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Development of a volatile amine sensor for the monitoring of fish spoilage

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Abstract

A sensor with potential for the development of a "chemical barcode" for real-time monitoring of fish freshness is described. This on-package sensor contains a pH sensitive dye, bromocresol green, that responds through visible colour change to basic volatile spoilage compounds, such as trimethylamine (TMA), ammonia (NH_3) and dimethylamine (DMA) collectively known as Total Volatile Basic Nitrogen (TVB-N). The sensor characteristics were studied as well as its response with standard ammonia gas. Trials on cod and under-utilised species have verified that the sensor response correlates with bacterial growth patterns in fish samples thus enabling the "real-time" monitoring of spoilage in various fish species. The sensor response can be interrogated with a simple, inexpensive reflectance colorimeter that we have developed based on two light emitting diodes (LEDs) and a photodetector.

Keywords: TVB-N; Volatile amines; Fish freshness monitoring; Chromoreactive dyes; Smart packaging; Chemical bar-coding

1. Introduction

Since the recent successive food crisis of the bovine spongiform encephalopathy (BSE) virus, foot-and-mouth disease and chicken flu virus, food safety assurance has become critically important to rebuild damaged consumer confidence. The increasing importance of food quality "traceability" is emphasized by a new European Union directive which has just come into place [1]. Within the fisheries industries, there is consequently great interest in developing accurate methods to evaluate real-time freshness of fish and seafood products.

At present, fish and seafood freshness judgment largely relies on sensory assessment of freshness attributes, such as appearance, texture, smell and colour, by trained assessors. These qualities are then compiled together according to a certain grading scheme to produce a quality index (QI) [2,3]. The fish industry and retailers are interested in methods that are objective, cost effective, rapid, reliable and non-destructive. Various approaches have therefore been used for the determination of fish quality. Typical methods include sensory (odour, taste, texture), microbiological (total viable count or TVC), physical (texture, electrical properties) and chemical (K and K_1 values, Total Volatile Basic Nitrogen or TVB-N, lipid oxidation). The K and K_1 values reflect the extent of ATP degradation after death and they have been extensively used as freshness indicators [4,5]. An excellent review of these methods has been provided by Olafsdottir et al. [6].

Generally after death, the number of microorganisms on the skin and gill surfaces, known as specific spoilage organisms (SSO), increases gradually and spreads within the various tissues [4]. These microorganisms, regardless of the origin of the fish, are usually *Pseudomonas* spp. [7] and they have been successfully employed for shelf-life prediction of various aerobically stored fresh fish [8,9]. Volatile compounds such as $(CH_3)_3N$ (trimethylamine or TMA), $(CH_3)_2NH$ (dimethylamine or DMA) and NH_3 (ammonia) are products of microbial degradation and are collectively known as TVB-N. Hence, TVB-N levels are a

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potential indicator of fish spoilage. The standard EU method for determination of TVB-N levels in tissue samples consist of extraction of volatiles bases by a perchloric acid solution followed by steam distillation of the extract which is then collected in boric acid and titrated against standard HCl [10]. Although accurate when performed by experienced analysts, the method is nonetheless destructive and time consuming.

Recent methods include the development of a TMA vapor probe that was consistent with TVB-N levels, gas chromatography methods and various sensory measurements [11], a TMA biosensor for fish freshness analysis using flavin-containing monooxygenase type-3 (a major enzyme in the metabolisation of TMA in human liver) [12], and an array of semiconducting metal-oxide (SMO) sensors for the monitoring of freshness in Atlantic salmon, Atlantic cod and haddock [13]. The natural fluorescence of fish muscle was also studied as a rapid and non-destructive method for monitoring fish freshness [14]. The principal component analysis of the intrinsic fluorescence spectra may be used as fingerprinting criteria allowing the discrimination between fresh and aged filets. However, these devices require a physical contact between the fish tissue and the sensor or to be physically linked to another monitoring apparatus, thus preventing their use on individually packed food products without compromising the integrity of the packaging.

This paper reports the use of solid-state sensors based on bromocresol green (pK_a of 4.72) to monitor TVB-N levels in fish headspace. In previous work we established a correlation between dye colour and the population of *Pseudomonas* spp. [15,16]. More recently, the sensor formulation has been improved in terms of sensor sensitivity and colour stability. Bromocresol green was selected since a slight increase in pH would immediately trigger a visible colour change, thus giving in theory a highly sensitive device. As basic spoilage volatiles amines are gradually produced in the package headspace, a pH increase follows resulting in the sensor colour changing from yellow to blue, easily visible to the naked eye.

2. Experimental

2.1. Materials and chemicals

Bromocresol green (sodium salt), cellulose acetate (Mw approximately 30,000 g/mol) and all ammonium bromide salts used in this study, such as octadecyl trimethyl ammonium bromide, tetrahexyl ammonium bromide, tetraoctyl ammonium bromide or cetyl trimethyl ammonium bromide, were obtained from Sigma–Aldrich (Dublin, Ireland); dibutyl sebacate (DBS) and nitrophenyl octyl ether (NPOE) were obtained from Fluka Chemicals (Dublin, Ireland) and optically clear polyethylene teraphthalate (PET, 175 microns grade) sheets were obtained from HIFI Industrial Film Ltd (Dublin, Ireland).

2.2. Sensor fabrication

2.2.1. Formulation

A typical sensor solution contained a binder, such as cellulose acetate (63%, w/w), a dye, such as bromocresol green (2%, w/w), an ammonium salt (4%, w/w), such as octadecyl trimethyl ammonium bromide, tetrahexyl ammonium bromide, tetraoctyl ammonium bromide or cetyl trimethyl ammonium bromide and a plasticiser, such as DBS or NPOE (31%, w/w). The mixture was sonicated for 45–60 min until dissolution was complete. Optically clear PET discs were spin-coated (model WS-200-4T2 from Laurell Technologies Corporation (North Wales, PA, USA)), under constant nitrogen flow at 1000, 2000 and 3000 rpm for approximately 10 min. The coated discs were then placed in an open container, at room temperature and in a dark cupboard for at least two days to complete the drying process. Sensor discs of 6 mm diameter were punched out using a simple paper puncher taking great care not to damage the coated surface. Films generated at each rotation speed varied in terms of thickness. This variable was assessed in the experimental design.

2.2.2. Sensor assemblage

Sensors were assembled by placing the coated spot face down in a sandwich between a polytetrafluoroethylene (PTFE) gas permeable membrane and a clear protective adhesive cover above. The membrane protects the sensor from condensation of water vapor while allowing gaseous compounds to pass through. The optically clear PET allows reflectance measurement with minimum reflectance loss.

2.3. Sensor characterisation

When a pH indicator dye is placed in an environment that is basic enough so that deprotonation of the dye occurs, a shift in its absorption spectrum wavelength maximum (λ_{max}) takes place. In the case of bromocresol green, it occurs from 438 nm (acidic form of the dye) to 615 nm (basic form of the dye). This colour change was monitored with an inexpensive, in-house developed reflectance colorimeter based on light emitting diodes (LEDs) and a photodetector. The LEDs used were from Kingbright®, λ_{max} 590 nm which had excellent spectral overlap with the absorbance spectrum of the bromocresol green basic form (Fig. 1). An Ocean Optic 2000® spectrometer was used for to obtain the LED emission spectral range.

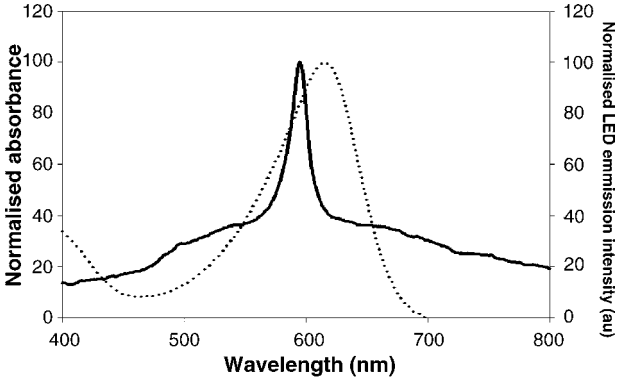

Fig. 1. (- - -) BCG absorbance (pH 6.5) and optical scanner yellow LED emission spectra (—). The yellow LED emitted the closest to the BCG basic form absorbance wavelength maximum (λ_{max}) and thus was selected as most suitable for its detection.

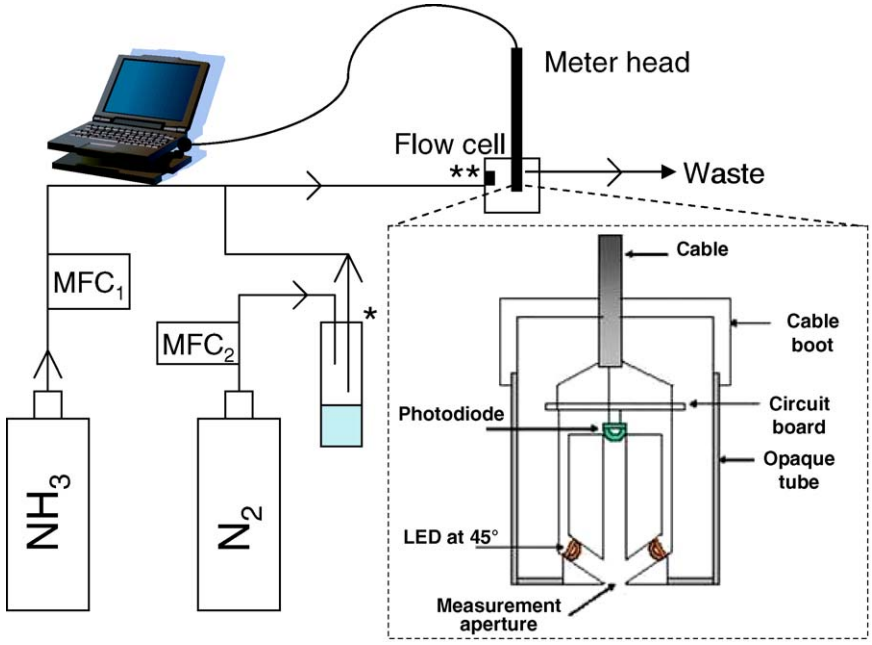

Fig. 2. Schematic of the ammonia calibration system, MFC: mass flow controller; (*) drechsel bottle; (**) gas disperser (colorimeter: Irish patent No S2004/0542 filed on 13 August 2004).

Sensor calibration was carried out by using various concentrations of ammonia gas obtained by mixing nitrogen and 100 ppm synthetic ammonia in nitrogen with mass flow controllers. The sensor was fitted to the aperture of the optical scanner head and placed, through an airtight rubber seal, into a ca. 57.4 cm^3 flow cell (4.7 cm × 4.7 cm × 3.3 cm: $L \times W \times H$) to monitor in real time the sensor responses to changing ammonia concentration. The data was logged by a PC connected to the optical scanner (Fig. 2).

2.4. Fish spoilage trial

2.4.1. Experimental setup

Three fish species, cod (Gadidae family), cardinal (also known as Bulls-eye, Epigonidae family) and Roundnose Grenadier (also known as Grenadiers or rattails, Macrouridae family), were selected for investigation. Cod is a species widely established while cardinal and Roundnose Grenadier are newly assessed deep-water species that show potential for inclusion into the current Irish fish market [17,18]. Three freshly caught whole cods were filleted at a local harbor market (Howth, Ireland) and placed in a sealed ice container. Aseptic techniques such as the use of disposable gloves, bactericide built-in cutting board and flame sterilized scalpel were used to avoid sample contamination. Nine replicate tissue samples of 1.05 ± 0.05 g were removed from the filets. Three sets of three replicate samples were tested using sensors of varying thickness produced by spin-coating at three different speeds: 1000, 2000 and 3000 rpm. Reproducing the experimental design proposed by Byrne et al. [15], the sensor response was monitored every 2 h with the reflectance colorimeter fitted with superbright yellow LEDs. Sensors not exposed to spoiling fish samples were used as reference sensors for these measurements. At each time interval, 5 measurements were performed per sensor so that a total of 15 measurements were carried out for sensors of each thickness.

2.4.2. Microbial analysis of cod samples

Simultaneously to the above spoilage trial, 21 samples of ca. 25 g were removed from the same cod filets, under the same aseptic conditions, and placed in zip lock freezer bags. They were also allowed to spoil at room temperature and every 2 h, along with every sensor response measurement, a sample was transferred into a freezer. Samples were in a frozen state in less than 1 h. TVC counts were determined, using the pour plate method, on plate count agar (Oxoid CM463) while the spread plate method was used on agar base (Oxoid CM733) with CFC selective supplement (Oxoid SR103) to give Pseudomonad counts. Plates were counted after 48 h incubation at 30 °C and results were correlated with the sensor response.

3. Results and discussion

3.1. Sensor optimisation

3.1.1. Film thickness

PET discs were spin-coated with the sensor solution at 1000, 2000 and 3000 rpm to generate films of different thicknesses

Table 1
Effect of spin-coating speed on sensor thickness

Speed (rpm)	Profilometry (microns)	Profilometry Stdev ($n = 2$)	SEM (microns)
1000	2.57	0.14	2.31
2000	1.36	0.13	NA
3000	1.01	0.2	NA

It was not possible to determine to satisfaction the thickness of the sensors spin-coated at 2000 and 3000 rpm under SEM.

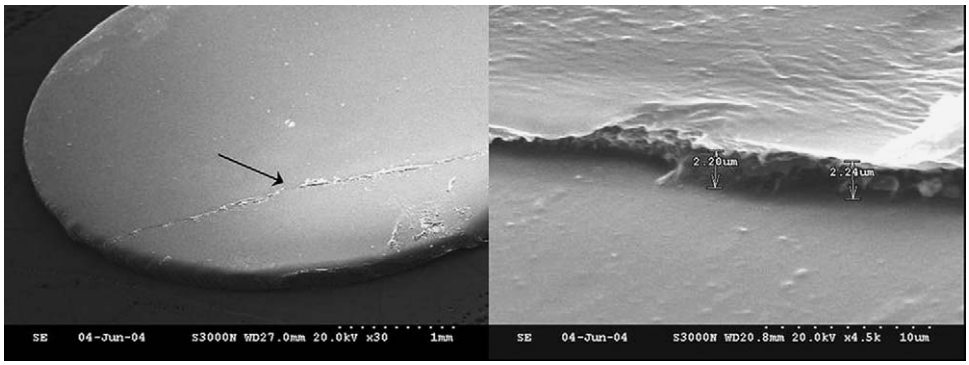

Fig. 3. SEM pictures of a sensor spin-coated at 1000 rpm. A section of the sensor was kept uncoated using a tape mask. The arrow shows the line separating coated and uncoated sections that allow measurements from the PET surface.

which were determined using a Veeco® profilometer model Dektak V200–Si (Table 1) and an Hitachi S-3000N scanning electron microscope (SEM, Fig. 3). Results indicated, as expected, that the higher the spin-coating speed, the thinner the sensor with the average profilometer results being 2.57, 1.36 and 1.01 μm ($n = 2$) at a spin rate of 1000, 2000 and 3000 rpm, respectively. At spin rates higher than 3000 rpm, such as 4000 rpm, the decrease in film thickness became marginal, whereas at spin rates less than 1000 rpm, such as 500rpm, the films proved hard to dry and became brittle and flaky.

Fig. 3 shows a SEM side view of a sensor (1000 rpm) with the coating stripped away using a tape mask (arrow). SEM data showed that the thickness of the coating is around 2.2–2.5 μm which is consistent with the profilometry data for coatings generated at this speed (Table 1).

3.1.2. Sensor response to ammonia

Fig. 4 shows typical sensor responses to ammonia gas. The response time of the sensor was found to depend on the flow rate of the gas supply (as this determines the rate at which the sample steady-state condition is established in the flow cell) and the relative humidity (as the dye protonation–deprotonation process requires the presence of an effective proton transport medium to ensure efficient shuttling of protons from the acidic dye (proton donors) to the basic ammonia (proton acceptors)). However, in a sealed package environment, both of these factors are relatively constant and therefore will have little influence on the sensors.

The third factor that affects response time is the concentration of the ammonia (a volatile base). As is often observed with bulk optrode-type devices, in terms of sample concentration, the response time depends of (i) the initial concentration (larger changes are faster) and (ii) the direction of changes (increases are faster). Fig. 4 shows that the response time (t_{90}) was estimated, in the flow cell described above, to be approximately 5 min when exposed to an ammonia concentration step from 0 to 14.29 ppm, at a flow rate of 350 ml/min and in a relative humidity level of 89%. This time is more than adequate as the increase in volatile amines in the food package will occur over a period of days. The initial baseline was re-established after 2 h of experiment. Colour measurements were taken from the onset of the response until no more increase was recorded. Sensors with the greatest thickness, coated at a spin rate of 1000 rpm, showed the biggest dynamic range. This is due to the greater amount of material on the sensor film compared to the thinner films, which increased the optical path length and resulted in increased absorbance values. With the manual setup described above we were able to vary the ammonia concentration in the range 0–15.0 ppm, and over this range, the sensor response was found to be linear (Fig. 5). We are currently working on extending the available calibration range of ammonia using a more sophisticated sampling system with automatic mass flow controls.

Responses were reproducible (coefficient of variation of 4% at 14.3 ppm, $n = 3$) and sensors, kept in dark and dry conditions, were found to be stable four months after fabrication

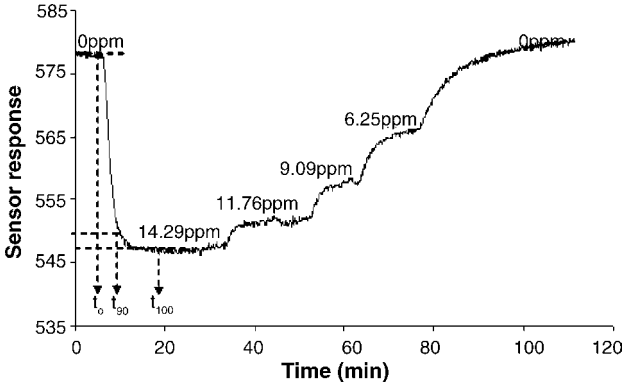

Fig. 4. Typical sensor response (spin-coated at 1000 rpm) to increasing ammonia concentration.

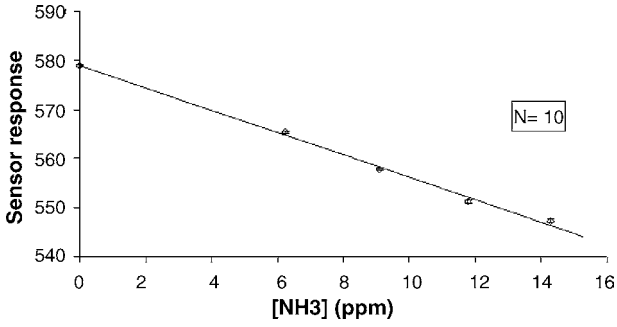

Fig. 5. Sensor responses (spin-coated at 1000 rpm) to increasing ammonia concentration monitored by the optical scanner. A linear range from 0 to 15.0 ppm was observed.

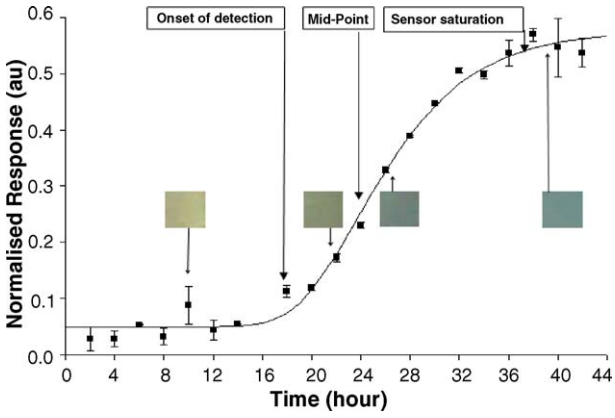

Fig. 6. Typical change of TVB-N level in cod at room temperature in bromocresol green spin-coated sensors. Data points are averages of fifteen measurements. The error bars are standard error of the mean values and may be hidden by the data point. Pictures of sensors show colour change over time.

(visual inspection and baseline measurements with the colorimeter scanner).

3.2. Fish spoilage trial

3.2.1. Cod

All sensors in contact with the tissue samples responded to the increasing volatile basic amines generated by spoilage with a very distinct colour change from yellow to blue. Sensors were monitored every 2 h until no further colour change was observed. Fig. 6 shows the rate of change in colour of sensors (1000 rpm) in contact with spoiling cod at room temperature. Prior to the first 18 h, no colour change was detected by the reflectance colorimeter. Thereafter, the sensors steadily changed colour from yellow to blue (approximately 20–38 h). Responses of the sensors spin-coated at 2000 and 3000 rpm were similar in response time and range. Furthermore, the reflectance colorimeter did not detect differences in signal steady-state changes between these sensors of various thicknesses. However, the thickest sensors, coated at 1000 rpm, provided a more intense colour change to the eye than those coated at 2000 or 3000 rpm allowing a better visualisation of the occurrence of spoilage. A spin speed of 1000 rpm was selected for further studies, as at slower spin speeds, the formulation did not form films of reproducible thickness.

3.2.2. Cardinal and roundnose grenadier

The onset of spoilage was detected in the region of 30 h in both cardinal and the roundnose grenadier samples, almost 10 h later than in the cod samples (Fig. 7). This indicates that the cardinal and roundnose grenadier samples released volatiles amines at a slower rate than cod. Similar results were found by Byrne et al. [15] when comparing another under-utilised fish species, orange roughy, and cod.

3.3. Microbial analysis of cod samples

The TVC counts slowly increased from ca. 10^4 cfu/g during the initial 10 h but rose sharply from then on before stabilising

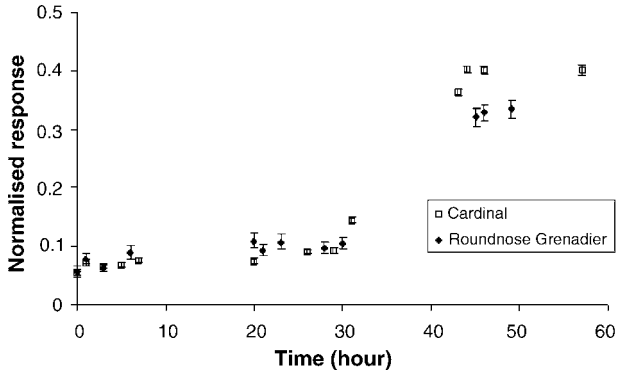

Fig. 7. Typical bromocresol green sensor response to spoiling roundnose grenadier and cardinal samples over time at 20 °C. Data points are averages of 15 measurements. The error bars are standard error of the mean values and may be hidden by the data point.

at 26 h at approximately 10^8 cfu/g (Fig. 8). Initially, the *pseudomonas* counts were at approximately 65% of the TVC counts rising to approximately 100% at around 18 h. Similarly to the TVC counts, they remained unchanged for the initial 8–10 h but rose sharply up to almost 26 h with ca. 10^8 cfu/g.

When compared to the sensor response in the same figure, it can clearly be seen that not only does the sensor response correlate with the changes in both bacterial populations (TVC and *pseudomonas* in tissue) but the onset of sensor colour change also correlates with the level of product rejection (10^7 cfu/g). It is difficult to use reported bacterial counts in the literature to define exact spoilage thresholds as they can vary depending on the catch season, geographical location and above all fish species. However, Gram and Huss [7] suggested that when stored aerobically, spoilage of iced fish is reached at levels of 10^8–10^9 cfu/g of specific spoilage organism while Koutsoumanis [8] and Olafsdottir et al. [6] both reported TVC and *pseudomonads* values of 10^7 cfu/g for fresh fish samples to reach end of shelf life. These levels were reached after about 18 h in our experiments (Fig. 8).

A delay between the rise in microbial population and the sensor response curves is also apparent from Fig. 8. This delay is inherent as volatile base generation follows the increase in SSO population. Indeed, a delay between rise in microbial popula-

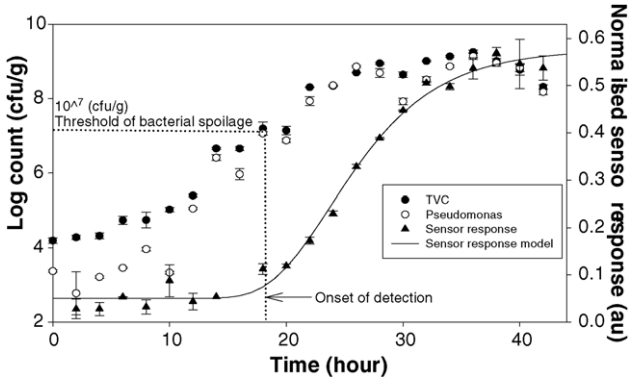

Fig. 8. Correlation of sensor response (1000 rpm) and changes in bacterial population of fresh cod kept at 20 °C over time. Bacterial data are averages of two replicates while sensor data are average of 15 measurements. The error bars are standard error of the mean values.

tion and appearance of the chemical spoilage markers (including TVB-N) has been previously observed by Gram and Huss [7] and Hamada-Sato et al. [4]. Thus, the sensors accurately tracked the increase in volatile base concentration in the package headspace and since the region of change in sensor colour coincides with higher levels of SSO in the fish tissue, the scanner measurements of on-package sensor colour are useful indicators of approximate SSO population and therefore spoilage of the fish samples.

It is however important to mention that since the observed colour change always lags behind the rise in microbial population, this also means that it will not precede it and that false positive results cannot occur. Clearly, this approach can be used to indicate the presence of high microbial populations in packaged fish and identify that it is unfit to eat. However, the correlation is approximate and there may be situations where the microbial population is high and rising, and the sensor colour change is only beginning to happen, i.e. false negatives may happen during this time window before the TVB-N levels have risen in the headspace.

4. Conclusions

We have developed a simple colorimetric sensor and inexpensive measurement device that may enable the condition of a fish product to be independently assessed in terms of its freshness using an immobilised chemoreactive dye formulation and an inexpensive LED-based reflectance colorimeter. The sensor response was found to correlate with bacterial growth patterns in fish samples thus enabling the “real-time” monitoring of spoilage in various fish species. The approach is sensitive to volatile amines, with a linear response to ammonia gas concentration from 0 to 15 ppm.

Future development includes integration of dye deposition with commercial packaging technology. The resulting ‘smart packaging’ incorporating a ‘chemical barcode’ must be compatible with the packaging line and be capable of implementation at minimal cost to the producer.

The advantages of this technology are many. It allows the product to have a longer effective shelf life by allowing freshness to be measured visually along the best-before date, hence reducing margins of error. The added guarantee of product safety for the consumers is of course of primary importance, although wastage reduction (through more accurate estimation of best-before dates) and product confidence is certainly of interest to manufacturers and retailers, as the products must be disposed of whether they are fit to be consumed or not when the estimated best-before date is reached.

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