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# High wavenumber Raman spectroscopy in the characterization of urinary metabolites of normal subjects, oral premalignant and malignant patients

Elumalai Brindha [a], Ramu Rajasekaran [a], Prakasarao Aruna [a,*], Dornadula Koteeswaran [b], Singaravelu Ganesan [a]

[a] Department of Medical Physics, Anna University, Chennai, India
[b] Department of Oral Medicine and Radiology, Meenakshi University, Chennai, India

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ABSTRACT

Urine has emerged as one of the diagnostically potential bio fluids, as it has many metabolites. As the concentration and the physiochemical properties of the urinary metabolites may vary under pathological transformation, Raman spectroscopic characterization of urine has been exploited as a significant tool in identifying several diseased conditions, including cancers. In the present study, an attempt was made to study the high wavenumber (HWVN) Raman spectroscopic characterization of urine samples of normal subjects, oral premalignant and malignant patients. It is concluded that the urinary metabolites flavoproteins, tryptophan and phenylalanine are responsible for the observed spectral variations between the normal and abnormal groups. Principal component analysis-based linear discriminant analysis was carried out to verify the diagnostic potentiality of the present technique. The discriminant analysis performed across normal and oral premalignant subjects classifies 95.6% of the original and 94.9% of the cross-validated grouped cases correctly. In the second analysis performed across normal and oral malignant groups, the accuracy of the original and cross-validated grouped cases was 96.4% and 92.1% respectively. Similarly, the third analysis performed across three groups, normal, oral premalignant and malignant groups, classifies 93.3% and 91.2% of the original and cross-validated grouped cases correctly.



## 1. Introduction

Cancer has become one of the most common causes for the high morbidity and mortality rate next to cardiac related diseases. World Health Organization (WHO) has reported that, the incidence and mortality rate owing to cancers will be doubled by the year 2030 [1,2]. In India, 80,000 new oral cancer cases are diagnosed annually, where majority of them are detected only in the advanced stages [3]. Tobacco chewing, including exposure to smokeless tobacco products and excessive alcohol consumption accounts for about 90% of oral cancer risk and play a significant part in the etiology of oral cancer [1,4].

Tobacco related carcinogens may produce mutations in the tumor suppressor protein p53. As this protein plays a significant role in the cell proliferation and DNA repair mechanisms mutations in the gene due to the carcinogenic effects of tobacco may produce DNA damage in cells, thereby leading to the development of potentially malignant oral disorders (premalignant lesions) such as leukoplakia or erythroplakia and oral submucous fibrosis (OSMF) [5,6]. As the possibilities of transforming these premalignant lesions into malignant is high, early stage disease diagnosis is mandatory for better treatment [7,8]. Though the oral premalignant and malignant lesions have distinct clinical features from that of the healthy oral cavity, patients are reporting to the doctor only at the advanced stages which further increase the mortality rate [2]. Hence, the high mortality rate of oral cancers may be greatly reduced when the disease is detected at the early stages, as the probability of disease free survival rate is remarkably high in oral cancers [9].

The histology of oral cavity is highly complex as the tissue structure and biochemical contents may vary with respect to different sites and the detection and discrimination by conventional techniques may mislead the diagnosis as they are subjective [7,10]. Based on these, researchers are looking for an alternative diagnostic modality for the early diagnosis of the disease. In this context, molecular pathology has been considered to detect and understand the molecular mechanism behind the pathological transformation of normal cells into neoplasm [8]. During the transformation of normal oral tissues into malignant,

* Corresponding author at: Department of Medical Physics, Anna University, Chennai 600025, India.
*E-mail addresses:* aruna@annauniv.edu, arunaprakasarao@gmail.com (P. Aruna).

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both biochemical and structural variations take place at the cellular and tissue level with varying optical and biological properties, and these micro-environmental changes play a key role in providing the specific spectral signatures which could be exploited by the optical based diagnostic techniques [11,12].

Many optical techniques have been found effective in the field of diagnostic oncology [13–23]. Among these, Raman spectroscopy combined with highly developed chemo metrics has shown to be a potentially significant tool in diagnostic oncology, as it provides detailed information about the molecular composition, conformational and structural changes within the tissues under different pathological transformation [17,23–29]. Many researchers reported the feasibility of Raman spectroscopic characterization and diagnosis of premalignant and malignant tissues in the "fingerprint region (FP)" i.e. 600–1800 $cm^{-1}$, as Raman spectral signatures in the above said region provides wealth of information about the diagnostically relevant bio molecules such as proteins, lipids and DNA [14,26–44]. These studies emphasize the potential of using Raman spectroscopy in diagnostic oncology in the FP region at cellular and tissue level. Though fingerprint region offers wealth of information, Raman spectra in the high wavenumber region has geared up recent years in the characterization of biomaterials in diagnostic oncology as this region is almost free from fluorescence and provides relatively intense Raman signals from bio molecules [43–50]. Also, HWVN region has a larger Raman scattering cross-section in the NH, OH, and CH stretching region compared to FP region which allows the better assessment of the Raman signal [Reference 49]. Rolf Wolthuis et al. have measured the water concentration in brain tissue using HWVN region Raman spectroscopy and achieved appreciable results [47]. Mo et al. have demonstrated that HWVN Raman spectroscopy allows for non-invasive detection of cervical pre cancer [48]. Thus, several research groups have shown the possibilities of using HWVN Raman spectroscopy in diagnostic oncology at tissue and cellular level [49,50].

However, not much reports are available on the Raman spectroscopic analysis of bio fluids. Recently, attention has been gained towards the analysis of body fluids for disease diagnosis as it provides diagnostically relevant information under different pathological conditions [53–61]. Among various bio fluids, urine is also considered to be one of the important biological fluids, as it has many metabolites, and the physiochemical properties of these metabolites may vary under different pathological conditions [53]. Studies reported that during the transformation of normal cells into abnormal, excess metabolic end products are released into the blood which in turn is excreted in the urine through glomerular filtration with altered micro environment. These altered levels of metabolites under abnormal conditions may be used to discriminate malignant from normal conditions [62,63]. Based on this, preliminary studies have reported on the Raman spectroscopic characterization of urine samples in diagnostic oncology [58,64–67]. In this regard, we have reported elsewhere the possibility of using FP Raman spectroscopy combined with multivariate statistical analysis tool to discriminate the urine samples of oral cancer patients from that of normal subjects with the sensitivity and specificity of 98.6% and 87.1% with an overall accuracy of 93.7% [66]. To our knowledge, no work has been reported on the Raman spectroscopic characterization of human urine subjects in the HWVN region towards the diagnosis of cancer.

Hence, an attempt has been made to verify whether HWVN Raman spectroscopic characterization of urine samples of normal subjects and patients with oral premalignant and malignant conditions offers significant diagnostic information in the discrimination of the three groups based on the observed spectral signatures. Further, to verify the diagnostic potentiality of the present technique in discriminating the oral premalignant and malignant patients from the normal subjects, principal component analysis (PCA) and principal component based linear discriminant analysis (PC-LDA) were employed and the results were discussed.

## 2. Materials and methods

### 2.1. Samples

First voided morning urine samples were collected in a sterile sample container from normal volunteers and from pathologically confirmed oral premalignant and malignant patients, those who were admitted for treatment in Arignar Anna Memorial Cancer Institute and Hospital, Kanchepuram, Tamilnadu, India. A total of 80 normal subjects both male and female in the age group of 20 to 65 years and 57 oral premalignant and 60 oral malignant patients in the age group of 20 to 70 years were included. Samples were selected in such a way that they were free from other abnormalities like renal infection, diabetes, jaundice and bacterial infections. The raw urine samples were stored in the refrigerator at 4 °C and examined as such, within 48 h, from the time of collection after thawing it to room temperature. The present study protocol was ethically approved by Health and Welfare Department, Government of Tamilnadu, India vide reference no. 47846/E2/2011-1. The Raman spectra of standard chemicals such as flavins, tryptophan and phenylalanine (Sigma Aldrich Co, St. Louis, MO, USA) were measured in order to assign and explore the various vibrational modes of the prime urinary metabolites which are present in the urine. These standard chemicals were used in their supplied state (powder form) without any pre-processing.

### 2.2. Raman spectroscopy measurements

Raman spectroscopic characterization of raw urine samples were done with confocal micro Raman system (LabRAM HR800 spectrometer, Horiba Jobin-Yvon, France). Before collecting the Raman signal, wavenumber position and the detection system were spectrally calibrated with commercially available standard carbon tetrachloride (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) for the known Raman peaks at 218, 314, and 459 $cm^{-1}$. Raw urine samples without any further pre-processing were excited with 785 nm diode laser. The grating used was 600 g/mm, blazed at 500 nm and the laser power at the sample was maintained at 13 ± 0.05 mW. The backscattered Raman signal was accumulated twice to remove the cosmic showers from external light sources if any, and the acquired Raman signal was integrated for 90 s over the spectral range from 2600 to 3500 $cm^{-1}$. Raman spectra of standard chemicals were measured using quartz plate with 50 × NIR objective (NA = 0.80) with the working distance of 0.5 mm. A Peltier-cooled CCD (1024 × 256 pixels) was used to minimize the effect of thermal noise. Instrument control, signal collection, and data pre-processing were done with Labspec5 software as provided by the instrument manufacturer (Horiba Jobin-Yvon, France).

### 2.3. Data analysis

Every Raman instrument has a unique spectral responsiveness due to various instrument factors, which includes microscope objectives, filters, gratings and CCD detectors. Without intensity correction, the spectrum may show differences in relative peak intensities in addition to the spectrum from samples. Hence, in order to obtain accurate and reproducible results, built in intensity calibration factor ("HORIBA ICS") was applied to every raw spectrum (as provided by Horiba Jobin Yvon) [66]. Also, to eliminate the background signal which may be due to the influence of laser induced emission and external influences, the spectra were subjected to baseline correction by fitting a fifth order polynomial function and smoothened by Savitzky–Golay method. Data collection and pre-processing were carried out using Lab Spec 5-HORIBA Scientific V2.0 and Minitab software. Statistical analysis was performed with SPSS/PC + 19 software.

By considering the variations in the Raman spectral signatures between the urine samples of normal, oral premalignant and malignant subjects, pre-processed data in the region 2600–3500 $cm^{-1}$ were