

Laser Raman spectroscopy: Some clinical applications

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Recent advances in lasers, multichannel detectors and PC-based spectroscopic instrumentation have made Raman spectroscopy an invaluable tool for characterization of clinical samples like tissue, body fluids and cytological smears. Efforts are being made at present to develop Raman spectroscopy techniques for optical pathology, early detection of neoplasia, analysis of body fluids, optical imaging, spectral markers for identifying clinical conditions, etc. Some of the results which will lead to such applications are presented in this paper.

IN physiological systems, biomedical changes often precede or cause the onset of a disease. The changes, if observed and interpreted properly, often provide an invaluable tool to monitor the various stages of disease progression and effect of therapy. The present method for such monitoring is mainly by pathology or clinical biochemistry, which requires samples from biopsy or other methods. Pathological examination depends on morphology and is often subjective. Clinical analysis depends on immuno or analytical chemistry, is time consuming, and may not lead to unambiguous results, being not always sufficiently sensitive in early stages of disease.

Optical spectroscopic methods, on the other hand, are usually extremely sensitive, quite specific, and highly objective and can be made non-invasive even for *in vivo* applications. Since spectral properties depend on both molecular composition as well as structural changes, spectral analysis can provide diagnosis in early stages of diseases allowing intervention and therapy sufficiently early. This is especially important in areas like cancer, coronary problems, etc. where early detection is necessary for successful therapy.

Realizing the advantages of spectroscopy techniques for clinical applications, several groups have started intense research in this area in the last one decade¹⁻⁵. Recent advances in lasers, detectors and spectroscopic instrumentation have prompted several investigators to

develop advanced biomedical applications of spectroscopy in areas like optical tomography, early detection of cancer, photodynamic therapy, drug cell interaction, etc. Laser Raman and laser-induced fluorescence spectroscopy techniques are now being developed extensively to derive qualitative and quantitative information on composition, morphology, biochemical interactions, etc. in tissues, cells and body fluids, leading to an exhaustive understanding of the process of biochemical changes during disease onset as well as therapy⁶⁻¹⁸.

In the present paper, we report some of our results of laser Raman spectroscopy of tissues, body fluids, and cell samples for clinical applications.

Tissue spectroscopy

Tissues are cooperative assemblies of different cells of multicellular organisms and as such exist in various compositions. The cells are held together by extracellular macromolecules like collagen. Depending on the location of the tissue, we have various kinds of tissues like epithelial, connective, smooth muscle, etc., their composition changing considerably from one type to another. In epithelial tissue for example, there is very little extracellular matrix, whereas in connective tissues the matrix is more plentiful. The matrix itself can vary from the calcified, rock-hard structures of bone and teeth, to the transparent corneas, or the rope-like structure of tendon.

The spectroscopy of tissues thus depends very much on the type and origin of the tissue. The extracellular matrix consists primarily of fibrous protein embedded in a hydrated polysaccharide gel. The fibrous protein can be structural (collagen, elastin) or adhesive (fibronectin, laminin). The polysaccharides, glycosaminoglycans, are the other major constituents of the extracellular matrix, and form less than 10% by weight compared to the proteins.

Since cancer is a disease of the cell, it may appear that information on the extracellular matrix may not be of much importance. But the matrix plays a very important and complex role in regulating the behaviour of the cells

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in contact with it, like influencing their development, migration, proliferation, metabolic functions, etc. In view of this, information on cellular as well as extracellular matrix is equally important in understanding the biochemical processes which precede or accompany onset of disease.

The cells of tissues contain various molecular systems like lipids, DNA, enzymes, NADH, heme protein, etc., so that it may appear that the interpretation of spectra of tissue will present insurmountable difficulties. However, in practice, it is found to be not so, and spectral changes can be fairly well correlated with biochemical changes¹⁹.

In this section we will discuss the application of Raman spectroscopy in the study of tissues of the oral cavity, cervix, and ovary, mainly from the point of view of changes from normal to malignant situation.

Figure 1 *a* shows the spectrum of an oral tissue (buccal mucosa), which is typical of samples from different origins, such as different people, or different samples from one person. The bands at 1750 cm^{-1} (w), 1656 cm^{-1} (s), 1304 cm^{-1} (s) and the $1120\text{--}1081\text{--}1065\text{ cm}^{-1}$ triplet (m) characterize unsaturated lipid derivatives with a liquid crystal type of structure. This shows that the major part of the spectrum arises possibly from the membrane layer of the epithelial cells, and any protein spectra that may arise from the cellular protein and connective tissue are only weakly present. This is further confirmed by the C-H stretch region spectrum (Figure 1 *b*). Here also, the

normal tissue spectrum resembles that of a liquid crystal lipid.

Comparing normal spectrum with the malignant tissue spectrum in Figure 1, even a casual inspection reveals several drastic changes in the spectra. These include (i) the disappearance of the sharp 1751 cm^{-1} band, (ii) the broadening or doubling of the 1650 cm^{-1} band, (iii) the lowered intensities of the 1450 cm^{-1} (note the frequency shift from 1440 cm^{-1}) band, (iv) disappearance of the 1303 cm^{-1} band, the region now representing the pattern of the typical protein amide III band, (v) the appearance of sharp peaks at 1128 cm^{-1} and 1004 cm^{-1} , and (vi) the disappearance of the 1081 cm^{-1} group of bands. It should be mentioned here that several normal and malignant samples have been studied spectroscopically, and the spectra in all the cases were very close to those shown in Figure 1 *a* and *b* respectively. Also in the C-H stretch region the spectrum now resembles a Type I collagen, rather than lipid. It is surprising that, in spite of the fact that the total tissue contains both lipids and proteins and the 785 nm laser line can penetrate at least several hundred microns in the tissue, the normal spectrum is predominantly lipid, while the malignant sample spectrum is mainly protein-like. Though there are other weak bands also in the spectrum, a complete assignment will be possible only by sophisticated spectral analysis methods like principal component analysis (PCA), which require results of several samples. This is being done at present.

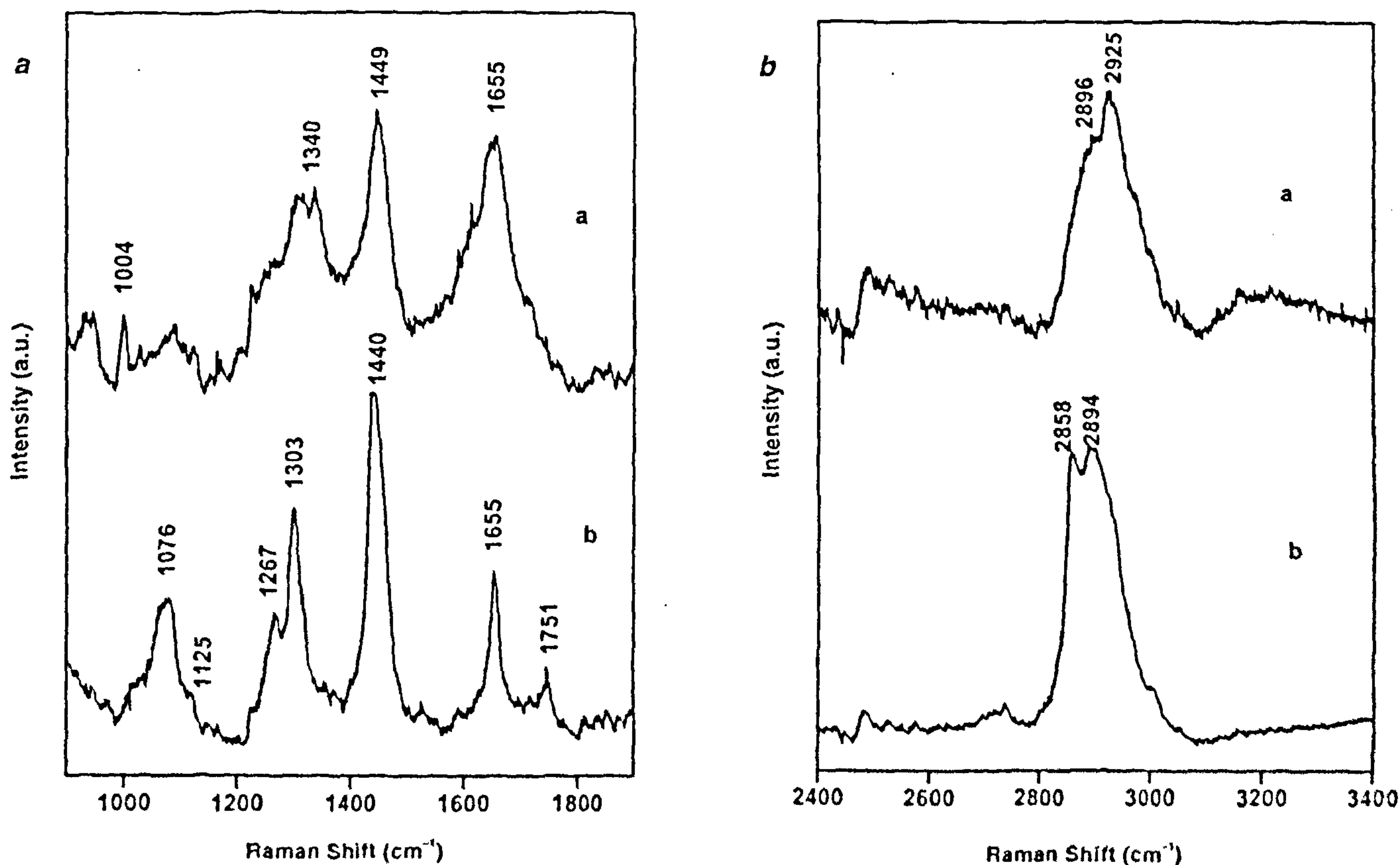


Figure 1. Raman spectra of buccal mucosa; *a*, malignant, *b*, normal.

The data thus clearly show that the epithelial membrane structure might have been considerably damaged, and a more protein-like spectrum has appeared at the onset of malignancy. That these changes are genuine structural effects arising from malignancies can be seen from Figure 2, where the spectra from the edge and centre of a surgically removed malignant tissue section are juxtaposed. While the spectrum of the edge resembles more a normal tissue, the spectrum from the centre is clearly that of a malignant specimen. All the samples we have studied are tumours of epithelial origin. There could be several reasons for the observed spectral differences between normal and malignant tissue samples in these cases. Normal tissue consists of well-differentiated and architecturally highly ordered groups of cells, an example being the progressive maturation of tall cells in the basal layer to flattened sequences on the surface. On the other hand, very often, malignant tissues will consist of anaplastic cells (undifferentiated) with no recognizable patterns of orientation with respect to each other. They may grow in sheets without any stratified squamous structure. Similarly in dysplasia, the uniformity of the individual cells as well as their architectural orientation is lost. The cells exhibit pleomorphism, with considerably large nuclei. Dysplastic changes are found adjacent to foci of cancerous transformation. In general, the structural organization and well-differentiated character of cells in

normal tissue (as well as benign tumours) are lost in malignancy or dysplasia. These might have led to the observed spectral changes, since, in one case, we are looking at a well-organized system of cells with their lipid bilayers presenting a uniform architecture, while in the other case, pleomorphism and architectural anarchy expose different regions of the different cells to the probing beam. These changes in spectral characteristics can be thus effectively utilized for early detection, identification of CIS, marking of surgical boundaries, and evaluation of therapy. Finally, we should also note that the stroma made up of connective tissue and blood vessels is crucial to the growth of neoplasms. Certain cancers induce a dense, abundant fibrous stroma (desmoplasia) which will enhance the spectrum from the corresponding fibrous components, viz. collagen-like protein. Correlations of such changes with histological evidence will help in identifying their origin and the possibility of using them for detection of early stages of malignancy. To the best of our knowledge these results are the first that have been obtained for oral cancer diagnosis, using Raman spectroscopy.

The Raman spectrum of squamous cell carcinoma of tongue tissue (results not shown) was more or less the same as that of buccal mucosa, except for a very sharp peak at 1224 cm^{-1} . The assignment of this band is not very clear at present, but it seems to be a possible marker for early stages of carcinoma, when the other bands are starting to show only minor changes. This has to be confirmed by further studies.

We have also studied adenocarcinoma of the cervical tissue. Once again the five bands discussed earlier show drastic changes from normal to malignant tissue spectra, showing that they can be used for cancer detection.

Mahadevan-Jansen *et al.*¹⁶ have selected 6 peaks of cervical tissue for diagnostic purposes in squamous intraepithelial lesion (SIL). Though our spectra for adenocarcinoma show some similarity with the qualitative intensity changes described by them, there are noticeable differences, presumably due to the different types of malignancy studied. A comparison of the results and assignments for these peaks is given in Table 1.

Another group of tissue we investigated is ovarian tissue with dysgerminoma. The spectra of normal and malignant tissues in such systems also are very similar to cervical and oral tissue spectra.

In general, tissue samples of different internal organs in normal and malignant states show spectra which are similar in their respective groups, i.e. all normal spectra resemble one another, while all malignant tissue spectra are similar to each other, while very different from the normal spectra. The spectral changes are quite noticeable and our preliminary studies indicate that even a visual examination of the spectra is sufficient to detect different stages of malignancy. Statistical analysis of the spectra with large enough data can thus be used for *in situ*

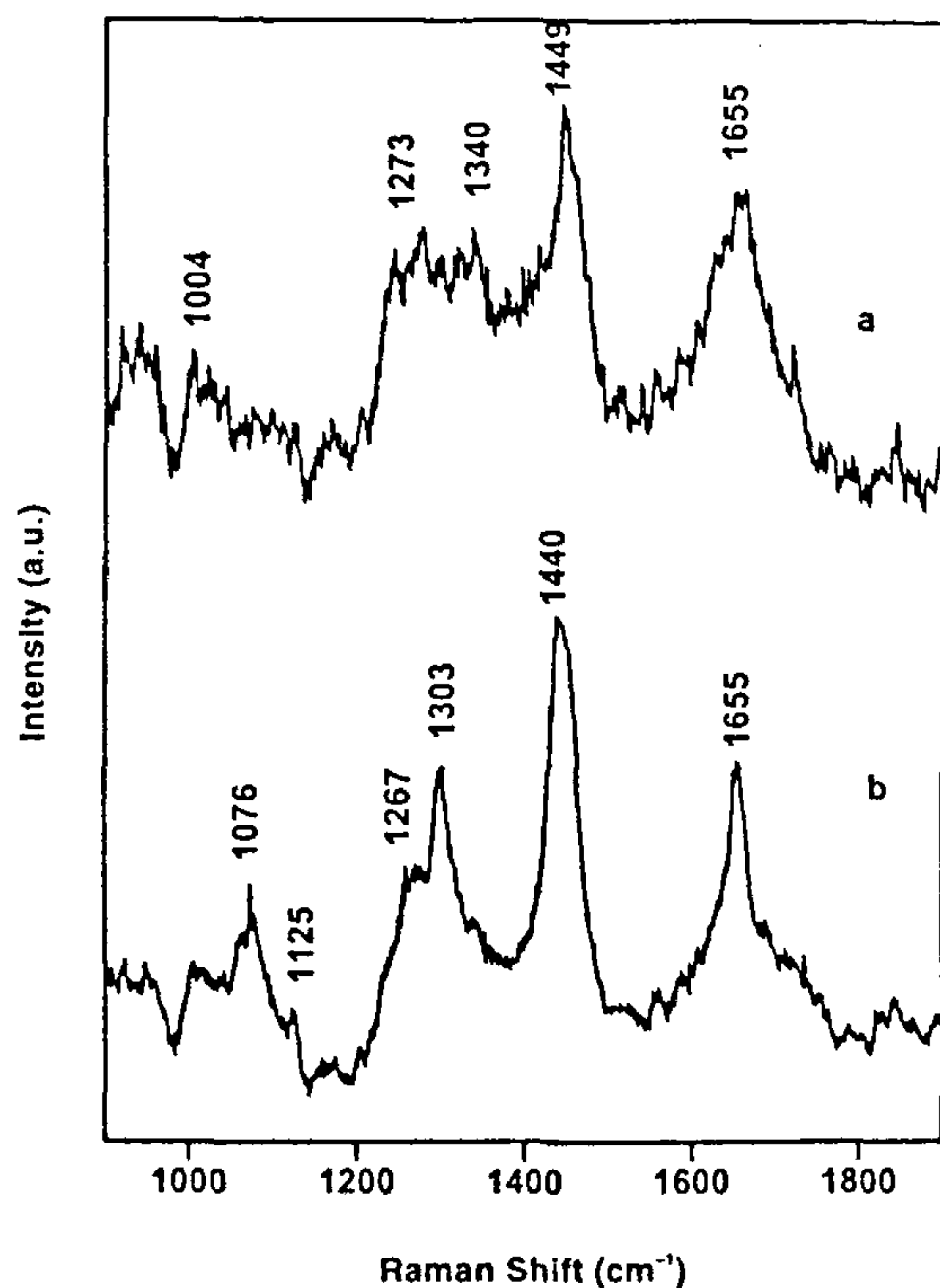


Figure 2. Raman spectra of (a) central portion and (b) edge of a surgically resected buccal mucosa tissue.

(*in vivo*) diagnostic purposes in early detection of oral, cervical, ovarian malignancies. We are now developing a fiber optic probe for such studies. All the spectra recorded in this work were obtained in a few minutes, with an F/4 spectrometer. With a fiber optic probe and spectrometer of better F values, it is possible to get very good spectra in a few seconds, making *in situ* studies quite easy.

Raman and surface-enhanced Raman scattering spectroscopy of biological fluids

Raman and infrared spectroscopy of biological fluids studied so far includes blood and serum¹⁸, urine²⁰ and synovial fluid²¹. Routine clinical analysis of several other fluids is being done at present for diagnostic purposes. These include amniotic fluid, pleural fluid, ascitic fluid, cerebrospinal fluid (CSF), etc. Even analysis of saliva has been proposed for early detection of cancer²². We have, for the first time, applied Raman and surface-enhanced Raman scattering (SERS) for the study of pleural fluid, ascitic fluid and CSF.

Most of the pleural fluid and ascitic fluid samples showed only typical protein spectrum (albumin) and it

was not possible to observe any major changes due to malignancy. However, there seem to be minor differences between the pathologically assigned malignant and normal samples. The spectra recorded by us so far for these samples have been weak and we are planning to get better spectra by changing over to shorter wavelengths and using enhancement techniques like SERS, multiple passing, spherical integration type cell and other better collection optics²⁰.

In addition to the minor differences, very rarely, the pleural and ascitic fluid samples showed drastic changes from the normal spectra. This is shown in Figure 3. The reasons for these are not clear at present, but if confirmed and understood, Raman spectroscopy of these fluids will give a unique tool to identify unusual physiological conditions, leading to diagnostic applications. We are planning fluorescence and HPLC studies to understand such anomalous spectra, and identify the components which give rise to the newbands.

In contrast to pleural and ascitic fluids, CSF samples gave complex spectra, consisting of many bands superposed over the weak protein spectrum of the albumin in CSF. CSF is normally examined for various pathological conditions. The usual analysis is for protein, sugar and

Table 1. Raman peaks of oral, cervical and ovarian tissues for diagnostic applications

Raman shift (cm^{-1})	Assignment*	Spectral change from	
		Normal	Malignant
2894 (Not reported)	CH ₂ asy. stretch	Very strong	Strong
2858 (Not reported)	CH ₂ sym. stretch	Very strong	Very weak
1751 (Not reported)	C=O Lipid	Sharp, weak	Disappears
1656 (1656)	C=C in normal Amide I in malignant (collagen)	Sharp, strong	Broadening or doubling (decrease in intensity)
1452 (1454)	CH ₂ protein (collagen)	-	New strong band (increase)
1440 (Not reported)	CH ₃ and CH ₂ phospholipids	Sharp, very strong	Disappears
(1330)	(Phospholipids, DNA)	Not observed	Appears (increase)
1302 (Not reported)	(Phospholipids CH ₂ twist)	Sharp, strong	Disappears
1267 (Not reported)	Phospholipids	Medium	Disappears
(1246)	(Collagen, DNA)	-	Broad, appears (increase)
1224 (Not reported)	Not assigned	-	sharp, medium new band
1125 } 1081 } 1065 }	Phospholipids, gauche conformation	Strong	Disappears
(Not reported)			
(1070)			
1004 (Not reported)	Phenyl alanine	-	Sharp, medium new band

*Raman shift, assignment and intensity changes in parentheses correspond to data in Mahadevan Jansen *et al.*¹⁶.

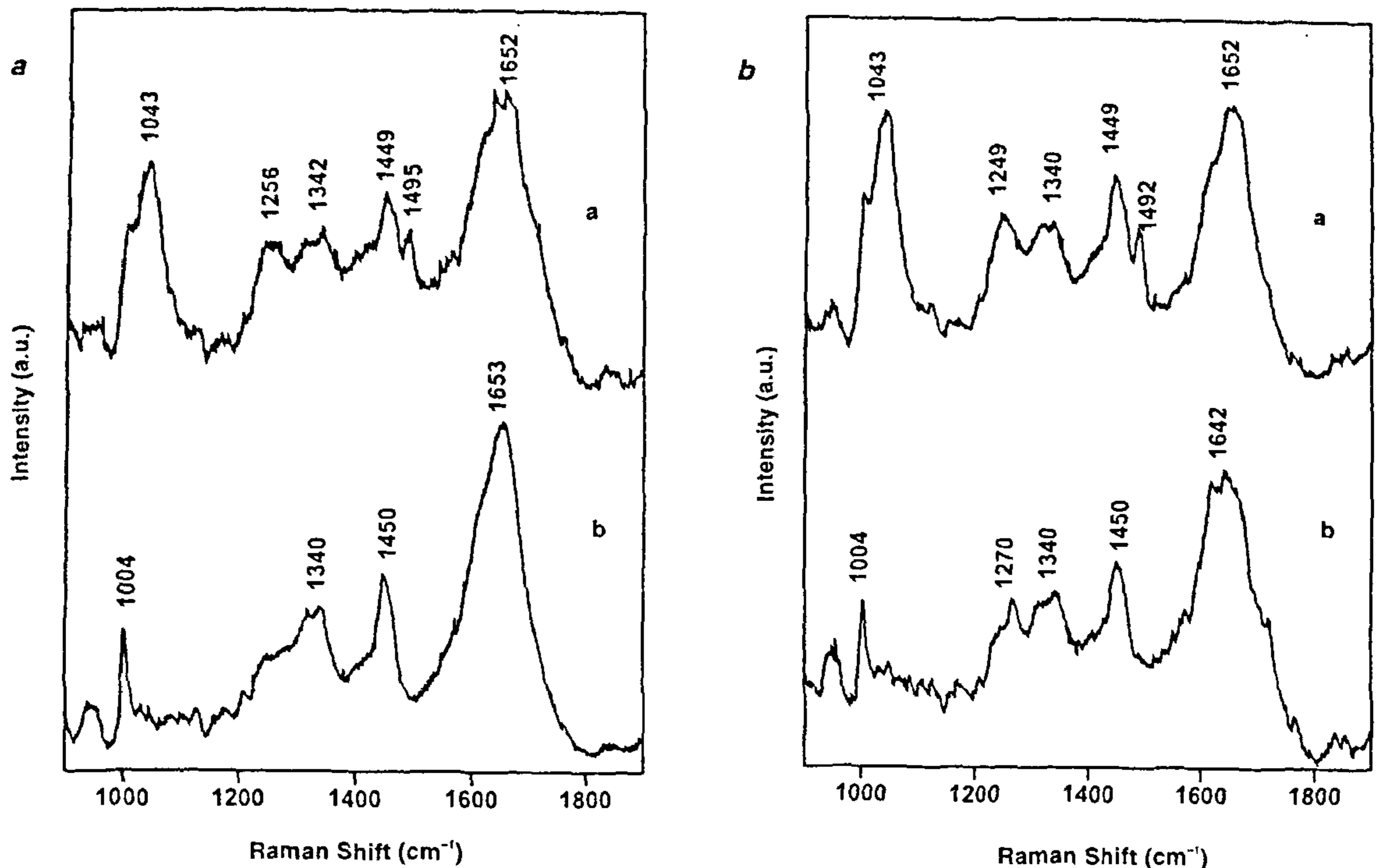


Figure 3. Raman spectrum of: *a*, two different samples of pleural fluid; and *b*, two different samples of ascitic fluid.

chloride. CSF is also cultured for bacterial presence. But none of these tests lead to unambiguous diagnostic result for various reasons.

We have obtained the Raman spectrum of several samples of CSF. The variety of spectra is shown in Figure 4 'a', 'b', 'c'. Whereas 'a' shows only a very weak protein background, with practically no sharp, strong peaks, 'c' shows several prominent Raman bands superposed on the protein background, and 'b' shows still another pattern. Table 2 shows the clinical history and analytical results of the three samples shown in Figure 4. It illustrates the variety of conditions under which CSF analysis is required and the difficulty of clear diagnosis based only on biochemical analysis. The wide variation in spectra shows that it may be possible to use Raman spectra of CSF for diagnostic purposes. However, although it is possible to assign the observed bands to appropriate molecular groups, correlation of the presence of these groups to the physiological and biochemical changes is very difficult, without many more samples and comprehensive analysis of the data.

The very fact that the Raman spectra are quite different for the three samples shows the utility of Raman spectroscopy in CSF analysis. Much more interesting results are obtained by looking at the SERS spectra of the CSF samples. Figure 5 shows the SERS spectra of the same samples shown in Figure 4. While sample 'c' showed practically no change in SERS, sample 'a' showed all the

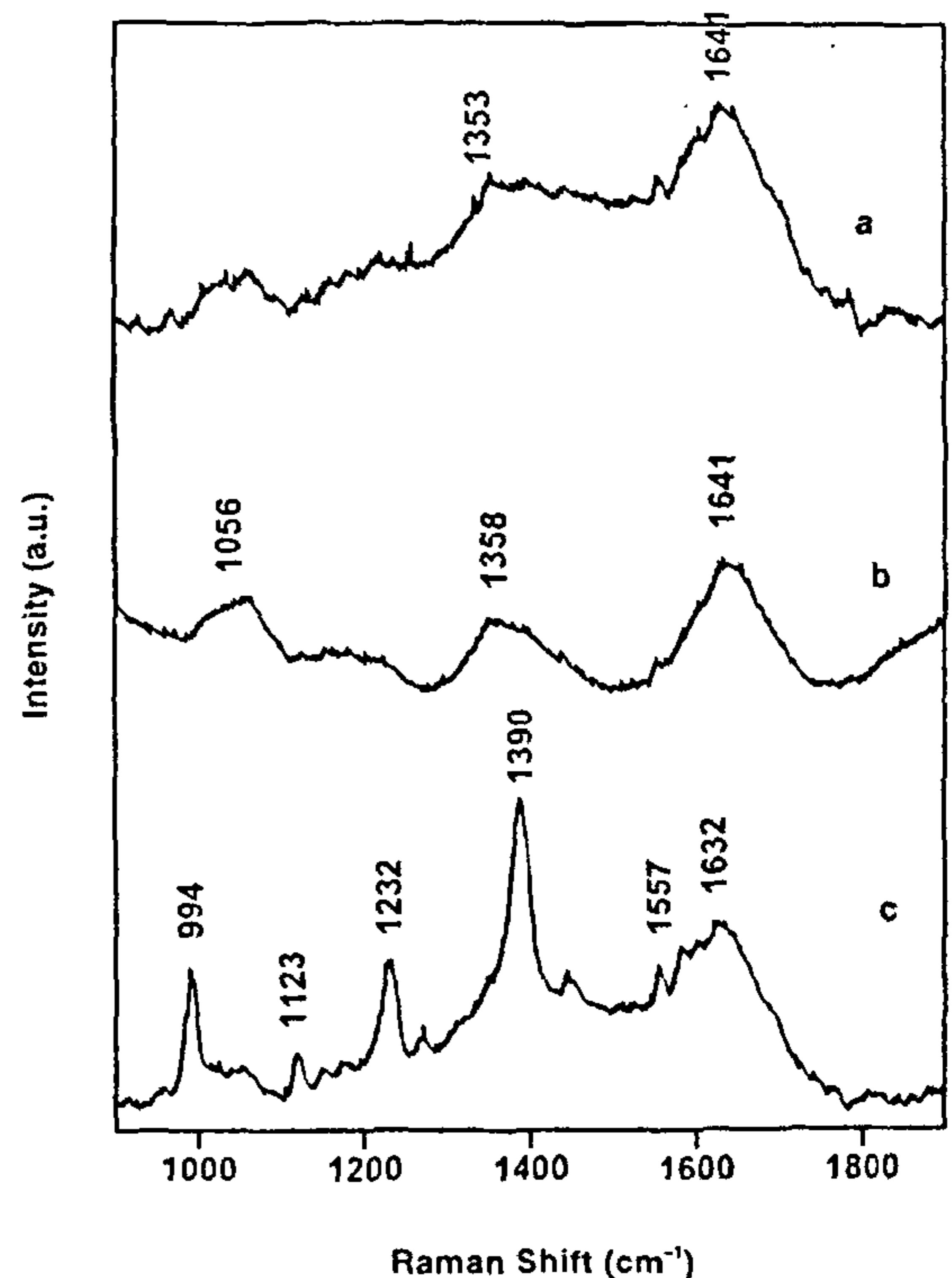


Figure 4. Raman spectra of 3 cerebro spinal fluid samples (see text for details).

Table 2. Clinical history and analytical results of the three samples shown in Figure 4

Sample	Clinical history	Analytical results				
		Total protein (mg/dl)	Glucose (CSF) (mg/dl)	Glucose (blood) (mg/dl)	RBC (cells/mm ³)	WBC (cells/mm ³)
a	Chronic organic brain syndrome (old history of Herpes Simplex encephalitis). Patient with fever and altered sensorium. C.T. normal (mild hydrocephalus) other investigations within normal limit. Patient developed hypokalemeia and died	95	61	116	51600	30
b	Vertebro basilar insufficiency with complete stroke. Brain stem plus cerebellar infarction with obstructive hydrocephalus	14	115	222	700	No cells
c	Hyponatrimic convulsion	35	125	149	Occasional	No cells

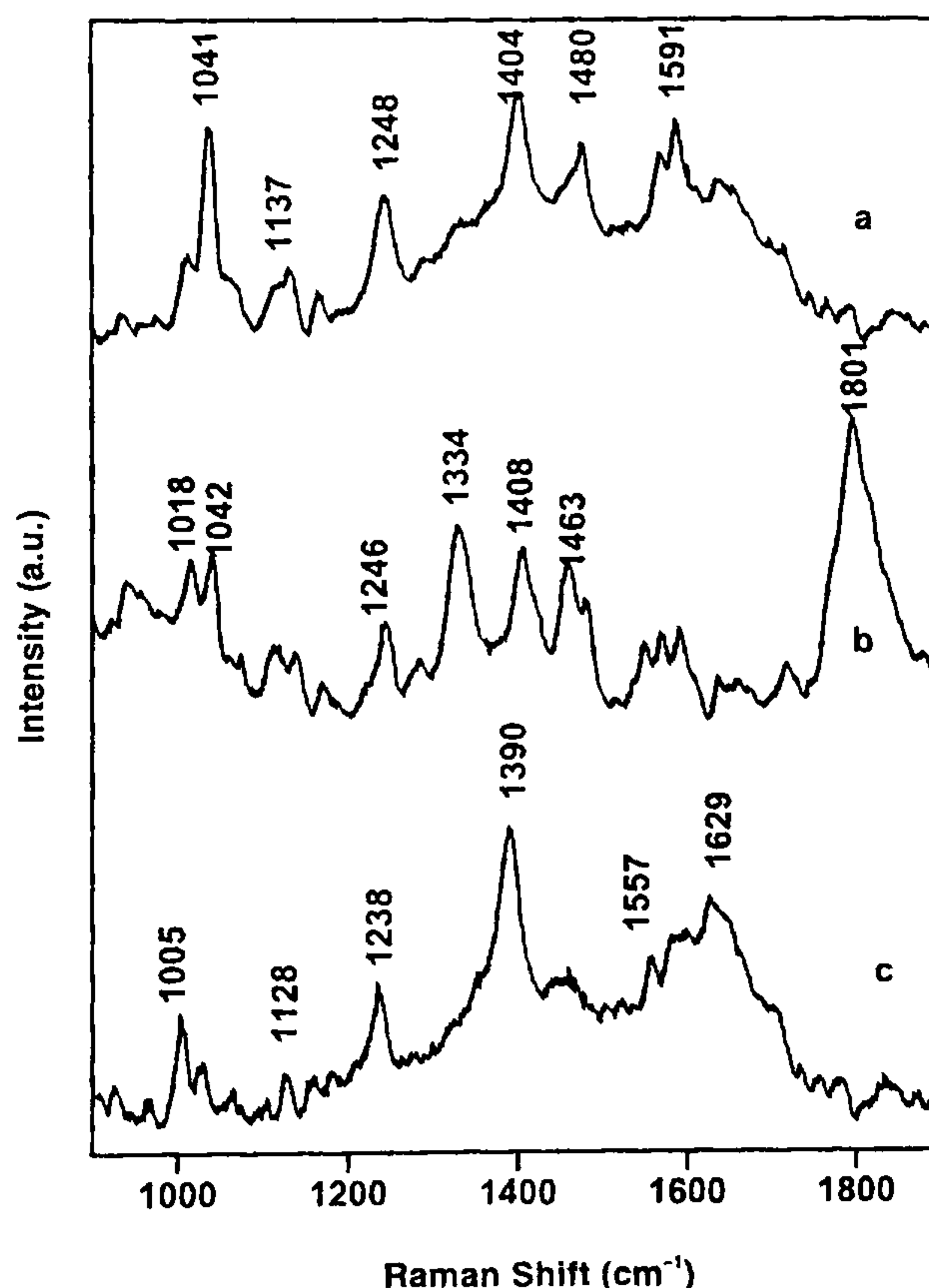


Figure 5. Surface-enhanced Raman scattering of the same cerebrospinal fluid samples of Figure 4.

bands corresponding to those of 'c' which were masked in the non-SERS spectra. Sample 'b' also showed some major changes.

The major components of CSF are sodium chloride, glucose, proteins (albumin), urea, and MHPG, with smaller amounts of lactate, bicarbonate, etc. Bands of glucose, urea, lactate, and bicarbonate are practically absent in the spectra because of their low concentrations, while the presence of protein, and other molecules like Flavo proteins, Cytochrome P450, etc. are suspected from the observed bands.

A detailed analysis of the spectra and correlation with clinical symptoms are being carried out at present.

Summary

It is seen from the Raman spectra presented above that Raman spectroscopy can be used very effectively for various clinical applications. These include early detection of neoplasia, optical pathology, decision making in demarcation of surgical boundaries, identification of

spectral markers of various clinical conditions, etc. In addition, the Raman spectra can also give information on the biochemical changes taking place prior to or during onset of disease. This will lead to a better understanding of the correlation between such reactions and the immunological processes due to them. Finally, in view of the very short time required to acquire Raman spectral data, portable Raman instruments²³ coupled to fiber optic Raman probes can be very effectively used in screening of population for early detection of oral cancer, cervical cancer, colon cancer, etc.

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