Chemical Analysis of Mimic Urine in Pathogenic Conditions using ATR-FTIR Spectroscopy

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Abstract. Urine is commonly used for the diagnosis and prognosis of several illnesses, particularly renal diseases. Quantitative analysis specifying urinary compounds provides valuable information for clinical applications. ATR-FTIR spectroscopy is a non-destructive method based on vibrational bonding that is currently being explored as a potential point-of-care test (POCT) for detailed analysis of both compound types and their concentrations. This study aimed to identify chemical compounds in simulated urine samples spiked with glucose, albumin, and hemoglobin using an ATR-FTIR spectrometer. The method involved measuring urine samples spiked with glucose, albumin, and hemoglobin at concentrations ranging from 1.000 – 0.005 g/dL, followed by multivariate statistical analysis. The study found that spectral patterns between 1073 cm^{-1} and 983 cm^{-1} (C-O stretching), 1390–1370 cm^{-1} (N-H stretching), and 1550–1540 cm^{-1} (N-H bending) were specific for urine spiked with glucose, albumin, and hemoglobin, respectively. The lower detectable concentrations of glucose, albumin, and hemoglobin in spiked urine were 0.029, 0.079, and 0.071 g/dL, respectively. This detection capability could be applied to identify abnormal urine, such as that found in CKD patients. Therefore, ATR-FTIR spectroscopy shows potential for use in distinguishing biomolecular differences without requiring reagents or complex sample preparation steps. However, this method requires further validation for clinical applications, particularly for monitoring and disease progression.

1 Introduction

Urine is a body fluid that contains thousands of compounds. It is formed by the kidneys, which filter blood through the glomerulus. The major component of urine is

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water (over 92%), along with nitrogenous compounds such as creatinine, urea and uric acid, and other metabolites [1]. Urine analysis (Urinalysis) is an important routine test for metabolite detection and diagnostic purposes [2]. Urinalysis allows for low-cost, effective analysis, and holds promise as a key resource for health monitoring. It can be conducted effortlessly on a daily basis without special equipment [2–4]. However, urinalysis has many limitations, including limited sensitivity and specificity, cross-reactivity with other agents, interference, false positive and false negative, low reproducibility, and qualitative as well as semi-quantitative measurement [5].

Automated urinalysis is often used for high throughput analysis and enhanced testing performance compared to manual methods. It is useful for the quantification of metabolite levels, imaging, and interpretation of sediment using machine learning (ML). Moreover, it can increase the sensitivity for the screening, diagnosis, and monitoring of diseases such as urinary tract infections and urolithiasis. Recently, advanced systems such as automated microscopy, flow cytometry, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, and laboratory-on-a-ship have been developed for reducing analytical errors and improving the quality of sediment and chemical test analysis [6]. However, while suitable for quantitative analysis, these methods are expensive, have long turnaround times (TAT), and are not suitable for point-of-care testing (POCT). Therefore, point-of-care devices should be applied for chemical component detection in routine applications.

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, which is non-destructive, user-friendly, cost-effective, label-free reagent, and offers high sensitivity and specificity, measures molecular bonding vibrations using mid-IR radiation [7,8]. Molecules exhibit vibrational modes corresponding to specific IR frequencies, providing information on the chemical compositions of samples [9].

Many studies have applied ATR-FTIR spectroscopy to determine compounds in samples, including blood and urine. Previous studies also reported that ATR-FTIR spectroscopy has been used to detect compounds such as drugs (e.g. lidocaine, bambuterol, and terbutaline), and biomolecules (protein) with specific spectral patterns, high sensitivity, and limit of detection (LOD) in ppm levels [10–13]. Thus, this method has the potential to detect individual molecules with high sensitivity and specificity.

Accordingly, this study aims to identify chemical compounds in urine samples spiked with glucose, albumin, and hemoglobin, mimicking the pathological conditions of renal diseases that may lead to the excretion of these components, thus demonstrating the capability of the ATR-FTIR spectrometer method.

2 Materials and Methods

2.1 Standard solutions preparation

Standard solutions were prepared for the experiments, including albumin, glucose, and hemoglobin. A bovine serum albumin (BSA) solution was prepared at a concentration of 5 g/dL by dissolving 2.5 g of BSA (Capricorn Scientific) in 40 mL of 0.90% normal saline (Klean & Kare Normal Kare). The mixture was allowed to sit at 4 degrees Celsius for 10 minutes to ensure that BSA did not precipitate, after which the solution was filled up to

a total volume of 50 mL. A glucose solution was prepared at a concentration of 4 g/dL by dissolving 2.0 g of glucose powder (D-glucose, APS Finechem) in 50 mL of 0.19% benzoic acid solution. A hemoglobin solution was prepared at a concentration of 2 g/dL by dissolving 1.0 g of dried hemoglobin (BD and BBL™ Hemoglobin Bovine, Becton, Dickinson) in 50 mL of distilled water. Then, all solutions were stored at 4 degrees Celsius before being used in experiments.

2.2 Ethical approval and simulated urine sample preparation

This study was approved by the local institutional ethics committee at the Center for Ethics in Human Research, Khon Kaen University (ECKKU), under HE664019. The experiments were conducted following the relevant guidelines and regulations, adhering to the ethical standards of the 1964 Declaration of Helsinki. All specimens were collected as leftover specimens from Srinagarind Hospital. Inclusion criteria comprised normal urine with negative urinalysis test results, estimated glomerular infiltration rate (eGFR) greater than or equal to 60 mL/min/1.73 m^2, and age at least 18 years old.

Two milliliters of randomly selected normal urine samples were pooled. The pooled urine was then prepared as mimic urine under four conditions: non-spiked and spiked with three individual chemicals (albumin, glucose, and hemoglobin) at ten varying concentrations (Table 1.). The simulated urine samples were frozen at -20 degrees Celsius before utilized.

Table 1. Preparation states of mimic urine: non-spiked and three spiked samples

Conditions	Concentrations (g/dL)
Non-spiked urine	-
Albumin-spiked urine	1.000 0.800 0.600
Hemoglobin-spiked urine	0.400 0.200 0.100 0.050
Glucose-spiked urine	0.025 0.010 0.005

2.3 ATR-FTIR spectral acquisition and data processing

The frozen urine samples were thawed and centrifuged at 10,000 RPM for 5 minutes at room temperature. The supernatant was collected for ATR-FTIR measurements. Three microliters of supernatant were deposited onto the diamond ATR crystal, dried for 10 minutes, and measured using a portable Agilent ATR-FTIR spectrometer from the 4500 series (Agilent Technologies, CA, USA). Measurement parameters included 64 co-added

scans for both background and sample with a 4 cm^{-1} spectral resolution in the 4000–650 cm^{-1} (mid-infrared) range. Each sample was measured for ten replicates. Spectral data were carried out using an Agilent MicroLab PC and analyzed with the Unscrambler® X (Camo Software, Norway) packages.

Preprocessing methods were applied to all ATR-FTIR spectral data. Initially, all spectra were processed by the baseline correction to adjust the spectral offset. Then, the second derivatives ($d^2\lambda/d\lambda^2$) with 3-order polynomials and 17 smoothing points using the Savitzky-Golay algorithm were used to resolve adjacent peaks and sharpen minor spectral features. The preprocessed data were analyzed in the fingerprint region (1800-800 cm^{-1}) and CH region (3000-2800 cm^{-1}). Multivariate analysis was performed by Partial Least Square Regression (PLSR), a supervised classification and multivariate modeling approach for the determination of linear correlations between the X-matrix variable and Y-matrix variable. Four-fifths of the preprocessed data were performed as a calibration set, and the remaining data were utilized as the validation set.

3 Results

3.1 ATR-FTIR spectra of standard solutions

The standard solutions of glucose, albumin, and hemoglobin were prepared and analyzed by an ATR-FTIR spectrometer. ATR-FTIR spectra showed differences due to composition variations (Fig. 1).

The average raw spectra showed a specific pattern of glucose standard in the 1200-900 cm^{-1} region, while albumin and hemoglobin exhibited a similar pattern. The second derivatives were performed to enhance minimal peaks. The difference was most noticeable in the glucose standard, which showed a majority of components in 1200-900 cm^{-1}, correlated to the raw data. The main peaks of glucose standard were observed at 1104, 1073, 1023, 1010, and 983 cm^{-1}, which are prominent in carbohydrates.

Albumin and hemoglobin standards represented a similar pattern in all regions due to their protein components. However, some different peaks were observed in both solutions. A shoulder at 1625 cm^{-1} was found in hemoglobin, while a sharper peak at 1512 cm^{-1} was observed in albumin. Additionally, there was a peak shift from 1394 to 1389 cm^{-1} in albumin and hemoglobin standards, respectively.

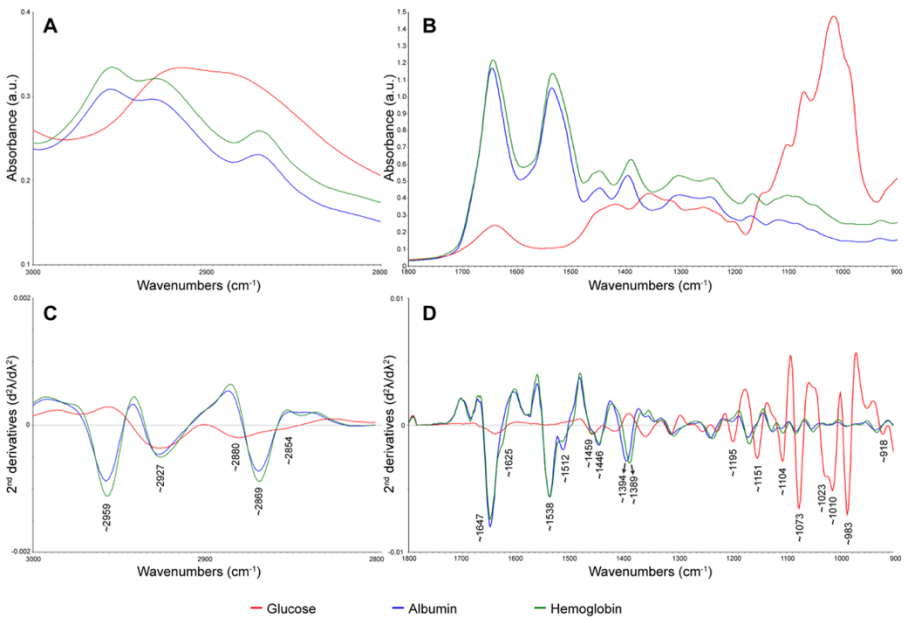

Fig. 1 Average ATR-FTIR spectra of standard solutions showing ATR-FTIR spectra of glucose (red line), albumin (blue line), and hemoglobin (green line) for (A) raw spectra in the CH region, (B) raw spectra in the fingerprint region, (C) second derivatives spectra in the CH region, and (D) second derivatives spectra in the fingerprint region.

3.2 ATR-FTIR spectra of mimic urine samples

To identify the specific patterns corresponding to their spiked standard solutions, the average raw spectra of spiked urine samples were analyzed and compared with non-spiked urine.

In spiked glucose urine, distinct peaks were dominant in the 1200-900 cm^{-1} region (Fig. 2A and Fig. 2B) related to a major characteristic region of the glucose solution (Fig. 1). The raw data for spiked glucose urine showed higher intensity compared to non-spiked urine. Observable average spectra peaks of spiked glucose urine were at 1073 and 983 cm^{-1}.

In spiked albumin urine, the spectra were not significantly different compared to non-spiked urine (Fig. 2C and Fig. 2D), although the raw data showed higher intensity at 1686, 1651, 1610, 1389, 1169, and 1132 cm^{-1}, but not discernibly. The only noticeable difference was the 1614/1588 ratio, which was higher in spiked albumin urine.

The spectra of spiked hemoglobin showed similarity to the spiked albumin conditions (Fig. 2E and Fig. 2F). The raw data also showed higher intensity in spiked hemoglobin urine compared to non-spiked urine. Additionally, 1539, 1515, and 1441 cm^{-1} and a higher 1614/1588 ratio was observed in spiked hemoglobin urine.

To define the specific spectra pattern of spiked albumin and spiked hemoglobin urine, a spectra comparison was performed. The consensus informative peaks were identified as 1390-1370 cm^{-1} in spiked albumin urine, and 1550-1540 cm^{-1} in spiked hemoglobin urine.

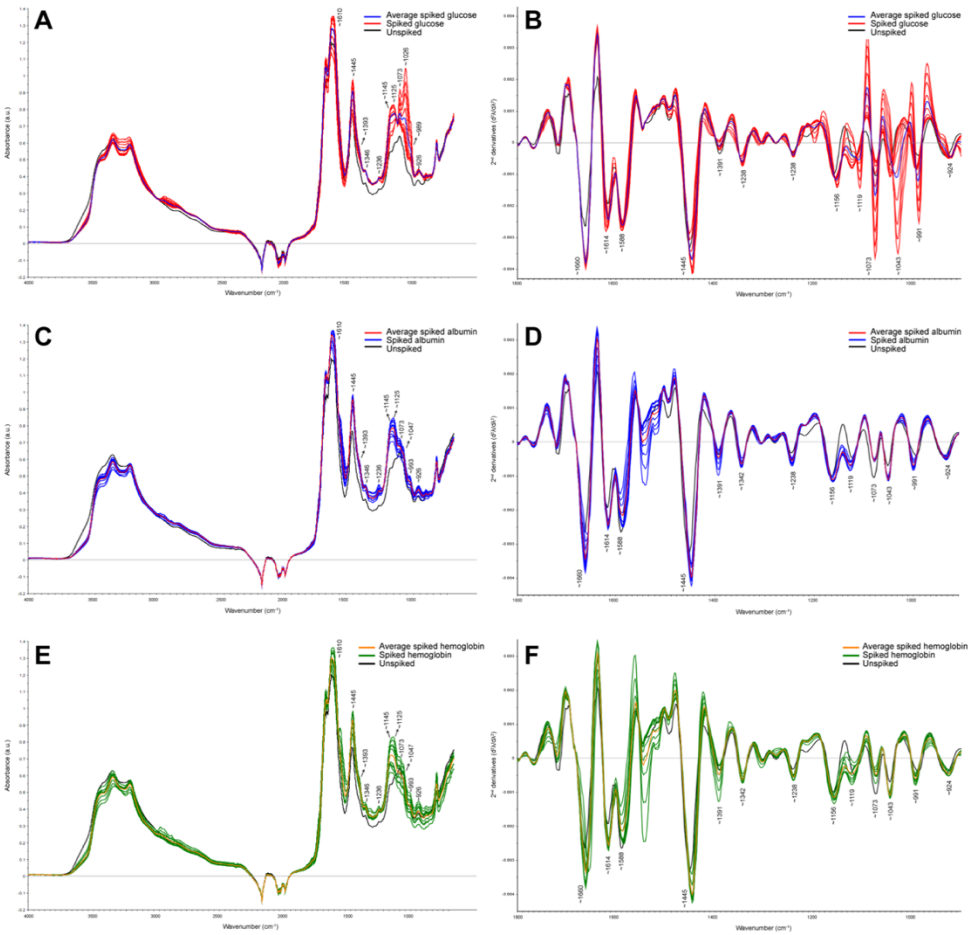

Fig. 2 Average ATR-FTIR spectra of spiked urine conditions showing spectra of unspiked urine (black line), spiked glucose urine (red line), spiked albumin urine (blue line), and spiked hemoglobin urine (green line). Raw spectra (A, C, and E) are presented for the entire region, while the second derivatives spectra (B, D, and F) are shown for the fingerprint region. Panels (A, B) compare unspiked urine and spiked glucose urine, while (C, D) compare unspiked urine and spiked albumin urine, and (E, F) compare unspiked urine and spiked hemoglobin urine.

3.3 Lower limit of detection (LOD) and Lower limit of quantification (LOQ)

To identify the potential of the ATR-FTIR spectrometer, the lower limit of detection (LOD) and lower limit of quantification (LOQ) were analyzed. The LOD was calculated as 3.3 times the ratio of standard deviation (RMSE) to the sensitivity of the method (slope), and the LOQ was determined as three times the LOD. The regression resultants of the calibration set were expressed as a linear equation: $Y = mX + C$, where Y represents the predicted concentration result, X represents the calculated ATR-FTIR data, and m and C denote the slope and intercept (offset) of the calibration model, respectively. Each equation demonstrates a strong linear relationship (R^2) between the predicted and actual concentrations, highlighting the accuracy of the regression models.

In ten concentrations of spiked glucose urine, the prominence pattern was observed in 1200-900 cm^{-1} with an increased intensity corresponding to concentrations. However, the intensity of second derivatives spectra of non-spiked urine was higher than 0.100 g/dL spiked glucose condition (Fig. 3A), consistent with the scatter plots. The apparent discrimination of each glucose concentration was found above 0.100 g/dL (Fig. 3B). Moreover, the PLSR model with 7 factors was used to predict glucose levels. The model showed linear prediction in calibration data with $Y = 0.9993X + 0.1981$, and $R^2 = 0.9993$. The performance of the model enabled lower detection at 0.029 g/dL and lower quantification at 0.088 g/dL (Fig. 3C).

In ten concentrations of spiked albumin urine and spiked hemoglobin urine, the derivatives spectra did not exhibit a trend in intensity across subregions (Fig. 3D and Fig. 3G). The intensity of each concentration was not related to the spiked concentration. However, the scatter plot represented the trend of separation in each condition. The concentration of albumin and hemoglobin at 0.200 g/dL showed clear discrimination (Fig. 3E and Fig. 3H). In spiked albumin conditions, the linear regression in calibration was $Y = 0.9931X + 2.0029$ with a high correlation at $R^2 = 0.9930$. The model for predicting albumin could detect a lower albumin concentration at 0.079 g/dL and lower quantification at 0.239 g/dL (Fig. 3F). Similarly, spiked hemoglobin conditions represented a highly linear correlation with $R^2 = 0.9978$, $Y = 0.9978X + 0.6445$. The model performance was able to detect and quantify hemoglobin at lower concentrations of 0.071 g/dL and 0.217 g/dL, respectively (Fig. 3I).

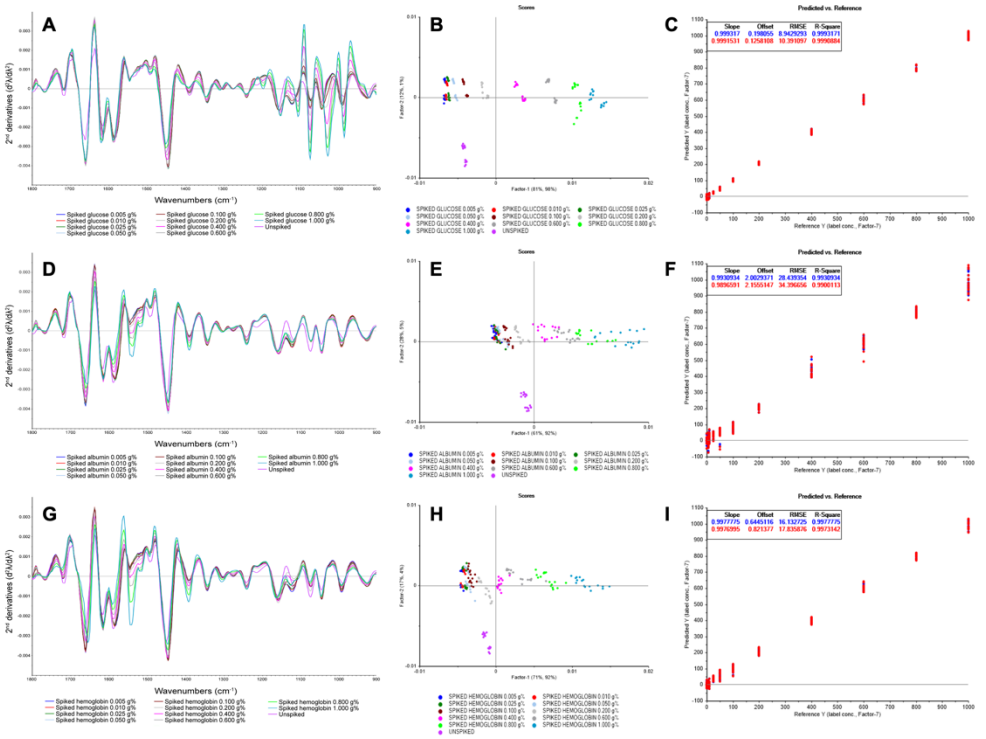

Fig. 3 Regression analysis for quantitative evaluation of ATR-FTIR spectroscopy. Average second derivatives ATR-FTIR spectra for ten concentrations of spiked urine: (A) spiked glucose

urine, (D) spiked albumin urine, and (G) spiked hemoglobin urine. The PLSR results for predicting chemical concentration are presented, where PLS models correspond to (A, B, and C) glucose, (D, E and F) albumin, and (G, H and I) hemoglobin. Scatter plots (B, E, and H) are labeled with ten different colors for each condition, and regression plots (C, F, and I) show the prediction for each concentration.

4 Discussion

This study aimed to evaluate the performance of the ATR-FTIR spectrometer concerning the identification of spectral patterns for chemical compound detection in urine. The spectral region of glucose substance was prominent in the 1200-900 cm^{-1} region, corresponding to C-O stretching vibrations [14]. The infrared spectra of glucose-spiked urine exhibited peaks at 1073 and 983 cm^{-1}, mainly representing the C-O stretching of glucose components. This finding can be used for monitoring glucose levels [14,15]. Albumin and hemoglobin solutions exhibited similar patterns in the amide region with slight differences in some minimal peaks. In spiked albumin and hemoglobin urine, only the 1614/1588 (amide I/amide II) ratio was higher. This higher ratio was associated with a greater secondary structure of protein, indicating potential changes in protein structure [16]. Albumin-spiked urine showed peaks in the 1390-1370 cm^{-1} region, referring to N-H stretching in the amide III structure [17]. Hemoglobin-spiked urine also exhibited peaks in 1550-1540 cm^{-1}, corresponding to N-H bending in amide II structure [18]. Non-spiked urine was representatively prominent in the 1640-1630 cm^{-1} region, which was assigned to C=O stretching and C=N stretching vibrations, referred to as major components of creatinine and urea in normal urine [19]. The prediction has shown errors between these two components due to the similarity in the amide structures of albumin [20] and hemoglobin [21], especially at low concentrations. This is likely caused by the interference of major components in urine, such as creatinine and uric acid [1]. However, this study only presented data from a single lot of spiked urine preparation, which may not be representative of all possible chemical variations. Therefore, various sources and variants of spiked samples should be explored. Additionally, further studies should focus on findings from clinical specimens for clinical application.

The lowest detection performances (LOD) for spiked glucose, albumin, and hemoglobin urine were determined to be 0.029, 0.079, and 0.071 g/dL, respectively. These values are relatively higher compared to previous studies, which could detect chemical compounds at the ppm level (1 ppm is approximately 0.0001 g/dL). Several studies have reported the LOD of ATR-FTIR spectrometer in urine samples as 6.7 ppm for microproteinuria [22], 1.5 to 1.7 ppm for lidocaine drug [10], and less than 100 ppm for bambuterol and terbutaline drugs [11]. The higher LOD obtained in this study may be attributed to the preparation steps and the general structures of the compounds. In comparison, other studies employed special steps for sample preparation. Therefore, advanced sample pretreatment may be necessary for urinary composition analysis. Moreover, numerous studies have applied ATR-FTIR data combined with machine learning, such as neural networks and multivariate analysis, to identify significant infrared signature patterns and enhance the effectiveness of regression statistical analysis. However, the performance of this technique cannot be equally compared to routine tests,

such as a urine dipstick test, for some parameters, as the latter has a lower LOD (glucose: 0.050 g/dL, albumin: 0.030 g/dL, and hemoglobin: 0.010 g/dL). The LODs from this study may be useful in certain clinical scenarios, such as screening for urine abnormalities. Under normal conditions, glucose in urine can be up to 0.025 g/dL [23]. The LOD for glucose in this study could be suitable for early detection in patients who have glycosuria. Additionally, the LOD for albumin greater than 0.079 g/dL could be detected in microproteinuria to macroproteinuria from the urine albumin-to-creatinine ratio (UACR). This report could have potential practicality for the monitoring and prevention of disease progression to later stages.

5 Conclusion

This study demonstrated the spectral signatures of glucose, albumin, and hemoglobin in spiked urine samples, showcasing the ability of the ATR-FTIR machine to detect specific chemical components. However, this method requires further validation for clinical applications, particularly in terms of monitoring and disease progression.

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