Quantitative analysis of homocysteine in liquid by terahertz spectroscopy

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Abstract: Homocysteine (C₄H₉NO₂S) is a variant of the amino acid cysteine, a harmful substance to the human body, which is closely related to cardiovascular disease, senile dementia, fractures, et al. At present, conventional methods for detecting homocysteine in biological samples include high performance liquid chromatography (HPLC), fluorescence polarization immunoassay (FPIA), and enzymatic cycling methods. These methods have the disadvantages of being time-consuming, sample-losing, chemical reagent-using and operation-cumbersome. Here, we present a method for the quantitative detection of homocysteine in liquid based on terahertz spectroscopy. Considering the strong absorption of water for terahertz beam, we also put forward a pretreatment method for drying samples at low temperature. These methods make the detection limit for homocysteine reach 10 µmol/L (human normal concentration). Based on the linear relationship between the homocysteine concentration and the THz spectral intensity, we can successfully achieve quantitative, accurate and real-time detection of homocysteine. As compared to Raman spectroscopy, the correlation coefficient of THz spectrum (R²₁₆.₂₄THz = 0.99809) is much larger than that of the Raman spectrum (R²₂₅₅₈.₂₆cm⁻¹ = 0.80022, R²₂₉₃₇.₃₂cm⁻¹ = 0.8028). These results are greatly useful for the accurate evaluation of pathological stage.

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1. Introduction

Homocysteine is a sulfur-containing amino acid that functions as a key intermediate in methionine metabolism and it is produced as a byproduct of methyl-transfer reactions [1]. In healthy middle-aged adults, the concentration of homocysteine is 5-15 µmol/L in human blood [2] and 3.5-9.5 µmol/L in urine [3]. Once the concentration of homocysteine is larger than the normal value, it will be defined as homocysteinemia, and can be classified as moderate (16 to 30 µmol/L), intermediate (31 to 100 µmol/L), and severe (>100 µmol/L) [4–5]. High levels of homocysteine will lead to cardiovascular disease, stroke, increase the fracture rate in the elderly, and even lead to Alzheimer’s disease and schizophrenia [6–9].

At present, conventional methods for detecting homocysteine in biological samples include high performance liquid chromatography (HPLC) [10–11], fluorescence polarization immunoassay (FPIA) [12], and enzymatic cycling methods [13]. However, these methods have the disadvantages of being time-consuming, sample-losing, chemical reagent-using and operation-cumbersome [14–16]. In addition, modern vibration spectroscopy methods, including NIR/MIR spectroscopy and Raman spectroscopy, can also work. However, these methods mainly analyze the substances based on the vibrational modes of chemical bonds and functional groups, which don’t work well
for the substances originated from the same category (their chemical bond and functional group are greatly similar to each other) [17–18].

Different from the NIR/MIR and Raman spectroscopy, the frequency range of THz wave corresponds to the vibration and rotation frequency range of the molecule. When THz passes through the sample, resonance absorption will occur if the THz frequency is consistent with the vibration and rotation frequency of the molecule, and these characteristic absorption peak spectra are unique. Therefore, THz spectroscopy has become an attractive qualitative and quantitative detection technique [19–23]. In recent years, terahertz spectroscopy has been widely used to detect biological and chemical molecules in drugs and food, such as proteins [24–25], amino acids [26], DNA [27] and even characteristic substances related to diseases [28–30]. Therefore, using terahertz spectroscopy is expected to achieve real-time, accurate and low-cost identification of homocysteine in blood/urine. However, the terahertz beam will be attenuated exponentially when it encounters aqueous solution, causing extremely low signal to noise ratio (SNR) and then difficulties in detection. At present, there is no good solution to this problem.

In this paper, terahertz spectroscopy is applied to the quantitative identification of homocysteine in liquid. We first tested the terahertz absorption spectrum of homocysteine in aqueous solution and concluded that it does not bind with water molecules in aqueous solution. Then, combing with a pretreatment method for drying samples at low temperature, we measured the absorption spectra of homocysteine at different concentrations, summarized and analyzed the linear relationship between the concentration of homocysteine and the THz spectral intensity. Finally, the quantitative analysis of homocysteine in liquid was achieved, and the limit of detection concentration was 10 µmol/L, which has reached the normal concentration in the human body. This study provides a reference for the subsequent rapid detection of homocysteine content in human blood/urine, which is of great significance for clinical detection applications.

2. Experimental setup and materials

The experimental setup is a commercial Fourier transform infrared spectrometer (FTIR) (Vectex 80v, Bruker Optics). The light source is a water-cooled mercury lamp and is detected by a DLaTGS/polyethylene detector. The spectral region covers 0.9-20.0 THz effectively, and the signal-to-noise ratio (SNR) is better than 10000: 1. All spectra were measured with a resolution of 4 cm⁻¹, a scan number of 32, and a scan speed of 5 kHz.

Silicon wafers (d = 0.3 mm, R = 20000 Ω · cm) were purchased from University Wafer, Inc. It was used as a glass slide, and the corresponding spectrum was collected as a reference spectrum.

Pure homocysteine (HCY) samples (≥ 95%, CAS: 454-29-5) were purchased from Sigma Aldrich. Considering that the content of homocysteine in human blood and urine is very low and the content of water is very high, but water has a great absorption of terahertz wave. Therefore, we dissolved homocysteine powder in pure water for quantitative analysis. Ultrapure water was provided by the laboratory water purification system (ELGA, UK).

3. Results and discussion

3.1. Fingerprint spectrum of homocysteine in aqueous solution

First, we measured the fingerprint spectrum of pure HCY powder in the range of 0.9-20.0 THz. The HCY powder was uniformly covered and fixed on the Si wafer with transmission tape for detection. The corresponding molecular formula and characteristic spectrum are shown in Fig. 1 (a)&(b), respectively. It can be seen that HCY has obvious characteristic absorption peaks at 1.91 THz, 3.47 THz, 6.24 THz, 9.36 THz, 11.19 THz, 12.57 THz, 13.41 THz, 16.24 THz and 19.42 THz.

Considering that HCY molecules may dissociate or bind with water molecules to form hydrates in the liquid state such as blood or urine, its THz absorption peaks standing for the vibration and
rotation frequency of the combined molecules will change. In order to verify this, we tested the change process in the experiment: took 20 µL of 0.1 mol/L HCY aqueous solution, dropped it on the Si wafer and placed it in the FTIR sample chamber to vacuumize for continuous detection. Here, the time interval between each test was 1 minute, until the spectrum was no longer change. The THz spectra of continuous detection are shown in Fig. 1 (c). It can be seen clearly that during the entire process of vacuum drying, the absorption peaks of HCY gradually appear as the water decreases, but the frequency points corresponding to the absorption peaks don’t have any change. Therefore, we can conclude that HCY molecule doesn’t form any hydrate with water molecules. Next, we extracted the time variable amplitude of each absorption peak [see Fig. 1 (d)]. It was found that the amplitude of the absorption peaks reached a stable level in about 7 minutes. According to this result, in the following experiments, we propose to dry HCY solution at low temperature (no more than 40 °C) before testing, saving the time required for water removal. The temperature is set below 40 °C to avoid protein mutate due to excessive temperature, for which may exist in the actual blood/urine solution.

3.2. Quantitative analysis of homocysteine in liquid at different concentrations

After clarifying the HCY absorption peak in the aqueous solution, we tested the homocysteine spectra in liquid at different concentrations to extract the relation between the absorption peaks and concentration. Using pure water as a solvent, we configured a set of samples with HCY concentration range of 1-5 mmol/L, each sample took 100 µL, and then put the Si wafer containing the sample into the oven (BG2-30) for drying at 40 °C for 15 minutes before testing. The time of low-temperature drying can be adjusted freely according to the volume of samples. As shown in Fig. 2 (a), it is obvious that the amplitude of absorption peak gradually increases with the
increase of sample concentration. To further quantify the change of the absorption peaks with the increase of HCY concentration, we extracted the peak amplitude to do the quantitative analysis. Considering that the amplitude of three peaks at 6.24 THz, 9.36 THz, and 16.24 THz are larger than the other characteristic peaks, the analysis error will be small, and the peaks change will be more obvious when the concentration is lower. Therefore, these three peaks are selected for the further analysis. As shown in Fig. 2 (b), linear fittings on the data were performed according to Beer-Lambert Law. The corresponding linear fitting function expressions are:

\[
y = 0.0063x + (4.38 \times 10^{-5}), R^2 = 0.99794 \tag{1}
\]
\[
y = 0.0060x + (1.48 \times 10^{-4}), R^2 = 0.99802 \tag{2}
\]
\[
y = 0.0104x - (4.52 \times 10^{-4}), R^2 = 0.99717 \tag{3}
\]

x is the sample concentration (mmol/L), and y is the amplitude in the expression (arb.units), $R^2$ is the correlation coefficient between HCY concentration and its THz amplitude. Equations (1), (2), and (3) correspond to the amplitude at 6.24 THz, 9.36 THz, and 16.24 THz, respectively.

Fig. 2. (a) Terahertz absorption spectra of homocysteine samples at the concentration of 1-5 mmol/L measured experimentally, (b) Absorbance at 6.24 THz (Pentagram), 9.36 THz (triangle) and 16.24 THz (square) for different concentrations of homocysteine samples, error bars have been labeled on each data.

We notice that the amplitude at 16.24 THz and its change slope are much higher than that of the other two absorption peaks, which is beneficial to the accurate recognition of weak signals and the accurate determination of samples at different concentrations. Therefore, we select the absorption peak at 16.24 THz to further analyze the THz amplitude change corresponding to the human concentration. We configured HCY aqueous solution in the concentration range of 10-150 µmol/L, of which 10 µmol/L is the normal concentration in human blood/urine. Considering that the content of homocysteine in human body is very low, we selected 1mL of each sample and put it into ep tube for 40 °C drying in the oven. When there was very little water in the samples, we transferred all the remaining samples onto Si wafer for testing. The spectra are shown in Fig. 3 (a). Similarly, we extracted the amplitude at 16.24 THz for linear fitting [see Fig. 3 (b)]. The corresponding linear fitting function expression is:

\[
y = (9.83 \times 10^{-5})x - (1.68 \times 10^{-5}), R^2 = 0.9988 \tag{4}
\]

x is the sample concentration (µmol/L), and y is the amplitude (arb.units) in the expression, $R^2 = 0.9988$ is the correlation coefficient between HCY concentration and the amplitude of 16.24 THz. These results prove that our method can detect low concentration HCY effectively and can realize the rapid quantitative analysis with high sensitivity. This will be very useful for later real-time fast and high-sensitivity clinical trials.
3.3. Comparative analysis of Raman spectroscopy

After quantitative analysis of HCY samples, we also compared our detection method with other technology. Here, we mainly evaluate the accuracy of the test results. We selected the Raman spectrum to repeat the above experiments. Compared with terahertz, Raman spectrum also belongs to physical detection, and there is no loss in the samples during detection, so the comparative analysis is more reasonable.

In the experiments, we used the laser confocal micro Raman spectrometer (LabRAM HR Evolution, HORIBA Scientific) with the excitation wavelength of 532 nm. To prevent the sample from being damaged, the power of laser source in Raman spectrometer is chosen as 250 mW, with the acquisition time of 5 s and spectral resolution of less than 2 cm\(^{-1}\). The experimental complete Raman spectrum of 575 cm\(^{-1}\) - 3200 cm\(^{-1}\) is shown in Fig. 4 (a), in which 672.57 cm\(^{-1}\) (corresponding to the -CS bond), 2558.26 cm\(^{-1}\) (corresponding to the -SH bond) and 2937.32 cm\(^{-1}\) (related to the stretching vibration of CH and CH\(_2\) groups in the range of 2900 - 3000 cm\(^{-1}\)) are selected as the Raman characteristic peaks of HCY [31–32]. Since the peak of 974.36 cm\(^{-1}\) is the fingerprint spectrum of silicon wafer, and the peak of 672.57 cm\(^{-1}\) is too weak, therefore we chose 2558.26 cm\(^{-1}\) and 2927.32 cm\(^{-1}\) for quantitative analysis. Similarly, the peaks at these two points were extracted for linear fitting. The results are shown in Fig. 4(b) and 4(c).

Considering the unit difference between the two technologies, we normalized the terahertz absorbance and Raman intensity, and compared their correlation coefficient R\(^2\), which reflects

![Fig. 3.](image-url) 

Fig. 3. (a) Experimentally measured THz absorption spectra of homocysteine samples at the concentration of 10-150 µmol/L. (b) Absorbance at 16.24 THz as a function of HCY concentrations, error bars have been labeled on each data. Note: The gray box indicates the range of human normal concentration.

![Fig. 4.](image-url) 

Fig. 4. (a) Raman spectra of homocysteine samples at 10-100 µmol/L, (b) and (c) intensity of characteristic peaks at 2558.26 cm\(^{-1}\) and 2937.32 cm\(^{-1}\) for different concentrations of homocysteine samples.
the linear correlation between variables. The theoretical prediction is based on the fitting line of Beer-Lambert Law. As shown in Fig. 5, the THz spectral results are in good agreement with the theoretical prediction results, and the correlation coefficient ($R_{16.24\text{THz}}^2 = 0.99809$) is much larger than that of the Raman spectrum ($R_{2558.26\text{cm}^{-1}}^2 = 0.80022$, $R_{2937.32\text{cm}^{-1}}^2 = 0.8028$). Therefore, THz spectroscopy has higher accuracy than that of Raman spectroscopy for the detection of low concentration samples.

Fig. 5. Comparison of homocysteine measured by THz and Raman spectroscopy. The purple solid line represents the standard value of concentration, and the corresponding methods are marked with different symbols. $R^2$ is the correlation coefficient.

4. Conclusion

In this paper, we realized the quantitative identification of homocysteine in liquid by using terahertz spectroscopy. Basing on the test of homocysteine in aqueous solution and at different concentrations, we find the limit of detection concentration was 10 µmol/L, which has reached the normal concentration in the human body. In addition, the linear relationship between the homocysteine concentration and the spectral intensity can be used to realize the quantitative, accurate and real-time detection of homocysteine. As compared to Raman spectroscopy, the correlation coefficient of THz spectrum ($R_{16.24\text{THz}}^2 = 0.99809$) is much larger than that of the Raman spectrum ($R_{2558.26\text{cm}^{-1}}^2 = 0.80022$, $R_{2937.32\text{cm}^{-1}}^2 = 0.8028$). Based on the above results, this study provides a reference for the subsequent rapid detection of homocysteine content in human blood/urine, which is of great significance for clinical detection applications.
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Disclosures

The authors declare no conflicts of interest.

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