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VOC profiling of *H. influenza Type B* in human serum using electronic nose for application in the diagnosis of early sepsis

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Abstract

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in response to infection and is associated with high rates of morbidity and mortality if not treated quicker. New methods are needed that reliably speed up the diagnostic process and limit costs. Odour-based assays could potentially fill this diagnostic niche. An E-Nose is an instrument that consists of a sensor and pattern recognition software that quantifies and classifies the gas. The sample peaks are produced mostly in the first ten minutes and thus is rapid compared to the gas chromatographic (GC) techniques. In this study, an E- Nose GC-sensor system (OdoReaders) was used as diagnostic tool *for Haemophilus influenza Serotype B* (Hib) sepsis. E-Nose sampled the headspace VOCs originating from Hib cultures grown on human serum. The first test showed strong baseline drift and no baseline recovery and thus a process of method development with different E-Nose setting was carried out and the sensor responses were analyzed using the automated retention time function in the software. The results mostly showed differences between control yet there was no Hib dose dependent increase or decrease in peak area. In addition, there were no viable cell counts and thus it could not be demonstrated whether VOCs detected were from live Hib. The project apart from detecting VOCs in sample provided information about the two crucial components of E-Nose. One the growth condition (less volatile) of Hib and the other is the detection software in E-Nose.

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VOC profiling of *H. influenza Type B* in human serum using electronic nose for application in the diagnosis of early sepsis

1.0 Introduction

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in response to infection and is associated with high rates of morbidity and mortality. For example, it is estimated that 4 million out of 13 million septic patients die worldwide each year (Ebrahim, 2011). When microbes begin to reproduce in the circulatory system, and if the immune system is unable to remove them at an adequate rate, septicemia develops. The risk for death in septic patients increased 7.6% per hour after the first six hours of documented hypotension (Kumar *et al*, 2006). Thus, an early identification of the causative agent is crucial for directing treatment decisions towards an evidence-based antimicrobial therapy (Won *et al*, 2010). Diagnosis relies upon blood culture followed by morphologic and biochemical species identification, with further culture to determine antibiotic sensitivity, thus taking 24-72 hours, depending on the microbe.

Culture has the major advantage that living organisms can be characterized for antimicrobial susceptibility testing (Bruins *et al.,* 2009). But it is essential to obtain rapid results for prevention and early treatment of sepsis. Current molecular techniques have the advantage of a reduced time to identify the microorganism but still require blood culture incubation. In addition, the molecular tests are expensive and the data generated by these tests need to be interpreted with caution (Nowakowska *et al.,* 2006). A summary of the principal properties and limitations of the available assays for the identification of pathogens is given in Table 1

Table 1: Properties and Limitations of the available techniques used in detection of pathogens

	ASSAY	PRINCIPLE	TURN AROUND TIME after culture (h)	LIMITATIONS	REFERENCES
Conventional Technique	Bacterial Culture	Culture of a sample in enriched broth, isolation of the pathogen after incubation and identification through its metabolic properties and susceptibility to antibiotic.	16	Time-consuming. Inhibition by antibiotics. Low sensitivity for fastidious organisms.	Peters *et al*, 2004 Paolucci *et al*, 2010
Amplification Techniques for whole blood samples	VYOO/ LOOXSTER	Multiplex PCR with gel electrophoresis	8	Only small quantity of DNA available. Labour-intensive.	Leehmann *et al*, 2009.
	SeptiFast	Broad-range PCR followed by sequencing	8-12		Mancini *et al*, 2010.
	SepsiTest	Multiplex real-time PCR assay using dual-fluorescence energy transfer (FRET) probes to detect and identify bacterial and fungal pathogens through ITS sequencing	6		Wallet *et al*, 2010
Hybridisation Techniques for positive blood culture	PNA-FISH	Fluorescence-based hybridization with peptide nucleic acid probes	1.5–3	Pathogenic specific identification, in some cases only at the genus level. Only after Gram stain information (2.5 h). No susceptibility data (yet) for antibiotics. Difficult technique.	Pelag *et al*, 2004
	AccuProbe	Chemiluminescent cDNA probes that detect rRNA	2.5		Lindholm and Sarkkinen, 2004

Amplification Techniques for positive blood cultures	Prove-it Sepsis	Multiplex PCR combined with Microarray	2.5	Requires technical expertise. One or few pathogens detected. Costly. Risk of laboratory contamination. Background bacterial DNA in blood. Detection of DNA instead of living organisms	Tissari *et al*, 2010
	Hyplex BloodScreen	Multiplex PCR with subsequent hybridisation on an ELISA plate	3		Wellinghausen *et al*, 2004
	PLEX-ID BAC Spectrum	Broad-range PCR combined with electrospray ionisation mass spectrometry	8		Ecker *et al*, 2010
	StaphPlex	Multiplex PCR and characterization of the amplicons by a Luminex suspension array	5		Tang *et al*, 2007
	Staph SR	Multiplex real-time PCR assay that amplifies a specific target sequence of *S. aureus* and a specific target to detect meticillin resistance	2.5 - 3		Grobner *et al*, 2009
	Xpert MRSA/SA	Real-time PCR assay that detects a sequence in the staphylococcal protein A gene and determines meticillin resistance	1		Wolk *et al*, 2009
Mass spectrometry for pathogen-specific peptide profile	MALDI-TOF MS	Mass spectral signals obtained from post-culture specimens compared with organism's proteomic profile from a database of standard reference spectra.	NA	Difficult for mixed populations of bacteria	Seng *et al*, 2009

CoNS, coagulase-negative staphylococci; ELISA, enzyme-linked immunosorbent assay; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; PCR, polymerase chain reaction; ITS, internal transcribed spacer; MALDI, matrix assisted laser desorption/ionisation; TOF-MS, time of flight mass spectroscopy; NA, not available.

An ideal diagnostic technology would identify the infecting organism(s), and ideally any antibiotic resistance, in a timely manner so that appropriate pathogen-driven therapy could begin promptly. None of the currently available conventional and molecular methods are sufficiently rapid, accurate or informative to achieve this (Ecker *et al,* 2010). There is an increasing world-wide awareness that bionics and artificial intelligence could play an important role in many aspects of human activity (Adam *et al*., 2012). Given the clinical impact of bacterial infections in general, new methods that could reliably speed up the diagnostic process and limit costs are essential. Odor-based assays could potentially fill this diagnostic niche.

Since ancient times, it has been documented that some infections liberate specific characteristic odors. Gases and liquids in their vapor phase are called volatiles and these can be odorous or odorless (Knobloch *et al.,* 2010). Volatile organic compounds (VOCs) are produced by bacteria as primary metabolites (e.g. acetone, ethanol, or acetic acid), or as secondary metabolites (e.g., signaling molecules) in different quantities (Zhu *et al,* 2010). VOCs could be used as fingerprints of each bacterium. For example, *P. aeruginosa* synthesizes 2-aminoacetophenone that has a grapelike odor (Cox *et al,* 1987) and indole is responsible for the characteristic odor of *E. coli* (Wang *et al,* 2001).

Numerous technological advancements have been utilized to accurately characterize microbial VOCs having not only the potential as markers for presence/absence of microbial growth, but also as a diagnostic tool (Thorn *et al.,* 2011). Bacteria need to be actively metabolizing to produce volatiles, if produced in sufficient quantities these could potentially be

used to accurately identify bacterial species within a timeframe far shorter than the existing traditional culture methods.

In past few decades, gas chromatographic (GC) techniques have been used for the rapid detection of volatile biomarkers. Studies of GC derived microbial VOCs found they were dependent on the growth substrate present (Zechman *et al.*, 1986 and Mitruka., 1975). It was quickly realized that 'head-space analyses of bacterial cultures using gas chromatography mass spectrometry (GC–MS) provided more information. For example, head-space GC–MS has been shown to yield quantitative data enabling species level identification of anaerobes (Julak *et al.*, 2000), and facultative bacteria, (Zechman *et al.*, 1986). Although GC-MS enabled the comprehensive study of VOC markers, it never evolved to a fully operational and accredited diagnostic tool. Some of the limitations of GC-MS includes that it is expensive, requires highly skilled operators, complexity of human volatile samples used and reliance on the use of internal standards to identify and quantify each VOC component (Phillips., 1997).

The intelligent gas sensor arrays and the subsequent development of artificial nose-like machines, able to mimic the mammalian olfactory system, gave hope to the attempts of rediscovering the diagnostic power of odors and volatiles in clinical practice (Pavlou and Turner., 2000). Electronic nose (E-Nose) using biosensor technology could at least determine the morphological groups of the microorganism. In near future, advanced intelligent bio-electronic noses could be configured as bedside instruments and might monitor a person's health.

An E-Nose is an instrument that consists of an array of gas sensors, typically 4-32 sensors with pattern recognition software that quantifies and classifies the gas. The inclusion of multiple sensors with different properties increases gas selectivity (Wulsin *et al.*, 2011). The principle of a

chemical gas sensor is that the analyte molecules come into contact with a chemically sensitive material that causes a property change in the material, resulting in an electrical signal change. Typically the sensors are contained in an instrument that regulates air-flow. Sampling is done in three phases: baseline phase, sampling phase, and recovery phase. The gas to be analyzed is exposed to the sensor array in the sampling phase while a reference gas is used during baseline and recovery phase in order to return the sensor values to the initial state. Valuable information is not only obtained in the in the sampling phase but also in the dynamic appearance of the recovery phase (Dutta *et al*., 2002).

Mostly the operation of E-Nose could be resolved (Harun *et al.,* 2012) into four simple steps. Firstly, the gas flows to the sensors in the manifold. Secondly, an electronic circuit takes the voltage signals from the electrochemical sensors and amplifies or conditions the signals as necessary. Thirdly, the output from the circuits is taken through a data acquisition board that has been added to a personal computer. Finally, pattern recognition program then analyzes the data thus collected. Data analysis can be done with tables and graphs.

E-Nose even detect difficult, odor-less, dangerous and/or unpleasant gases. Concerning the technical detection of odors; trace components and concentrated gases need to be distinguished. In the ideal case, the high-concentration substances are responsible for the odor impression and the odorless components which are also present are negligible regarding the measurement results (Rock *et al.,* 2008). Otherwise, the odorless background interferes with the measurement. Evidence so far indicates that E-Nose systems could detect and discriminate volatile fingerprints of microbes. TABLE 2 summarizes the *in vitro* and *in situ* results obtained using E-Nose.

Table 2: Potential E-Nose application for human disease diagnosis via detection of VOCs produced by bacterial pathogens.

Pathogen	Disease	Sample source	Potential VOCs	References
C. jejuni	Gastroenteritis	Faeces	Hexanal, (E)-2-octenal, pyrrole, ethyl ethanoate, methyl alcohol, 2-heptanone	(Garner *et al*., 2007)
M. tuberculosis	Tuberculosis	Human sputum cultures	Specific complex mixture of VOCs	(Fend *et al*., 2006)
P. aeuroginosa	Localized infections	Skin, lung	Butanol, methyl ketones (2-2-nonanone, 2-undecanone), heptanone, 2-aminoaceto-phenone	(Qureshi *et al*., 2009)
P. peroiens, P. taetrolens	Food poisoning	Stomach	2-methoxy-3-isopropylpyrazine (MIPP)	(Cheng *et al*., 1991)
S. aureus	Localized & systemic infections	All parts of the body	Isobutanol, isopentyl acetate ketones, I-undecene	(Kodogiannis., 2005)

The usefulness of E-Nose devices depends on two crucial components. First, the sample must be given in the correct format to optimize the interaction of volatiles in the headspace with the sensor. The sample should be presented to the E-Nose in the same way for each sample which is difficult to achieve during manual injection of samples. The humidity, temperature and sample size must be standardized to ensure that the data sets can be analyzed by comparison (Turner and Magan., 2004). Usage of appropriate standards for calibration would minimize drift problems over time.

Second, pattern recognition must enable large data sets to be analyzed rapidly. Further, E-Nose data must be interpreted in a similar manner and therefore requires a generalized method. It is not suitable for each application of E-Nose to have its own features for classification (Dutta *et*

al., 2005). Effective data management is necessary using techniques such as discriminant function analysis (DFA).

The main challenge of the practical application of E-Noses is that the gases of interest are part of a complex background, which might include water vapor. Humans have no receptors for water vapor whereas technical sensors might be sensitive to these background gases. The two relationships, one between detectable and not detectable substances and other between relevant and not relevant ones is also a crucial point for every E-Nose (Rock *et al.*, 2008). Independent of the detection methodology used, sampling is also another important issue. The blood, sputum, urine or the breath are used as samples in relation to the disease. This diversity of detection sites makes a universal sampling system impossible.

Instead of direct measurements requiring complex sample processes, classical microorganism cultivation methods can be used with further analysis by an E-Nose (Lykos *et al.*, 2001). In spite of diminishing the analysis speed advantage for the whole procedure, the resulting bacterium cultures could decrease the interferences problem. Sometimes in E-Nose, even if it produces encouraging results, there is still a lack of knowledge about the source of VOCs detected and specific VOCs are not identified. Also it provides no absolute quantification of VOCs by relying on comparative detection patterns.

With fewer limitations, E-Nose is found to be a desired system that can be developed for a specific disease with the required level of sensitivity. By collecting enough background (control) data and by considering sensor drift and nonlinear data sets, the group to which the sample belongs can be accurately predicted (Fend *et al.*, 2005). E-Nose devices are more flexible

and can be trained rapidly with large amounts of sensor data from controls to provide useful background volatile fingerprints. That could eventually make differentiation of infected samples easier and more rapid. Although for different microbial diseases, specific analysis systems might need to be developed. In future (Adam *et al.,* 2012), the development of robust instrumentation coupled with remote data acquisition and central processing powered by hybrid intelligence systems, could see E-Nose technology in common use.

This report summarizes the project work wherein an E-Nose GC-sensor system called OdoReaders was used as a diagnostic tool for *Haemophilus influenza Serotype B* (Hib) sepsis. The Hib sample used in this project is one among the most common bacterial pathogens responsible for sepsis. The GC sensor system comprised SRI instruments (SRI8610C) gas chromatograph fitted with a 30 m SPB1 sulphur (Supelco, Sigma Aldrich) GC column. OdoReaders was used to sample the headspace VOCs originating from Hib cultures grown on human serum. The sampling method was standardized to obtain reproducible headspace samples to allow direct correlation of the results obtained. A process of method development was carried out to profile VOCs for serial dilutions of bacteria with different E-Nose set and the sensor responses were analyzed using the automated retention time function in the software. The software generated a series of retention times versus peak areas from the chromatogram of resistance versus time.

1.1 Aim

To determine the threshold doses for detection of Hib in human serum by VOC analysis using E-Nose.

2.0 Materials and Methods

2.1 Bacterial cell culture

Haemophilus influenza Serotype B Strain Eagan (Cruickshank *et al*., 1975) was stored frozen at -80°C. When required, bacteria were streaked onto chocolate agar from frozen stocks and cultured overnight (14-16 hours) at 37°C in 5% CO_2 to reduce phenotypic changes in bacteria over time. Single colonies were picked and suspended in 5ml calcium and magnesium free PBS-B (Tezera *et al*., 2010). Optical densities of bacterial suspensions at 600nm were used to determine CFU/ml, using a previously defined conversion scale. Suspensions were used to inoculate human serum at specific multiplicities of infection (MOI) ranging from 1 CFU/mL, 10^2 CFU/mL, 10^4 CFU/mL, 10^6 CFU/mL and 10^8 CFU/mL. The non-inoculated universal (reference human serum) and the inoculated universals (bacterial solutions) were incubated at 37°C in 5% CO_2 for 24 hours to allow the bacteria to grow and to produce volatiles potentially.

2.2 Bacterial viability

After overnight incubation of inoculated human serum, the numbers of viable bacteria present in the human serum were confirmed by plating out 10µl of serial dilutions of bacteria (Hib10e0, Hib10e02, Hibe04, Hib10e06 and Hib10e08) and colony counting after overnight incubation on chocolate agar at 37°C and 5% CO_2.

2.3 Head space sampling technique

After 24 hours of incubation, the samples were transferred into specialized glass vials (McEntegart *et al*., 2000) to enable headspace analysis. Samples were placed in a water bath at 50°C - 55°C for 30min in order to derive off the volatiles and to kill the bacteria prior to sampling.

2.4 Electronic Nose (E-Nose) Sensory Unit

The E-Nose (OdoReaders – GC sensor system) used for the sepsis work were as follows. OdoReaders comprises a SRI instrument SRI8610C gas chromatograph fitted with a 30m SPB1 (Supelco, Sigma Aldrich) sulphur GC column with internal diameter - 0.32mm and film thickness - 4 microns. GC column was used or otherwise all the volatiles from the sample would hit the sensor instantaneously which would usually result in a large change in the sensor and would not give sufficient information about the sample (Rivai *et al*., 2010). Synthetic air was used as the carrier gas (BOC) and a flow rate of 12ml/min was maintained through the column and a temperature ramp was also used.

The temperature was held at 40^{o}C for 3 minutes and then ramped to 100^{o}C at 5^{o}C/min and was held again for 30 minutes and then ramped back to 40^{o}C at 10^{o}C per minute. The column is heated to drive off all the sample volatiles that have entered the column and to avoid longer elution time for these compounds (Michishita *et al*., 2010). Therefore, heating increases the information about the sample within a specific time window. The column has to be cooled again in order to inject the next sample. If it was maintained at 100°C for the whole run then the peaks would show no separation between the compounds and the sensor would not recover

sufficiently. Thus VOCs were let to elute and then the column was heated to encourage less volatile compounds to elute.

The end of the column exited the GC oven and was interfaced to an in-house produced gas sensor housed in an aluminium chamber (heated metal oxide 50:50 mass% tin oxide:zinc oxide, maintained at a temperature of 450^{o}C) and the sensor chamber had a purge of clean air maintained at 100ml/min to push the eluting gases (after reacting with sensor) out of the chamber and thereby inhibits the built-up of compounds within the chamber. The sensor resistance versus time was monitored and displayed using an analogue to digital converter interfaced to in-house written software. The initial GC was fitted with a split/splitless heated injection port (temperature - 110^{o}C), and was run either in splitless mode assuming that there would be small concentrations of volatiles from the samples (Haugen *et al*., 2006) or with a split of 100ml/min.

2.5 Test procedures:

Data were gathered as follows

For the sepsis test, the OdoReaders with a single purge was introduced to a sterile glass vial (McEntegart *et al*., 2000) containing 4.5ml of pre-treated (incubated in a 50^{o}C water bath for 30min) samples. Firstly using the manual injection system, 2cm^{3} of the headspace from the vial containing human serum was injected via the splitless heated injection port. The same operation was repeated four times with the dilutions (Hib 10e02, Hib 10e04, Hib 10e06, Hib 10e08) of Hib. The second test was done with a minor change in the gas column (GC) that included a minimal split heated injection port to try and avoid overloading the system and to increase peak resolution.

The latter tests were run on the same GC chassis (minimal split) fitted with an autosampler to avoid manual errors. For the third test, the samples were incubated at 55^{o}C for 30min to drive off the volatiles prior sampling. Each sample was put in the sample loading system and 1ml of the sample was injected onto the GC via a series of internal valve operations. The injected samples were subjected to 45min of heat up, 6min to cool down and 10min to recover. The fourth test was performed as above but in increased injected time ie. slower injection from the minimum allowed (0.1 seconds) to 1.2 seconds by the valve operation (which opens and shuts the valve within this time) and was retained for 1hr and 15min as opposed to 50min for previous experiments. The actual run time was not changed but allowed the baseline to recover for longer. The fifth test was performed by including an extra purge in the machine to push the entire VOC sample through the column and then the samples were retained for 1hr and 30min for baseline recovery. In the process of method development that setting was considered as the most appropriate. The final test was performed as above for reproducibility.

2.6 Data Analysis

The results were analyzed using the automated retention time function in the GC Detector ANNGenPL1216W2V3 (Version 3.0.0) software. The software generated a series of retention times versus peak areas from the chromatogram of resistance versus time. The data tables were interpreted using graph charts to show the differences between the peaks of the control and the peaks of the samples. In the process of method development, each test was done with a different E-Nose set up and thus a statistical analysis was impossible.

3.0 Results

3.1 VOC profile of Hib in human serum using E-Nose

3.1.1 One example E-Nose (Odoreaders) spectra of all six tests to show baseline drifts

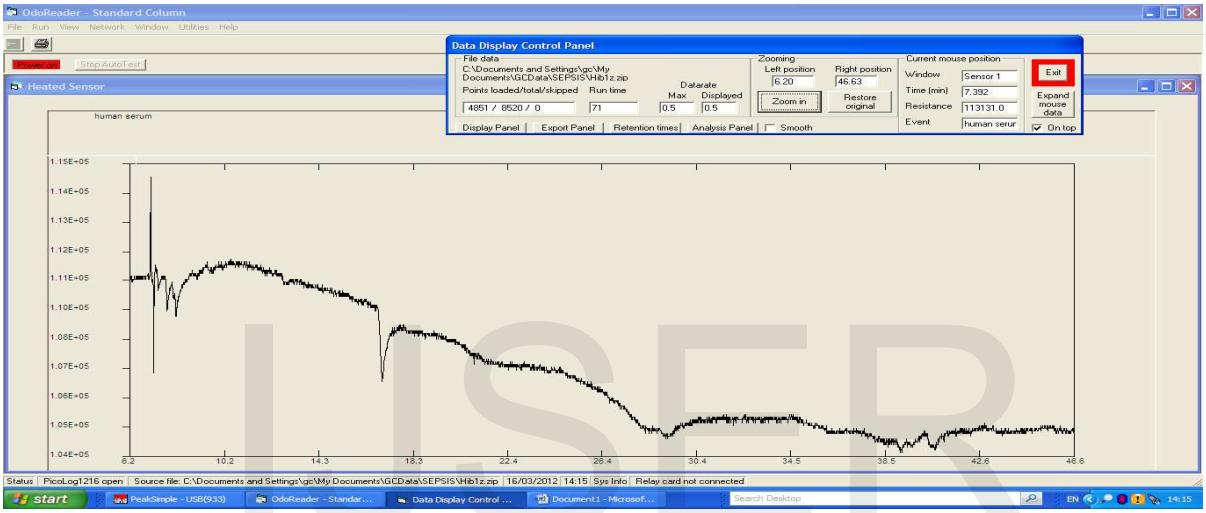

Figure 1: E-Nose (Odoreaders) spectra of human serum in Test 1.
Spectra of other samples are in appendix 7.3.1

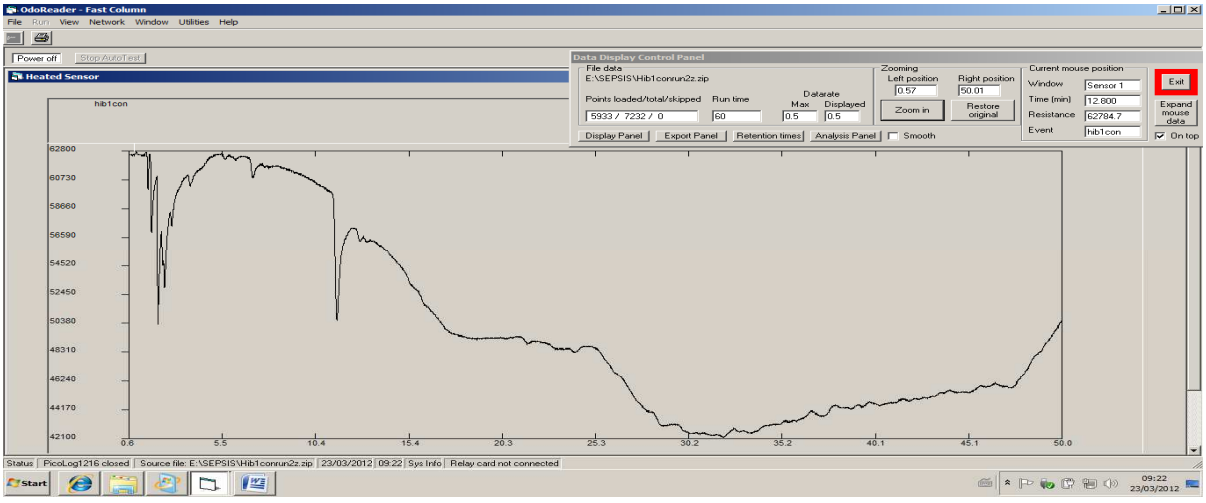

Figure 2: E-Nose (Odoreaders) spectra of human serum in Test 2.
Spectra of other samples are in appendix 7.3.2

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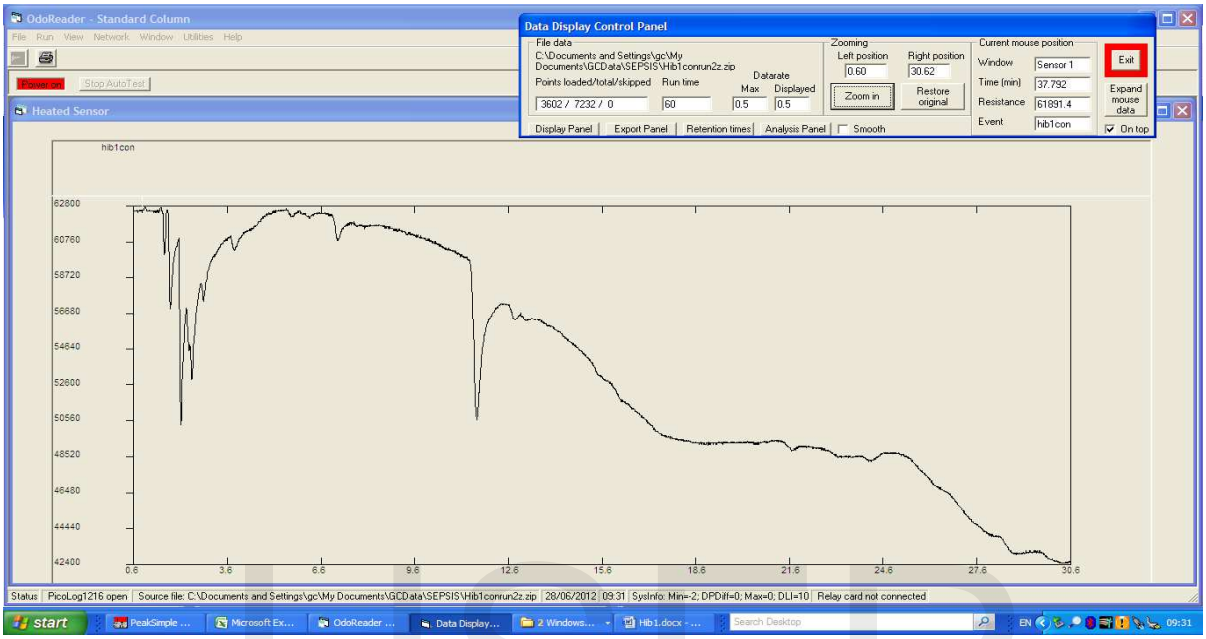

Figure 3: E-Nose (Odoreaders) spectra of human serum in Test 3.
Spectra of other samples are in appendix 7.3.3

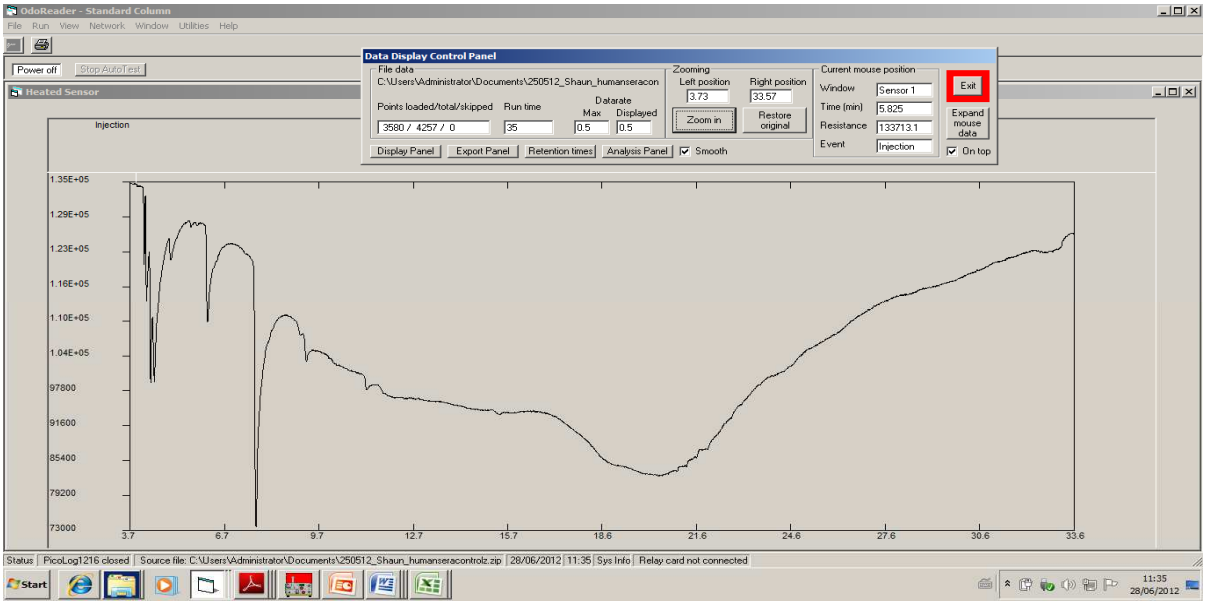

Figure 4: E-Nose (Odoreaders) spectra of human serum in Test 4.
Spectra of other samples are in appendix 7.3.4

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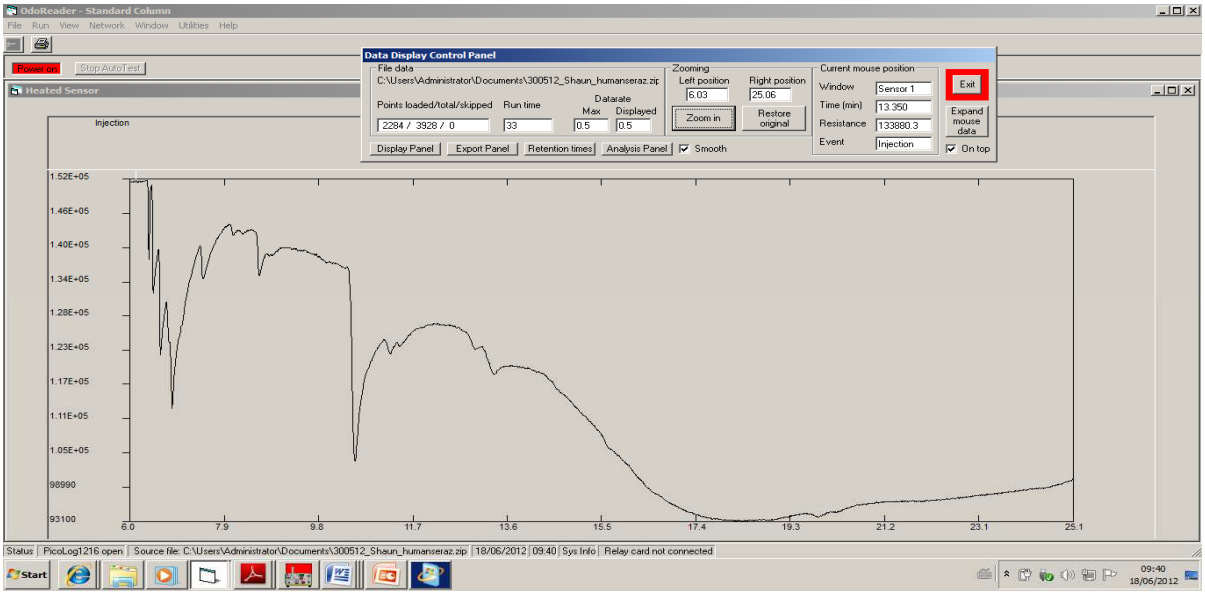

Figure 5: E-Nose (Odoreaders) spectra of human serum in Test 5.
Spectra of other samples are in appendix 7.3.5

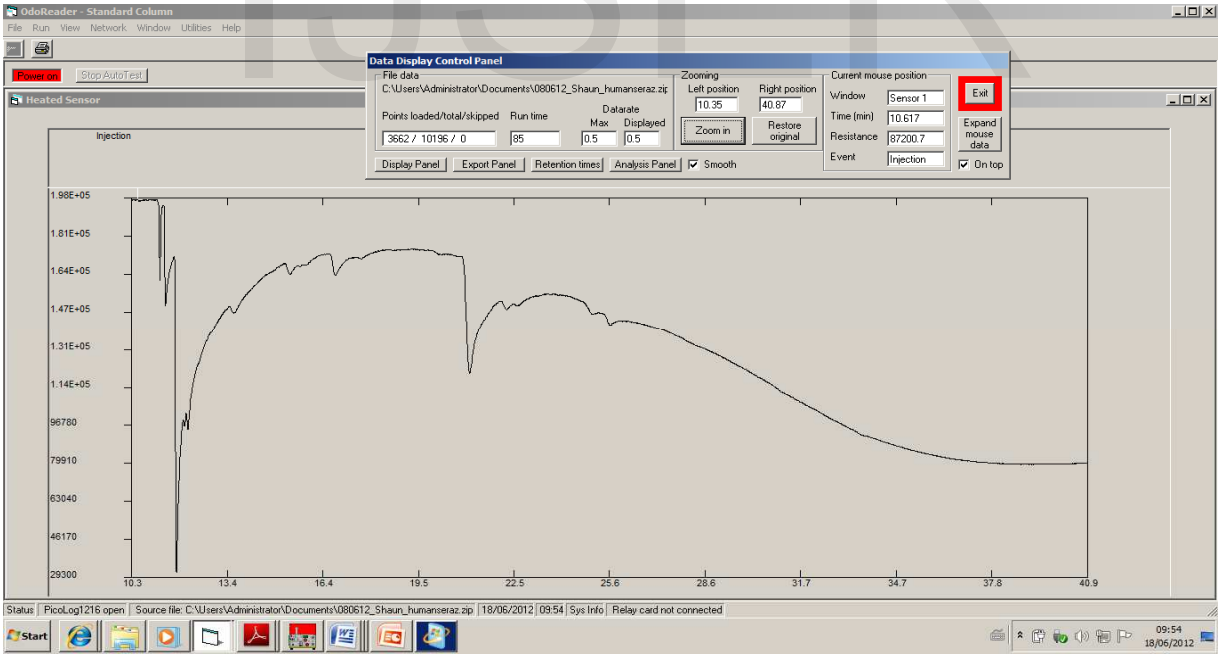

Figure 6: E-Nose (Odoreaders) spectra of human serum in Test 6.
Spectra of other samples are in appendix 7.3.6

In the first test, the samples were manually injected and the machine was set to a single purge with the split vent closed. These results had strong baseline drift in the peaks (Figure 1). Therefore, method development was carried out in order to find the optimal split to maximise both sensitivity and resolution of the E-Nose instrument so that volatiles released by the bacteria (peaks) could be observed above background noise.

The peak area vs retention time was recorded by the GC Detector software for different samples (Appendix 7.4.1). A graph chart was drawn to compare the dilutions of bacteria and to distinguish it from the human serum. Figure 7 represents that the peak VOC for each range of Hib infectious doses with background noise (serum control) subtracted. For example, the headspaces of Hib $1*10^6$ had relatively higher VOC concentration at 10.5min, compared with headspaces of Hib$1*10^8$. There was a key peak at 1.5min for all the samples (arrow) with slight difference in the peak areas.

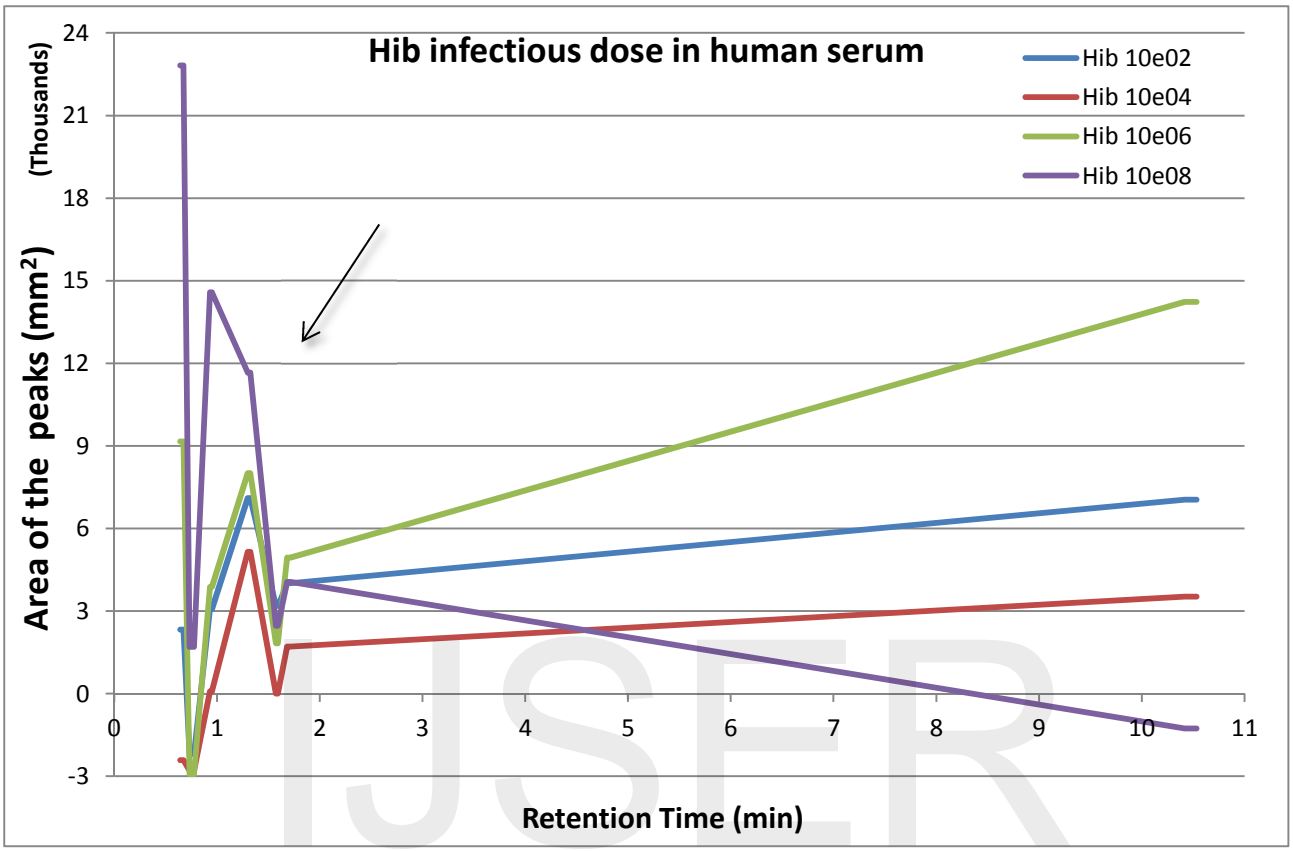

Figure 7: VOC profile of Hib in human serum using E-Nose in splitless mode and manual injection. Area values are blank (serum control) subtracted. Colours to identify Hib doses in lines.

The second test was done with a minimal split heated injection port as opposed to splitless injection port in the first test to avoid overloading the system and to increase peak resolution. The results from this E-Nose also had some baseline drift (Figure 2). The graph of peak area vs retention time (Appendix 7.4.2) shows interactive peaks among the samples. Figure 8 show that the except for one peak of Hib1*10^0 at 1.5min, other peaks of all samples till 3min and between 7min – 11min distinguishes itself from control yet the peaks are not consistent among samples.

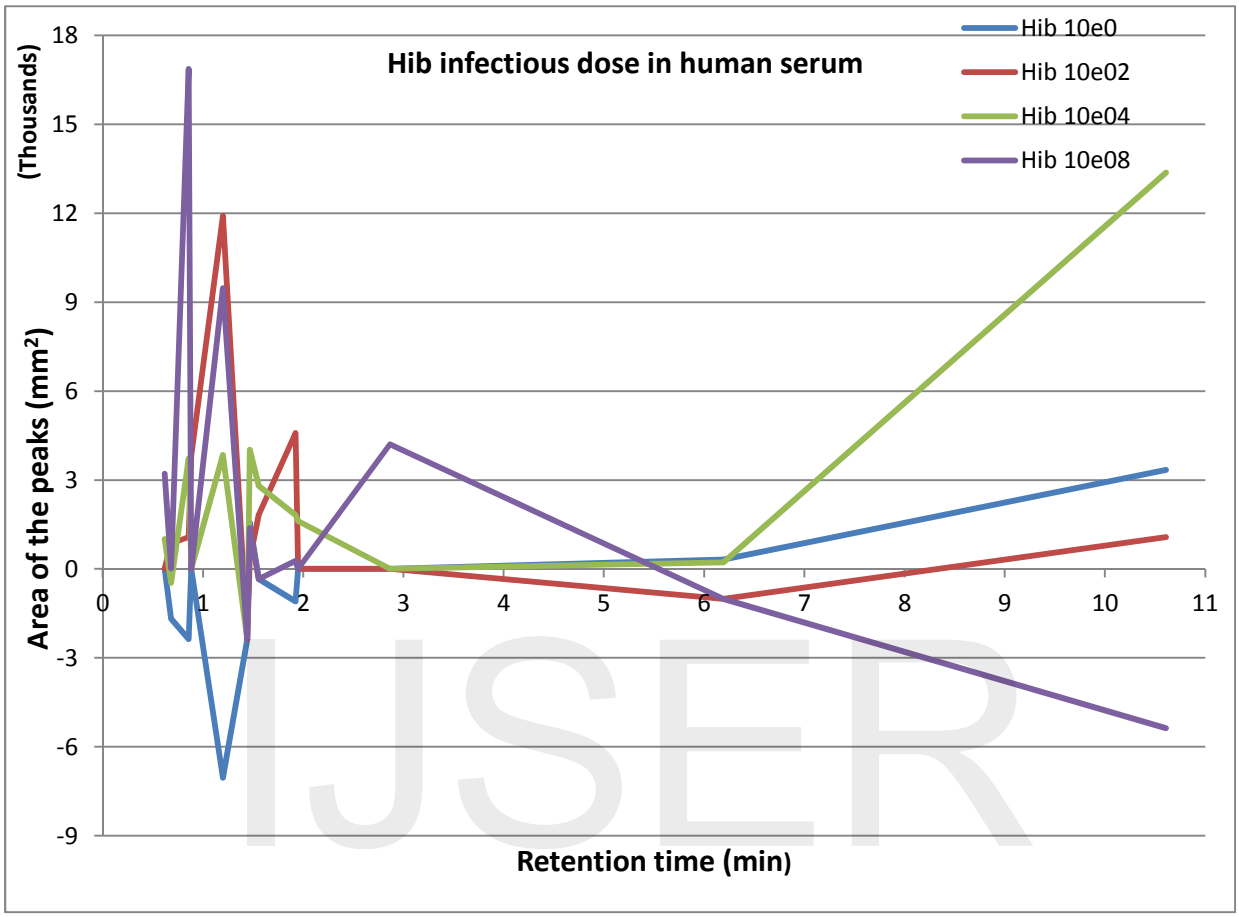

Figure 8: VOC profile of Hib in human serum using E-Nose with minimal split and manual injection. Area values are blank (serum control) subtracted. Colours to identify Hib doses in lines**.**

The third test added an autosampler to the above E-Nose setting in order to avoid errors in manual injection and that greatly reduced baseline drift (Figure 3). Figure 9 does not represent significant difference of the samples from the control yet show few interactive peaks in the first 2 min. The fourth test had an increased injection time into the E-Nose and minimised drifts totally (Figure 4). Figure 10 show few sim peaks (arrows) of Hib dilutions yet distinguishable from control at 0.5min, 2.5min and 3.5 min.

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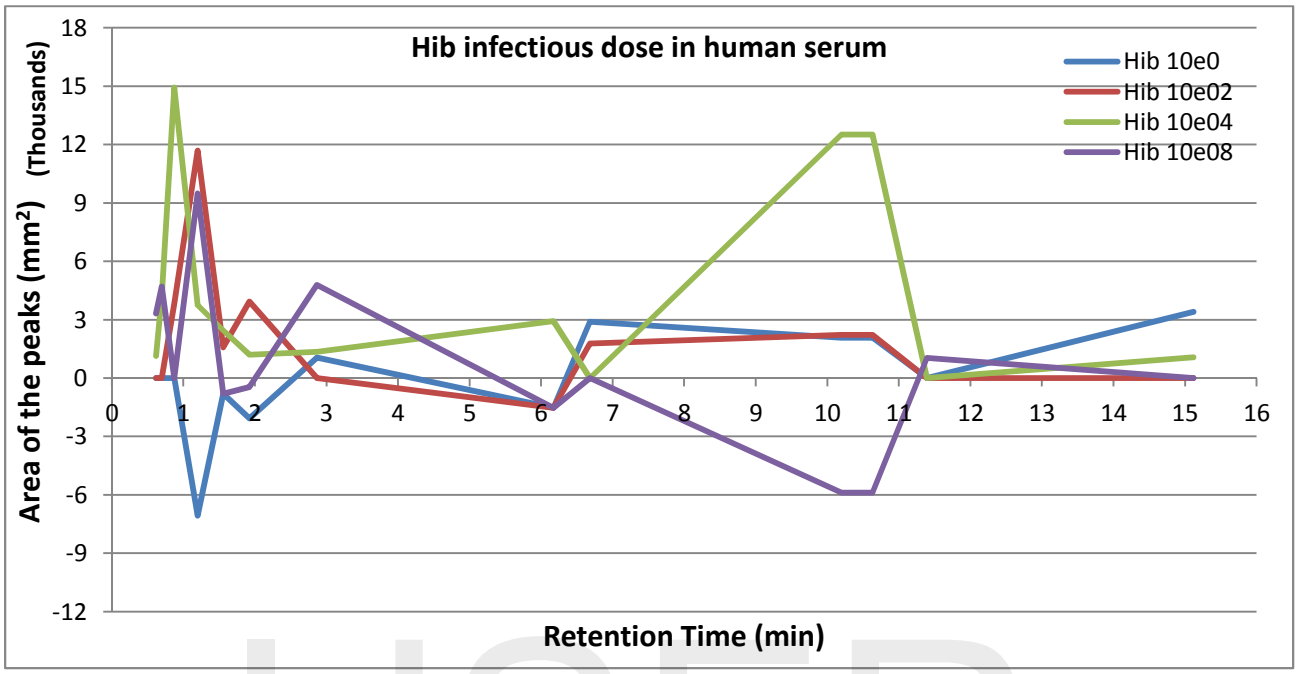

Figure 9: VOC profile of Hib in human serum using E-Nose with minimal split and autosampler. Area values are blank (serum control) subtracted. Colours to identify Hib doses in lines.

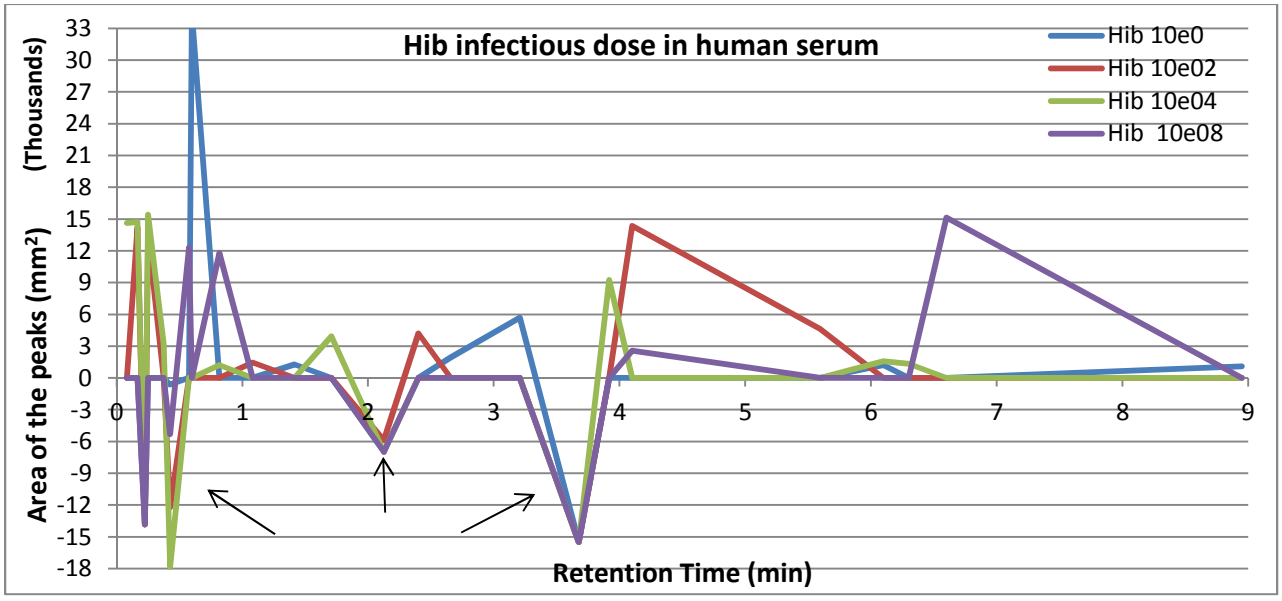

Figure 10: VOC profile of Hib in human serum using E-Nose with minimal split, autosampler and increased injection time. Area values are blank (serum: control) subtracted.

The fifth test used an E-Nose with an extra purge to push the entire VOC sample through the column and further increased the injection time (1hr and30min) which resulted in no drift in the peaks at all (Figure 6) which is optimal. Figure 11 show key peaks only in first 2 min (arrow) and with time Hib $1*10^6$ shows opposite trend to Hib $1*10^2$ and Hib $1*10^4$.

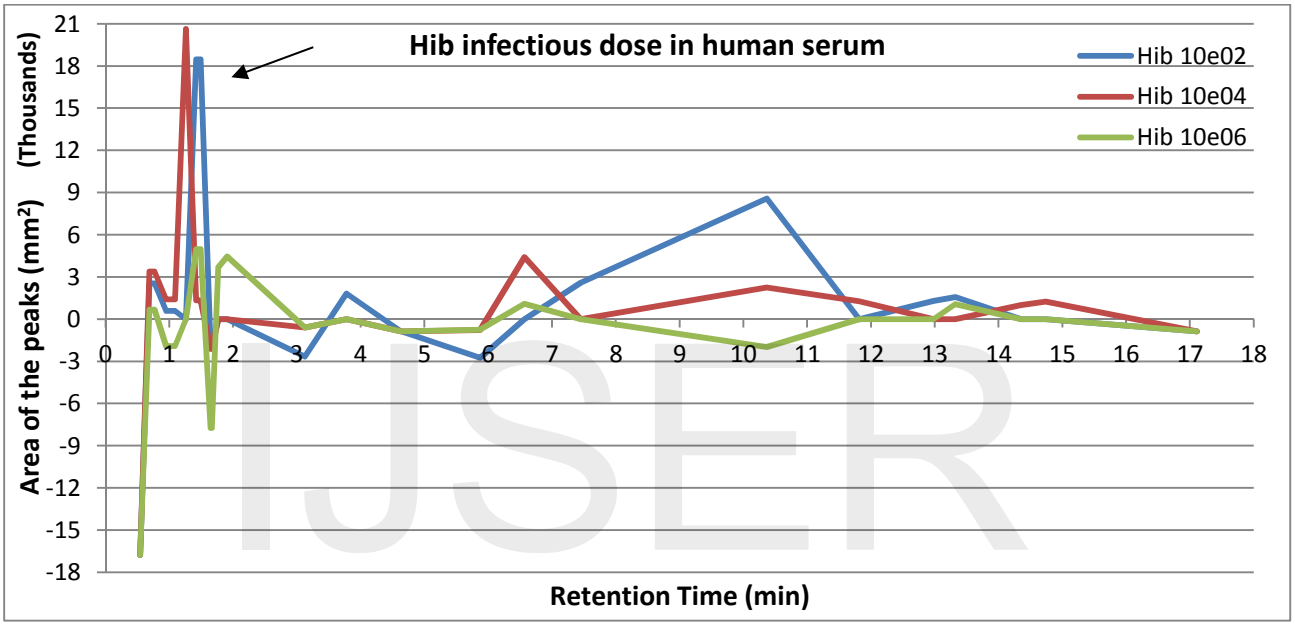

Figure 11: VOC profile of Hib in human serum using E-Nose with minimal split, autosampler, extra purge and increased injection time. Area values are blank (serum control) subtracted.

The final test included an E-Nose system with an autosampler, a minimal split, an extra purge and with increased injection time and that was considered as the best setting as the resulting spectra showed baseline recovery and no drifts. Figure 12 was drawn from final test data (Appendix 7.4.6) and it show clear peaks of all the samples mainly at 1.3 min greatly distinguishing it from control. Since all Hib dilutions show peaks (arrows) at 2min, 5.7min and 10.2min, it could be a VOC from Hib that might be useful for its detection. Similar peaks were

found for all Hib dilutions at these times however slightly differing in area which are dose-independent.

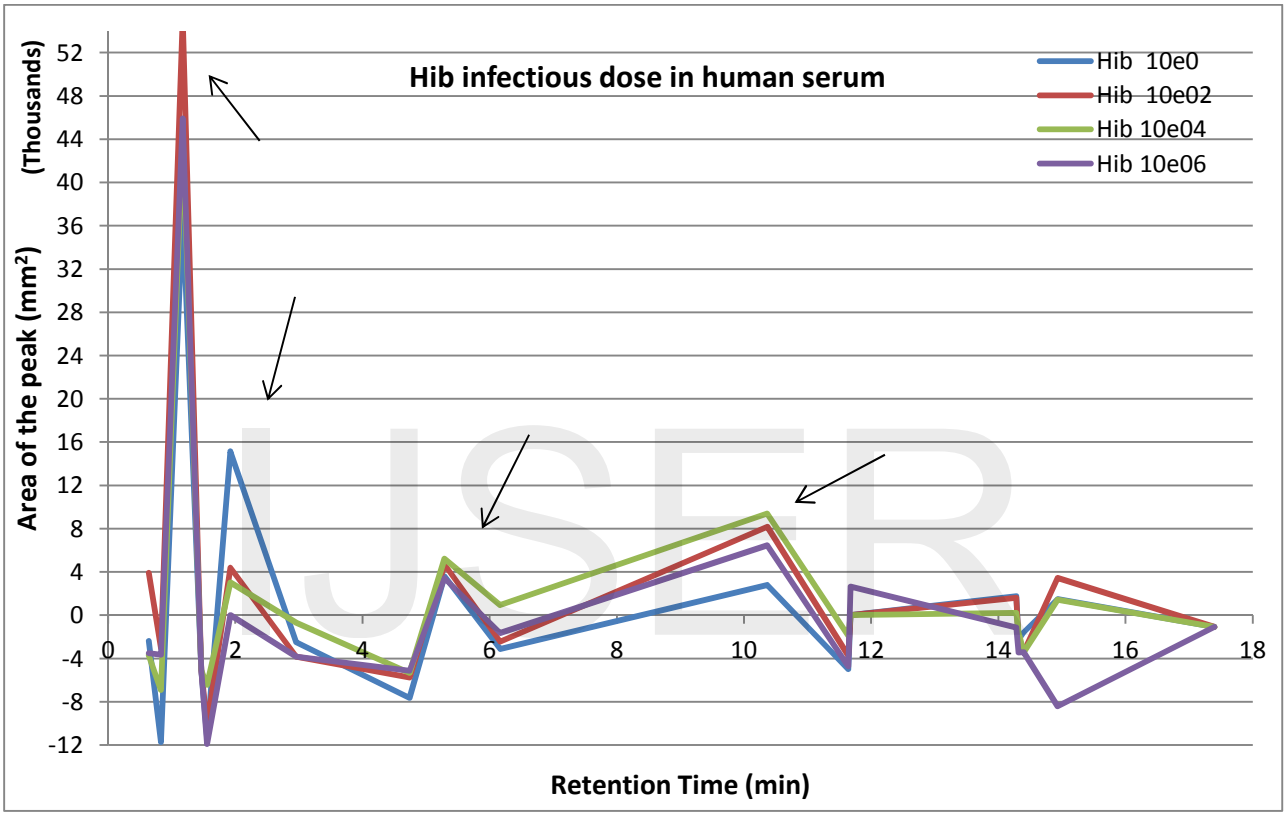

Figure 12: VOC profile of Hib in human serum using E-Nose with minimal split, autosampler, extra purge and increased injection time. Area values are blank (serum control) subtracted.

3.2 Key peaks from Hib dilutions in E-Nose spectra

The table below shows the level of VOC present in each dilution of Hib across all the six tests. Each result was graded arbitrarily for size of response (-, +, ++, +++) across each Hib dose and for different test protocols. Results for Hib dilutions were compared to serum control and * was used to represent samples that were not tested.

Table 3: Key peaks from dilutions of Hib E-Nose spectra

From the table above, it shows that there are few differences between the peaks of control and the peaks of samples. Test 1 show the Hib dependent increase in peak area. Test 2 and 5 show

Test	VOC identity through Retention time (min)	Human serum	Hib $1*10^0$	Hib $1*10^2$	Hib $1*10^4$	Hib$1*10^6$	Hib$1*10^8$
1	1.683-1.717	-	*	++	+	++	++
2	1.442	+	-	-	-	*	-
3	1.2	++	+	+++	+++	*	+++
4	0.167	-	-	++	++	*	-
5	0.546	++	-	-	-	-	*
6	1.925	-	++	+	+	-	*

loss of VOC. Test 3 show positive increase in VOC yet are Hib dose independent as similar increase is found in three doses of infection. Test 4 and 6 likely to show Hib dependent increase in peak area. The results are not consistent and the size of response could not be co-related exactly with the infectious doses of Hib.

3.3 Process of method development reduced baseline drifts with tests

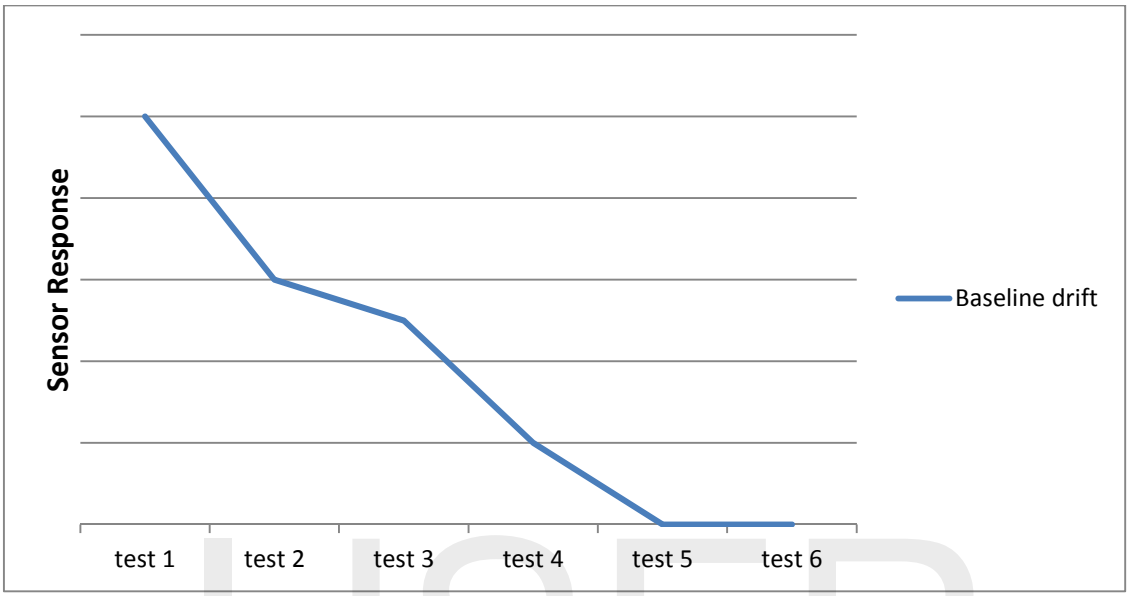

Figure 13: Process of method development reduced baseline drifts with tests.

Figure 13 shows the E-Nose sensor response of the tests considering the baseline drift (Figure 1-6). The process of method development mainly demanded to reduce or nullify baseline drift and the figure represents that the final test achieved it. The process of method development included changes in the machine setup like splitless to split mode or minimal split in the E-nose, from manual injection to auto sampler, normal to slower injection time and the samples was retained longer for baseline recovery.

4.0 Discussion

There are many papers (Gibson *et al*., 1997; Qureshi *et al*., 2009; Kodogiannis., 2005) that describes the identification and classification of bacteria with E-Nose devices. In most cases, good diagnostic results were achieved and there are bright prospects for commercial applications. The present study suggests that the E-Nose technology may have a small value as a screening test for Hib sepsis particularly in conditions where usage of expensive and labour-intensive machinery might be difficult. The separation step in E-Nose is faster than conventional analytical methods because most of the peaks occur within 10 minutes whereas conventional GC runs can take upto 1 hour (Ida *et al.,* 2006). Thus once the equipment is set up, the test is quick and simple yet the results are a bit difficult to understand.

The aim of the experiment was to investigate the possibility of developing a system for early detection and monitoring growth of Hib that causes sepsis. In order to develop such a system it was found necessary for the sake of simplicity, to work with VOC profiling of Hib dilutions in human serum. It would be necessary to use an array of 16 or more sensors to expose the sensor directly to the sample (Bruins *et al*., 2009). All sensors with different selectivity and sensitivity to VOCs could give more information about the volatiles in the sample due to the differential responses of the sensors. Even though sophisticated software is required for that approach yet might not produce enough information (Julian *et al*., 2000) to differentiate between infected and non-infected samples. Therefore E-Nose system considered in this study included only a single gas (tin oxide and zinc oxide) sensor and a GC column to help samples in reaching the sensor.

The first test was conducted with a manual injection syringe and the machine was set to a single purge and splitless injection port. The results had strong baseline drift in the peaks (Figure 1). In splitless mode, all the sample including non-volatile/semi volatile compounds are swept onto the column and therefore the column became flooded resulting in no sharp peaks (lesser resolution). The parameters were used from other work (McEntegart *et al*., 2000) assuming to be optimal since it would take many days from the test schedule to find out the optimal one. Even though each Hib sepsis test in E-Nose was done with changes in the machine setting like the split or splitless heated injection port, method of injection (manual injection and autosampler), addition of an extra purge and increased injection time (slower injection) to reduce drifts and to improve baseline recovery that were found in first test. Thus a process of method development was carried out throughout the project.

Therefore, in these tests, the desired results were to observe any new peaks and to see large differences between samples and whether E-Nose compromised to human serum sample. The second test replaced the splitless mode to minimal split to avoid overloading the system and to increase peak resolution since long retention time values in first test showed signs of improper recovery. Even though, the spectra had some baseline drift (Figure 2).

The third test included an autosampler to E-Nose thereby avoiding the errors that could happen in manual injection.

The fourth test allowed extra time for the system (1hr 15min as opposed to 40min in the previous tests) to recover since sensor baseline was suppressed compared to the initial value in the previous tests. The fifth test included all the above setting with an extra purge to push the entire VOC into the column else multiple samples might built-up within the chamber. In addition

the injection time was further increased to 1hr and30min which resulted in no drift in the peaks. The final test was the repeat of the above which was done with the E-Nose system including an autosampler, a minimal split, an extra purge and had increased injection time (slower injection). That setting was considered appropriate as the results showed baseline recovery and no drifts.

There can be no correlation between the sensor readings and the viable cell counts of the sample. For the first test, overnight incubated human serum samples were plated out by taking samples directly from incubator and the viability count showed a ten-fold growth in 10^8 and 10^6 Hib sample. But for the latter tests done in conditions such as refrigerate after overnight incubation of samples and then plating up samples in 2 hr intervals (before taking samples to VOC analysis) showed no growth and thus no viable count. Therefore the study demonstrates that if refrigerated, it is not favourable for Hib in human serum. Thus for the later tests either bacteria did not grow in the human serum or might have died in refrigeration and therefore it could not be demonstrated that the gas sensor is detecting the VOCs produced by the live Hib during growth.

A sensor signal is obtained at a specific time after injection of the sample into the column which is usually indicative of a specific compound (which has a specific retention time on the column) (Qureshi *et al.*, 2009). The GC Detector software in E-Nose generated retention times versus peak areas (Appendix 7.4) for a representative set of 4 dilutions of Hib and was put in graphs (Figure 7-12) to see the difference between the peaks of the control and the peaks of the Hib diltuions. The volatile profiles in these graphs (Figure 7-12) are mostly unique, possessing distinctive features that can be used to distinguish these Hib groups from one another and from

control however the peaks were not consistent. A number of key VOCs peaks were identified from these four Hib dilutions, and are listed in Table 3 yet no significant data were obtained as there were no infectious dose dependent increase or decrease of VOC.

Since all tests were conducted with different E-Nose settings, the results obtained was just collated rather than compared with each other through a statistical analysis. The study did not identify what the volatile compounds are by use of standards. Some of the compounds that are present in the headspace of these Hib dilutions might give rise to more than one peak in the E-Nose spectrum (Bachinger *et al*., 2002). The normal peaks or those additional peaks were not reproducible in test 6 which was a repeat of test 5. In the tests, VOCs generated during growth of Hib in human serum however might be lost by conventional aeration when transferring the sample into glass vial which was then heated (50°-55°C for 30min) to drive more volatiles. The choice of sensor by their sensitivities to detect the volatiles produced and the selection of growth media (human serum in this case) play a role in E-Nose to be a diagnostic tool. Growth medium should be chosen with lesser volatile materials such that the background contributed by the medium does not overwhelm the analytical signal (McEntegart *et al*., 2000).

If E-Nose should have the great potential for clinical application like diagnosis of sepsis, it must be noted that different bacterial growth conditions, for example medium and oxygen levels could result in the production of different volatile species as well as different quantities of VOCs. Most of the above parameters were not standardised and human serum might contribute to the background signal (Psychogios *et al*., 2011). No membranes or drying agents were used to remove water vapour and to suppress background signal. In addition infections like sepsis are

sometimes poly-microbial in nature (Mackowiak *et al*., 1980) and thus there could not be any standard VOC profile of one microbe such as Hib to diagnose sepsis.

The data from the E-Nose analysis of the headspace volatiles produced by the Hib samples demonstrate that E-Nose if combined with a statistical analysis method and if more sensors and standards were used to know the volatile compounds, it would have had great potential for Hib identification *in vitro*. The project might be continued to explore bacterial headspace VOCs *in vitro* under a variety of growth conditions (other than human serum) and machine set-ups developing this technique into a clinical diagnostic tool for detecting sepsis *in vivo*.

5.0 Conclusion

In summary, the results of this study show that E-Nose gave results quickly when compared to the conventional blood culture system and GC-MS. Even though, VOC profiles detected from different dilutions of Hib in human serum suggest that area peaks might not provide more useful information for identifying the threshold doses of Hib present. Further studies need to be undertaken with a greater number of species and strains to ensure that these volatiles production in human serum is conserved in the broader microbial population. It is clear from this study that culture media and conditions will need to be highly standardized if this approach is to be a success.

Thus in near future, the early detection and identification of blood infections like sepsis based on E-Nose technology may provide clinical advantages over conventional methods.

6.0 References

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7.0 Appendix

7.1 Hib culture plate

7.1.1 Chocolate agar

- Per 500ml
- Mix 475ml distilled water + recommended amount of Columbia agar base in 500ml flask
- Autoclave
- Leave in 70-80C water-bath for approximately 30minutes
- In laminar flow cabinet pour in 25ml (5%) sterile horse blood and return to the 70-80C water-bath
- Mix by agitating occasionally until the molten agar turns daily milk chocolate brown (approx 10 min)
- Leave to cool in a 55C water bath (or at room temp for 15-20mins). Pour out as usual.

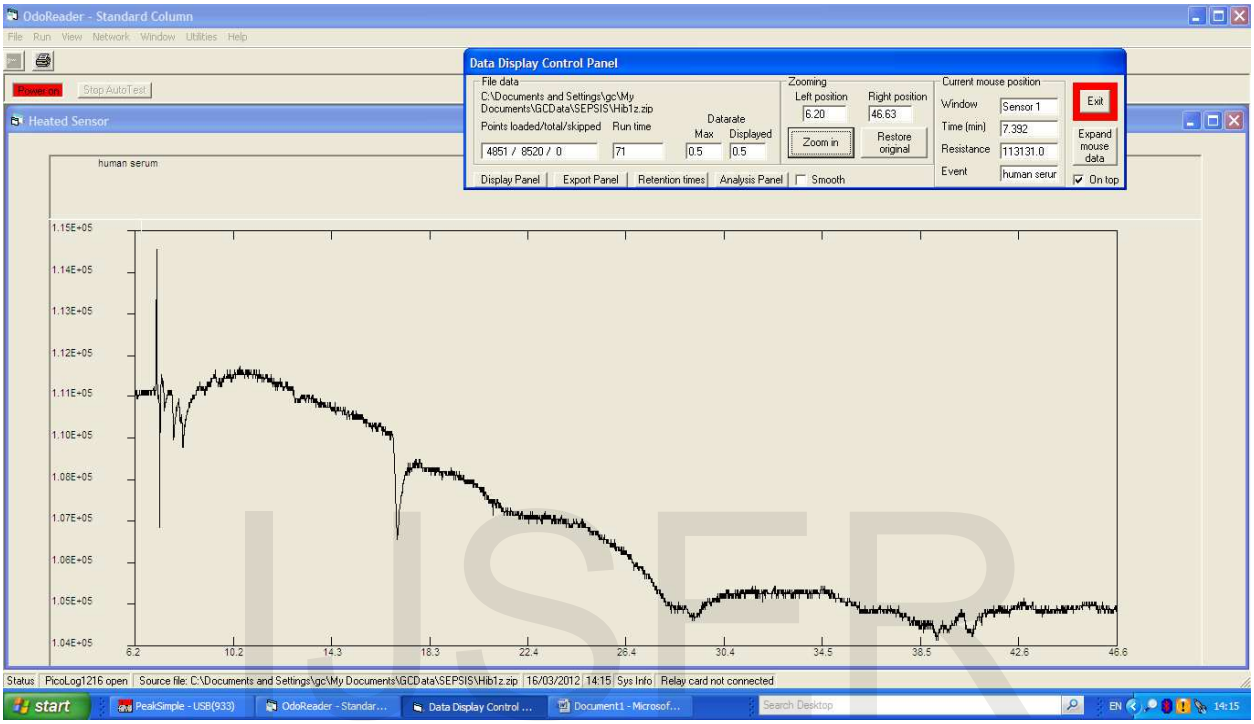
7.2 Solutions

7.2.1 PBS-B stock solution

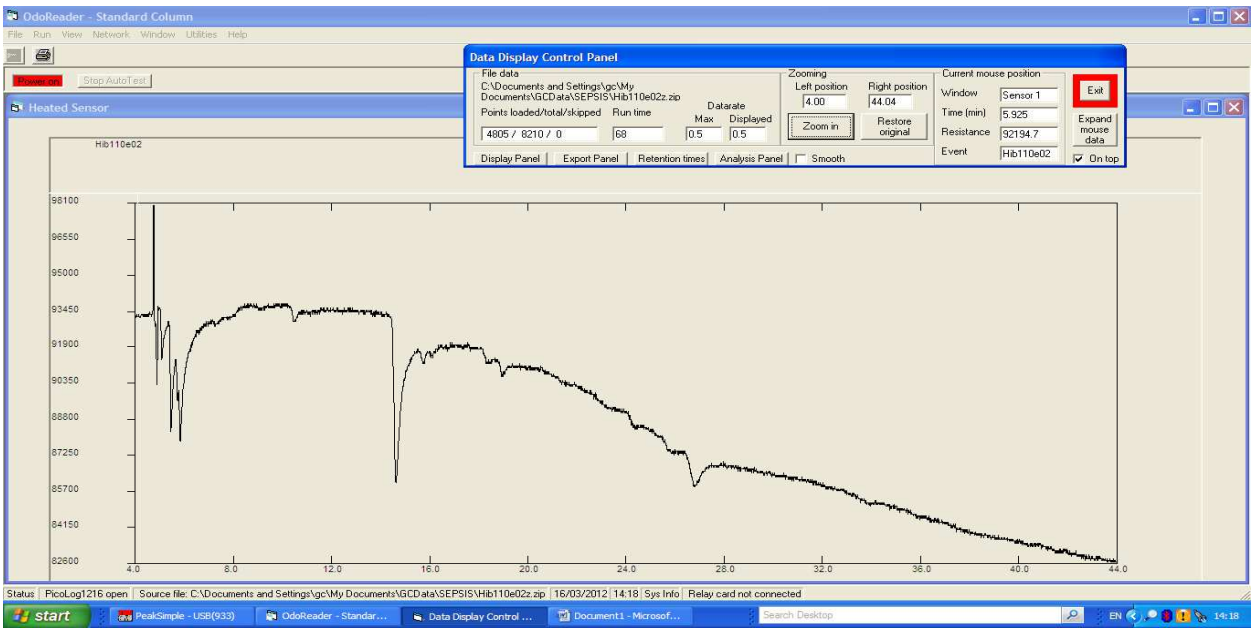
- Add 0.13g $CaCl_2$ –dihydrate and 0.21g $MgCl_2$ hydrate to 100ml distilled water and mix well
- Adjust pH to 1.46-1.48
- Add 2.5ml PBS-B stock solution to 500ml PBS-A or 1.25ml PBS-B stock solution to 250ml PBS-A to make working solution
- Now autoclave flask of PBS-B working solution

7.3 E-Nose (OdoReaders) spectra

7.3.1 Experiment 1

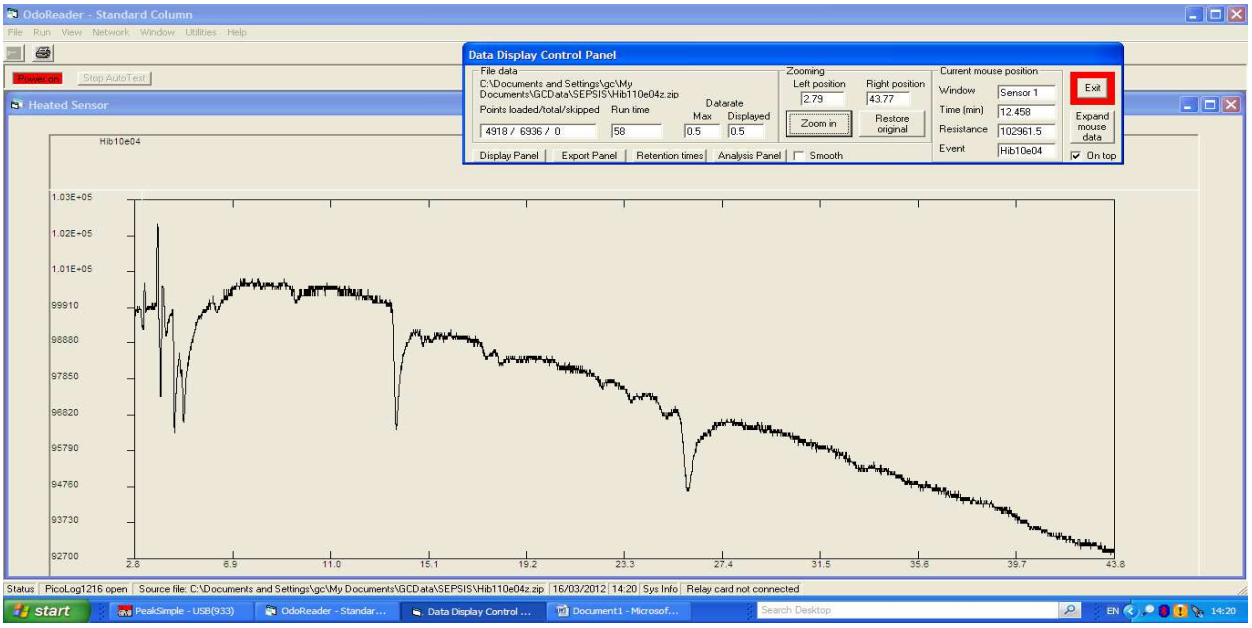


Human Serum (control)

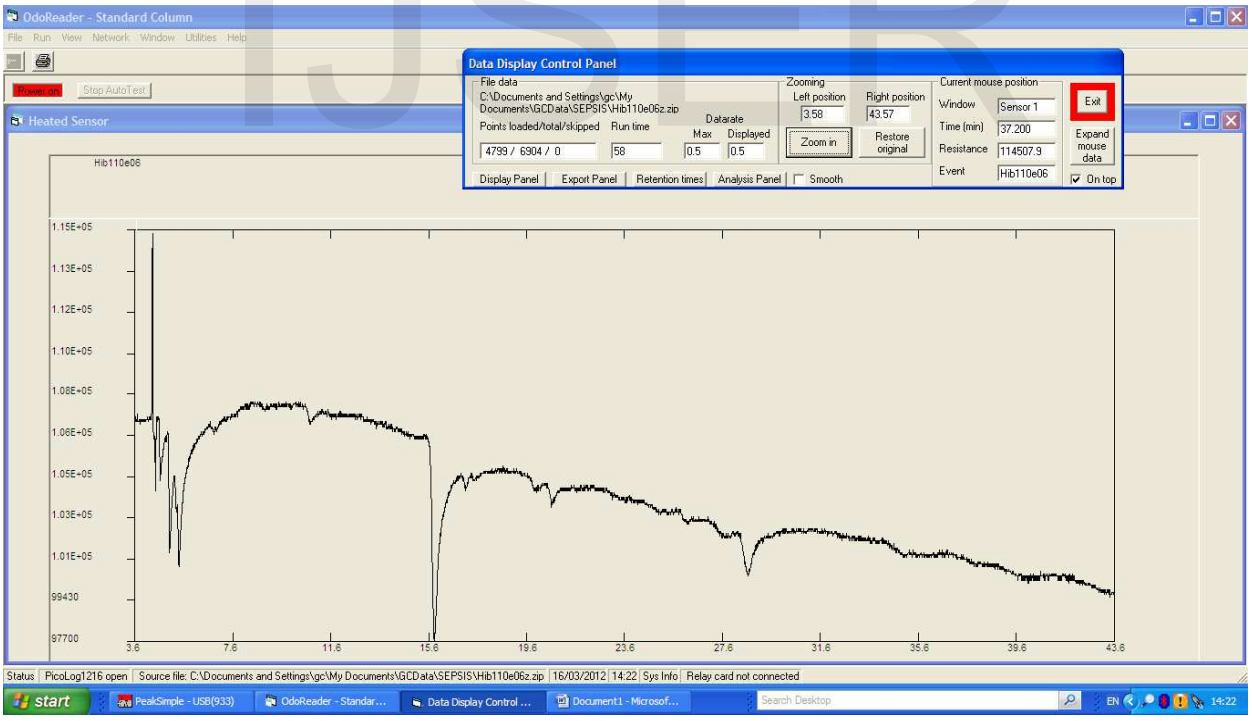


Hib (conc $10e^{02}$)

University of the West of England
BRISTOL



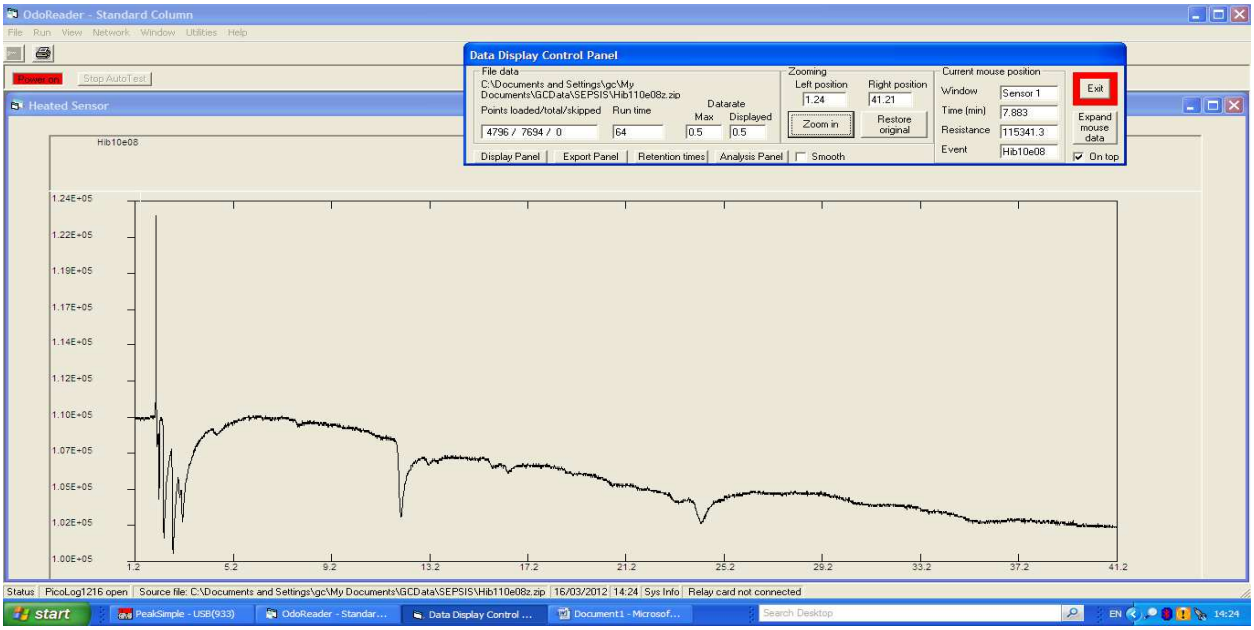
Hib (Conc 10e^{04})



Hib (conc 10e^{06})

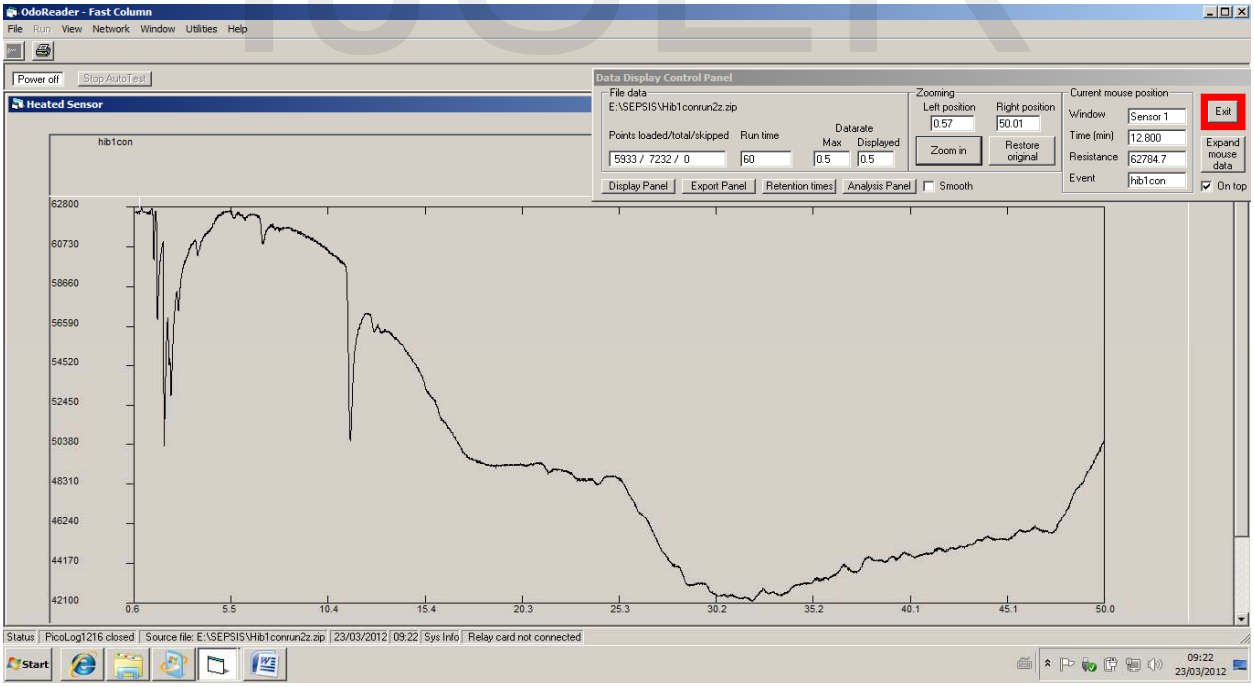
University of the West of England
BRISTOL

Rajeswari Jayavaradhan



Hib (conc 10e^{08})

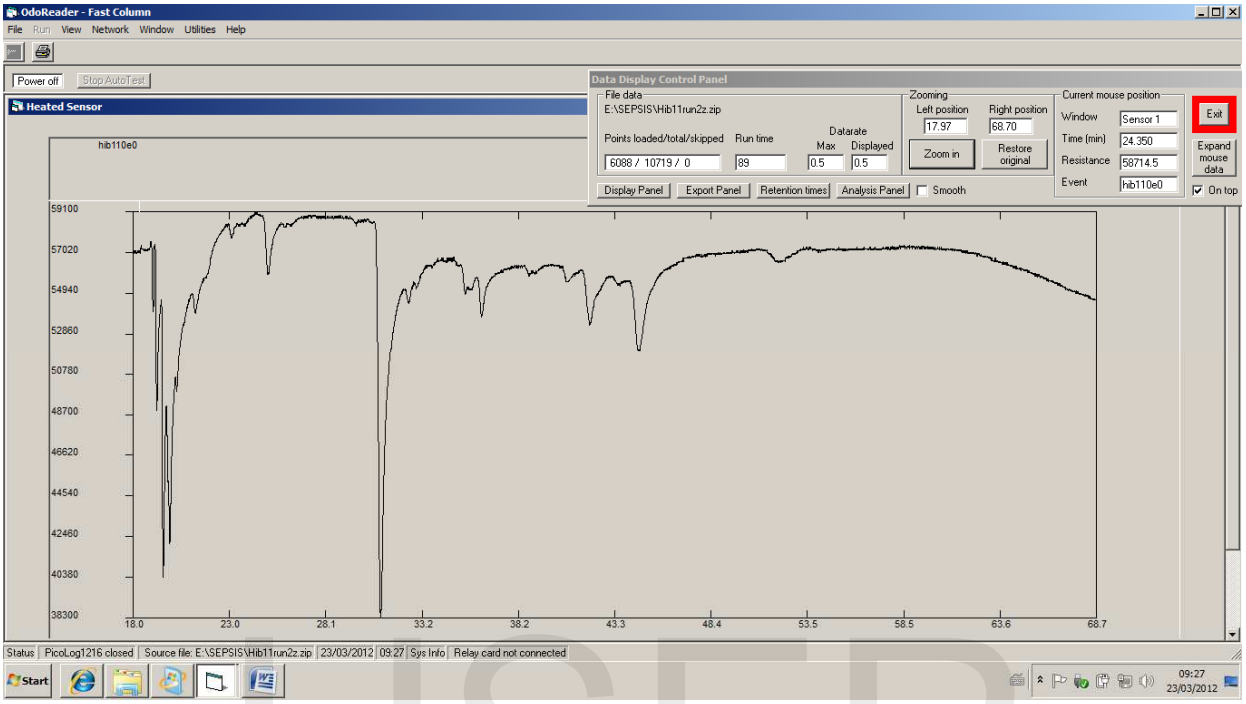
7.3.2 Experiment 2



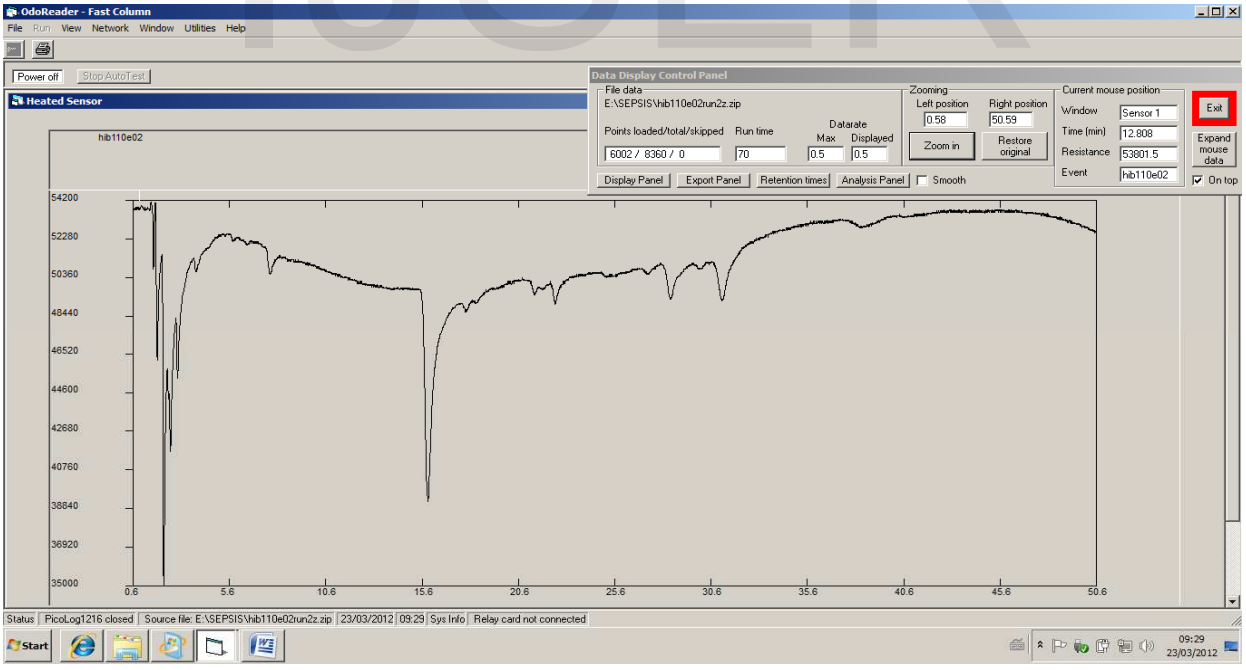
Hib control (human serum)

University of the West of England
BRISTOL

Rajeswari Jayavaradhan



Hib 10e0



Hib 10e02

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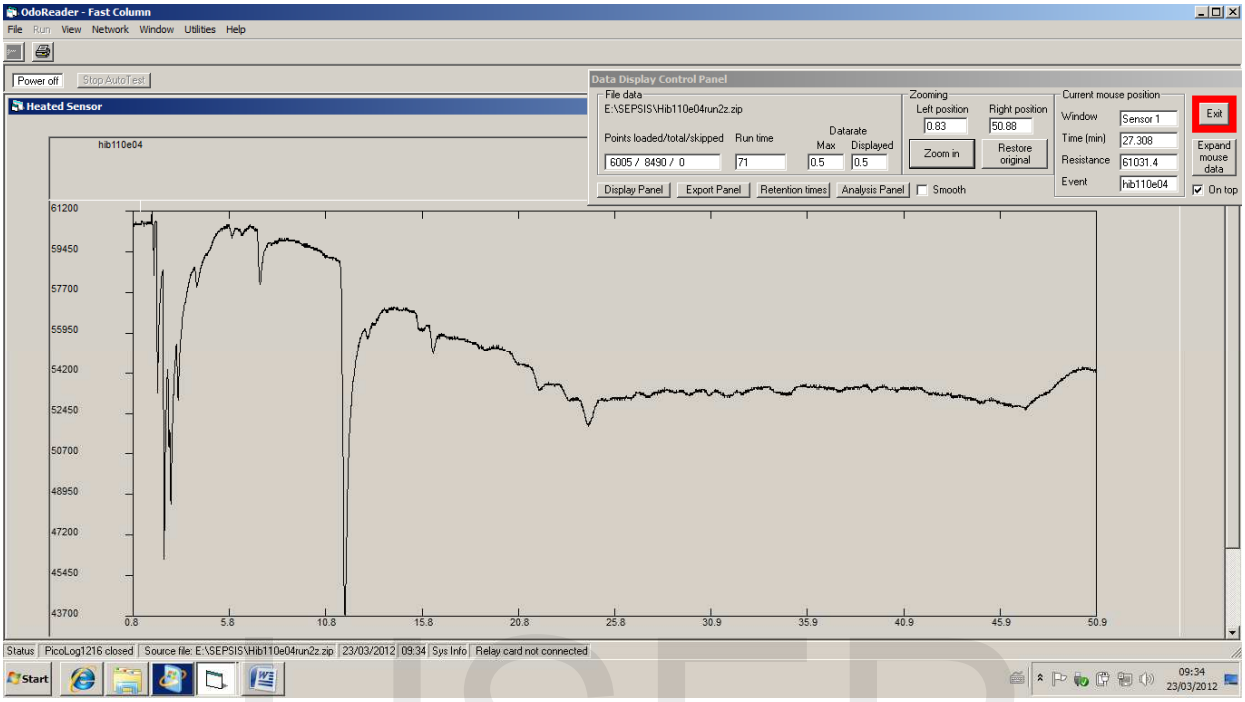
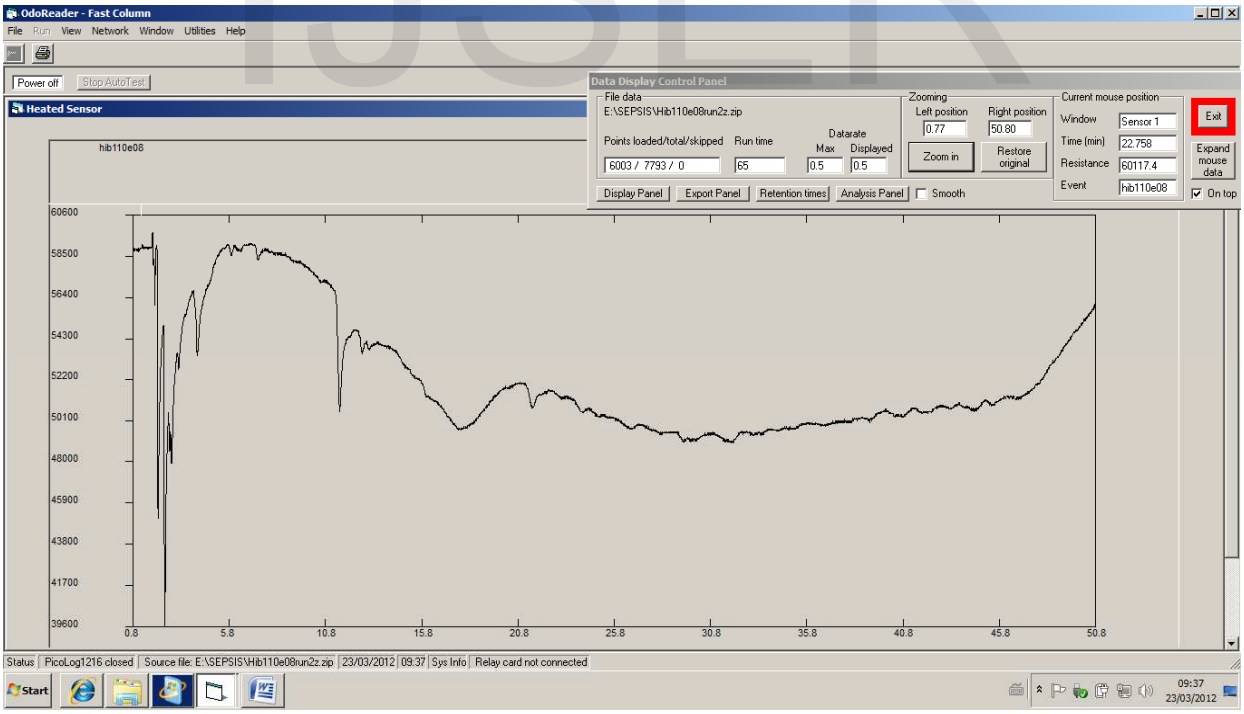
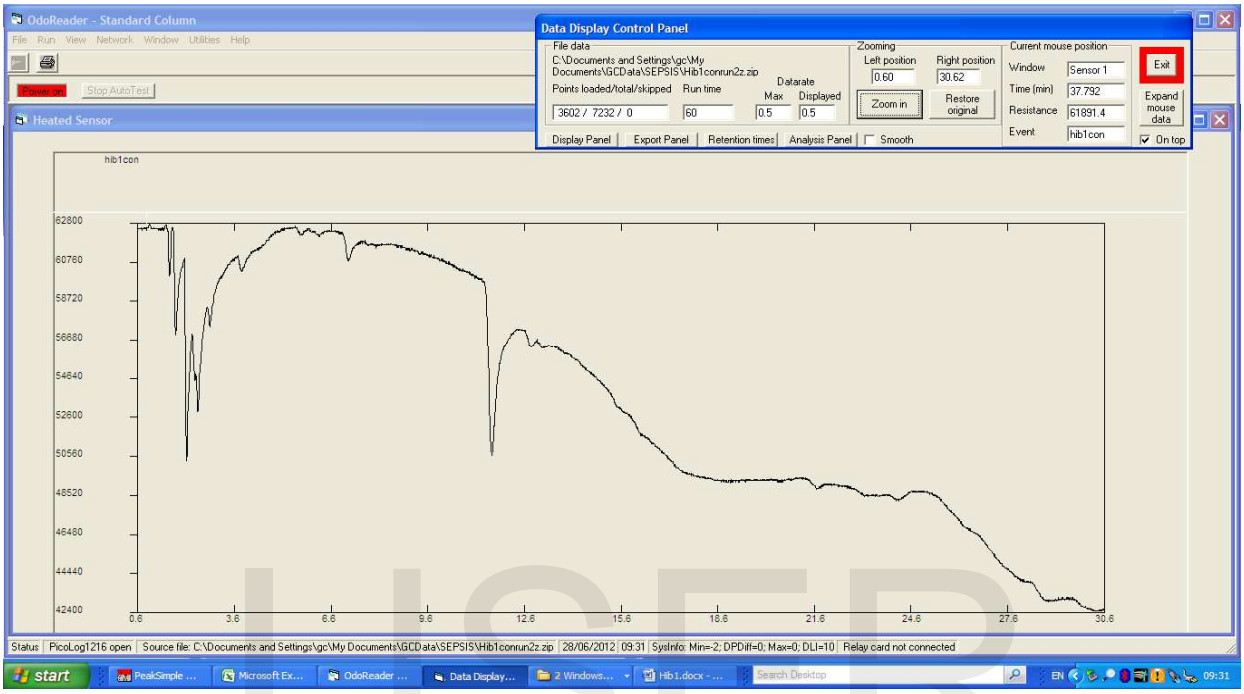


Hib 10e04

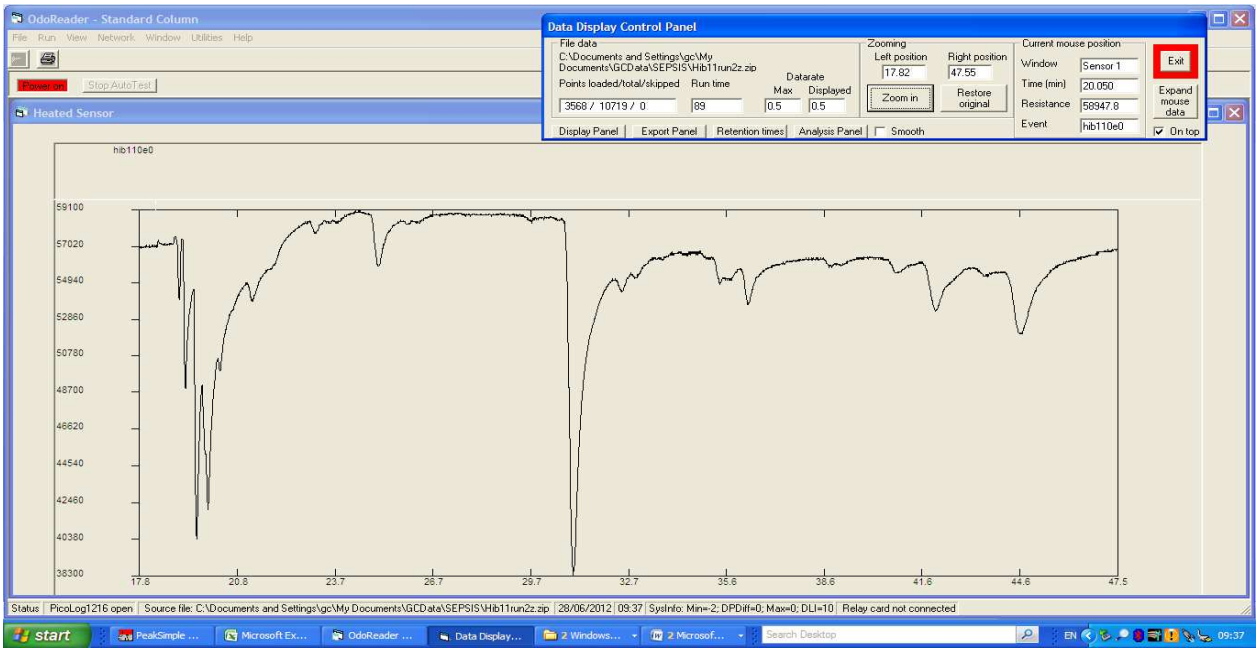


Hib 10e08

7.3.3 Experiment 3



Hib control



Hib 10e0

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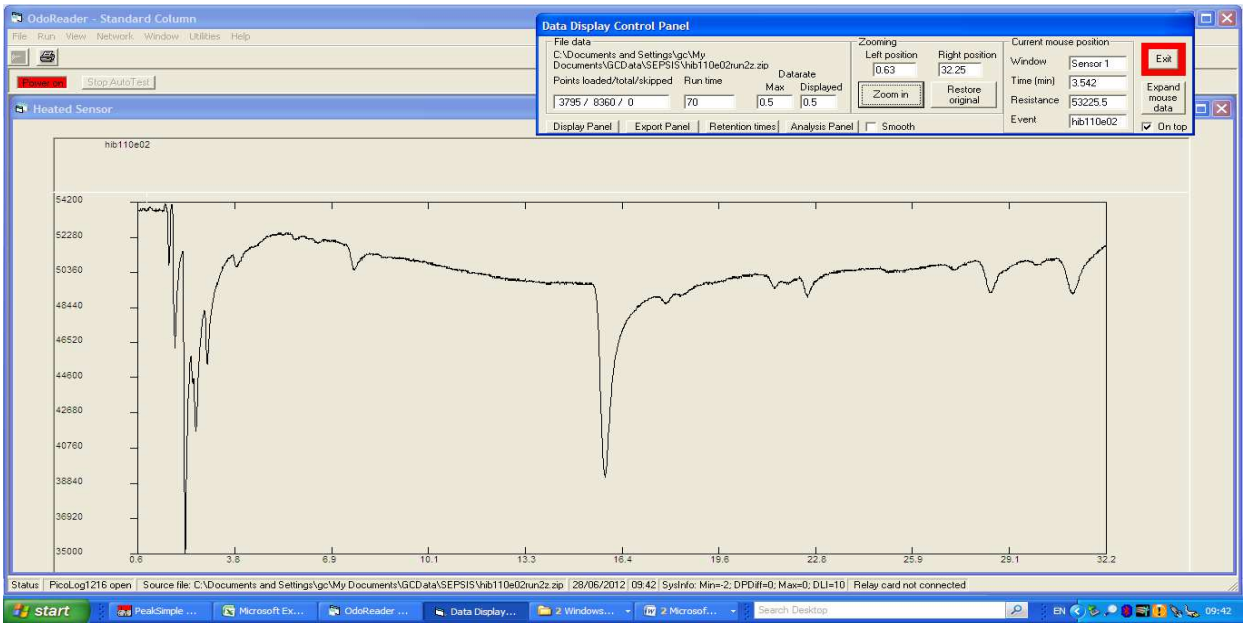
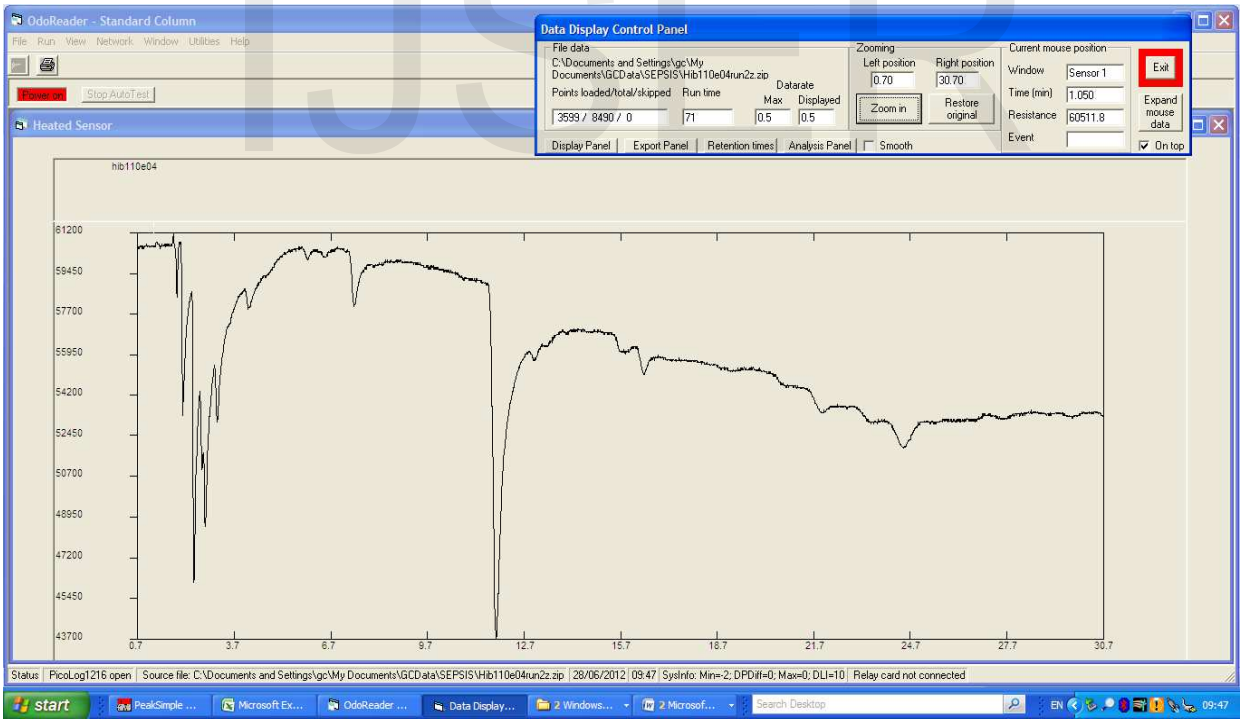


Hib 10e02



Hib 10e04

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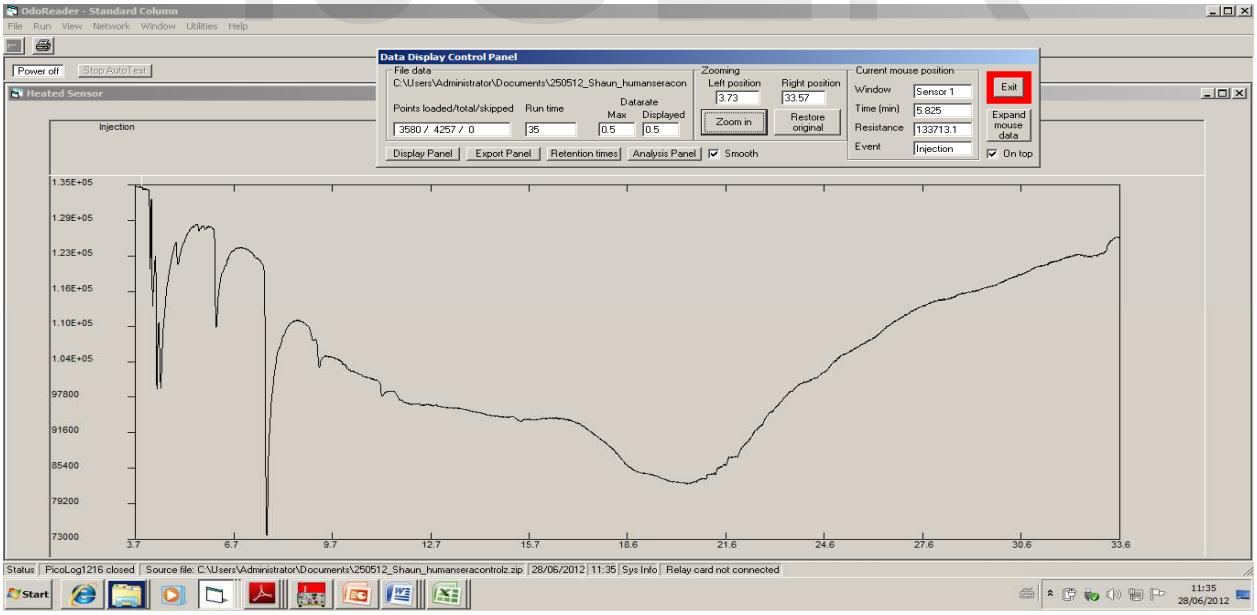
Hib 10e08

7.3.4 Experiment 4



Human sera

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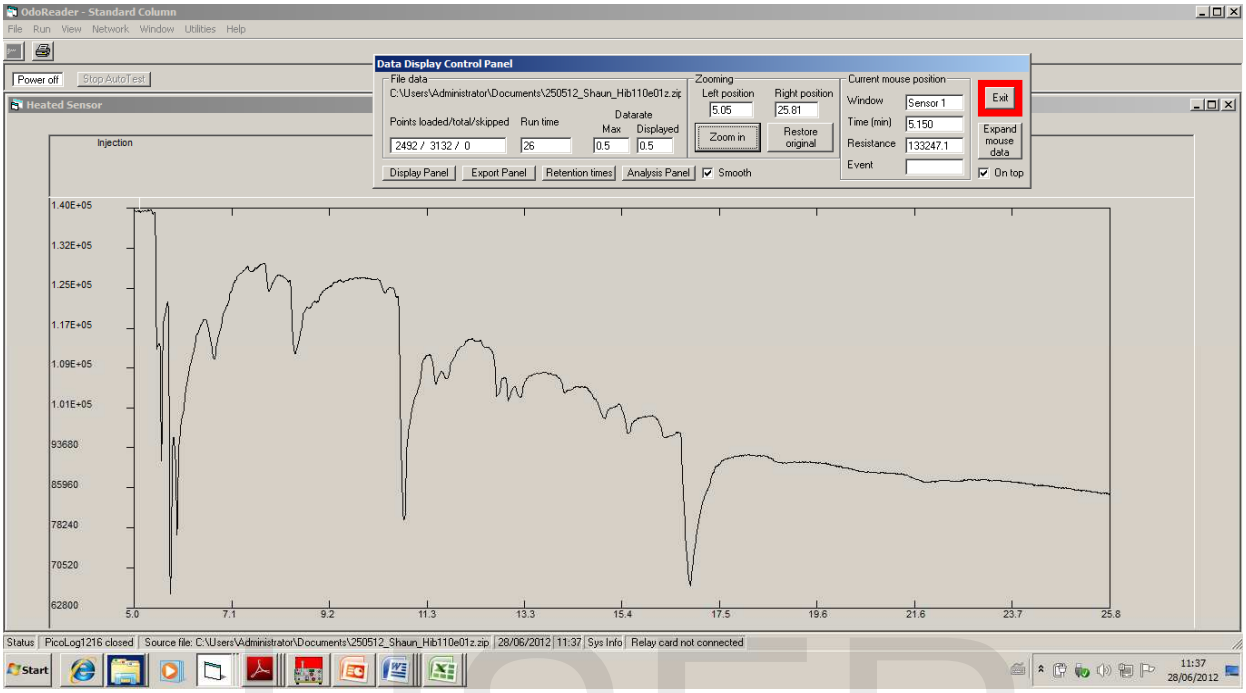

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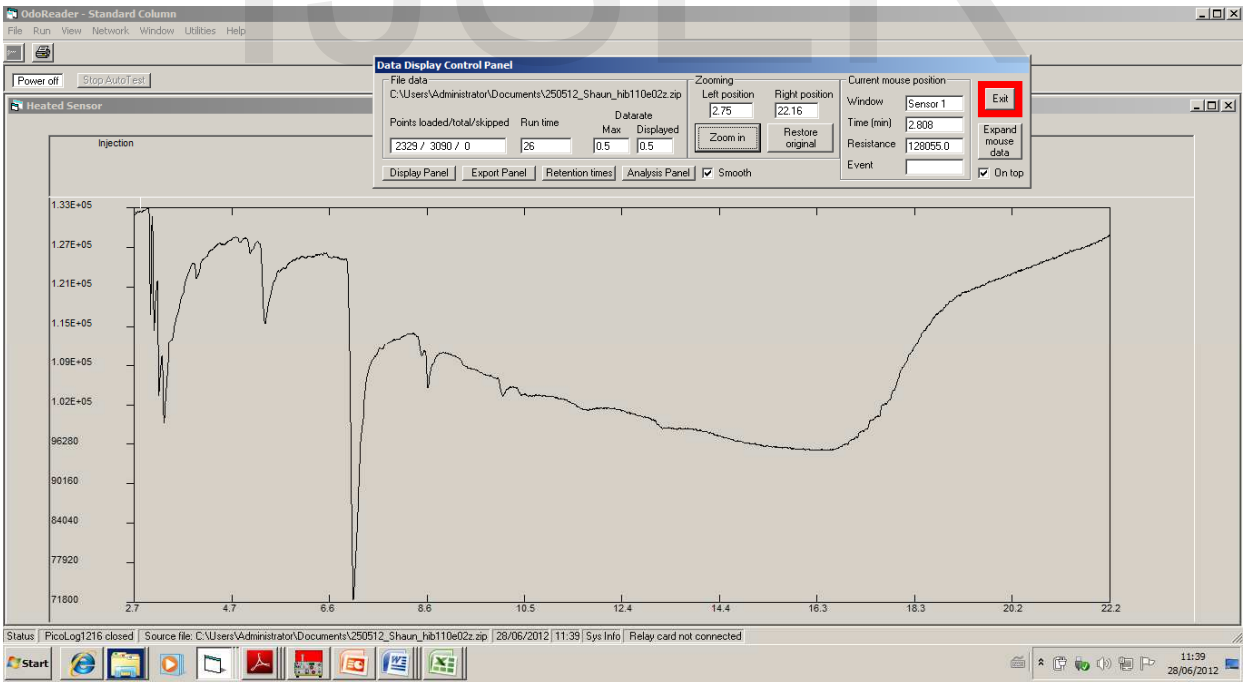

Hib 10e02

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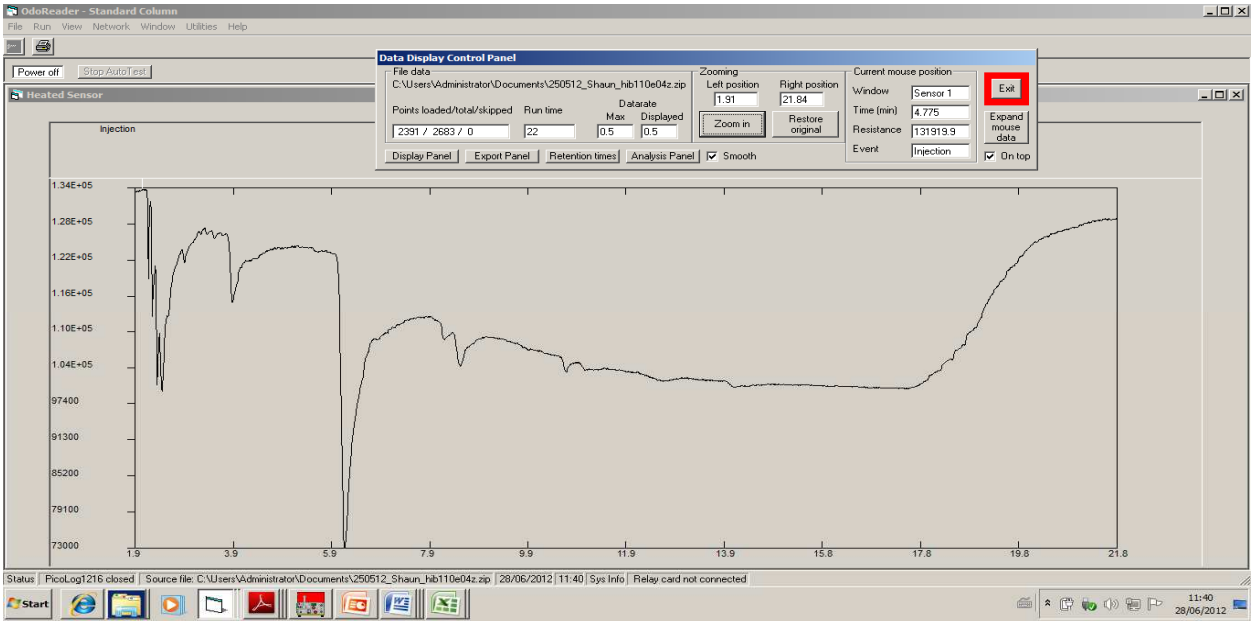
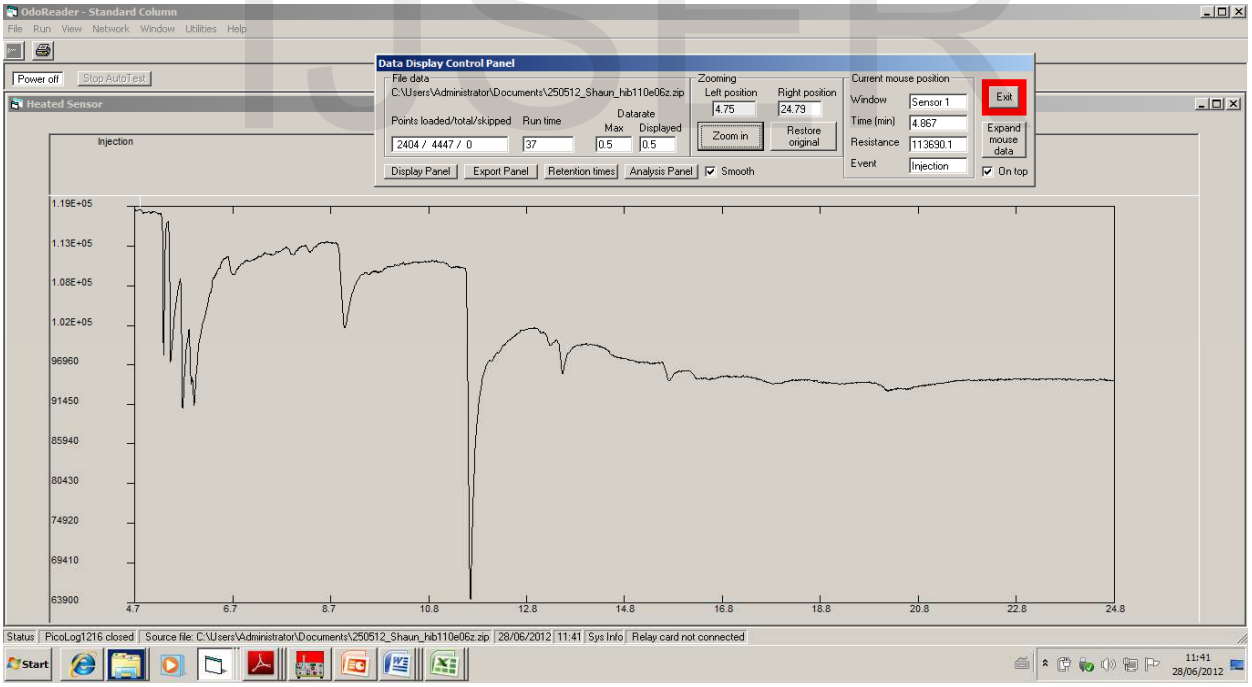


Hib1 10e04



Hib 1 10e06

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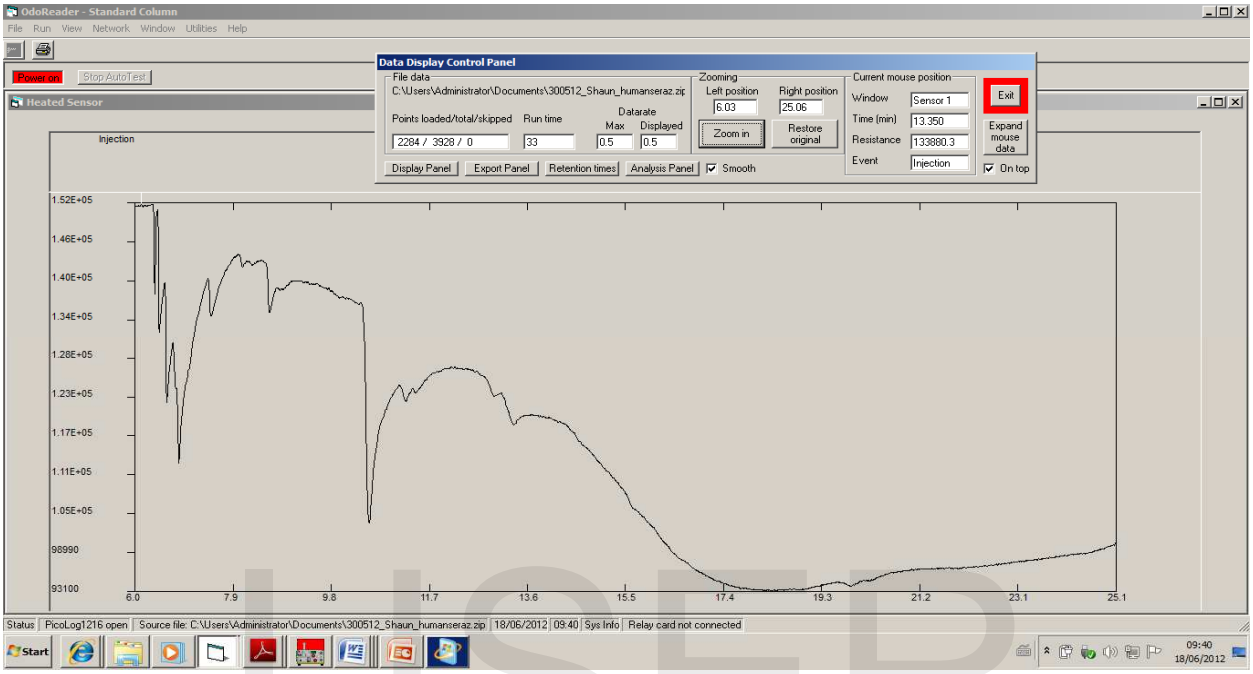
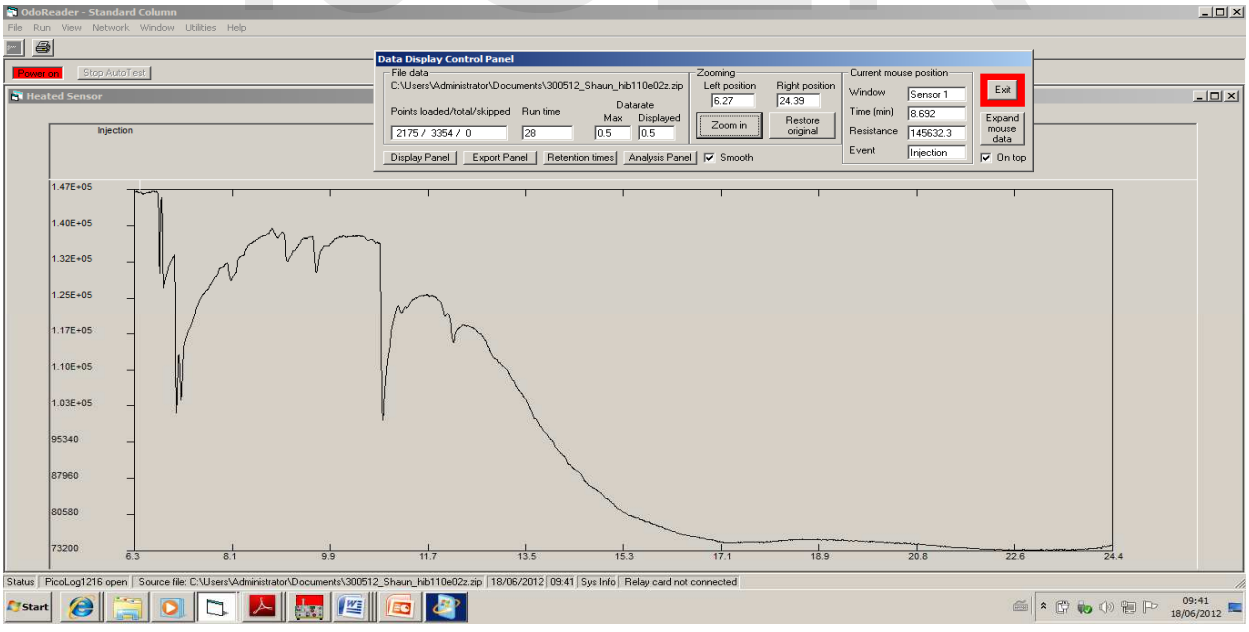
7.3.5 Experiment 5





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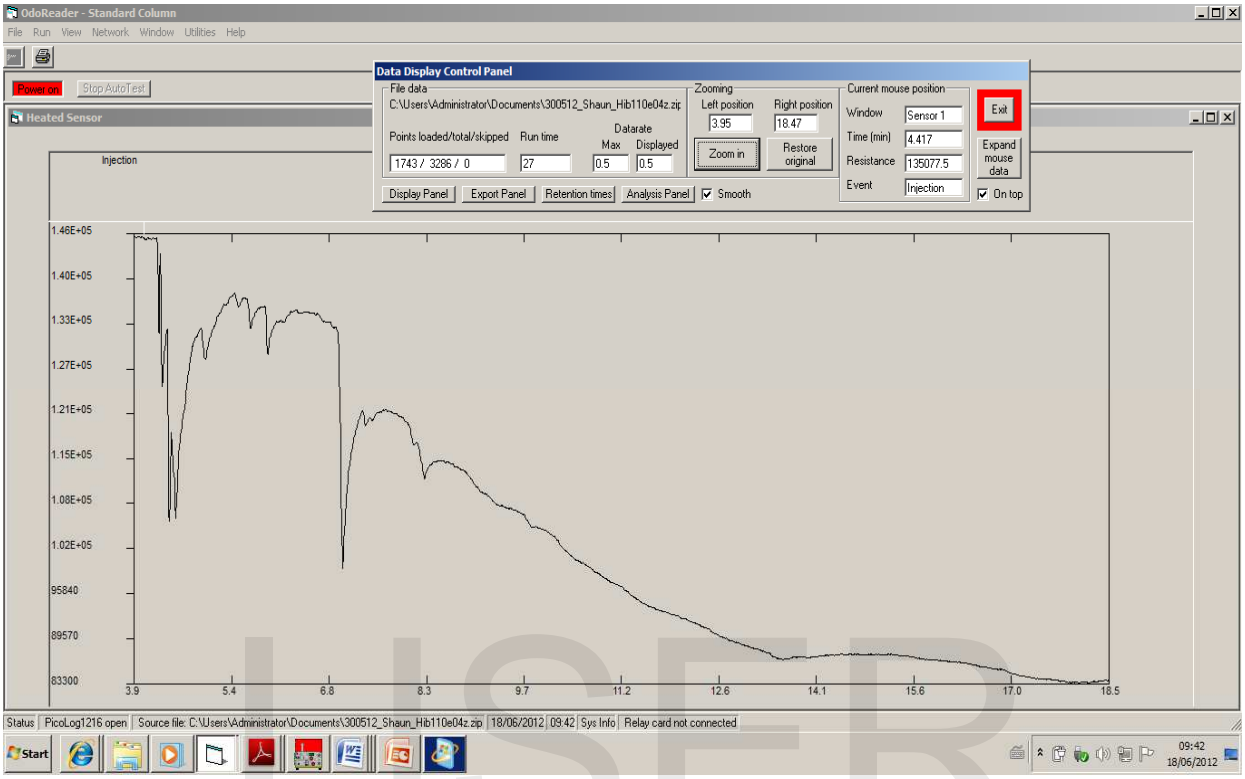
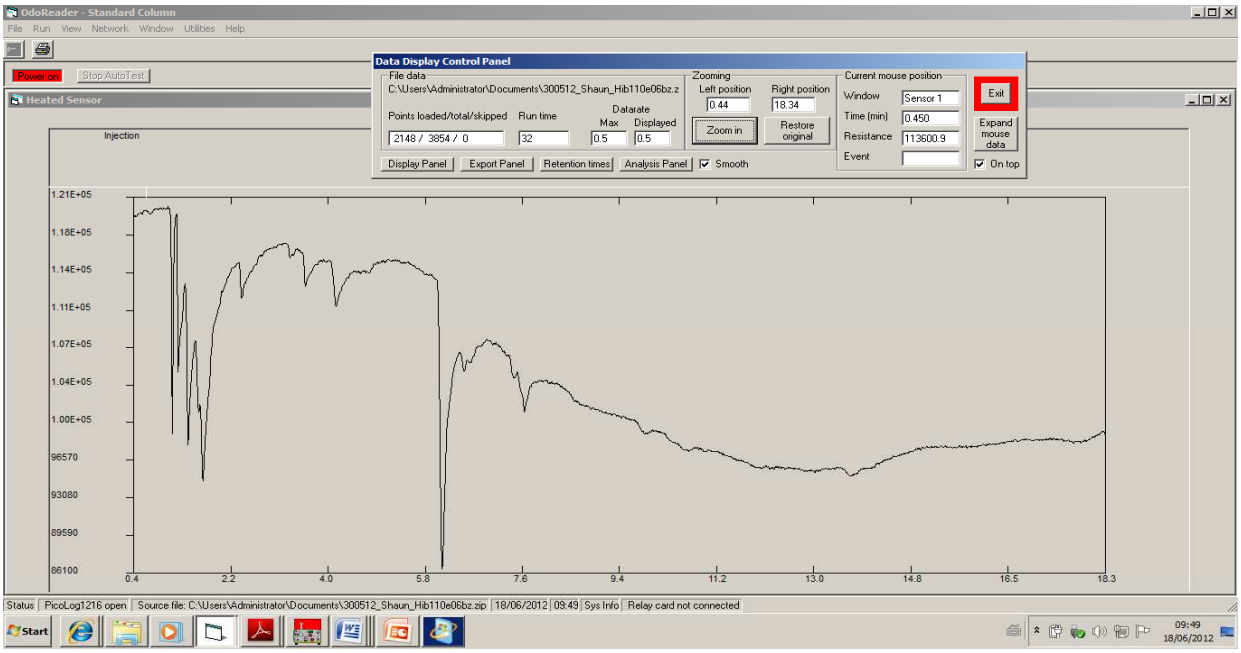


Hib 1 10e04



Hib 1 10e06

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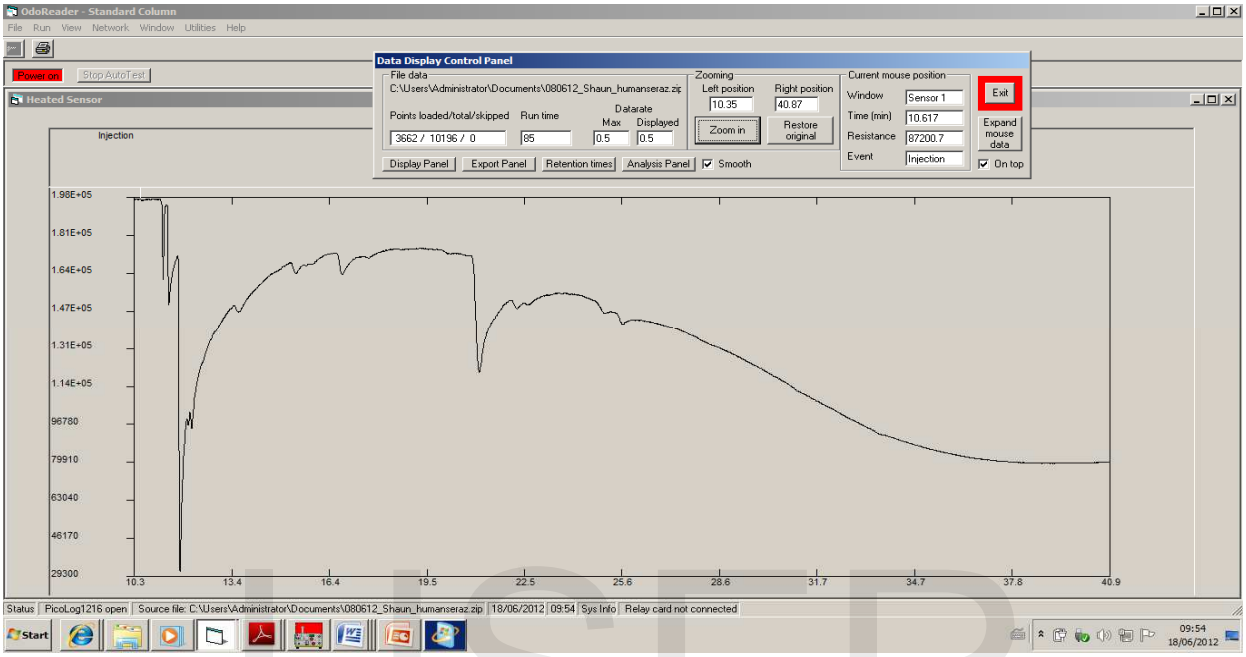
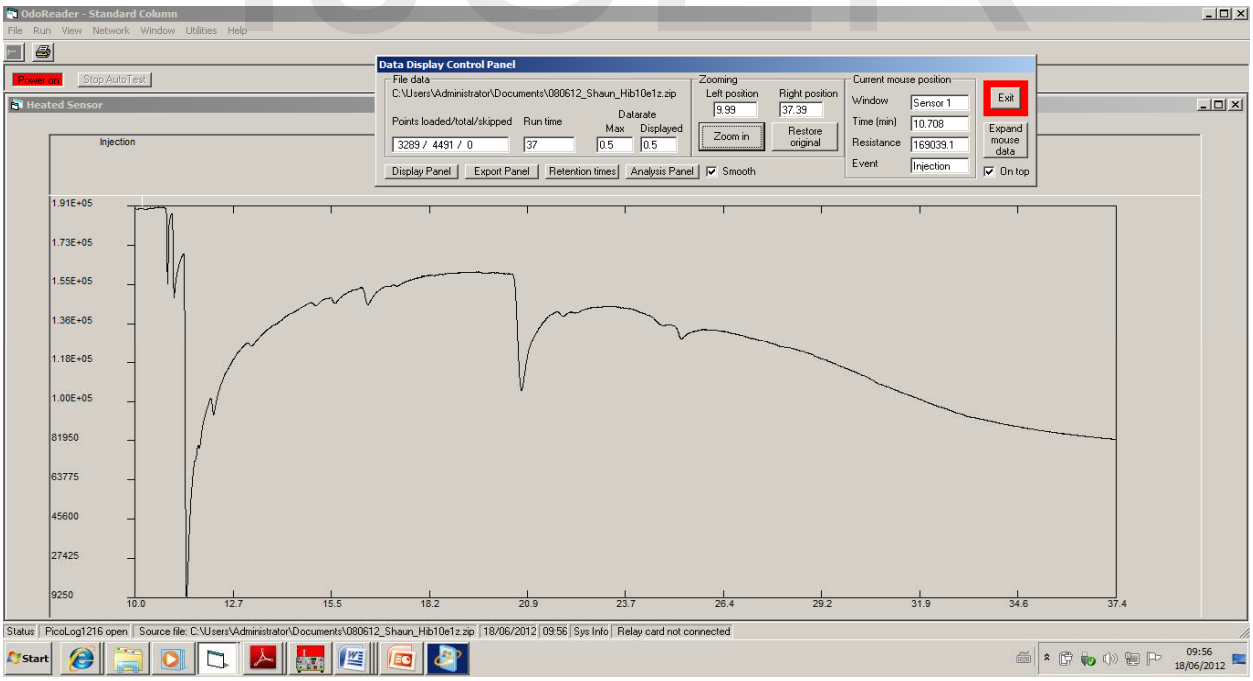
7.3.6 Experiment 6



Human sera



Hib 10e01

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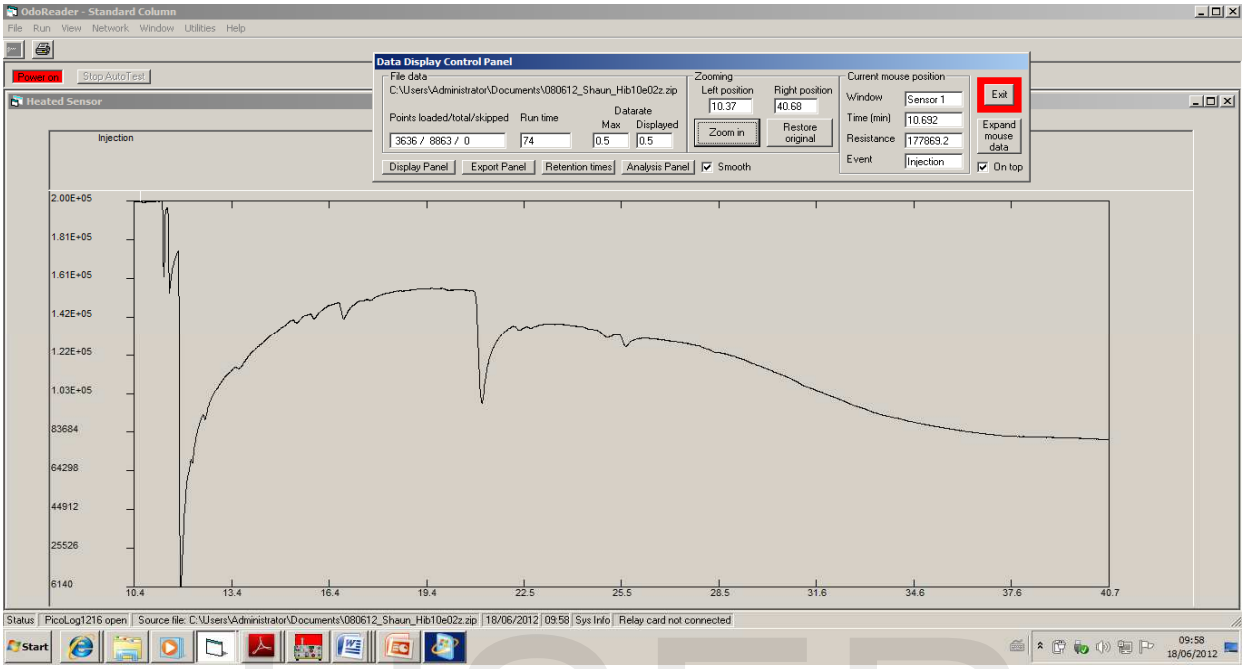
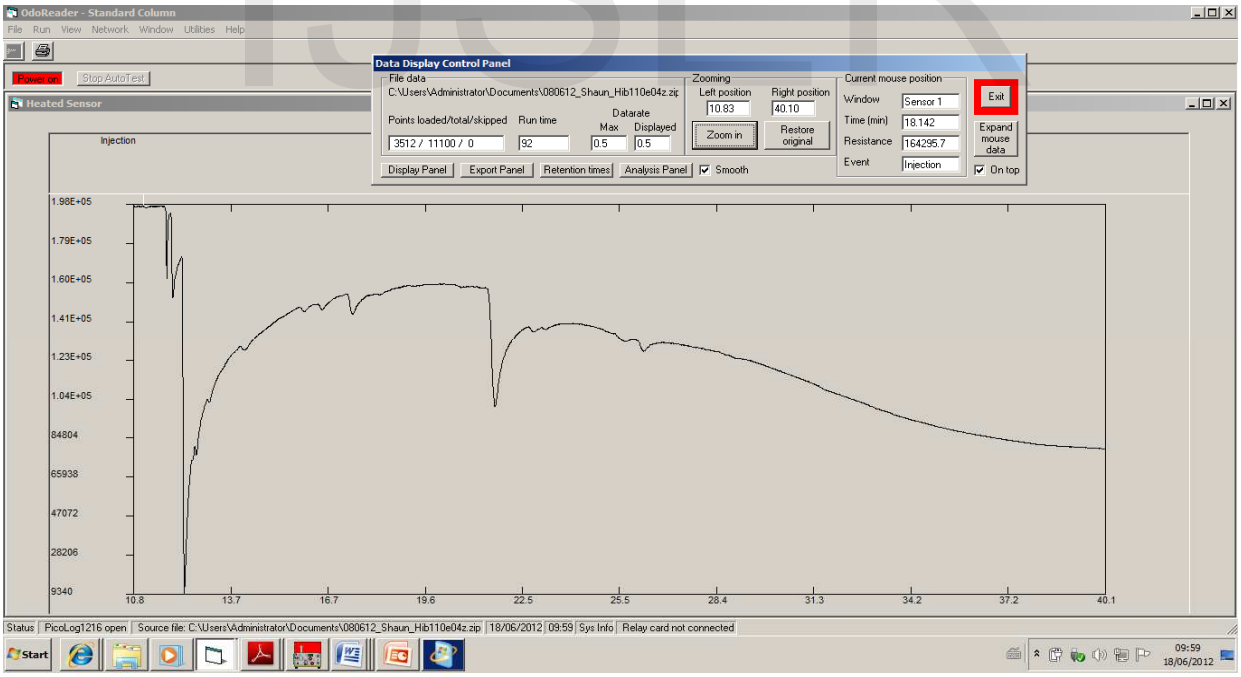


Hib 10e02



Hib 10e04

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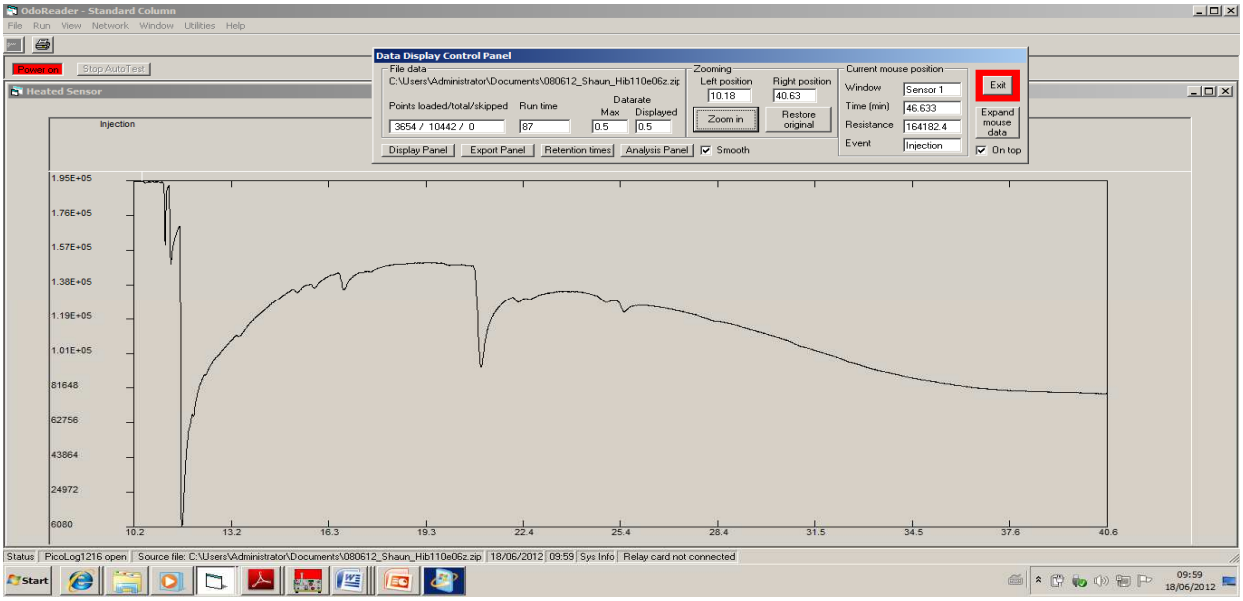

Hib 10e06

7.4 Software results showing retention time vs area peaks

7.4.1 Experiment 1

Retention time (min)	sample				
	Human serum	Hib-1 $10e^{02}$	Hib-1 $10e^{04}$	Hib-1 $10e^{06}$	Hib-1 $10e^{08}$
0.642-0.675	7940	10260	5516	17101	30764
0.733-0.775	7032	4835	4233	4107	8730
0.933-0.950	1083	4157	1168	4958	15660
1.300-1.325	1941	9041	7089	9949	13606
1.575-1.592	absent	3189	absent	1827	2461
1.683-1.717	absent	4009	1711	4940	4054
10.417-10.533	1264	8303	4785	15492	8440

7.4.2 Experiment 2

Retention time (min)	sample				
	Human serum	Hib-1 $10e^{0}$	Hib-1 $10e^{02}$	Hib-1 $10e^{04}$	Hib-1 $10e^{08}$
0.617				1011	3206
0.683	4695	3018	5548	4222	4705
0.858	10845	8476	11923	14572	27715
0.883			3807		
1.200	21052	14009	32967	24904	30521
1.442	2386				

1.467	2233	3804	2568	6255	3604
1.558	3470	3126	5301	6275	3132
1.925	1095		5682	2916	1374
1.950				1609	
2.867					4201
6.192	1016	1334		1232	
10.608	16400	19745	17468	29770	11031

7.4.3 Experiment 3

Retention time (min)	sample				
	Human serum	Hib-1 $10e^{0}$	Hib-1 $10e^{02}$	Hib-1 $10e^{04}$	Hib-1 $10e^{08}$
0.617				1137	3328
0.683	4695	3555	5770		
0.700				4222	4705
0.858	11215	8467	11923		27715
0.875			3807	14928	
1.200	21273	14202	32967	25027	30756
1.467	2350	3858	2979	6716	3930
1.558	3932	3126	5505	6383	3132
1.925	2070		6000	3270	1604
2.742					1671
2.867		1048		1346	4787
6.175	1527			4453	
6.6833		2901	1781		
10.200-10.63	18084	20159	20310	30588	12200
11.392					1043
15.117		3400		1062	
21.467		2569			
24.033		3406			

7.4.4 Experiment 4

Retention time (min)	Sample				
	Human serum	Hib-1 $10e^{0}$	Hib-1 $10e^{02}$	Hib-1 $10e^{04}$	Hib-1 $10e^{08}$
0.083				14616	
0.167			14189	14730	
0.225	13841				
0.250			13516	15412	
0.292	13662				
0.333		17698	13046	4534	
0.375				3164	

0.425	17838	17217	5605		12534
0.517	4983				
0.575					12292
0.600		34435			
0.725		10233			
0.817				1201	11775
1.000					4571
1.058	1867				2729
1.083			1457		
1.417		1264			
1.708				3942	
2.125	6984		1089		
2.400			4217		
2.658		1924			
3.208		5680			
3.675	15498				
3.917				9251	
4.100			14360		2580
5.283	1435	10727			
5.6			4610		
6.10		1206		1573	
6.3				1337	
6.6					15141
7.2	1161				
7.475		4103			
7.800		2733			
8.550					1514
8.950		1096			
10.225		1524			
11.571		4430			

7.4.5 Experiment 5

Retention time	Human sera	Hib 10e02	Hib 10e04	Hib 10e06
0.546	16778			
0.69-0.77	13042	15576	16419	13729
0.95-1.09	12000	12589	13401	10074
1.26			20629	
1.42-1.49	3641	22106	4987	8611
1.65-1.67	7737	5812	5619	
1.77				3682
1.91				4443

3.13	2665		2070	2071
3.78		1820		
4.63	863			
5.87	2762		1972	2017
6.57			4407	1098
7.45		2592		
10.368	10497	19079	12751	8503
11.817			1264	
12.98		1283		
13.32		1564		1061
14.35			1007	
14.74			1253	
17.11	879			

7.4.6 Experiment 6

Retention time	Human sera	Hib 1 10e01	Hib 1 10e02	Hib1 10e04	Hib1 10e06
0.642	71386	68993	75310	67709	67892
0.833	88895	77166	85852	81993	85248
1.175	280963	318317	336200	325245	326888
1.467	5032				
1.558	14135	2820	3871	7693	2222
1.925		15144	4401	3015	
2.958	3833	1337		3134	
4.742	7638		1871	2288	2512
5.292		3586	4671	5221	3405
6.167	18043	14928	15604	18976	16384
10.367	104211	107011	112401	113612	110666
11.642	6026	1045	2267	4140	1298
11.675					2658
14.283	1130	2877	2744	1350	
14.317	3458	1195	1052	1859	
14.4	3406	1826			
14.933	8417	9898	11871	9856	
17.4	1081				