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Optical Assay for Biotechnology and Clinical Diagnosis

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Abstract— **In this paper, we present an optical diagnostic assay consisting of a mixture of environmental-sensitive fluorescent dyes combined with multivariate data analysis for quantitative and qualitative examination of biological and clinical samples. The performance of the assay is based on the analysis of spectrum of the selected fluorescent dyes with the operational principle similar to electronic nose and electronic tongue systems. This approach has been successfully applied for monitoring of growing cell cultures and identification of gastrointestinal diseases in humans.**

Index Terms— **chemometrics, clinical applications, fluorescence dyes, optical diagnosis.**

I. Introduction

SINCE the last decade the research associated with the development of new sensor assays to detect multiple analytes has been growing intensively [1-3]. The use of such an intelligent device with the capability of accurate and reliable diagnosis could help to decrease probability of harm, minimize clinical intervention, costs of medical treatment and as a consequence improve long-term public health. This requires new developments and implementation of suitable indicators to estimate potential risk factors and their effects on health, identification of early symptoms of diseases or monitoring the progress of treatment [4, 5].

Current trends in the development of sensing technologies mimic perception of mammalian smell and taste (schematically shown in Fig. 1a). Known as electronic noses (e-noses) and tongues (e-tongues), these techniques [6, 7] are based on the 'smelling/tasting' array of cross-sensitive chemical receptors and interpretation of detected composite signals with suitable statistical methods [8, 9] (see Fig. 1b). The specificity of such a methodology is related to the pattern recognition in response to signals produced by interactions of the sample with all sensing elements [10, 11]. The obtained patterns are unique to particular conditions and therefore can be considered similar as the fingerprints.

The analytical capability of such an approach is less likely to lie in the measurements of analytes' concentrations than in profiling the chemical or biological processes and qualitative sample analysis. Although both e-nose and e-tongue technologies are currently commercially available, they suffer from significant limitations, such as poor stability, limited selectivity, low reproducibility, demand for frequent calibration, complexity of generated information, and high fabrication costs of sensor arrays [6,12].

Lately there has been a surge of attention to optical diagnostics, especially in the field of real-time non-invasive *in vivo* detection [13]. This is explained by the facts that optical techniques are often non-destructive, do not require physical contact with a sample during analysis and enable rapid response [14,15]. They are able to provide information at the molecular level through tissues and living organs originating from both animals and humans. Light can also be used as a tool for manipulating or modifying living cells and can be focused at a tiny spot, which allows precise localised and minimal invasive treatment [16]. By providing more effective, cheaper and easy accessible service, biophotonics and optical diagnostic technology can have a huge and crucial impact on clinical practice.

Recently we described a fluorescence-based assay with the operating principle similar to e-noses and e-tongues used for simultaneous measurements of several physicochemical parameters [17]. Those preliminary experiments have been performed to investigate the analytical capability of the proposed assay and validate its potential for quantitative analysis of samples. Additionally, previous publication includes the details on the methodology and data evaluation. A schematic illustration of the concept of optical/fluorescent assay is shown in Fig. 1c. The mixture of five fluorescent dyes interacts with the sample and generates excitation-emission matrix (EEM) fluorescence spectra (Fig. 2) distinctive for biochemical or medical conditions.

EEMs are generated by passing white light (typically from a Xe source) through an excitation polychromator which splits the light into different wavelengths at a pre-determined range which then irradiate the sample in the cuvette. The fluorescence light emitted by the sample passes through an emission polychromator which also splits the light into

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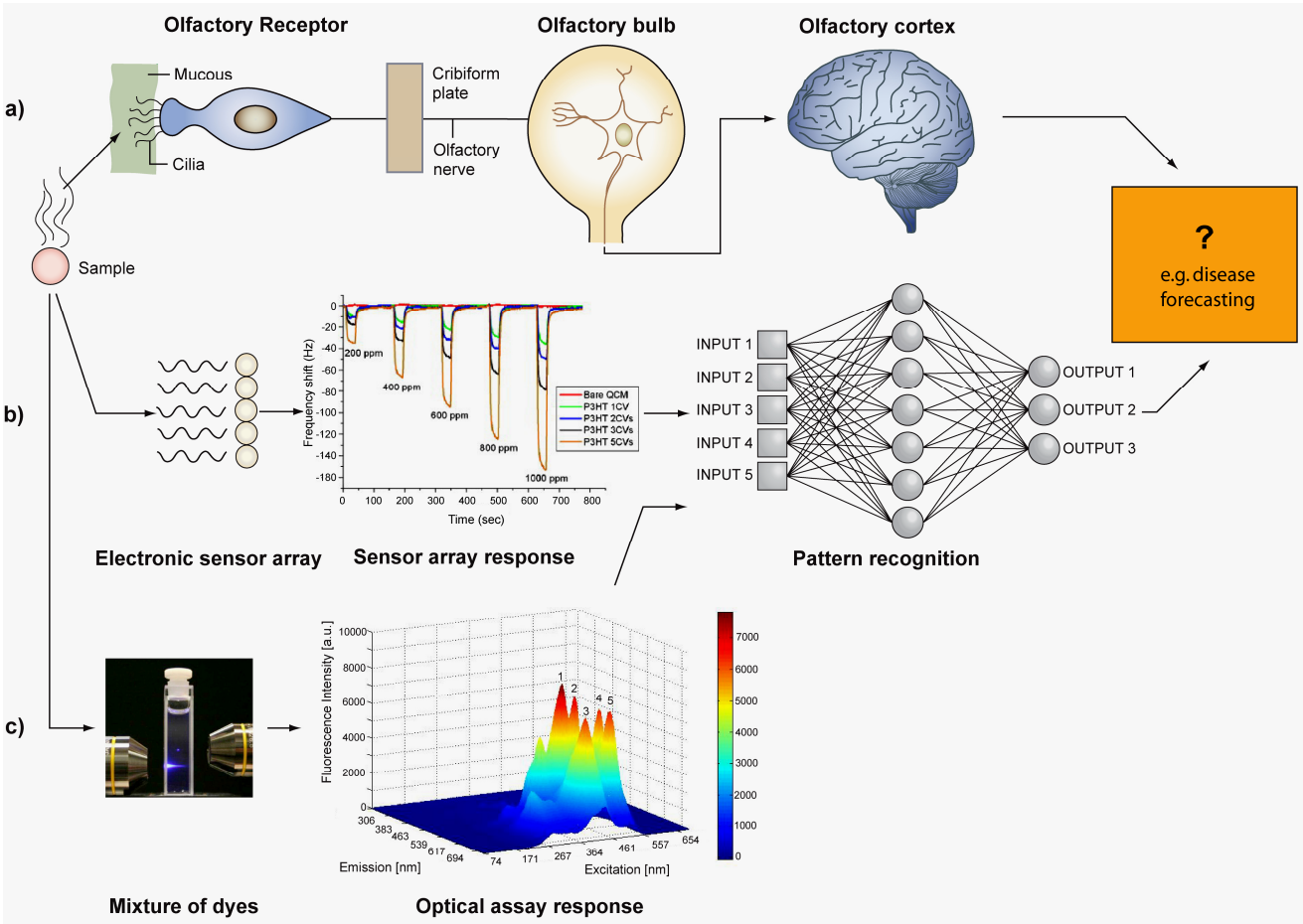


Fig. 1. Schematic representation of sensing systems: (a) human olfactory system, (b) electronic analogue and (c) optical/fluorescence analogue [17].

different wavelengths from which the split light is captured on a charge-coupled device (CCD) camera, thus generating an image or fluorescence profile as shown in Fig. 2. Using chemometric approaches these patterns are further analyzed and the optical signal is transferred into analytical characteristics of the samples. Additionally, for the minimum interference with biological samples, selected fluorescent dyes are responsive in the VIS-NIR range.

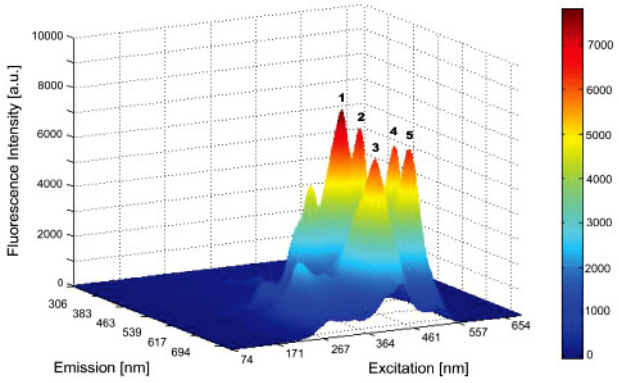


Fig. 2. 3D color mapped surface of the excitation-emission spectral pattern for selected 5 fluorescent dyes: Dye 1 is 8-hydroxypyrene-1',3,6-trisulfonic acid, Dye 2 is Oregon green 514, Dye 3 is Rhodamine B, Dye 4 is Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium dichloride, Dye 5 is Thionin acetate [17].

In the current study we investigate the potential of the technique developed in [17] for qualitative examination of biological and clinical samples.

The feasibility of this approach has been tested for monitoring development phases of growing bacterial cultures and for analysis of urine samples from healthy volunteers and patients diagnosed with gastrointestinal diseases.

In the first issue we take into account that certain types of bacteria, fungi, viruses or parasites have significant impact on human life considering their beneficial or harmful effects. Therefore, controlling the growth of microorganisms is necessary for many practical reasons including medicine, prevention or treatments of diseases, production of drugs, etc. This involves the identification of the phases of microbial growth to inhibit the process or recognize and learn favorable environmental conditions, which they need to live and reproduce.

The second test was performed on human urine samples for recognition of three gastro-intestinal diseases (irritable bowel syndrome, Crohn's disease and ulcerative colitis). They are currently serious public health problems, which have been forecasted to further increase in most countries around the world [18, 19]. The identification of the symptoms, early diagnosis and treatment of these diseases is still challenging because the etiology and factors causing these complex

disorders are yet not well known. Therefore, their mechanisms are difficult to explain, various types difficult to discriminate and therapeutic targets hard to identify [20, 21]. Typically, their diagnosis involves many analytical tests which are often costly and invasive. Therefore, it is of the great importance to search for the suitable and sensitive method for controlling gastrointestinal microbiota and early detection of dangerous pathogens [22].

II. Materials and Methods

The assay composed of five commercially available and inexpensive fluorescent dyes has been successfully developed for measurements of pH, temperature, dissolved oxygen and ionic strength of a solution (given by a buffer concentration) [17]. The results obtained have demonstrated, with high accuracy, quick, simultaneous identification and calculation of several physicochemical parameters (see Table I). Analytical performance of the assay was demonstrated with relatively low root means square error (RMSE), which describes the quality of fitting of a regression model.

TABLE I
PERFORMANCE OF FLUORESCENT DYES ASSAY

	Parameter	Dye Name	Sensitivity Range	RMSE
1	pH	8-Hydroxypyrene-1',3,6-trisulfonic acid, Oregon Green 514	5 - 9	0.004
2	temperature [^{o}C]	Rhodamine B	25 - 40	0.437
3	dissolved oxygen [ppm]	Tris (4,7-diphenyl - 1,10-phenanthroline) ruthenium dichloride	0 - 21.6	0.049
4	buffer concentration [mM]	8-Hydroxypyrene-1',3,6 - trisulfonic acid	5 - 150	6.818

A. Composition of dyes mixture

The mixture of fluorescent dyes were prepared as a stock solution of following dyes: 0.15 mM 8-Hydroxypyrene-1',3,6-trisulfonic acid, 0.1 mM Rhodamine B, 2 mM Thionin acetate, 0.025 mM Oregon Green 514, 6 mM Tris (4,7 – diphenyl - 1,10 - phenanthroline) ruthenium dichloride. Solutions of dyes were prepared in deionized water and stored in ~5 ºC, covered with aluminum foil to protect them from light. The 200 µl of each water/dye stock solutions were mixed together and used in further experiments.

B. Samples preparation

The bacterial strain used in the experiment was Escherichia coli (JM 83) provided by Cranfield University (UK). Bacteria were recovered from frozen state by growing them in a Miller LB broth (Fluka Biochemica, Cat No.1.10285), solution of 12.5 g of the medium in 0.5 L of Milli-Q water, overnight at 37 ºC and subcultured. The bacteria colonies were transferred into centrifuge tubes filled with 20 ml of liquid medium and incubated for 60 hours at 37 ºC [23]. The tubes with bacteria cells were collected at different intervals and centrifuged at 2800 rpm for 20 min. The supernatant was filtered through a 0.22 µm filters and 3 ml of the filtrate were transferred into 4 ml quartz cuvettes. The optical/fluorescent assay was then applied to supernatants taken from suspensions of growing bacteria. Control fluorescent measurements were performed using samples without growing bacteria. Additionally, bacterial growth was monitored by measuring the absorption of the suspensions [24].

Further experiments have been performed with urine samples obtained from healthy volunteers (CTR) and patients diagnosed with Crohn's disease (CD), ulcerative colitis (UC) and irritable bowel syndrome (IBS). All volunteers were given information, consent forms to read and sign, and a questionnaire, which provide details on their diet, exercise, sleep, medication and general health. The samples, provided by Addenbrookes Hospital (UK), have been obtained prior to any medical treatment and/or conservation. They have been stored at -80 ºC to maintain sample integrity. Before the measurements each sample was defrosted, centrifuged at 2800 rpm for 20 minutes and filtrated through 0.45 µm glass fiber filters. The mixture of fluorescent dyes was added into this supernatant, and then measured.

C. Instrumentation

The measurements of fluorescence intensity have been performed using three-dimensional spectrofluorimeter Jobin Yvon – SPEX FL-3D (Instruments SA, Stanmore, Middlesex, UK) at 0.5 s of time exposure. The spectra have been recorded over a range of excitation (74-691 nm) and emission (227-724 nm) wavelengths. The range of wavelengths was based on the technical specification of the spectrofluorometer. The fluorescence measurements were performed using quartz cuvettes with stoppers and a light path of 10 mm.

Absorption spectra of bacteria suspensions at different growing phases were measured with Spectrometer (UVPC 2100, Shimadzu, Japan) at 550 nm.

D. Data evaluation

The changes in fluorescent patterns (see an example in Fig. 2) caused by interactions with the dyes surrounding media have been analyzed using an artificial neural network (ANN). ANNs were implemented in MATLAB (version 7.3.0, MathWorks Inc., 2006) using the Neural Network Toolbox (version 5.0.1) and trained on the data patterns of samples of known identity (45 samples with known time of bacteria growth from 0 to 60 hours) using Bayesian back-propagation of errors [25]. Once the ANN was trained, the approach was applied for evaluation of 18 unseen samples. Based on learning experience, the network was capable of identifying unknown fluorescent fingerprints and predicting outputs (identifying different test solutions).

The total number of urine samples provided was limited to 32. We admit that this number is small yet for statistical

purposes it is suitable to generate preliminary results leading to a proof of concept. Table II summarizes the sample distribution between the disease states. In order to classify the three gastrointestinal diseases pertaining to the urine samples via their EEM spectra, two approaches were undertaken.

TABLE II
NUMBER OF DISEASE STATES IN URINE SAMPLES

Disease	Control (CTRL)	Irritable bowel syndrome (IBS)	Crohn's disease (CD)	Ulcerative colitis (UC)
Number of samples	9	11	6	6

The first involved performing principal components analysis (PCA) [25] on each sample's EEM spectrum in order to attain the most influential emission profiles. This was achieved by taking, for each sample, the loadings profile of the first principal component (PC1) which is generated from the decomposition of the respective EEM spectrum. The PC1 loadings profile is taken because it will have a high percentage of variance and have no contribution from noise within the EEM spectrum. The collection of profiles were inputs for the ANN probability network [26] to classify each of the three diseases from the healthy controls by assigning class values to the states: 1 for healthy; 2 for disease. Leave-one-out cross-validation (LOO-CV) permitted each sample to be classified leading to an overall success of classification. In order to perform a thorough analysis, and thus to ascertain the best results, different scaling techniques were employed such as mean-centering, auto-scaling and range-scaling. Each have their own merits: mean-centering removes any offset from the data originating for example from instrumental drift; auto-scaling permits the analysis of the data by correlation and not covariance; range-scaling make all the samples equally important therefore biological activity dominates. The linear classification technique of partial least squares discriminant analysis (PLSDA) [27] was also employed for comparison against ANN. The technique of PLSDA attempts to maximize the covariance between the classification (healthy vs disease) and the loadings profiles.

The second approach involved the use of a multi-way technique called parallel factor (PARAFAC) analysis [28]. It is a multi-way extension of PCA and thus permits data reduction of a data cube (collection of EEMs) into a scores matrix and two loadings matrices: the first containing profiles pertaining to excitation wavelengths; the second containing profiles pertaining to emission wavelengths. The key advantage with this technique is that given the correct number of components have been selected, the pure fluorescence emission spectra for each dye can be captured within the PARAFAC loadings [29]. The loadings corresponding to the emission spectra were combined with the scores to reproduce the sample matrix in which the number of rows corresponds to the number of samples and the number of columns to the emission wavelength range. This matrix formed the input parameters for PLSDA and the ANN probability network. Classification was performed as described above.

Finally, in order to ensure that the results were valid, a Monte Carlo simulation was performed [30]. This involves generating random class values for each sample and then classifying via the leave-one-out cross-validation (LOO-CV) method. The number of runs was set to 500. This is to guarantee a normal distribution is attained. If the overall classified result is beyond the 95% confidence limit, then that result can be deemed valid.

III. RESULTS

In the analysis of bacteria growth the mixture of dyes was added to supernatants taken from suspensions of growing microrganisms. Samples were collected at different intervals of time. During their growth the concentration of nutrients, oxygen and pH were changing. These reflected in the modification of dyes spectra which were further analyzed by ANN. Bacterial growth was monitored by measuring the absorption of the suspensions. Additionally, control measurements were performed using samples consisting of the mixture of fluorescence dyes in solutions of only the medium (LB broth), without growing cells. They were kept in the same condition as diagnosed samples. The results for control measurements showed no changes in fluorescence signal, whereas the changes in fluorescence caused by the presence of bacteria have been clearly identified.

The results of ANN predictions are presented in Fig. 3. The graph illustrates the correlation between real (measured) time of growth of bacteria culture (x axis), and the time predicted by ANN (y axis). Circles (○) indicate the mean values of data points of the network prediction and error bars indicate standard deviation.

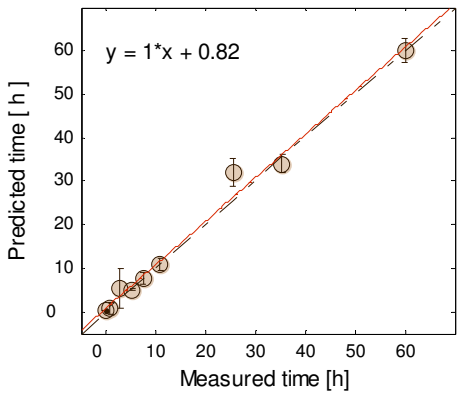
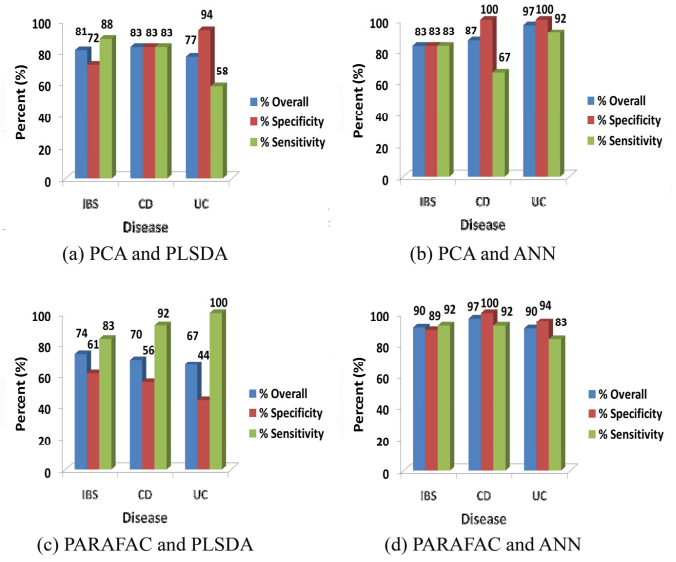


Fig. 3. Correlation between the time of bacterial growth measured experimentally and predicted by ANN: circles (○) indicate mean values of data points of ANN simulations: solid line (—) shows the best linear fit and the line (– • –) presents ideal response of ANN with zero error. Error bars indicate standard deviation.

These results demonstrate capability of the technique to identify detected fluorescent fingerprints and predict outputs.

The RMSE is 6.412 and indicates very accurate prediction and the potential of the dye assay application to analyze biological samples.

Classification of the urine samples (Fig. 4) shows great promise particularly when performed with artificial neural networks (ANNs) and in both cases, outperform the linear approach of partial least squares discriminant analysis (PLSDA). Fig. 4 also shows that, overall, the ANN probability network has performed better than PLSDA. However, with regards to PCA, the sensitivity of PLSDA is slightly superior to that of ANN (Fig. 4a and 4b) which means that it can distinguish the respective disease better with the exception of ulcerative colitis (UC).



(a) PCA and PLSDA (b) PCA and ANN

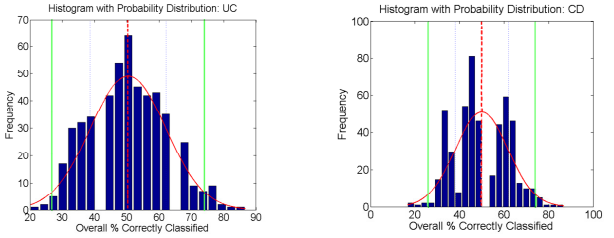
(c) PARAFAC and PLSDA (d) PARAFAC and ANN

Fig. 4. Comparison of the PLSDA (a and c) against the ANN probability network (b and d) to discriminate the three diseases from the healthy controls in terms of % Overall*, % Specificity** and % Sensitivity*** after application of PCA (a and b) and PARAFAC (c and d) to each EEM.
*__Overall__ means the overall success of discrimination; **__Specificity__ represents the number of *true negatives* (e.g. healthy samples). If the percentage of true negatives was 90% (90 out of 100 healthy samples were classified correctly) then there would be 10% *false positives* (10 out of 100 healthy samples incorrectly classified, i.e. classed as diseased); ***__Sensitivity__ represents the number of *true positives* (e.g. diseased samples). If the percentage of true positives were 95% (95 out of 100 diseased samples correctly classified), then there would be 5% *false negatives* (5 out of 100 diseased samples incorrectly classified, i.e. classed as healthy).

However in terms of specificity we observe 100% which implies that the healthy control can be completely distinguished from the diseased samples. With regards to PARAFAC (Fig. 4c and 4d) the ANN probability network has performed better than PLSDA but also has resulted in a better outcome than the application of PCA. On this occasion, the sensitivity pertaining to UC in PLSDA (100%) is better than ANN (83%) yet the corresponding specificity is much worse (Fig. 4c).

In order to confirm that the results are indeed valid, Monte Carlo simulations were performed. In this context, the word "simulation" does not refer to a hypothetical situation (emulation of reality) but the fact that repeated sampling is performed. In this case, the classification vector is randomly generated, and then used in the classification procedure with either PLSDA or ANN.

Fig. 5 shows two examples of plots attained which affirm that the results are valid. Both plots (Fig. 5a and 5b) show a normal distribution and that the overall percent classified is beyond the 95% confidence limit, in other words the results are significant and not due to chance. The reason for the lack of results, e.g. ~42% and ~68% in Fig. 5a and ~53% in Fig. 5b, is due to the fact there is an odd number of samples in the control dataset in conjunction with the respective data sets not balanced.



(a) PCA and ANN (96.67%) (b) PARAFAC and ANN (96.67%)

Fig. 5. Monte Carlo simulations applied to the urine samples in which very good overall classifications were attained for PCA (a) and PARAFAC (b).

IV. Discussion

These promising results indicate that it is possible to use the fluorescence signals of a dye assay combined with an artificial neural network (ANN) model in the determination of bacterial presence and their growth phases. This would be helpful for analysis of the quality of clinical, pharmaceutical or biomedical samples. Further analysis can be improved by investigation of specific factors which affect bacteria growth, such as pH, oxygen, salt, sugars or nutrients concentration. Usually, these parameters are optimal for one strain but they tend to vary for others. They also can change the natural environment and affect the growth of particular strain of microorganisms. Therefore it is important to know the actual effects of single factors on the growth of microbial. The current results indicate that it is possible to control the growth rate of microorganisms implying that in the future it will be comprehensively possible to inhibit unwanted strains from growing such as human pathogenic bacteria. Conversely, it can lead to the improvement of the development of the beneficial strains once the impact of certain factors on their growth is better understood.

The work here has also demonstrated that ANNs can be employed as a classification tool to distinguish three gastrointestinal diseases (irritable bowel syndrome, Crohn's disease, and ulcerative colitis) from healthy controls with extremely good overall accuracies of >90%. ANNs also performed better than partial least squares discriminant analysis (PLSDA). This can be partly attributed to the fact that PLSDA can only model linear relationships where as ANNs are able to model both linear and non-linear relationships [26].

Furthermore, classification via PLSDA has produced very good sensitivities but poor specificities which imply that many false positives were predicted (Fig. 4c). In contrast, the classification via ANN has resulted in high sensitivities and specificities, especially for Crohn's disease (CD) at 92% and 100% respectively (Fig. 4d).

However the success in distinguishing the diseases from the controls is also attributable to the data pre-treatment, that is in the employment of parallel factor (PARAFAC) analysis to extract the pure emission profiles from the EEM spectra – a major advantage of PARAFAC provided that the correct number of components are selected [29]. This was shown to be better than using principal components analysis (PCA). This is because PCA only focused on the EEM of one sample therefore a PCA loading (PC1) was generated for each sample. However, PARAFAC was able to act on all of the EEMs simultaneously, i.e. across all the samples, therefore revealing other trends and characteristics within the data that cannot be detected by PCA alone. It must be stated that PCA is not a classification technique; it is exploratory. In this capacity it acts as a data reduction technique with the aim of removing noise. PC1 is thus used because it contains the emission profile that will have captured the most variance in the data and have zero contributions from noise.

The success of the classification of the three diseases against the control is fortified by the application of the Monte Carlo simulation [30]. This has statistically demonstrated that the respective overall accuracies attained are not due to chance (Fig. 5). This also implies that the respective models generated have good accuracy. It is therefore feasible to infer that there are significant and distinctive characteristics contained within the excitation-emission matrix (EEM) spectra (e.g. Fig. 2) to permit the classification of the diseased samples.

Leave-one-out cross validation (LOO-CV) is a thorough yet time-consuming procedure, particularly if there are a large number of samples (e.g. in excess of 100). Other forms of cross-validation exist such as venetian blinds cross validation (VB-CV) and block cross validation (B-CV) [30]. The former involves omitting, for example the odd-numbered samples, building a model with the even-numbered samples then classifying the odd-numbered samples; the next iteration would involve omitting the even-numbered samples, etc. Other permutations could also be implemented. B-CV is more similar to LOO-CV but instead of omitting one sample, omits for example 10 consecutive samples and builds a model with the remaining samples. These work faster than LOO-CV yet are not as accurate.

In recent years the technique of bootstrapping has become commonly employed [30]. A specified number of samples are selected randomly from the dataset to form a validation set. The remaining samples thus make up the bootstrap set. The latter is randomly split into a training and test set. A model is created from the training set then the test set is classified. This is repeated *N* times, e.g. where *N* could be 250. The model that resulted in the best classification is used to classify the validation set from which the accuracy and performance are assessed. Given that accuracy tends to be better, bootstrapping can however be computationally intensive. For this work, the low number of samples present in each subset (as shown in Table II) did not permit its use therefore LOO-CV was employed.

Although the total number of samples used in this study are low (32), the study has illustrated the potential of this proposed method. The next phase of the work would involve substantially increasing the number of samples, for example in excess of 100. This would lead to improved clinical relevance since every patient or healthy volunteer will have different characteristic responses as indicated by the metabolites contained in their urine. Furthermore, the ability to be able to distinguish the diseased samples from each other, for example, a "one-against-all" approach such as classifying irritable bowel syndrome in the presence of healthy controls, Crohn's disease and ulcerative colitis, should also be investigated. Finally, making use of the metadata collected via the questionnaire (diet, lifestyle, medication, etc) could also be incorporated into the classification model.

V. Conclusions

In this study, we demonstrated an optical form of e-nose and e-tongue, which offers a promising alternative to electrochemical systems providing highly sensitive, easy and inexpensive measurements where no reference signal is needed. The system also gives a possibility of remote sensing and further assay miniaturization. Potential of the proposed analytical tool is wide and diverse and can have great impact on a variety of applications including non-invasive diagnostics of tissues, cosmetics testing, early warning of ultraviolet radiation abuse, general health monitoring, therapeutic management, fundamental physiological investigations and disease diagnosis.

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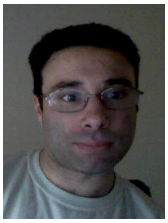
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Optical assay for biotechnology and clinical diagnosis

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