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VIBRATIONAL SPECTROACOPY IN BODY FLUIDS ANALYSIS

Andrei A. BUNACIU,[a] Oana Mihaela ANTOCHI (BALTAG),[a] Șerban FLESCHIN,[b] Vu Dang HOANG[c] and Hassan Y. ABOUL-ENEIN[d*]

[a] SCIENT – Research Center for Instrumental Analysis, (CROMATEC_PLUS S.R.L.), 077167, Tâncăbesti – Snagov, Roumania
[b] Department of Organic Chemistry, Biochemistry and Catalysis, Faculty of Chemistry, University of Bucharest, 060114, Bucharest, Roumania
[c] Department of Analytical Chemistry and Toxicology, Hanoi University of Pharmacy, Hanoi, Vietnam
[d] Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Centre, Dokki, Giza 12622, Egypt

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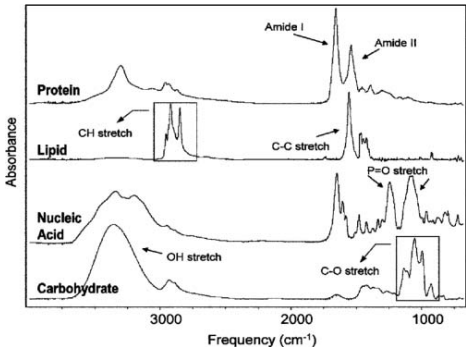
Raman and infrared (IR) spectroscopy are two complementary vibrational spectroscopic techniques that have experienced a tremendous growth in their use in biological and biomedical research. Vibrational spectroscopy offers a unique opportunity to investigate the composition of unknown substances on a molecular basis. The spectroscopy of molecular vibrations using mid-infrared or Raman techniques has been applied to samples of body fluids. This article reviews the fundamental operating principles of these two techniques and attempts to provide a comprehensive survey of recent studies that have used vibrational spectroscopy for some applications related to body fluids published in the period 2005-2016.



INTRODUCTION

Vibrational spectroscopy is certainly one of the most important analytical techniques available to today's scientists since is able to investigate simultaneously organic and inorganic compounds.

In a medical practice, blood or urine samples are taken from many patients. Analysis of these samples is usually centralized, either in clinics or in specialized laboratories. This leads to a time lapse between taking the sample and the availability of results for the blood or urine constituents, which may delay therapy. Test kits which enable point-of-care-testing are too inaccurate and are also frequently avoided because of a high cost.

Rapid detection of diseases enables the early administration of a therapeutic strategy when the treatment is most effective, thus saving health expenditure and lives. For this purpose, vibrational spectroscopy is a suitable technique as it is nondestructive, label-free, rapid, cost-effective, easy to operate and requires simple sample preparation. Moreover, the use of serum spectroscopy for diagnostics has the advantage for patients to be relatively non-invasive compared to current diagnostic methodologies such as biopsies.

Vibrational spectroscopy provides information on the composition and structure of matter. The principle of IR spectroscopy is based on the interaction of IR light with matter. Molecular bonds absorb the IR radiation at the resonant frequency of the bond or group, exciting vibrational modes. The resultant spectrum is a biochemical fingerprint of the analysed sample,

* Corresponding author: haboulenein@yahoo.com

each absorption peak/band corresponds to a specific vibration or combination of vibrations of a molecular bond. This absorption phenomenon obeys the Beer–Lambert law, thus allowing to obtain both quantitative and qualitative information.[1]

Vibrational spectroscopy offers an attractive alternative to conventional clinical chemistry analytical methods, with the spectra themselves providing the basis to recognize various components within a mixture and quantify them individually. One obvious advantage is that no reagents are required; and in principle, once a particular analytical method has been established, that analysis may be carried out repetitively with no resources other than the spectrometer itself. The most common analysis in the clinical chemistry laboratory is serum, blood, and urine tests. By coincidence, many of the most important analytes lie in concentration ranges that make them suitable for analysis by IR spectroscopy. They are routine techniques for fingerprinting and identifying chemicals and act as standard methods in analytical chemistry and pharmacy.[2]

The main function of the clinical chemistry laboratory is to perform quantitative and qualitative analyses of body fluids such as serum, blood, urine, and spinal fluid, as well as other materials such as tissue, calculi, and faeces. The aim of the present review is to present recent advancements in the potential use of vibrational spectroscopy for discriminating between normal and malignant body fluids, with varying degrees of dysplasia. Biological and medical applications, in particular, have progressed significantly in recent years, many reviews covering this field being published.[3-15]

The objective of this article is to review new developments in applications of vibrational spectroscopy in biomedical investigations, covering the period between 2005 and 2016. Prior to a review on this subject, it is useful to give a short introduction to the theoretical aspects related to vibrational spectroscopy and body fluids followed by discussion of the quantitative and qualitative biomedical investigations of the technique.

1. Theoretical aspects related to vibrational spectroscopy

This section provides a brief overview of the basic fundamental principles behind Raman and IR spectroscopy.

Vibrational spectroscopy includes several different techniques, but the most important techniques are mid-infrared (MIR), near-IR (NIR), and Raman spectroscopy. Both mid-IR (MIR) and Raman spectroscopy provide characteristic fundamental vibrations that are employed for the elucidation of molecular structure and are the topic of this review. The first method (infrared) measures the absorption, transmission, or reflection of mid-infrared (MIR) radiation (with wavelengths in the range 2.5 to 25 μm), which is caused by the interaction of the electric dipole moment of the molecule with the infrared radiation. The second method (Raman) illuminates the sample with radiation of much shorter wavelengths - *i.e.* far away from the vibrational resonance - and measures that fraction of scattered radiation for which the energy of the photon has changed.

Every molecule has a unique fingerprint of vibrational frequencies, which makes Raman and Fourier transform infrared (FTIR) spectroscopy highly specific techniques for molecular identification. Both techniques can be employed noninvasively, making them ideal for biomedical applications. Raman and FTIR spectroscopy are sometimes referred to as sister techniques and provide complementary information about molecules, but they differ in several fundamental ways. Almost any compound having covalent bonds, whether organic or inorganic, absorbs various frequencies of electromagnetic radiation in the infrared region of the electromagnetic spectrum.

Raman spectroscopy of biological samples is based on the inelastic scattering of photons by intrinsic molecular bonds in the sample, which leads to the generation of photons that are shifted in energy from that of the incident photons by an amount equal to the characteristic vibrational energy of the bond. A laser operating in the near-infrared region (*e.g.* 785 nm) is generally used as the excitation light source in order to minimize the generation of autofluorescence, which would otherwise overwhelm the much weaker Raman signal. This is because most biological materials have a window of low absorption in the near-infrared spectral region (about 700–900 nm). Another reason to choose near-infrared excitation light is to avoid any potential photodamage or thermal damage to biomolecules such as DNA, which has been observed when shorter wavelength light is used[16, 17].

Infrared spectroscopy is a complementary technique to Raman spectroscopy that also has been used to probe the vibrations and rotations of molecular bonds in biological samples. While Raman spectroscopy probes the bonds via a scattering

process and measures shifts in the energy of the scattered photons, IR spectroscopy acquires molecular and structural information of a sample by measuring the absorption of light in the mid-infrared region (4000 cm^{-1} to 300 cm^{-1}), which is the spectral range where most molecular vibrations of biomolecules absorb. Due to the difference in the physical principles of these two techniques (i.e. Raman is based on a change in polarizability while IR is based on a change in dipole moment), molecular bonds can either be Raman active, IR active, or both. Thus IR and Raman can yield complementary spectral information. IR spectroscopy is typically performed using an interferometric Fourier transform (FT) scheme rather than a dispersive detection mode because of its multiplexed and high throughput advantages. The core components of this setup are a broad spectral light source covering the mid-IR region and an interferometer (*e.g.* a Michelsen interferometer) with a scanning mirror.

The use of a synchrotron source for FTIR[18, 19] has gained considerable interest because it offers 100–1000 times more brightness, the result of a small source that restricts the emitted light to small angles to enable a more collimated beam.

2. Theoretical aspects related to the body fluids

Biofluids, such as serum and plasma, represent an ideal medium for the diagnosis of disease due to their ease of collection, that can be performed worldwide, and their fundamental involvement in human function. The ability to diagnose disease rapidly with high sensitivity and specificity is essential to exploit advances in new treatments, in addition, the ability to rapidly profile disease without the need for largescale medical equipment (*e.g.* MRI, CT).

Vibrational spectroscopy has been investigated as a diagnostic tool and has shown great promise for serum spectroscopic diagnostics. However, the optimum sample preparation, optimum sampling mode and the effect of sample preparation on the serum spectrum are not very well known.[20]

Biological samples are composed mainly of proteins, lipids, sugars and deoxyribonucleic acid (DNA). All those compounds are active in the IR range, as can be seen in figure 1, and any change produced in the composition or the structure can be evaluated by IR measurements.[21]

Serum is composed of water, organic substances, and inorganic salts; it can reflect human beings' physiological and pathological conditions and is easier to collect and more suitable for rapid diagnosis.[22]

Water is the major molecular component within biological matrices, and strongly affects the utility of selected electromagnetic spectral regimes due to strong O–H absorptions, especially in the MIR region. However, water has a relatively broad transmission window in the NIR, thereby enabling direct measurement of the biological specimen. However, despite the merits of operating in the NIR region, the information content and data interpretation of biological NIR spectra are frequently affected by relatively weak and highly convoluted absorption features[4]. It is no surprise that the IR spectra of biological fluids are dominated by water absorptions, with features from the dissolved species superimposed on the water absorption profile. Despite the overwhelming strength of the water absorptions, the absorption pattern for the dissolved species can be recovered, as illustrated by the mid-infrared spectrum (Figure 2a) and by the near-infrared spectrum (Figure 2b) of a typical serum specimen.

A typical measurement scheme is to sandwich a small volume of the sample between demountable calcium fluoride or barium fluoride windows that are separated using a Teflon® ring spacer to provide an optical pathlength of 6–10 μm. This pathlength is short enough to bring the strong water absorption at 1645 cm^{-1} into an absorbance range of 1–1.5, which is low enough that solute absorptions in the same wavenumber range may be recovered by subtracting the spectrum of pure water. Attenuated total reflectance (ATR) spectroscopy provides an alternative means to measure absorption spectra by using the experimental arrangement illustrated in Figure 3.

The difficulties associated with strong water absorptions can be eliminated by simply eliminating water from the sample. Typically 5–50 μL of liquid is spread on a suitable substrate and allowed to dry, and a transmission spectrum is acquired for the resulting film. In addition to eliminating the spectral interference of water, this approach can provide inherently better spectral resolution by virtue of eliminating the water/solute interactions. A representative spectrum of a dry serum film is illustrated in Figure 4. The specimen was first diluted twofold in aqueous 4 g L^{-1} potassium thiocyanate (KSCN) solution. The absorption of SCN- at 2060 cm^{-1} was used for subsequent normalization of the spectra as part of the development of quantitation models[23].

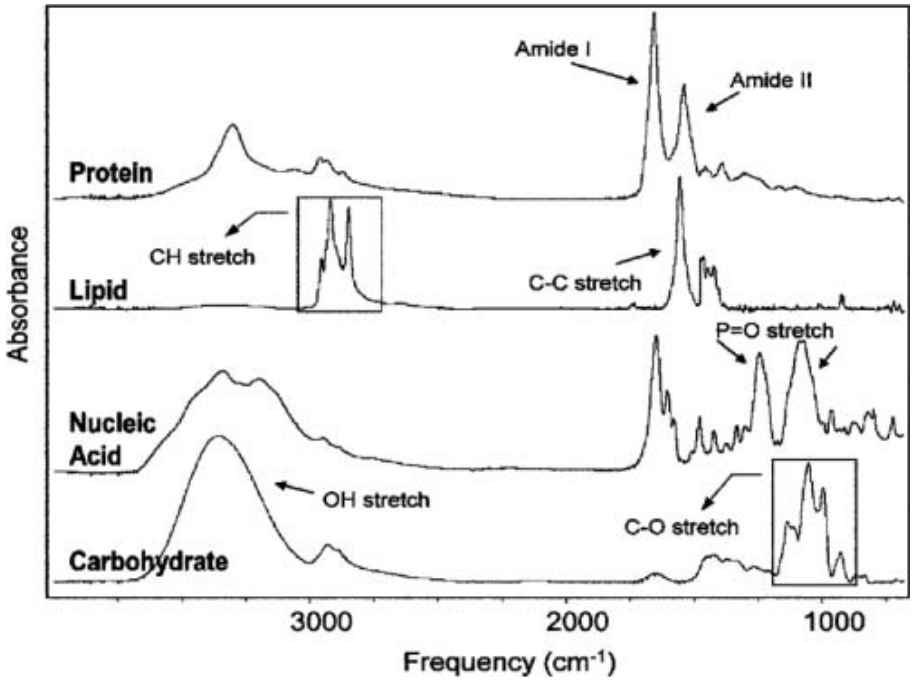


Fig. 1 – Infrared spectra for different cellular components.

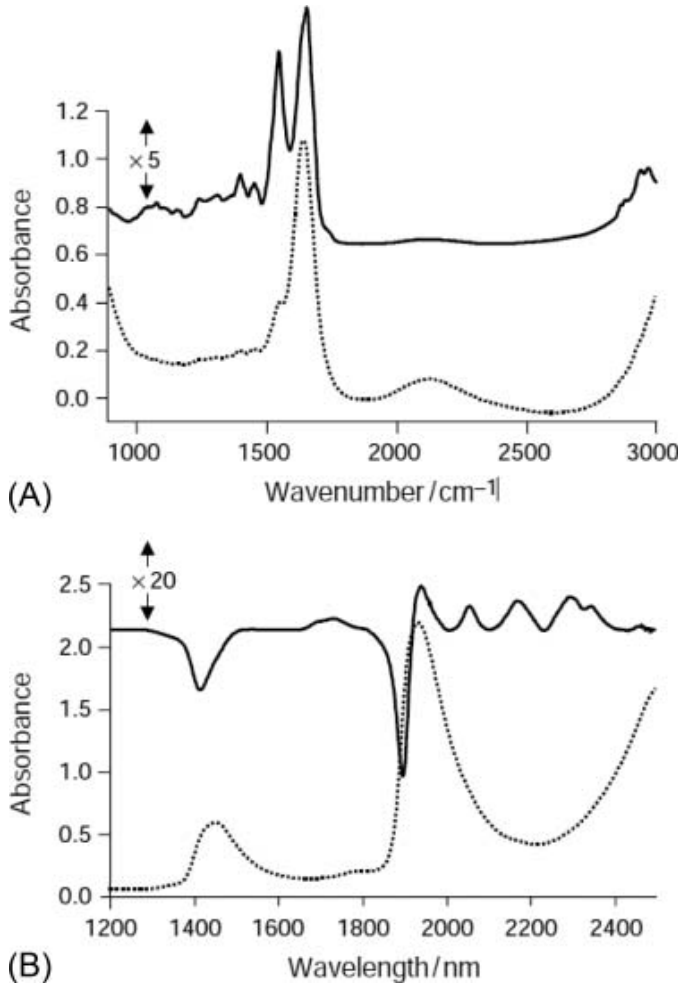


Fig. 2 – Mid-infrared (a) and near-infrared (b) absorption spectra of serum (dashed lines) and the residual spectra with the spectrum of water subtracted from each (solid lines).

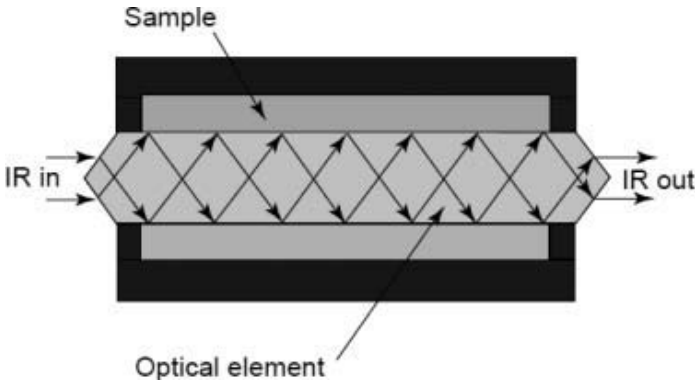


Fig. 3 – Apparatus to measure the ATR spectrum for a liquid specimen.

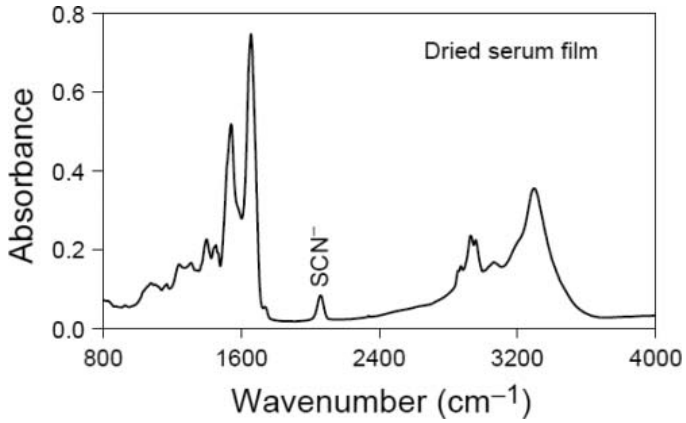


Fig. 4 – Absorption (transmission) spectrum for a serum film dried onto a barium fluoride window.

In order to realise the full potential of IR spectroscopy as a leading healthcare tool, some issues need to be understood prior to clinical translation[24]. One issue, in particular, related to biofluid spectroscopy is due to the strong IR activity of water. As such, the most common protocol for analysing liquids such as bio-fluids is the drying of drop deposits. However, it has been shown by the optical and spectroscopic assessment that this deposition is not all the times *e.g.* homogenous.[25] Fingerprint spectra may diagnose the origin and grade of pathology based on a classification algorithm.

The potential of FTIR and Raman spectroscopy has been widely investigated for diagnostic purposes for cell and tissue analysis and the feasibility to use them for serum sensing has been suggested and applied to a wide range of body fluids,[4] ranging from serum,[25,26] tears,[27] urine or saliva.[11]

3. Body fluids analysis applications

Diabetes, a disorder in the control of the blood glucose level is considered to be one of the most important metabolic diseases worldwide[28]. Nowadays, self-monitoring of blood glucose is based on painful finger pricking. Therefore, many researchers have aimed at the development of a non-invasive sensor to monitor the blood glucose level continuously. Although several *in vivo* blood glucose measurement studies have been performed by different research groups using near-infrared (NIR) absorption and Raman spectroscopic techniques, the prospective prediction has proven to be a challenging problem. An important issue, in this case, is the demonstration of causality of glucose concentration to the spectral information, especially as the intrinsic glucose signal is smaller compared with that of the other analytes in the blood–tissue matrix.

Thus, Near-Infrared Spectroscopy (NIR) coupled with chemometrics is one of the most used method.[29] NIR radiation is essential for assays using diffuse reflectance spectroscopy of skin for non-invasive glucose quantification and diabetes screening based on glycation effects. It was found that the first overtone band, 1500-1800 nm, is most informative for aqueous solutions while for glucose measurement of serum samples the combination band was found to be the better choice. Also, Raman spectroscopy was used for quantitative, non-invasive (transcutaneous measurement of blood analytes, using glucose as an

example.[30, 31] Finally, the results suggest that the incorporation of chance correlations for *in vivo* cases can be largely attributed to the uncontrolled physiological sources of variations. Such uncontrolled physiological variations could either be intrinsic to the subject or stem from changes in the measurement conditions.

Raman spectrum peaks, using 532 nm laser system, for diabetic blood serum are observed and were attributed to carbohydrates, proteins, lipids, collagen, and skeletal C-C stretch of lipids acyl chains.[32] This *in vitro* glucose monitoring methodology will lead *in vivo* non-invasive on-line monitoring having painless and at the same time, the data will be displayed on-line and in real time. The measured Raman peaks provide a detailed biochemical fingerprint of the sample and could confer diagnostic benefit in a clinical setting.

Infrared spectroscopy may represent an appropriate tool with which to identify novel diseases mechanisms, risk factors for diabetic complications and markers of therapeutic efficacy. Human blood, saliva, semen and vaginal secretions could successfully be detected and differentiated from one another, as can be seen in Figure 5, when analysed with ATR FT-IR spectroscopy.[33]

This enabled identification based on the unique spectral pattern, combination of peaks and peak frequencies corresponding to the macromolecule groups common within the biological material, such as proteins, sugars and phosphates. This study and other similar[34-37] proved the discrimination and identification of each body fluid and demonstrate the potential for FT-IR and Raman spectroscopy to be utilized as a confirmatory method for body fluid identification with high confidence.

In the same field of applications, forensic analysis, it was found that vibrational spectroscopy can be used for determination of drugs of abuse (cocaine, diazepam, methamphetamine, cotinine and benzoylecgonine) in oral fluids.[38] Comparing IR, Raman and NMR spectroscopy techniques, was proved that Surface Enhanced Raman Spectroscopy (SERS) is the most sensitive technique for the detection of illicit drugs in oral fluid. The use of IR spectroscopy for determining drugs of abuse in oral fluid is growing although the LODs obtained until now do not yet satisfy the necessities in the forensic field. Finally, NMR spectroscopy has been seldom used to determine drugs in oral fluid. Furthermore, those techniques that already dispose of good portable instrumentation, in particular Raman and IR spectroscopy seem the most promising spectroscopic tools for determining drugs in oral fluid.

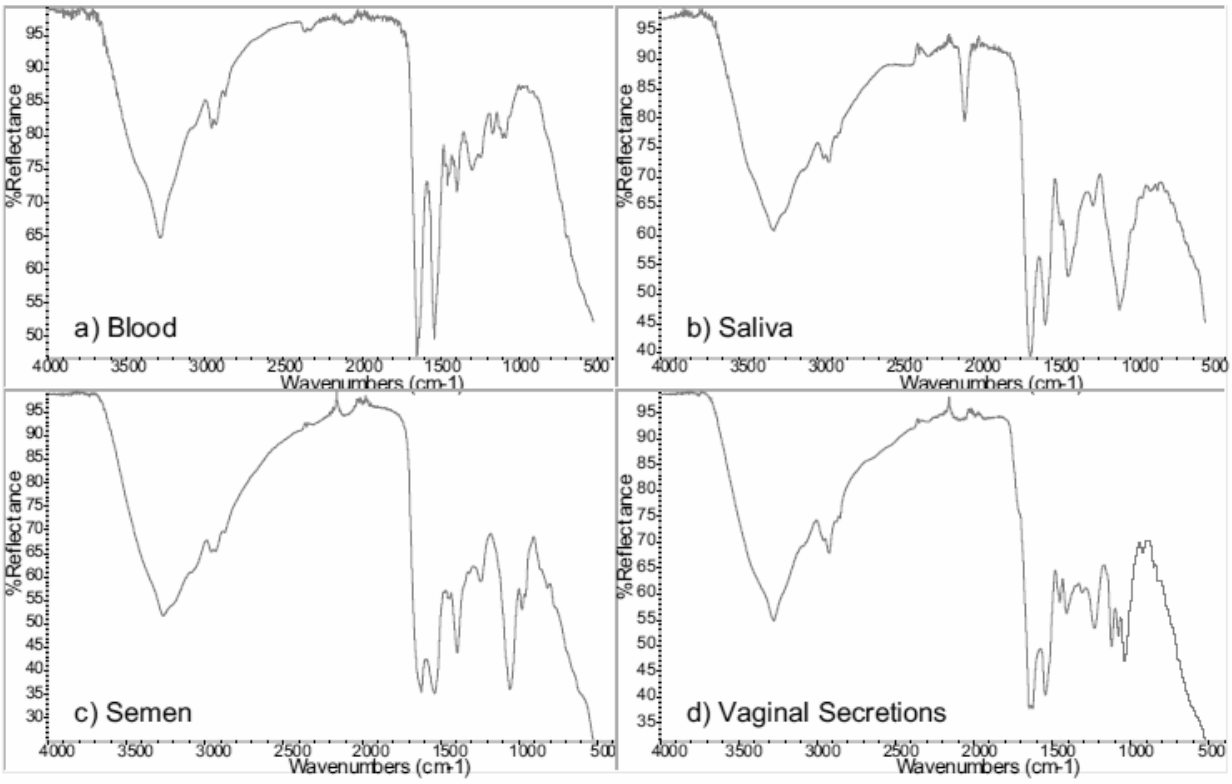


Fig. 5 – ATR FT-IR spectra of blood (a), saliva (b), semen (c) and vaginal secretions (d).

Immunoglobulin G (IgG) is crucial for the protection of the host from invasive pathogens. The concentration of IgG in blood and other biofluids is directly related to the level of humoral immunity where by abnormal IgG concentrations are often indicative of disease or the risk of susceptibility to infection. Due to its importance for human health, tools that enable the monitoring of IgG levels are highly desired. Consequently there is a need for methods to determine the IgG concentration that are simple, rapid, and inexpensive.[39] The results showed that ATR infrared spectroscopy is potentially a simple, quick, and inexpensive method to measure IgG concentrations in human serum samples. The results also showed that it is possible to build a united calibration curve for the umbilical cord and the venous samples.

In addition to immune system disruption, HIV/AIDS infection is also known to cause metabolic abnormalities ranging from dyslipidemia, hyperglycemia, insulin resistance, and diabetes.[40] ATR-FTIR spectroscopy coupled with chemometrics successfully distinguished sera from HIV infected patients and uninfected controls with distinctions visible in the presence of treatment.[41] The study provided original insights for novel systems diagnostics for HIV/AIDS.

Asthma is a chronic inflammatory disorder of the airways characterized by airway hyper responsiveness and reversible air flow obstruction that fluctuates over time.[42] According to World Health Organization, 300 million people suffer from asthma. In a recent study, was explored the feasibility of detecting asthma and determining treatment response in asthma patients, through Raman spectroscopy of serum.[43] Differences like changes in protein structure, increase in DNA specific bands, and increased glycosaminoglycans-like features were more prominent with an increase in asthma severity. Overall promising results were obtained, and a largescale validation study on random subjects is warranted before the routine clinical usage of this technique.

Biostructure disorders (*e.g.*, uncontrolled cell division, invasive cell growth into adjacent tissue. and metastatic implantation to other body sites) are called - cancer. Cancer is becoming the leading cause of death all around the world. It is well known that a precise, accurate diagnostic report is very helpful for drawing up strategies for treatment. Innovative diagnostic methods that provide indications complementary to conventional histopathology, in particular the early biomolecular alterations under malignant conditions are under scrutiny. One such candidate method is infrared (IR) spectral imaging, which has the potential to provide, in a nondestructive and label-free manner, a biochemical fingerprint of cells and tissues.[44, 45]

IR spectroscopy of blood plasma or serum is a rapid, versatile, and relatively non-invasive approach that could characterize biomolecular alterations due to cancer and has the potential to be utilized as a screening or diagnostic tool.[46]

The main spectral changes between breast cancer and healthy patients were recorded in the area of the CH stretching vibrations, the C–O ribose, the ribose backbone and the P–O vibrations.[47] The natural detection limit of IR spectroscopy is in the area of 0.1–0.01%. So the changes which were observed in the spectrum must lie in this area. These examinations show that it is possible to make a qualified diagnosis from a small amount of serum on breast cancer. Breast cancer can also be differentiated from other diseases.

Another simple and rapid method for the detection of breast cancer with IR spectroscopy was developed.[48] The method needs only 1 μL of a serum sample. The serum sample is dried on a suitable sample carrier such as a Si plate. After drying the IR spectrum is measured. Every disease leaves a typical fingerprint in the IR spectrum of serum. This typical fingerprint can be used to identify different patient groups, as can be seen in Figure 6. The identification system can be trained by classification methods, using two independent classification methods, cluster analysis and artificial neural networks.

Cervical cancer screening programs have greatly reduced the burden associated with this disease. However, conventional cervical cytology screening still lacks sensitivity and specificity. There is an urgent need for the development of a low-cost robust screening technique.[49] Following FT-IR spectroscopy, derived spectra were examined for segregation between classes in score plots generated with subsequent multivariate analysis.

Since serum can reflect human beings' physiological and pathological conditions, FT-IR spectroscopy was used to compare gastric cancer patients' serum with healthy persons' serum.[50] The H2959/H2931 peak height ratio might be a standard for distinguishing gastric cancer patients from healthy persons; the result showed that the RNA/DNA ratios of gastric cancer patients' serum were obviously lower than those of healthy persons' serum, as can be seen in Figure 7. The results suggest that FT-IR spectroscopy may be a potentially useful tool for diagnosis of gastric cancer.

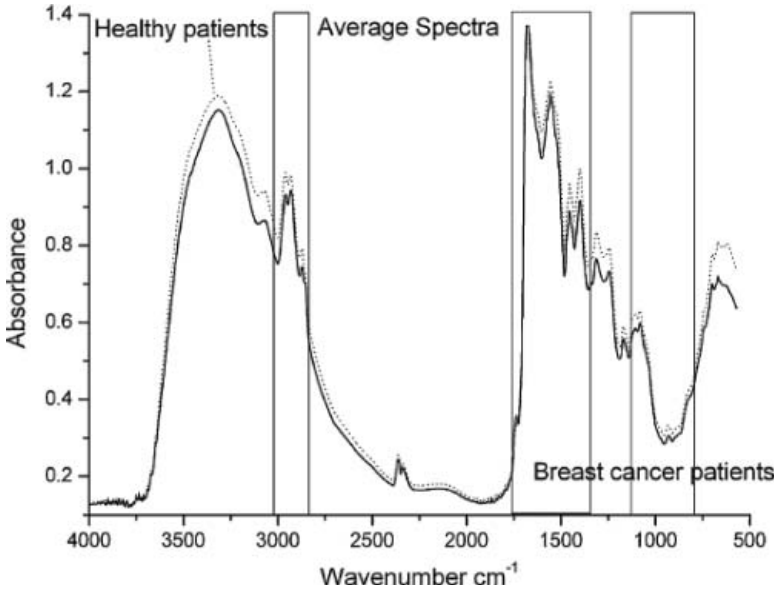


Fig. 6 – Overview spectra in transflection, spectral region 4000–500 cm^{-1}.

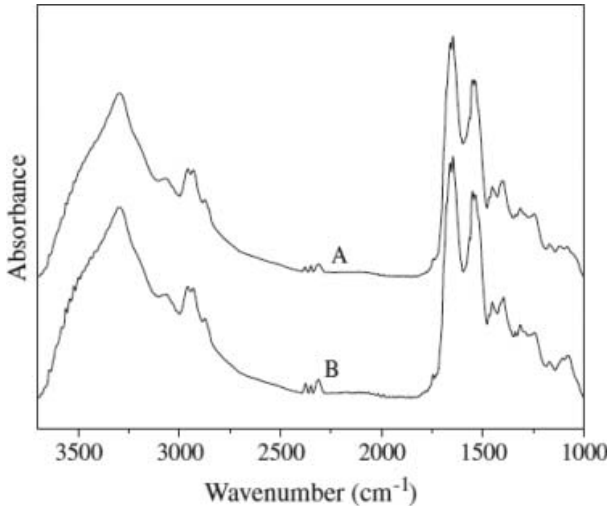


Fig. 7 – Average IR spectra of gastric cancer patients' serum (A) and healthy persons' serum (B).

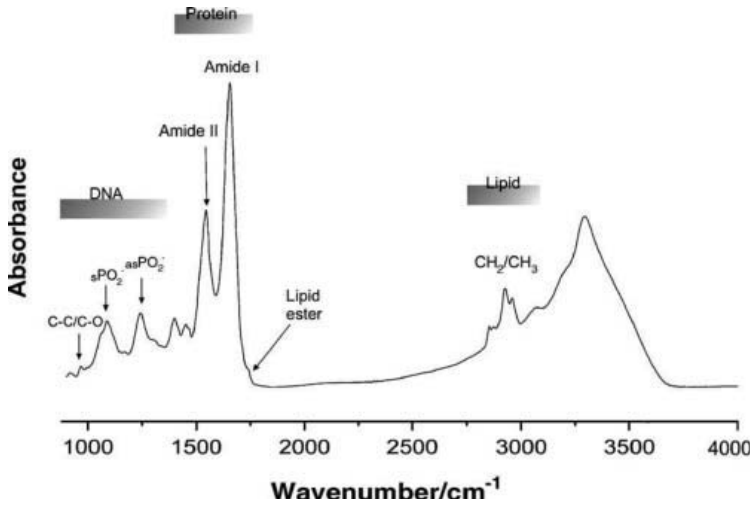


Fig. 8 – Representative IR spectrum of normal lymphocytes revealing basic cellular molecules such as proteins, lipids and DNA as marked.

Several other studies were performed in order to compare the serum from healthy persons with the serum from different types of cancer patients, such as: leukaemia,[51-55] lung,[56, 57] prostate,[58] oral cavity,[59-61] cervical[62] as well as for monitoring the effectiveness of drugs during chemotherapy.[63-66]

Like many other biomedical sciences, the development of the field of hematology has evidently been driven by technology. Hematology is a discipline devoted to understanding and exploiting information in blood in order to understand basic physiological functions and to facilitate the prevention, diagnosis and treatment of hematological diseases and disorders. IR spectroscopic-based techniques can be used to analyse DNA alterations, secondary structural changes in proteins, and to profile cellular lipids[67] as can be seen in Figure 8.

IR-based methods hold several attractions for consideration in the hematology laboratory: (1) No chemical reagents or specific molecular probes are required—the infrared "spectral patterns" of the species of interest can provide the basis for detection and quantitation; (2) only small amounts of sample (of the order of microliters of fluids, or small numbers of cells (about 103) are required, leaving ample material for other clinical tests; and (3) it is suited for automation - IR analysers can yield test results within 15 min, with little training required of the operator.

In this way vibrational spectroscopy can be used for the determination of: Hb oxy-deoxy transition in erythrocytes under stretching conditions,[68] biochemical parameters in human serum.[11, 12, 69, 70]

CONCLUSIONS

Vibrational spectroscopic methods are multipurpose techniques that offer advantages in simplicity, rapidity, low-cost and minimal sample preparation. Both techniques, have significant potential in the field of biomedical analysis, as they can give molecularly specific biochemical information without the use of extrinsic labels and without being invasive to the system studied. The greatest benefit of these techniques lies in the high molecular sensitivity combined with a spatial resolution down to a few micrometers. Another advantage is the ability to probe samples under native conditions, which allows new insights into samples without the need for fixation, stains, or an additional marker. Advances in instrumentation have made FTIR spectroscopic imaging the tool of choice for an increasing number of applications. It was demonstrated that the vibrational properties of water are sensitive to the cellular environment of human tissue and are capable of distinguishing between cancerous and normal human breast tissues. These properties can be treated as hydration fingerprints to discriminate between cancerous and normal tissues, but a definite assignment of the origin and uniqueness of these bands remains and further studies are necessary. The development of Raman spectroscopic signatures of body fluids has demonstrated the potential for routine spectroscopic examination of biological samples. FT-IR and ATR FT-IR spectroscopic techniques produce spectra containing bands, or peaks, representative of the vibrations of structural bonds and functional groups within biological samples. The positioning of the peaks are specific to particular interactions with molecular bonds and provide specific information relating to the biochemical composition[71]. The proposed methods overcome the problems associated with currently used biochemical methods, which are destructive, time consuming and expensive.

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