STUDY OF PHENYLALANINE NIR SPECTRA FOR PHENYLKETONURIA DETERMINATION**

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The aim of this study is to investigate the spectroscopic characteristics of phenylalanine in the NIR spectral range as a preliminary step towards the development of an in vivo method for the determination of phenylalanine in the blood of newborn babies. The first steps in this study included in vitro experiments with mixtures of phenylalanine, saline, and whole blood. Results showed absorption bands in the NIR spectral range that are assigned to overtones of C-H, N-H, and O-H in the range of 1400 to 1700 nm, which together with the melanin absorption lines in the visible spectra range, demonstrate the potential of NIR/VIS spectroscopy as an inexpensive, non-invasive, rapid, and accurate alternative to routine methods for determining of phenylketonuria.

Keywords: *Near infrared spectroscopy, phenylketonuria, phenylalanine.*

ПРИМЕНЕНИЕ ИК-СПЕКТРА ФЕНИЛАЛАНИНА ДЛЯ ОПРЕДЕЛЕНИЯ ФЕНИЛКЕТОНУРИИ

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Изучены спектроскопические характеристики фенилаланина в ближнем ИК-диапазоне спектра с целью разработки метода in vivo для определения фенилаланина в крови новорожденных. Первые шаги в этом исследовании включают в себя эксперименты in vitro со смесями фенилаланина, физиологического раствора и цельной крови. Получены полосы поглощения в ближней ИК-области, соответствующие обертонам CH, NH и OH в диапазоне 1400—1700 нм, которые вместе с линиями поглощения меланина в видимой области демонстрируют возможности спектроскопии видимого/ИК-диапазонов в качестве недорогой, неинвазивной, быстрой и точной альтернативы рутинным методам диагностики фенилкетонурии.

Ключевые слова: *спектроскопия ближнего ИК-диапазона, фенилкетонурия, фенилаланин.*

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Introduction. Phenylketonuria (PKU) is a genetic metabolic disorder characterized by the accumulation of phenylalanine in the blood, leading to mental retardation. It is caused by a deficiency in the hepatic enzyme phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH) that prevents normal metabolism of phenylalanine [1]. The frequency of this disorder is approximately 1 in 10 000 Caucasian newborns and it is commonly included in the newborn screening panel. Babies are screened for PKU 2–7 days after birth using dried blood spot (DBS) samples drawn by neonatal heel prick. Like all bio-chemical techniques used to measure phenylalanine levels, this method also requires drawing a few drops of blood from the baby's heel [2]. Such methods generally involve long analysis times, in the range of a few days, and complicated manual sample preparation procedures. The heel-prick test also carries risks that include bleeding and infection.

Newborn babies with PKU cannot convert phenylalanine into tyrosine, which is normally incorporated into all proteins and is a precursor of thyroxine, melanin, and the neurotransmitters dopamine and norepinephrine. In PKU, the metabolic process stops without producing the needed end products, while phenylalanine continues to accumulate, and melanin levels decrease. As a result, newborns with PKU tend to have blond hair and generalized hypopigmentation. Even children with darker skinned parents have a light skin colour. Figure 1 presents normal and PKU metabolic pathways.

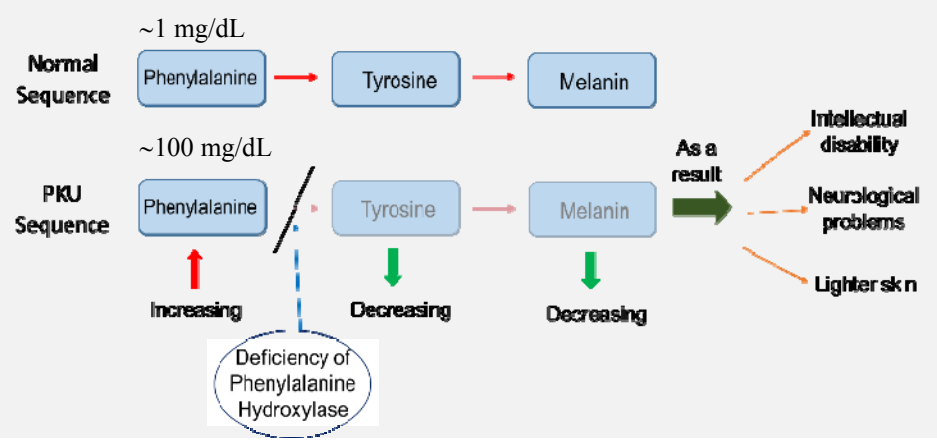


Fig. 1. Normal and PKU biochemical metabolic pathways.

The quest for a non-invasive, simple, and accurate method of PKU determination led us to develop a spectroscopic method that we hope has the potential to replace the conventional method currently used.
To do so, we asked ourselves two questions. Which substance do we want to detect? Which *in vivo* spectroscopic method is the best choice for that? To answer the first question, one must take into account that normal blood phenylalanine levels are in the range of 1 mg/dL; in newborns with PKU, the levels may reach 100 mg/dL, and in addition, the melanin level is decreased. So, the parallel measurement of elevated phenylalanine levels and decreased melanin levels can give us a measure for determining PKU.

For the second question, possible *in vivo* spectroscopic methods that can be used are UV, Vis, NIR, MIR, and Raman.

The criteria for selecting the preferred spectroscopic method depend on the detected substances. We have for measuring the increase in phenylalanine:

a) MIR spectroscopy is an excellent and very sensitive technique that can be used to study the fundamental vibrations and associated rotational-vibrational structures. Since human tissue is a highly absorbent medium in this spectral range, infrared light does not penetrate and is not expected to reflect from blood vessels that contain phenylalanine [3]. Thus, although phenylalanine produces intense absorption lines in the blood, the method is unsuitable for detecting phenylalanine *in vivo*.

b) The NIR bands are assigned to overtones of C-H, N-H, and O-H stretches and combinations of the stretches. The region between 700 to 1600 nm is typically assigned to the overtones, while the region between 1600 to 2500 nm is assigned to the combinations [4]. In addition, it is well known that the visible to NIR range, from 700 to 1300 nm, is an optical window in which optical penetration can reach up to 2 cm, offering deep information about tissues [5]. To the best of our knowledge, the question of the phenylalanine absorption lines in the NIR has yet to be investigated; we dedicate the next paragraph to this issue.

c) Raman spectroscopy is a vibrational spectroscopic technique that provides molecular-level information based on inelastic scattering [6]. The tissue is illuminated by a monochromatic laser beam that interacts with the tissue molecules, creating a scattered light signal. In our special case of illuminating newborn skin, the irradiation dose of the excitation wavelength is a significant parameter for the analysis of the scatter signal. With UV/Vis sources, Raman peaks are more intense than with NIR sources but carry a higher risk to the infant's skin. NIR lasers generate lower autofluorescence compared with VIS lasers but at the same time produce weaker scattering signals [7]. As a result, although Raman spectroscopy yields good absorption lines, it is unsuitable for determining PKU.

d) UV and Vis spectroscopy – No significant absorption lines are obtained for phenylalanine.

Measuring the decrease in melanin:

a) MIR and NIR spectroscopy – Absorption bands for melanin are either non-existent or very weak in these spectral ranges.

b) UV and Vis spectroscopy – The absorption spectrum of melanin in these ranges has been previously investigated [8]. The peak absorption of human melanin pigment is around 335 nm and decreases with increasing wavelength. Melanin absorption is almost completely attenuated for wavelengths longer than 700 nm.

In summary, we concluded that the best way to determine PKU by *in vivo* spectroscopic means is to use the NIR spectral range to measure the increase in phenylalanine in veins and the Vis spectral range to measure the decrease of melanin levels in the skin, as presented in Fig. 2.

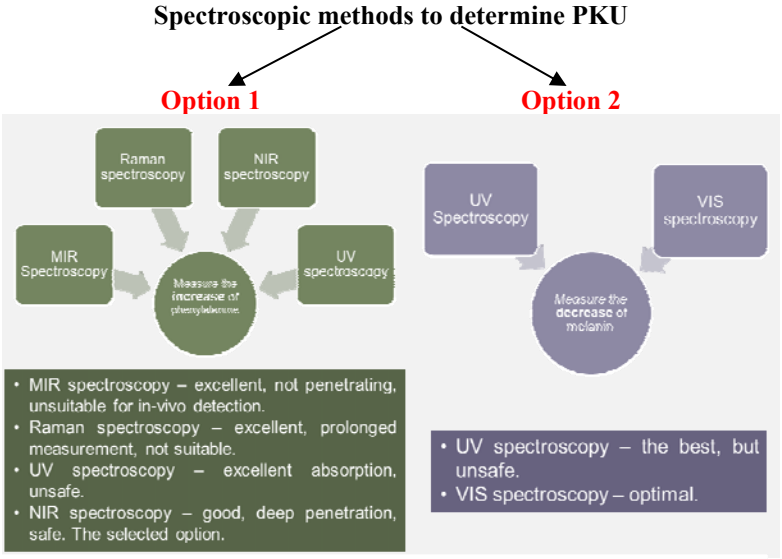


Fig. 2. Spectroscopic alternatives for PKU determining.

Our roadmap for accomplishing the goal of determining PKU consisted of the following stages:

In vitro experiments with various concentrations of phenylalanine in saline solution presented in this paper.

In vitro experiments with various concentrations of phenylalanine in blood presented in this paper.

In vivo tests on newborn babies is the next stage of this research not presented in this paper.

Materials and methods. MIR absorption experiments were performed to detect the spectral bands of L-phenylalanine in saline solution that may be potential candidates for the NIR measurement in the next phase of the study.

Solutions of L-phenylalanine (minimum purity 98%, obtained from Sigma Aldrich) in saline were measured in the MIR spectral range using the ArcOptix FTIR Rocket equipped with an ATR accessory (PIKE model HATR with ZnSe crystal). All spectroscopic measurements were performed at room temperature with 4 cm^{-1} spectral resolution and an average of 15 scans. ATR-FTIR spectra are shown with an absorbance scale.

Figure 3a illustrates the absorbance spectrum of 25 mg/mL L-phenylalanine in saline solution in which the band around 3075 cm^{-1} is attributed to the antisymmetric stretching of $[NH_3]^+$ and the band around 2960 cm^{-1} is attributed to CH_2 stretching modes. The first overtones of these frequencies are expected to be

observed in the NIR spectral range. The CH_2 bending mode is observed around 1445 cm^{-1}, the O–H in-plane bending vibration is observed around 1350 cm^{-1}, and the peaks in the 1625–1500 cm^{-1} region are attributed to the $[NH_3]^+$ group. These results are in good agreement with earlier reports [9–11].

To study the NIR spectra of L-phenylalanine, we prepared two sets of samples with varying concentrations of L-phenylalanine ranging from 10 to 5000 mg/dL: the first set was a solution of L-phenylalanine in saline, and the second set was a solution of L-phenylalanine in 70% red blood cells and 30% saline. The samples were measured in transmission mode in quartz cuvettes of 1 mm optical path length, over the NIR spectral interval from 0.9 to 1.7 μm using a NIRQuest Ocean Optics spectrometer and eFTIR software for the subsequent analysis.

Figure 3b presents the absorbance spectra of L-phenylalanine in saline solution in the NIR spectral range from 900 to 1700 nm in which three main bands, denoted *A*, *B*, and *C*, are evident.
The *A* band centred at 1405 nm is assigned to the first overtone of the O-H group [12], the *B* band at 1560 nm is assigned to the first overtone of the N-H group (in the range of 1530 to 1600 nm) [13], and the *C* band at 1670 nm is assigned to the first overtone of the C-H stretching.

Figure 3b demonstrates that the absorbance bands *B* and *C* increase with increasing L-phenylalanine concentration, while the *A* band, which is associated with water, increases with saline concentration, or in other words, with decrease in L-phenylalanine concentration. The appearance of such changes in opposite directions indicates that these bands originate from different molecules.

Next, L-phenylalanine was mixed in a variety of concentrations with 70% of red blood cells and 30% of saline solution, and the absorbance spectra were measured in the NIR spectral range from 900 to 1700 nm (Fig. 4a).

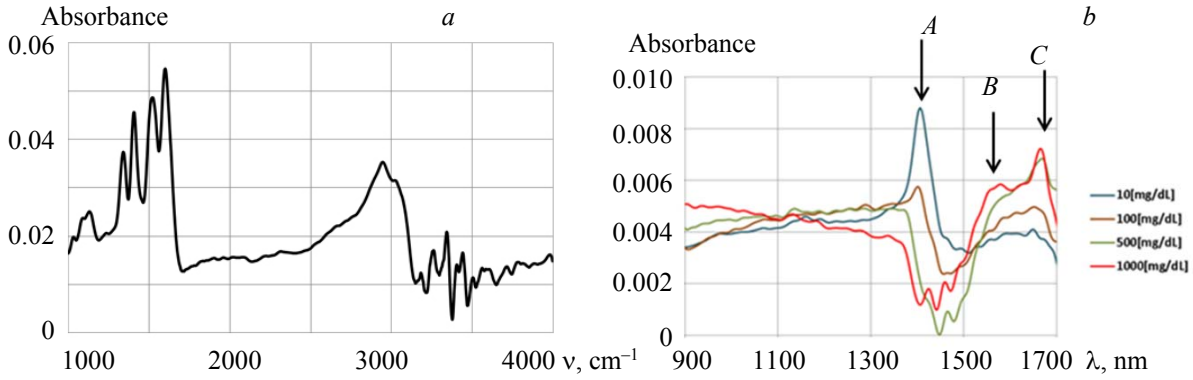


Fig. 3. (a) MIR absorbance spectrum of 25mg/mL phenylalanine in saline solution;
(b) NIR absorbance spectra of phenylalanine in saline solution.

Again, three bands, *A*, *B*, and *C*, are identified, where bands *A* and *C* are centered at the same wavelengths as in the previous, saline experiment (Fig. 3b), and *B* is centered at 1520 nm, which is also related to the first overtone of the N-H group like the 1560 nm band.

In order to detect the sharper and more accurate peaks of the absorption spectra of the samples in Fig. 4a, the 2nd derivative was calculated for the spectrum of the highest L-phenylalanine concentration.

Figure 4b illustrates the 2nd derivative of the absorption spectra of 56 mg/dL phenylalanine in the blood-saline solution in which six main bands, denoted *A*-*F*, are evident: band *A*, centered at 1405 nm, is assigned to the first overtone of the O-H group, band *B*, centered at 1495 nm, is assigned to the stretching overtone of the N-H group, bands *C* and *D*, centered at 1520 and 1560 nm, respectively, are both assigned to the first overtone of the N-H group, band *E*, centered at 1620 nm, is assigned to the NH_2 combination band, and band *F*, centered at 1670 nm, is assigned to the first overtone of the C-H group.

In addition to the direct spectroscopic analysis shown above, a PLS regression analysis was performed using the Unscrambler (Camo, Norway).

Partial least squares (PLS) is a widely used technique in spectroscopic analysis that can be used to construct a linear predictive model for the phenylalanine concentrations based on the spectrum. In our case, each absorption spectrum comprises 541 different frequencies.

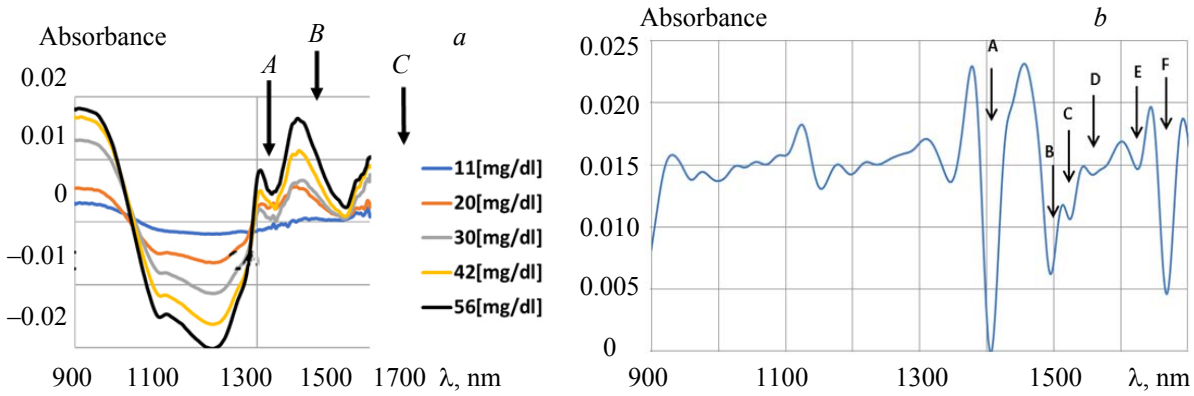


Fig. 4. NIR absorbance spectra of L-phenylalanine in a 70% blood and 30% saline solution. (a) Pure absorbance; (b) second derivative of the absorbance for the 56 mg/mL phenylalanine concentration.

PLS is used especially in cases in which the number of the independent variables (number of wavelengths) is significantly larger than the number of samples, as is the case here. Also, the conventional ordinary regression fails in the case of a large number of correlated but independent variables, as in our case.

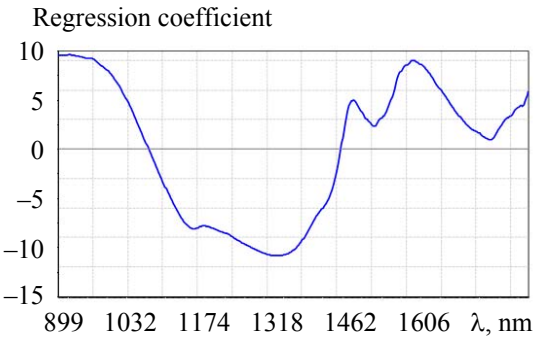
The main idea behind PLS is to first perform a principal component analysis on the independent (wavelength absorption data) and dependent (phenylalanine concentrations) variables, and then use only the first k principal components for the regression.

The spectral absorption of the NIR spectra was used to develop the PLS calibration model using the cross-correlation option. Figure 5 presents the calculated regression coefficients computed from the data. Figure 5 enables us to predict the phenylalanine concentration according to the equation:

$$\text{Predicted phenylalanine concentration} = b_0 + \sum_i b_i \left(\text{absorbance at } \lambda_i\right),$$

where the b_i is the regression coefficient at the ith wavelength.

Predicted phenylalanine values were calculated according to the regression coefficients and are plotted in Fig. 6 vs. the phenylalanine reference values. As can be seen in Fig. 6, the R-square of the regression analysis is 0.989.



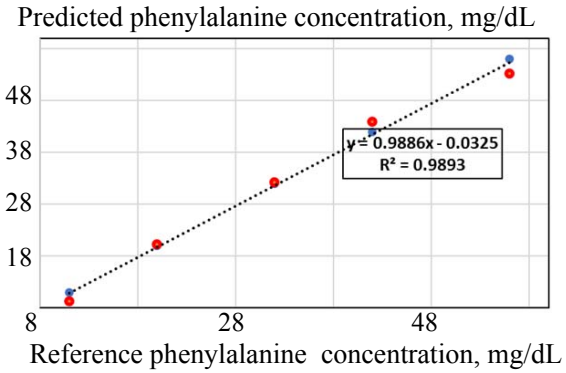


Fig. 5. Regression coefficient data derived from the PLS analysis.

Fig. 6. The predicted vs. reference phenylalanine concentration values.

Conclusions. We succeeded in demonstrating that L-phenylalanine can be detected in saline and in blood in the NIR spectral range, and especially in the range of 1400 to 1700 nm, with C-H and N-H overtones at the exact wavelengths of 1495, 1520, 1560, 1620, and 1670 nm. We must take into account that in order to develop an *in vivo* method for determining PKU, VIS spectroscopy must also be used in parallel for the detection of melanin. The current stage of the research addressed the L-phenylalanine spectra only. The first two *in vitro* stages were completed successfully. The final stage in the development of an *in vivo* me-

thod for determining PKU will consist of *in vivo* experiments on newborns in hospitals using the NIR spectral range to detect phenylalanine, simultaneously with the VIS spectral range, to detect melanin. We are also aware that the expected signals reflected from the skin will probably have lower SNR ratios, which will force us to use more complex clustering methods as well.

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