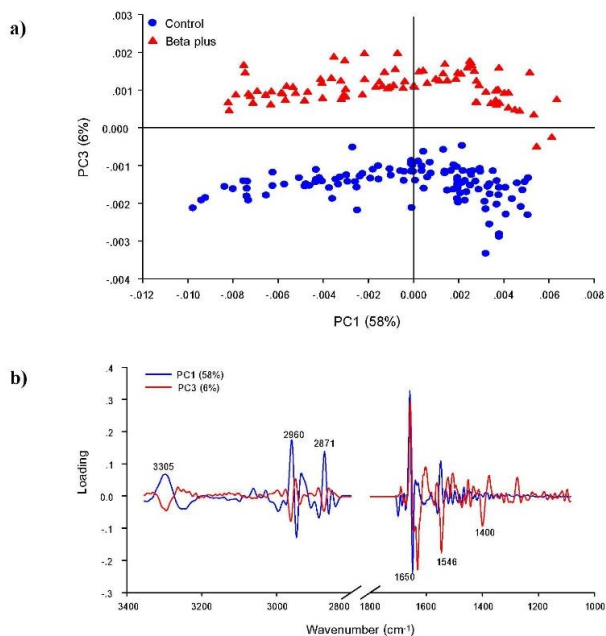


Figure 2. Scores (a) and loading plots (b) from PCA of the spectra data for normal (control) and β^+ -thalassemia (beta plus)

spectral regions from 3400-2800 cm^{-1} and 1800-1000 cm^{-1} (Figure 1(b)). The characteristic functional groups contributed to the formation of the protein side chains and backbone vibrations. The average second derivative spectra after 7 points of smoothing and normalization with extended multiplicative signal correction over the spectral ranges of 3400-2800 cm^{-1} and 1800-1000 cm^{-1} . There were 7 major bands comprising NH stretching, CH₂ stretching, CH₂ stretching, CH₃ stretching, CH₃ stretching, C=O stretching (amide I), and NH bending (amide II) derived from around 3300, 2960, 2929, 2871, 2852, 1650, and 1540 cm^{-1} , respectively (Rahmelow *et al.*, 1998; Barth, 2007). Principal component analysis (PCA) has proven to be useful in analysis, providing 2 types of information which are visualization of similar spectral clustering and identification of variables (spectral bands representing various molecular groups within the samples). The major variability between the spectra can be concentrated into a smaller set of values called principal components. To define the major

variability between the normal and abnormal of β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia, and α^+ thalassemia, the principal components were applied to the pre-processed spectra recorded in the 3400-2800 cm^{-1} and 1800-1000 cm^{-1} regions.

The comparison of the PCA scores' plot between the normal and abnormal Hb of β^+ -thalassemia, β^0 -thalassemia, HbE, α^0 -thalassemia, and α^+ -thalassemia can be clustered separately along PC1, PC2, and PC3. Scores and loading plots from the PCA of the spectra data for normal Hb (114 spectra from 24 samples) and β^+ -thalassemia Hb (166 spectra from 20 samples) explained 64% of the total variance in the data set (58% for PC1 and 6% for PC3) (Figure 2).

Fifty-one percent variance of PC1 and PC3 were explained by 5% for the group between the normal Hb and β^0 -thalassemia (70 spectra from 20 samples), as shown in Figure 3. A perfect separation between the normal Hb and HbE (105 spectra from 20 samples) was 60% of the total variance (52% of PC1 and 8% of PC3) (Figure 4), the same as the group

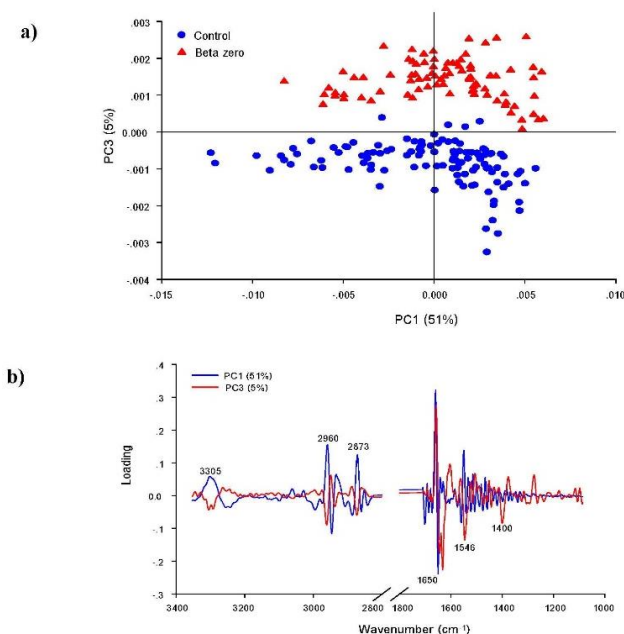


Figure 3. Scores (a) and loading plots (b) from PCA of the spectra data for normal (control) and β^0 -thalassemia (beta zero)

between the normal Hb and α^+ -thalassemia (108 spectra from 20 samples) (Figure 5). The last group between the normal Hb and α^0 -thalassemia (89 spectra from 20 samples) was clear separation which was explained by 33% variance of PC2 and 2% of PC3 (Figure 6).

The loading plot of PC1 and PC2 exhibited C=O stretching, NH bending, and NH stretching which effected on the amide I, amide II, and amines as a negative peak at 1650, 1540, and 3300 cm^{-1} , respectively. These showed the change in the level of the helix secondary structure, according to the thalassemic Hb which mutated as marked changes in the heme environment and resulted in distinctly different spectral bands for CO bound to the α and β subunits. The PC3 was focused to obtain the variations in minor components which were the bonds of the protein side chains.

The α^0 -thalassemic spectra had clearly high intensity in the CH bands at 2960, 2929, 2871, and 2852 cm^{-1} which were assigned more

protein side chains. Since the α^0 -thalassemic Hb had no α globin chain, it had excess β globin chains and became free β globin chains. These free β globin chains were not stable, and therefore the anemia was happening. The CH bands of the α^+ -thalassemic spectra were different from the α^0 -thalassemic spectra and showed low levels, because these thalassemias had less protein side chains than normal spectra (the synthesis of α globin chains was low).

For HbE, the phenomenon was the same as the α^0 -thalassemic spectra with high CH bands. According to the HbE, the Hb which mutated from glutamic acid to lysine at position 26 of the β globin chain had lower CH bands than lysine, but the C=O had equal normal spectra which were different from the α^0 -thalassemic spectra. This performance showed that these Hbs had functionality. The β^0 and β^+ -thalassemic spectra had low intensity in the CH bands at 2852 cm^{-1} , the same as the HbE spectra. The β thalassemia had a defect at the

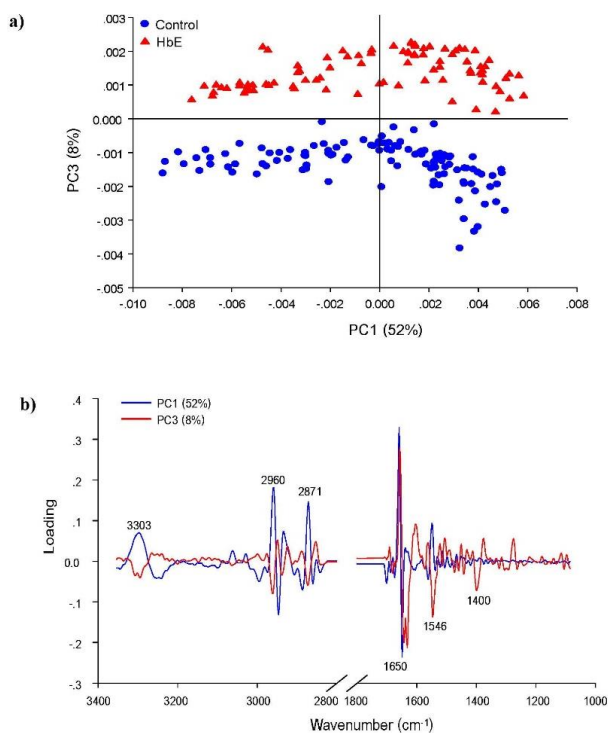


Figure 4. Scores (a) and loading plots (b) from PCA of the spectra data for normal (control) and HbE

β globin chains, but the δ and γ globin chains were combined with the α globin chains instead of the β globin chains which were defective. However, the occurrences were the same as the normal spectra in many bands that were C=O, NH, and other CH bands.

Unsupervised hierarchical cluster analysis (UHCA) was applied to examine similarities and differences between the spectra of the normal and abnormal Hb of β^+ -thalassemia, β^0 -thalassemia, HbE, α^0 -thalassemia, and α^+ -thalassemia using spectral information in the range of 3400-1000 cm^{-1} . The average spectra from the α^0 -thalassemia samples in branch A were clearly separated from the 5 groups (normal Hb, β^+ -thalassemia, β^0 -thalassemia, HbE, and α^+ -thalassemia) in branches B1 and B2. Branches B21 and B22 of β^+ -thalassemia, β^0 -thalassemia, HbE, and α^+ -thalassemia were classified as branch B1 of the normal Hb

(Figure 7). These results were used to confirm the PCA analysis.

Conclusions

In this study, the IR spectra of hemolysates derived from a cohort of patients with HbE, and α and β thalassemias revealed the important alterations in the protein secondary structures. The prominent spectral changes in the Hb of patients with thalassemia were as follows: decreased α helix content, increased parallel and antiparallel β sheet content (amide I), and decreased intensities of tyrosine absorption bands (-CH₂). Similar changes were observed in the hemolysate derived from a patient with the HbE disease. It should be noted that the major IR features indicated that the reduction of the α helix content and the increase of the

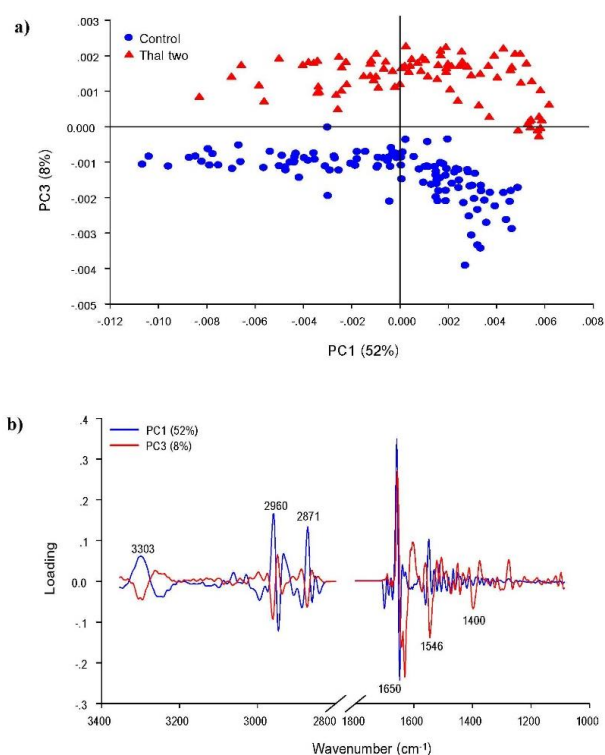


Figure 5. Scores (a) and loading plots (b) from PCA of the spectra data for normal (control) and α^+ -thalassemia (Thal_two)

β structure likely arose from the denatured unbound α chains characteristic of thalassemia.

These results explained that the amount of Hbs within the RBCs of thalassemic patients had lower amounts than non-thalassemic patients (amide I was the point of protein quantity). They related to the MCV and MCH indices which were lower than 80 fL and 27 pg, respectively, for thalassemic patients (Huisman, 1992; Clarke and Higgins, 2000; Wild and Bain, 2006). The peak of α^0 -thalassemic absorbance had clearly different CH₂ stretching around 2929 cm⁻¹ from the normal blood samples because these Hbs presented the combination of more β chains which found an increase in amino acid side chains. For β thalassemia, the γ and δ chains increased, and HbE had more glutamic acid in the β chain. This study extended the previous findings by using more samples

for each type of thalassemia and using the PCA and UHCA program analysis to designate individual spectra of the Hb from the normal controls and thalassemic patients and found that this technique could classify and differentiate the Hbs of HbE and all thalassemia (found in Thailand) from the normal controls. This is the first report to classify between normal Hb and the Hb of β^+ -thalassemia, β^0 -thalassemia, HbE, α^0 -thalassemia, and α^+ -thalassemia (which are clear categories of genotype) using only the FTIR. There are some of the samples into which thalassemic patient carriers can be classified. Nowadays, routine laboratory investigation cannot detect thalassemic carriers in 1 step, as it requires 4 methods which are the CBC, OF test, DCIP, and HPLC techniques. To complete these 4 methods is expensive, takes time, and requires experienced personnel to handle them, especially

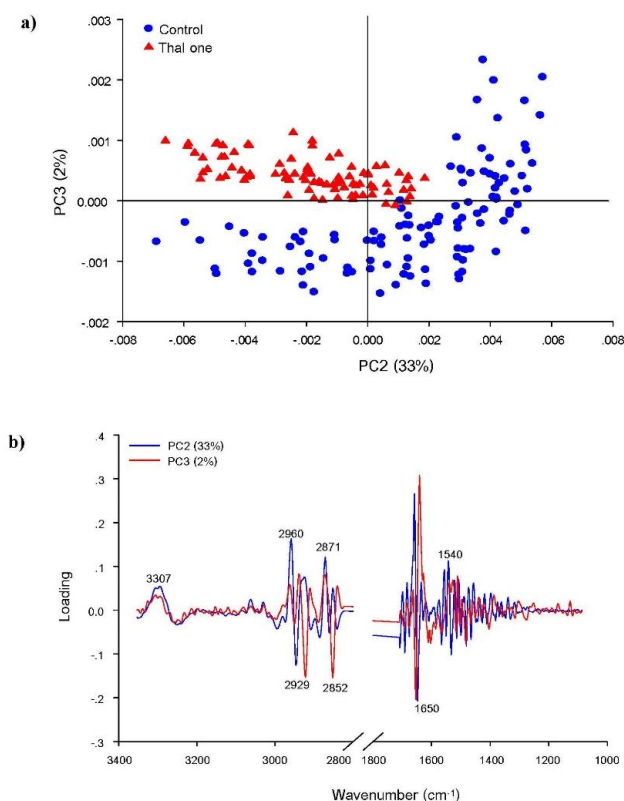


Figure 6. Scores (a) and loading plots (b) from PCA of the spectra data for normal (control) and α^0 -thalassemia (Thal_one)

the HPLC technique (National Institute of Health of Thailand, 2010). The CBC and OF techniques cannot detect α^+ -thalassemia, the DCIP reveals only HbE, and HPLC diagnoses β thalassemia but cannot investigate α^0 -thalassemia and α^+ -thalassemia (Rauf *et al.*, 2017). Those are the limits and pitfalls for each screening technique to overcome the issues for detection of thalassemia carriers (Barrett *et al.*, 2017). Thus, the IR method will be a very useful technique to screen for the carriers and can reduce the risk to the children from married couples who are both carriers, because 30-50% of the Thai population are thalassemic carriers, although they are unaware of it themselves and do not check before marriage.

There are several potential advantages and benefits in using the IR-based method to screen and discriminate between the types of thalassemia: it is 1 step and has a short turnaround time (completed within 30 min); it is reagent-free (it uses only the IR reflective slide to acquire an absorption spectrum), and the IR patterns of the species of interest provide the basis for detection and quantification; it uses a small sample volume (2 μ L), leaving sample material for other clinical tests which is particularly important in the fetus, for which collecting blood for sampling is difficult and limited; it is simple (the expertise can be acquired after minimal training); it can be

automated (it does not require more staff and it can be analyzed with high throughput samples); and it can resolve all the variants of the Hb of thalassemia which are found in Thailand. To simplify the method, it is not necessary to centrifuge after preparing the hemolysates, which will shorten the turnaround time for testing because other possible influences from cell membranes and other cellular components are less than 3% (Weed *et al.*, 1963). In the future, more clinical data of thalassemia patients should be analyzed to identify the types of thalassemia and confirmed by DNA analysis without the need for Hb typing to save time and costs. Therefore, it will reduce the expense significantly and also help clinicians to advise couples before pregnancy since the babies from thalassemic carrier couples are at risk of being severely thalassemic and will be public health problems in the future.

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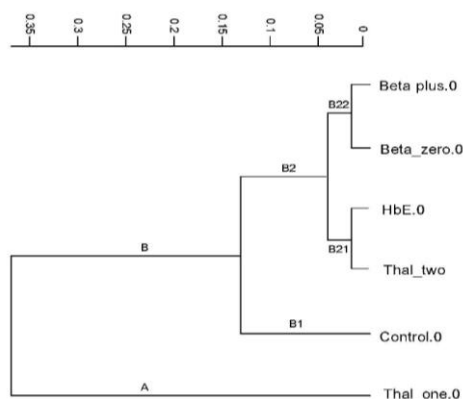


Figure 7. Dendrogram obtained by UHCA of average spectra from normal (control) and abnormal Hb of β^+ -thalassemia (beta plus), β^0 -thalassemia (beta zero), HbE, α^0 -thalassemia (Thal_one), and α^+ -thalassemia (Thal_two)

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