HPLC-LIF for early detection of oral cancer

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At present, the diagnosis of many cancers relies on the subjective interpretation of morphological changes in biopsy samples. This usually provides only late diagnosis. Early detection, which can provide more successful therapy, is expected to be possible by identification of tumour markers in physiological samples. Immunoassay used at present for this purpose has several drawbacks. It is applicable only for known markers, can usually detect only one marker at a time, and may also fail to detect a marker when there exist conditions, which may mask or prevent the interaction between antigen and the antibody. We have developed a high performance liquid chromatography-laser induced fluorescence (HPLC-LIF) technique to detect and record simultaneously spectra and chromatograms of physiological samples, which will enable the detection of multiple 'markers' in a single physiological sample in a short time. Samples of saliva and serum from normal and oral cancer subjects have been studied with the set up. The present studies show that body fluids like saliva and serum of normal, premalignant and malignant subjects have substantially different protein profiles. By simultaneous recording of the chromatographic peaks and corresponding fluorescence spectra, it is possible to carry out unambiguous discrimination between normal, premalignant and malignant cases even when markers are present in femto/subfemtomole quantities, which should assist in early diagnosis of neoplasia.

IT is recognized that even small primary cancers shed viable tumour cells into the circulatory system. In the progression of a tumour from a homogeneous proliferating clone to a group of heterogeneous sub-populations of cells, the cells generate an entire array of enzymes and surface molecules[1]. These molecules, as well as those produced by the tumour's host in response to the presence of the tumour are generally known as tumour markers. They can be used to detect the presence of malignancy when located in clinical samples like cytological smears and biopsied tissue samples or body fluids[2]. At present, the diagnosis of many cancers, especially oral cancer, depends almost completely on histomorphological analysis of stained tissue sections, which provide only late diagnosis. On the other hand, biomarkers could be used, not only for early detection, but also for identification of putative pre-malignant lesions, prognosis of disease, monitoring of therapy, and early detection of recurrence or development of second primary cancer[3]. At present, immunoassay is the only readily available technique for detection of tumour markers[4]. Unfortunately, so far no single marker has been identified in oral cancer that is not present in normal or benign lesions. Immuno methods are generally applicable for detection of only one marker at a time. Moreover, failure of immuno methods does not necessarily prove that the marker is absent. Anything that masks or prevents the interaction of the antibody and antigen will lead to a false-negative result. It is highly probable that a combination of several markers, detected simultaneously, will definitely improve the chances of success in early detection of cancer. By combining ultrasensitive optical methods like laser-induced fluorescence, with highly efficient separation techniques like HPLC or CE (capillary electrophoresis) we can detect ultra trace amounts of individual biomolecules in complex, multicomponent physiological systems[5–8]. The use of laser-induced fluorescence has several advantages over conventional fluorescence spectroscopy. They are (i) the limited influence of the stray light effects due to high monochromaticity, (ii) accurate focusing capability of the excitation source on to the microbore capillary flow cell, resulting in excitation of picolitre amounts of sample without loss of the excitation energy and (iii) complete elimination of excitation energy by use of dielectric laser mirrors, leading to very low background. In our laboratory, we have set up an HPLC-LIF system for detection of tumour markers in various forms of malignancy. The system is capable of detecting and recording the spectra of femtomoles (or still lower quantities) of proteins as they flow past a probing laser beam. The sensitivity can be enhanced by several orders of magnitude if needed. Typical studies on saliva and serum samples from normal and oral cancer subjects have shown that the set-up provides a very powerful technique for detection of several 'markers' in a single sample. The results in the present work demonstrate that the technique represents a possible powerful method for early detection of premalignancy, malignancy and recurrence, as well as monitoring of therapy.

Myoglobin, insulin, creatine kinase, bovine serum albumin (BSA), known tumour markers, as well as trifluoroacetic acid for HPLC were procured from Sigma and used as such. HPLC grade solvents acetonitrile, methanol, water and isopropanol were obtained from E. Merck.

The experimental system consisted of the following subunits that have been assembled together to form the final setup: HPLC, laser excitation source, CCD-spectrograph for recording fluorescence spectrum and monochromator-PMT-lock-in-amplifier for chromatographic

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peak-detection. Figure 1 shows the layout of the experimental set-up. The HPLC system (Hewlett Packard series 1100, USA) has a quaternary pump, a degasser, solvent reservoir cabinet, and a manual injector (Rheodyne). A reverse phase biphenyl narrow bore column (Vydac) was connected to this system using PEEK tubing (Upchurch Scientific). The effluent from the column was passed through a 75 μm i.d. quartz capillary (Hewlett Packard) using finger-tight fittings (Upchurch Scientific) and 1/16in.unions (Agilent).

An intra cavity frequency doubled Ar^+ laser (Innova 90C FreD) at 257 nm, obtained by doubling 514.5 nm with a BBO crystal was used for fluorescence excitation. The use of 257 nm for excitation has several advantages. All three fluorophores, phenylalanine, tryptophan and tyrosine present in proteins have reasonably good absorption at this wavelength. Also the Raman line from the solvents is close to the excitation line (~ 288 nm), and does not interfere with the fluorescence emission observed usually above 300 nm. The laser beam is tightly focused onto the capillary using a focusing lens (f = 2 cm). The plasma lines and fundamental were removed from the excitation line by using two dichroic mirrors, which reflect the 257 nm radiation to the lens transmitting all other wavelengths. A power of around 5–10 mW on the capillary surface was found to be sufficient for excitation. The fluorescence and Raman signals emitted were collected on both sides of the capillary at 90° to the excitation beam. On one side the collected signal was focused on to the spectrograph (Acton) through a third 257 nm reflecting dichroic mirror. The dispersed fluorescence and Raman signals were recorded with a CCD (Roper Scientific, Model-RTE/CCD-128-HB) interfaced to a PC. A 600 g/mm grating, blazed at 300 nm was used for recording the spectra. Fluorescence and Raman signals emitted on the other side of the capillary were collected using another lens system and focused on to the entrance slit of a DH10 (SPEX) monochromator. The signal was chopped using an EG&G Model 651 chopper and detected with a Hamamatsu R 453 Photomultiplier. Output of the PMT was fed into an EG&G preamplifier followed by a digital lock-in-amplifier (EG&G) interfaced to a PC.

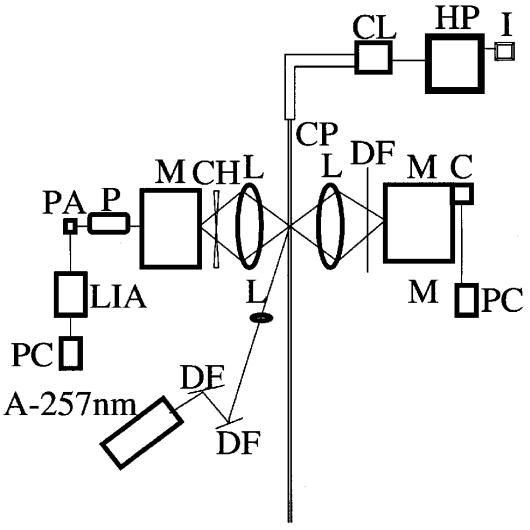

Figure 1. Experimental set-up for HPLC-LIF. I, Injector; HP, Pump; CL, Column; CP, Quartz capillary; L, Lens; F, Filter, M, Monochromator; C, CCD; CH, Chopper; P, PMT; PA, Preamplifier; LIA, Lock-in amplifier; PC, Computer; A, Ar^+ laser; DF, Dichroic filter.

The biological samples, i.e. blood and saliva of cancer patients were acquired from College of Dental Surgery, Manipal Academy of Higher Education. Normal samples were collected from healthy volunteers. These samples were analysed within a few hours of collection, though no changes in chromatogram or spectra are observed due to storage for several hours. Blood samples were stored at room temperature for about 30 min, centrifuged at 3000 rpm for 5 min and the separated serum was collected. The normal and malignant serum samples were usually diluted 100 to 500 times with ultra pure water. A 20 μl sample was injected into the HPLC. Both malignant and normal saliva samples were diluted 10 times with ultra pure water and 20 μl injected into the HPLC system. Because of the small dimension of the capillary and tight focusing of the laser (< 10 μm), the alignment of the system is rather critical. This is done conveniently, by maximizing the Raman signal from the effluent (water, acetonitrile, and methanol). As each chromatographic peak appeared in the lock-in system, corresponding fluorescence spectra were recorded using the spectrograph CCD system. A chromatogram of the sample and spectra of all the components in it are thus available at the end of a run.

The spectral recording and chromatographic detection sensitivity of the set-up was tested using proteins like insulin, myoglobin, creatine kinase and BSA. Figure 2 *A* shows the chromatographic peaks for standard solutions of BSA. Spectra from the blank and from 90 fm of BSA are shown in Figure 2 *B*. Plot of normalized chromatographic signal (background subtracted) of BSA versus the quantity of BSA injected into the HPLC is shown in Figure 2 *C*. The limit of detection taken as 3 times background noise is ~ 8 fmoles for the present system with lock-in detection. In the CCD-spectrograph the dark noise is 80 counts/s. The spectrum was recorded for 5 s and an estimate of sensitivity taken as thrice the dark count (1200 counts) is also of the same order, viz. ~ 7 fmol. As will be seen later, this sensitivity is more than needed for real-life samples. Further increase in sensitivity can be achieved on the spectrograph, if needed, by using a high NA microscope objective for collection of fluorescence[6], or replacing the *f*/4 spectrograph with one with lower f value[9]. The limit of detection under such conditions can be in the subfemtomole region. The sensitivity of the lock-in system can easily reach sub-femto-

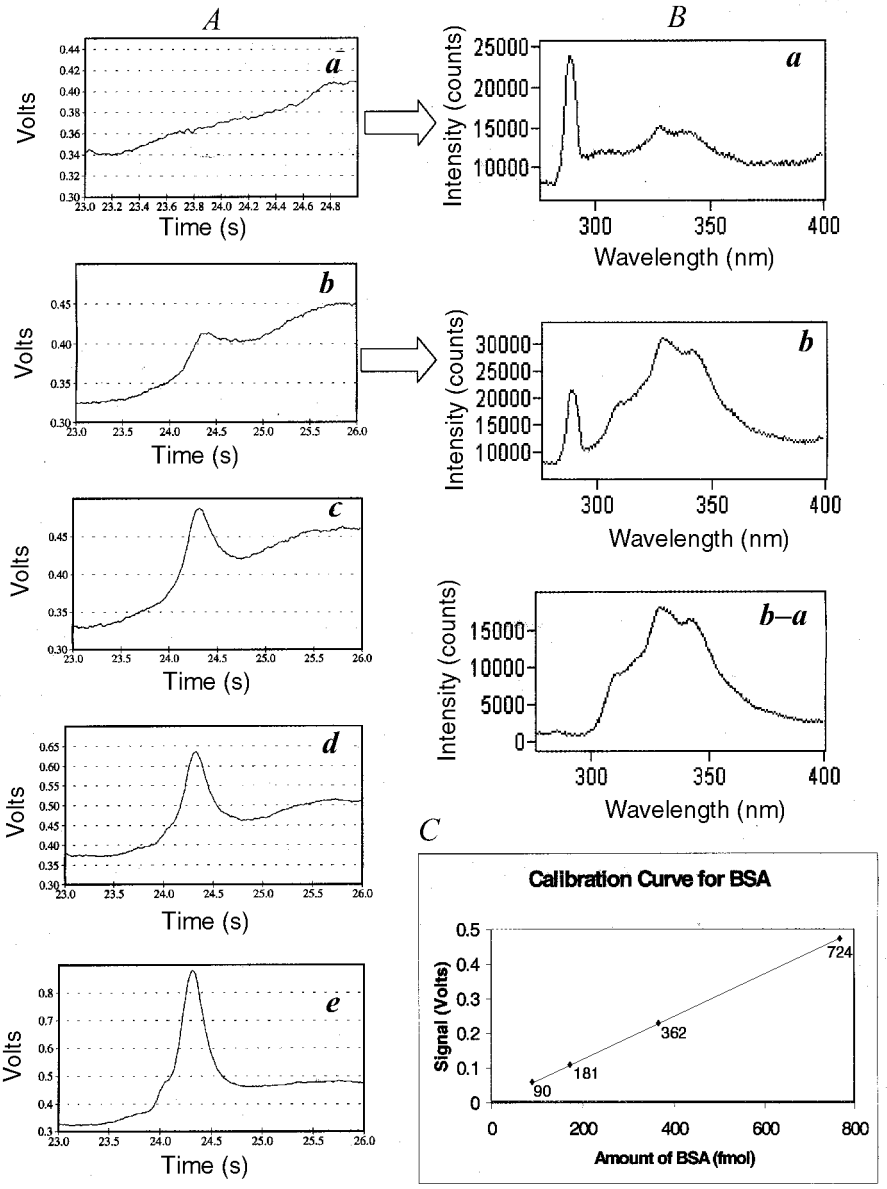

Figure 2. *A*, Chromatograms of different amounts of BSA, ***a***, blank; ***b***, 90 fm; ***c***, 181 fm; ***d***, 362 fm and ***e***, 724 fm. *B*, Fluorescence spectra of 90 fm BSA (100 µm slit width, 5 s accumulation). ***a***, Background spectrum; ***b***, Spectrum before background subtraction; ***b–a***, Spectrum after background subtraction. *C*, calibration curve for BSA.

molar level if needed, by replacing the monochromator with band pass filters, using high PMT voltages and laser power and better signal processing[10].

Figure 3 shows chromatograms of saliva samples from different subjects. Saliva from healthy volunteers with no oral malignancy or tobacco habit is taken as normal. Figure 3*A* shows chromatogram from normal volunteers belonging to different age groups, sex and reproductive stages. It can be seen that all the chromatograms are basically similar though there are some minor differences. The number of major peaks and relative intensities are very similar in spite of the fact that the subjects correspond to widely varying physiological conditions. There are only two major peaks in the early elution, (up to 15 min) and 5 major peaks in the later stages. Chromatograms of volunteers with tobacco habits but no malignancy did show some changes. However, they still do not show any major peaks other than those present in normal samples (Figure 3 *B*) in the early elution up to 15 min.

Pre-malignant phases of oral cancer include conditions like lichen planus, leukoplakia, erythroplakia, submucous fibrosis, etc. We have studied saliva samples from many such subjects. Figure 4 shows typical examples of these cases. The most noticeable difference from normal subjects is the appearance of several strong additional peaks in the early elution. In order to identify which of

these indicate progression to malignancy, several cases have to be studied and followed up. This is being done at present and will be completed over a period of 1–2 years.

Figure 5 shows similar results for established malignancy cases in various stages. Once again, several additional peaks are observed in these samples. Comparison of the normal, pre-malignant and malignant sample chromatograms clearly shows that while all normal saliva samples more or less matched with one another, pre-malignant and malignant samples are very different from normal. New peaks are observed and some of the peaks in normal saliva show drastic decrease in some of the malignant cases. The spectra of various fractions of normal, pre-malignant and malignant saliva are shown in Figure 6. As can be seen, there is considerable variation in peak positions, intensities, half widths and shapes of the emission bands among the different chromatographic peaks.

As seen from Figure 6, spectra of different protein components in saliva show subtle differences between normal, pre-malignant and malignant cases. Some of the spectra have very low intensity, while others are very strong. Many of them are similar in shape. It is thus obvious that without their separation on the HPLC column, no information on relative spectral characteristics or concentrations could have been obtained, even if a total fluorescence spectrum of the sample is available (Figure 6, TF).

It is well known that cancer is not a single disease. It is therefore to be expected that the expression of proteins in the system need not be the same under different conditions of malignancy or in different stages of malignancy. The number of peaks, and intensities which are proportional to their relative concentrations, as well as the retention times in the various samples cannot thus be expected to resemble each other closely. This is clearly shown by the results in Figures 3, 4 and 5. Thus any

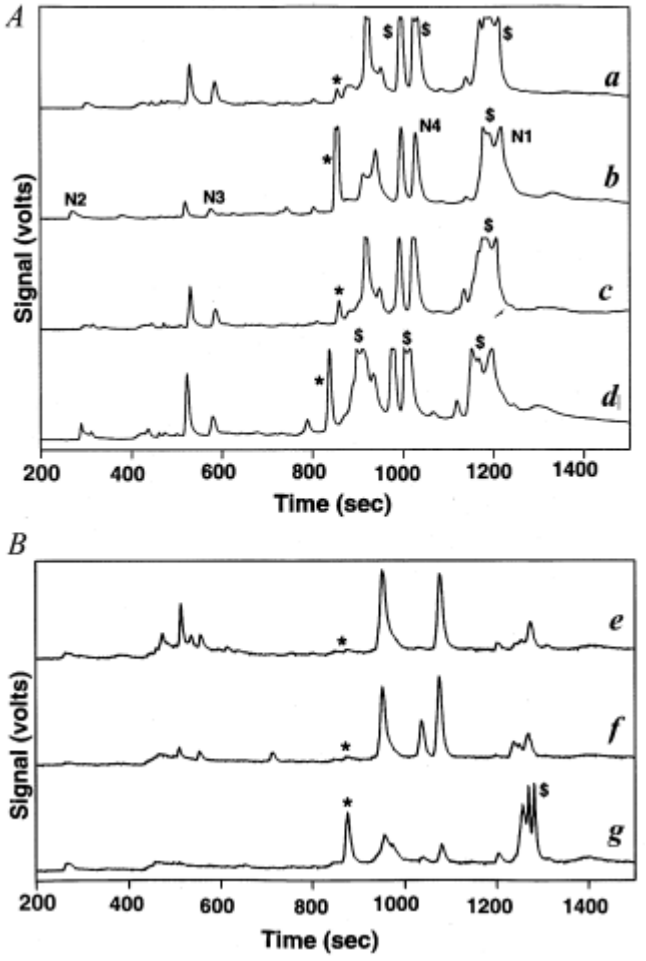

Figure 3. *A*, Chromatograms of normal saliva samples. ***a***, Female, 38 years, Mother of two; ***b***, Male, 23 years; ***c***, Male, 28 years; ***d***, Female, 33 years, Pregnant. *B*, Chromatograms of smoker's saliva samples. ***e***, Male, 38 years, 3–4 cigarettes a day; ***f***, Male, 43 years, 6–7 cigarettes a day; ***g***, Male, 28 years, 10–12 cigarettes a day. The * in this and subsequent figures indicates a peak coming from the solvents in some of the runs, and is variable from run to run. $, The dips observed at the strong peaks in this and subsequent figures result from shutting off of the recording system due to excess signal overloading.

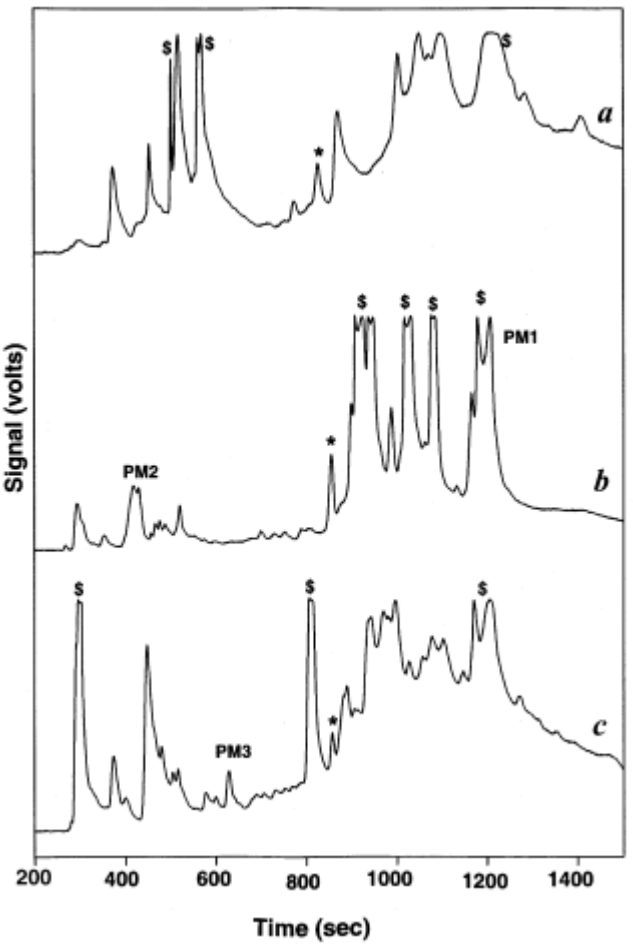

Figure 4. Chromatograms of oral premalignancy saliva samples. ***a***, Leukoplakia; ***b***, Lichen planus; ***c***, Submucous fibrosis.

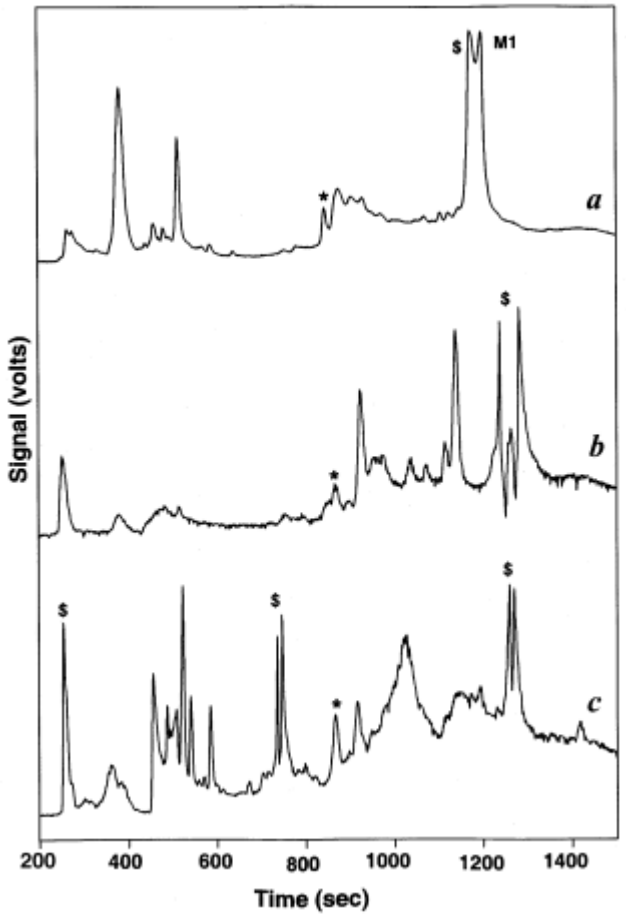

Figure 5. Chromatograms of oral malignant saliva samples. ***a***, Carcinoma buccal mucoa; ***b***, Oropharyngeal carcinoma; ***c***, Squamous cell carcinoma of lip.

pattern recognition analysis can be of limited use in statistical evaluation or classification of stages of malignancy, etc. The important results to be noted are that, (i) all normal samples have the same pattern, (ii) pre-malignant stages have a large number of extra peaks with low retention times while normal peaks are still observed, and, (iii) malignancy leads to drastic changes even in the normal components.

Oral cancer is one form of cancer for which not many markers have been indicated[2] till now. Squamous cell antigens (glycoprotein, 44–48 kDa), epithelial keratins, immunosuppressive cytokines and cell surface antigens[4,11] have been suggested as possible markers. The major proteins in normal saliva are mucin, α-amylase and lipase with trace levels of immunoglobulins[12]. Since the aim of the present work was to test the capability of our system to discriminate between normal, pre-malignant and malignant physiological samples, no attempt was made to correlate the 5 strong peaks in normal saliva to any possible salivary proteins. The enormous increase in many of the very weak peaks as well as the decrease in some of the peaks in malignant saliva compared to normal samples clearly show the capability of the system in detecting simultaneously even very small changes in the molecular species from normal to pre-malignant and malignant.

It should be noted that all the chromatograms were recorded with the monochromator set at 340 nm, with band pass less than 10 nm (2 mm slits). As can be seen from the spectra in Figure 6, some of the 'markers' have their peak intensities in the 300–320 nm, while some others have their peaks at 350 nm. Also all the spectra have widths greater than 30–40 nm. This shows that the detection sensitivity of the lock-in system can be increased by 1–2 orders of magnitude by using band pass filters for detection, with simultaneous measurement of background fluorescence at a suitable wavelength, say 450–500 nm, for background correction. We are incorporating these modifications at present. Normally all the spectra are recorded with 100 μm slit, 1 s accumulation and no binning of the pixels. For further sensitivity increase, slits can be opened up to even 500 μm, spectra can be accumulated for about 20 s and several pixels (even up to 100) can be binned. This will increase the sensitivity 2–3 orders of magnitude. We have combined all these ideas in typical test runs (data not shown) and have found that sensitivity increases about 1000 times.

Intrinsic protein fluorescence comes from the aromatic amino acids, tryptophan, tyrosine and phenylalanine. The excitation wavelength, 257 nm, in our case, is absorbed fairly well by all these three amino acids. However, the emission maximum of phenylalanine is around 280 nm, and its quantum yield is very low. Moreover, energy transfer to tyrosine and tryptophan by resonance energy transfer is highly likely, and so the bands observed in the present studies cannot be from phenylalanine groups. Tryptophan emission is known to be highly sensitive to the average environment of the molecule[13], starting with a structured emission at 300 nm in completely nonpolar environment, and changing to a broad emission around 350 nm when exposed to a polar environment. Tyrosine in water gives a fluorescence emission around 300 nm; the emission being relatively insensitive to polarity and more narrowly distributed, compared to tryptophan. Most of the 'markers' in the present runs are eluted early in the run, under highly polar environment (70–50% water/TFA). It is interesting to note that some of these markers do exhibit peaks around 300 nm. These peaks could arise from either a tyrosine residue or a tryptophan residue in a highly hydrophobic environment. However, hydrophobic proteins are usually eluted late. Moreover, since similar spectral features were recorded for insulin, which has similar retention time, 4 tyrosine moieties and no tryptophan[14], the former possibility is more probable. Detailed investigations including 'on-line' and 'off-line' Surface Enhanced Raman Spectroscopy (SERS) on these individual fractions are now being carried out, to get a better understanding of the nature of the proteins and the significance of their presence in malignancy.

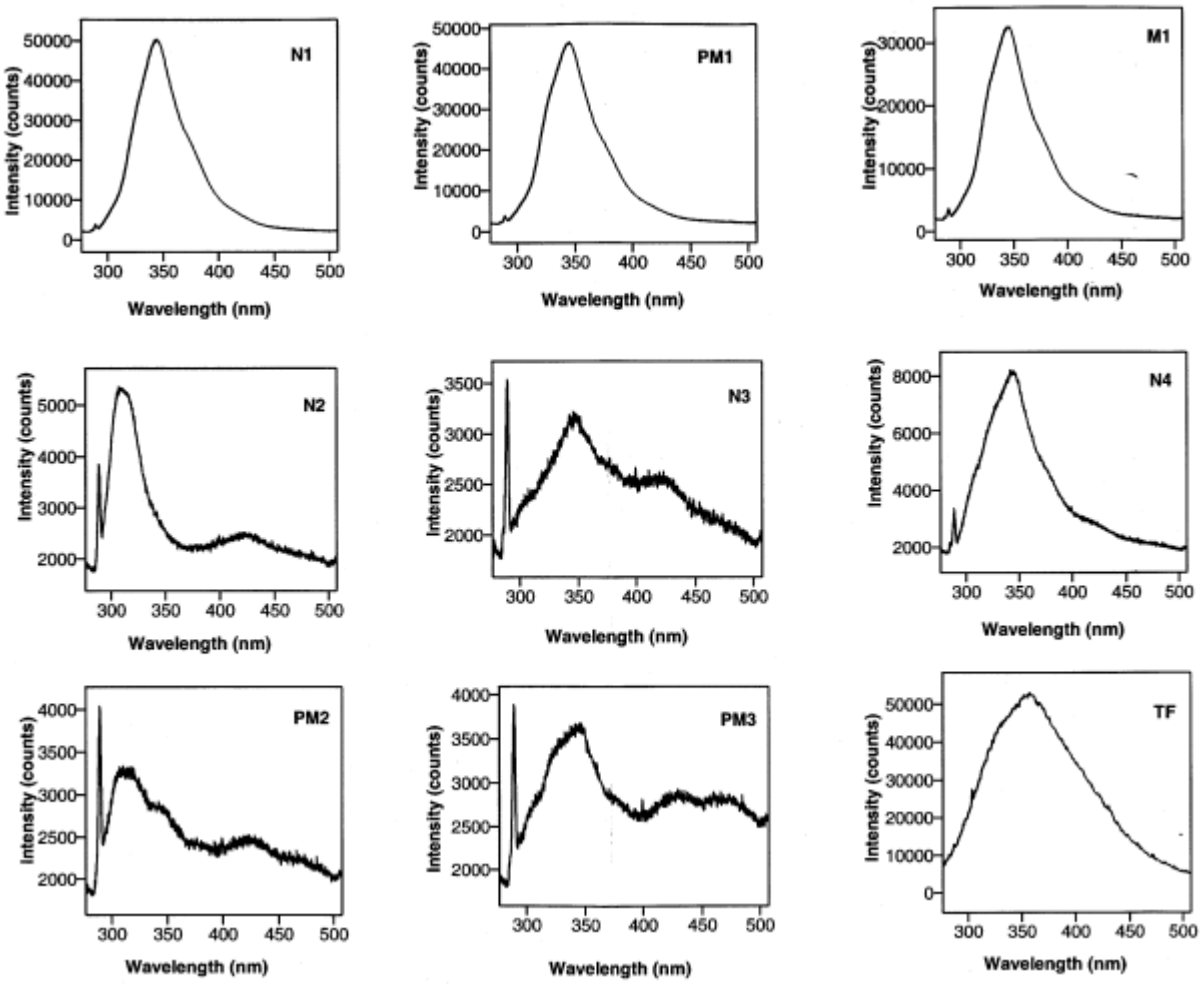

Figure 6. Fluorescence spectra of saliva components of different types N1, PM1 and M1, Fluorescence spectra of component with same retention time from Figures 3, 4, and 5; N2, N3 and N4, Fluorescence spectra of different components from same sample in Figure 3; PM2 and PM3, Fluorescence spectra from two components in different pre-malignant samples from Figure 4; TF, Total fluorescence spectrum of normal saliva excited by 257 nm.

We have carried out analysis of serum samples under similar conditions. As in the case of saliva, serum samples from normal subjects were all very similar to one another, while samples from malignant cases showed many additional peaks, as well as noticeable changes in relative concentrations of components. These changes can be understood only after correlating results from a statistically significant number of subjects under various stages of premalignancy, malignancy and metastasis, since expression of serum proteins may change significantly under such variations. These studies are being carried out at present.

HPLC-LIF, combined with on-the-run spectroscopy of saliva and serum are capable of providing spectra and chromatograms of multicomponent physiological samples for detection of tumour markers. The method is highly sensitive, capable of detecting femto/subfemto moles of 'markers', and is thus comparable to methods of immunoassay, employed at present for this purpose. However, unlike immunoassay, which can detect or estimate only one known marker at a time, the present technique can look at multiple markers in one run, thus improving the diagnostic capability. The time required for the test is also very short, being less than one hour after receiving the sample, since no preprocessing or immuno reactions are involved. The present results also show that though there are no established markers identified at present for oral cancer, it may be possible to select some of the many new proteins observed in premalignancy and malignancy, as suitable markers. This has to be done by analysing data from a sufficiently large number of samples in each category, like putative premalignancy, various stages of malignancy, recurrence, etc.

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