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Colourimetric plastic film indicator for the detection of the volatile basic nitrogen compounds associated with fish spoilage

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Abstract

The first example of an extruded polymer film containing the pH sensitive dye bromophenol blue (BPB) is described in which the polymer encapsulated dye changes colour from yellow to blue upon exposure to basic volatile nitrogen compounds, such as those given off by fish as it spoils. The latter include: trimethylamine (TMA), dimethylamine (DMA) and ammonia (NH₃), and are collectively known as total volatile basic nitrogen (TVB-N). The films' colourimetric response to specific levels of TMA, as measured using absorbance spectroscopy and digital photography coupled with RGB colour analysis, is reported. The indicator is then used as a fish spoilage indicator at 22 and 4 °C, whilst at the same time a microbiological study is carried out, and in both cases the results reveal a strong correlation between the change in colour of the indicator with the concentration of bacterial colony forming units on the fish; the latter is often used as a measure of fish freshness. The correlation arises because the increase in TVB-N in head space of the package is due to the gradual bacteria-induced decomposition of the fish. The colourimetric TVB-N plastic film indicator's potential as a spoilage indicator for packaged fresh fish is discussed briefly.

Key words: ammonia; amine; indicator; extrusion; colour; fish; freshness

1. Introduction

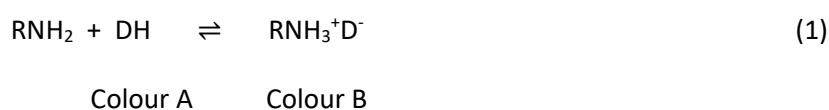
Intelligent packaging is 'a packaging system that is capable of carrying out intelligent functions (such as detecting, sensing, recording, tracing, communicating, and applying scientific logic) to facilitate decision making to extend shelf life, enhance safety, improve quality, provide information, and warn about possible problems' [1]. Often the sensing part of intelligent packaging involves at least one fluorometric or colourimetric indicator that responds to an important packaging parameter [2], such as: the combination of time & temperature [3], or the presence and level of a gas (wanted or unwanted) inside the package, such as: CO₂ [4], O₂ [5], volatile amines [6] or ammonia [7]. Colourimetric indicators are generally less common than their fluorometric counterparts [8], but have the advantage that they can be readily assessed by eye or using digital image technology which is now commonly available and inexpensive [9, 10]. Intelligent packaging is particularly relevant to the fisheries industries which face ever-increasing regulative constraints relating to the safety and traceability of fresh and processed fish [11]. As a consequence, there is a real need in the fish industries for a simple, easy to use, inexpensive method for determining fish freshness after packaging, i.e. a quality control indicator for packaged fish [6, 11, 12]. Such an indicator would be a useful quality control tool as the packaged product makes its way from the packager to the retailer and then onto the customer [13].

A European Union recognised indicator of freshness is the total volatile basic nitrogen (TVB-N) present in the flesh of fish [14], due to volatile amines, such as trimethyl amine (TMA), dimethyl amine (DMA) and ammonia (NH₃), that are responsible for the characteristic 'fishy' odour associated with spoiled fish; the EU TVB-N spoilage threshold is 35 mg per 100 g of flesh, anything above this level is deemed as unsuitable for consumption [11]. TVB-N level is a measure of fish freshness because food spoilage bacteria are responsible for the generation of the volatile amines and ammonia *via* the metabolic breakdown of amino acids in fish flesh [12]. Although a world-wide standard of fish freshness, the usual method for measuring the TVB-N level in fish involves a number of complicated steps and titrations which take typically 4h for one analysis [15].

Given the relationship that exists between TVB-N and bacteria levels, it is not surprising therefore, that another popular measure of fish freshness is the level of bacteria present in the fish. The surface microbial population on a test sample, assessed by taking swabs, is called the total viable count (TVC), with units of: colony forming units (cfu) [16] and it is usually suggested that fish is no longer safe to eat once the measured TVC level exceeds 10⁷ cfu g⁻¹ [17]. Note, however, that exact spoilage thresholds, i.e. the TVB-N and TVB levels employed to identify spoiled fish, can vary greatly since they depend on season, maturity and, in particular, species [18]. Unfortunately, the measurement of TVB-

N, or TVC levels, is non-trivial, time-consuming and destructive of the sample and/or package and so 100% quality assurance (QA) of packaged fish is not possible, and so QA is limited to regular sampling, usually at the packaging stage. It follows from the above discussion that a simple, inexpensive, disposable, non-toxic, quantitative indicator of either of these parameters would benefit greatly the fisheries industries and consumer.

Relevant to the development of a TVB-N colorimetric ink-based sensor is the fact that this group [19] and others [20] have reported the development of ammonia indicators, based on the response of a pH indicator dye *via* the following simple colour-changing general reaction:



where R = H and DH and D⁻ are the differently coloured protonated and deprotonated forms of a pH dye, respectively. Not surprisingly, this system is also sensitive to volatile amines, where R, in reaction (1), is usually an alkyl group, and so has led to their use by others [6, 12, 17, 21-25] as fish freshness indicators based on the assumption that the colour change exhibited by the indicator can be used to identify either the TVB-N [17, 25], or TVC [6, 12, 23, 25], threshold level. To date, a typical fish freshness indicator, based on reaction (1), comprises a pH indicator dye encapsulated in a water-soluble polymer medium (EP = encapsulation polymer) and covered by a gas-permeable, ion-impermeable, membrane (GPM) so as to prevent dye leaching from the indicator film into the food package itself. The latter feature, if exhibited, is obviously completely unacceptable and yet not at all unlikely without a GPM isolating layer since the inks used to make the TVB-N indicator films are usually water-based, and all fresh packaged fish has a water activity coefficient *ca.* 0.99 [26], so that the relative humidity, RH, inside the package is *ca.* 100%, making water contact with the label likely, either through direct contact with the fish or *via* condensate on all the interior surfaces of the package, including the lidding and any indicator that may be placed there.

Monitoring the change in colour of the indicator is usually carried out using absorbance spectroscopy or *via* L*a*b chromaticity measurements. The former technique is ideal for carrying out quantitative analysis of reaction (1) *via* the concentrations of DH and D⁻, i.e. [DH] and [D⁻], respectively, but is relatively expensive and not conducive to the routine and rapid analysis of an indicator incorporated inside a food package. In contrast, chromaticity measurements are much simpler and cheaper to make, as is digital image colour analysis, but, often when used, the relationship between absorbance and the colour measuring parameter is rarely established and so cannot be used to extract out relative values of the [DH] and [D⁻] and so probe reaction (1) in a quantitative manner. Instead, colour analysis methods are almost always employed simply to construct a calibration graph relating the measured

colour parameter to the concentration of the analyte of interest, such as ammonia or TMA, but rarely correlated to the actual TVB-N or TVC levels in the fish, as noted for most entries in Table 1, which lists most of the fish freshness indicators that have been reported to date. Note: in only one case [25] is the sensor response correlated to the measured TVB-N **and** TVC in the fish.

In a recent paper, this group reported a naked, i.e. no additional GPM layer, low density polyethylene (LDPE) plastic colour-based sensor for CO₂, which was created by blending in an extruder, LDPE with a CO₂-sensitive pigment, comprising a coating of a colour-based pH-sensitive, dye (thymol blue) on nanoparticles (principal particle size, ca. ~20 nm) of hydrophilic, fumed silica [27]. The CO₂-sensitive indicator plastic film used was tested using both **gaseous** and dissolved CO₂ and, importantly, in the latter case showed no signs of dye leaching into the aqueous solution because the LDPE encapsulation medium acts as an intrinsic gas-permeable membrane. The stability of these plastic film CO₂ indicators in aqueous solution is in marked contrast to CO₂ based ink films which tend to lose dye in aqueous solution and so require a GPM [27, 28]. Since polymer film extrusion is a very simple, easily scaled-up process, it follows that such indicator films are inexpensive to manufacture and, indeed, this technology forms the basis of the 'After Opening Freshness' indicator label recently produced by Insignia Technologies Ltd. and currently being trialled by Sainsbury's on their fresh ham packages [29, 30].

The above work suggests that it should also be possible to make an extruded plastic indicator film for ammonia and volatile amines, which does not require an additional gas permeable membrane unlike the ink indicators in Table 1, and, like the CO₂ plastic film indicators reported recently [27], will be inexpensive to mass produce and easy to use. Thus, this paper describes, for the first time, the production and characterisation of a new colourimetric extruded plastic film indicator, which has no need for an additional GPM layer, for the detection of the volatile amines and ammonia and its subsequent use as a potential fish spoilage indicator.

Table 1 – Reported amine/TVB-N indicators

Dye	EP	GPM	T (°C)	Analytical method ^a (for indicator)	Spoilage measurement	Comments	ref
BCG	CA	PTFE	20	Reflectance colorimetry	Cfu	2006/2007. Sensor optical response determined as a function of ammonia, but not correlated to TVB-N. Colour change starts as the fish spoilage reaches 10 ⁷ cfu g ⁻¹ . Cod, grenadier and whiting spoilage are studied.	[6, 12]
BCG	CA	PTFE	19-21	Chromaticity (CIE L*a*b colour space) [21]	Cfu	2014. Largely same indicator as above, although no PTA added, but this time a reflectance spectrometer is used to make the optical measurements necessary to allow the calculation of the colour difference parameter, ΔE* [22]. Sensor response determined as a function of the concentration of TMA in solution <i>via</i> the level of TMA in the vapour, although the actual TMA level in vapour was not determined. Fish spoilage (<i>via</i> TVC) was also measured, but no attempt made to correlate with optical response. The work is focussed on the spoilage of mackerel.	[23]
BCG and other dyes	Silica gel	none	4 and 20	Colour response converted to grey scale intensity.	Cfu and TVB-N	2016. Indicator dyes on silica gel plates used to create an array of indicators. Colour of different indicators determined using a flatbed scanner. Reported TVB-N levels in fish correlate with the optical response of the indicator arrays, although no calibration graph given. Atlantic salmon spoilage is studied here.	[17]
BTB/PR	Not given	PET	4, 10, 20	Chromaticity (CIE L*a*b colour space) [21]	NH ₃ (by gas chromatography)	2016. The colour difference parameter ΔE* is linearly correlated with ammonia concentration. Indicator changes colour as fish spoils but no attempt made to relate colour change to TVC or TVB-N in fish. Skate spoilage studied.	[24]
AC-beads	none	none	4, 25	Chromaticity (CIE L*a*b colour space) [21]	Cfu and TVB-N	2018. Beads fixed to lid of closed petri dish containing fish. <u>ΔE* correlated to TVB-N content and variation in bacterial populations.</u> Rainbow trout spoilage is studied.	[25]

a: always as a measure of TVB-N level in gas phase of packaging

EP: encapsulating polymer; BCG: bromocresol green; CA: cellulose acetate; PTA: phase transfer agent; BTB/PR: a mixture of bromothymol blue and phenol red; AC: anthocyanin (red cabbage dye) in alginate beads; PTFE: polytetrafluoroethylene; PET: polyethylene terephthalate.

2. Experimental

2.1 Materials

All chemicals were purchased from Sigma Aldrich, unless otherwise stated, and in the highest purity available. All solutions were prepared on the day, and all aqueous solutions were made up using double-distilled and deionised water. The gases were purchased from BOC. The fine, low-density polyethylene, LDPE, powder (MFI = 20) used for preparing the masterbatch was supplied by PW Hall (Glasgow). The SiO₂ (Aerosil® 130 hydrophilic fumed silica) nanoparticles, used to make the TVB-N sensitive pigment, was a gift from Evonik (BET surface area = $130 \pm 25 \text{ m}^2 \text{ g}^{-1}$, particle size ca. ~20 nm). The cod fillets used to test the indicator film and in the assessment of the degree of microbiological spoilage with storage time, were purchased from a local branch of the supermarket chain Tesco plc and used on the day of purchase, with a best before date 3 days in advance of their use.

2.2 TVB-N pigment preparation

In a 120 cm³ beaker were placed 100 cm³ of an aqueous dispersion of 2 g of Aerosil® hydrophilic silica, followed by 0.2 g of the protonated form of the pH indicating dye, bromophenol blue (BPB), i.e. HBPB; BPB has a pK_a = 3.85 [31]. The mixture was sonicated for 10 minutes in an ultrasonic bath to dissolve the dye and produce a homogeneous dispersion of the particles; the resulting clear, yellow-coloured suspension was then stirred vigorously using a magnetic stirrer for 2 hours. A bright yellow, dry powder pigment, comprising Aerosil® hydrophilic silica particles coated with BPB, was then obtained by spray-drying the suspension using a Buchi Mini Spray Dryer B-290. This pigment responds to the presence of volatile nitrogen species, such as ammonia or volatile amines, by turning from an initial bright yellow colour to dark blue, due to reaction (1). Photographs of the pigment before and after exposure to trimethyl amine (TMA) vapour are illustrated in the supplementary information, figure S1. The colouration of the pigment upon exposure to TMA vapour is virtually instantaneous, and, under quiescent, ambient conditions, the pigment recovers its original colour after ca. 24 h, once the source of TMA has been removed.

2.3 TVB-N indicator preparation

The BPB-coated hydrophilic silica pigment was blended in a 1:9 (w/w) ratio with fine LDPE powder until a uniform yellow colour was achieved. This 10 wt% pigmented polymer powder was then pelletised using a Rondol Microlabs Twinscrew extruder, with operating temperatures of 90, 115, 125, 135 and 125°C for the feed, zones 1-3 and pelletising die, respectively, and a pigment/LDPE mixture feed hopper rate of 41 rpm. The extruder screw speed was 80 rpm and the pelletizer speed was 0.5 m min⁻¹. The resulting pellets were then re-extruded as pellets to ensure that the pigment was

dispersed evenly throughout the LDPE polymer encapsulating material. These pellets were then extruded into a thin, clear yellow-coloured plastic film (ca. $42 \pm 2 \mu\text{m}$), with 10 wt% pigmentation using the Rondol Microlabs Twinscrew extruder, with operating temperatures of 90°C , increasing to $110\text{--}125\text{--}135^\circ\text{C}$ and finally 140°C for the feed, zones 1-3 and the sheet die, respectively. The film was cut into 25 mm diameter discs for testing. As illustrated in figure S2 of the supplementary information, when exposed to a stream of argon gas, which had previously passed through a 4 wt% aqueous solution of trimethyl amine (TMA), the indicator film changed from yellow to blue in about 21 min, due to reaction (1), where DH is the protonated form of the pH indicating dye, i.e. yellow-coloured HBPB, and D^- , the deprotonated form, blue-coloured BPB $^-$ and RNH_2 is TMA. Other work showed that the indicator responded similarly to ammonia and dimethyl amine. However, in order to simplify the study, this work focussed on the response characteristics of the indicator towards just one of these volatile nitrogen species, namely: TMA. Batch to batch reproducibility of the indicator film was $>95\%$ and the small difference in colour was attributed to a difference in polymer film thickness, rather than film composition.

2.4 Methods

All UV-vis spectra were recorded using a Cary 60 UV-vis spectrophotometer. All digital images were captured using a Canon EOS 700D equipped with an EFS 18-135 mm lens and analysed using freely available software (Fiji ImageJ) [32]. Digital image analysis was carried out by extracting the values of the digital colour defining parameters: Red, Green and Blue, i.e. RGB, each of which lie in the range 0-255, from the image of the TVB-N indicator film under test using the ImageJ software [32]. These values were then used to calculate the value of the normalised Blue parameter, nB , since here Blue showed the most striking change in colour and value when the indicator is exposed to TMA, where:

$$nB = B/(R + G + B) \quad (2)$$

The fish was packaged in a heat-sealed package (dimensions: 180 x 135 x 50 mm) made of amorphous polyethylene terephthalate (A-PET), using a Mantle Packing Rotopack SVR semi-automatic modified atmosphere tray sealer. The lidding material was PET, with a film thickness of $30 \mu\text{m}$.

2.5 Sensor Calibration

In order to measure the sensitivity of the indicator towards TMA, gas mixtures of N_2/TMA , containing different levels of TMA, were generated by bubbling nitrogen gas through a Drechsel bottle containing 100 cm^3 aqueous solutions with different wt% levels of TMA (0.1-4.0 wt%) The concentration of the TMA in the gas phase for each of these solutions was determined by trapping the TMA in the associated vapour (1 hour at a flow rate of 100 mL min^{-1}) with 100 cm^3 of a 0.5 M boric acid and then

titrating the latter with 0.01 M NaOH [15]. Thus, for example, a typical experiment revealed that a 4 wt% aqueous solution of trimethyl amine (TMA) yielded a gas-phase concentration of TMA of 0.125 mM. The same TMA amine vapours were then flowed through a gas cell placed in a UV-vis absorption spectrophotometer, containing a 25 mm disc of the plastic film BPB indicator, which allowed the spectral changes of the TVB-N indicator to be recorded as a function of exposure time for each of the TMA vapours tested. This data then allowed the construction of a calibration graph of the absorbance of the TVB-N film at 605 nm (due to the blue coloured deprotonated form of BPB, i.e. BPB⁻) after 2h as a function of the concentration of TMA in the vapour, i.e. $[TMA]_{vap}$.

2.6 Microbiology

All solutions for microbiological detection were prepared fresh and sterilised in an autoclave prior to use. A soybean casein lecithin polysorbate culture medium broth (SCDLP) and agar solution were prepared as described elsewhere [33]. The agar solution was sterilised in an autoclave, after which it was carefully poured into standard count plates (approximately 25 cm³ per plate) before being left to cool and set at room temperature. The agar plates were stored in the fridge at 4°C when not in use, and allowed to reach room temperature prior to being used.

The fish was cut into 100 g fillets which were then placed into individual 180 x 135 x 50 mm plastic trays which were then sealed in air with a PET plastic lid. 2 identical sets of the 100 g packaged cod fillets were kept at 22°C, one containing the TVB-N indicator film (which was photographed), and the other one was sampled for microbiological testing; both photography and sampling were carried out regularly and at the same time, t , after the initial sealing of the package. In the latter work, at each time interval, a 10 g sample of the fish was taken aseptically from the package (which was then resealed) and mashed into 10 cm³ of SCDLP solution. Six, ten-fold serial dilutions of the SCDLP/fish extract were then made using saline solution and 0.1 cm³ of the final diluted sample were added to sterile petri plates containing ca. 25 cm³ standard plate count agar, and spread using an L-spreader. After 10 minutes of drying, the plates were incubated at 37°C for 24 hours. The colony forming units (cfu's) were then counted to determine the original microbial content of the fish (units: colony forming units (cfu) per cm³) for time, t , after the initial sealing. The whole procedure was then repeated but this time at 4 °C, rather than at room temperature, i.e. 22°C.

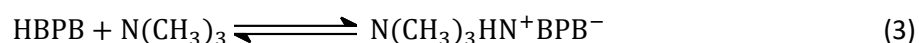
3. Results and Discussion

The TVB-N plastic film indicator, the preparation of which is described in section 2.3, was first tested for response to TMA vapours of different concentrations and then used to monitor fish spoilage at two different temperatures, namely, 22 and 4 °C, respectively, in order to explore the extent its response correlates with TVC level. The results of this work, and some additional experiments on dye leaching, are described and discussed in the following sections.

3.1 Correspondence between Indicator absorbance and colour analysis measurements

As noted earlier, with colour-based indicators absorbance measurements are highly desirable given that they can be used to probe reaction (1) in a more analytical manner, by providing a measure of the relative values of the [DH] and [D⁻], where, in this work, DH and D⁻ are, respectively, the protonated and deprotonated forms of the pH indicator dye BPB. In contrast, digital photography, coupled with colour analysis, is a less expensive and a more suitable technique for monitoring the response of colour-based indicators *in situ*, i.e. inside the package. However, as noted earlier, it is not often that the relationship between these two experimental parameters, i.e. absorbance and digital image RGB colour analysis derived values, like *nB* in this work, is explored and exploited so as to allow a more rigorous quantitative analysis of the system, although there is increasing interest to do so [9, 34, 35] and is the basis of this initial investigation of the optical response of the TVB-N plastic film indicator to TMA vapour.

The absorbance spectrum of a standard TVB-N plastic indicator film was monitored using UV-vis spectrophotometry, and photographically, as a function of time at room temperature (22 °C), over a period of 120 min, following its exposure to the amine rich vapour above a 4 wt% aqueous solution of trimethyl amine (TMA); as noted earlier under these conditions the measured value of the [TMA]_{vap} is 0.125 mM TMA. The recorded changes in absorption spectrum, and in appearance of the TVB-N plastic film indicator, as a function of exposure time are illustrated in figure 1(a). The observed colour changes are due to reaction (1), or more precisely, so as to reflect the actual amine and pH indicator dye used:



Thus, the initial yellow protonated form of BPB in the TVB-N indicator film, HBPB, which has an absorption spectrum wavelength maximum (λ_{max}) at 450 nm is slowly, over 2 h, converted to its blue deprotonated form, $\lambda_{\text{max}} = 605$ nm, due to the equilibrium reaction (3). The sluggish response of the TVB-N plastic film indicator, i.e. ca. 120 min, is attributed to the low permeability of TMA in low density

polyethylene, coupled with the relatively thick ($\sim 42 \mu\text{m}$) nature of the plastic film.

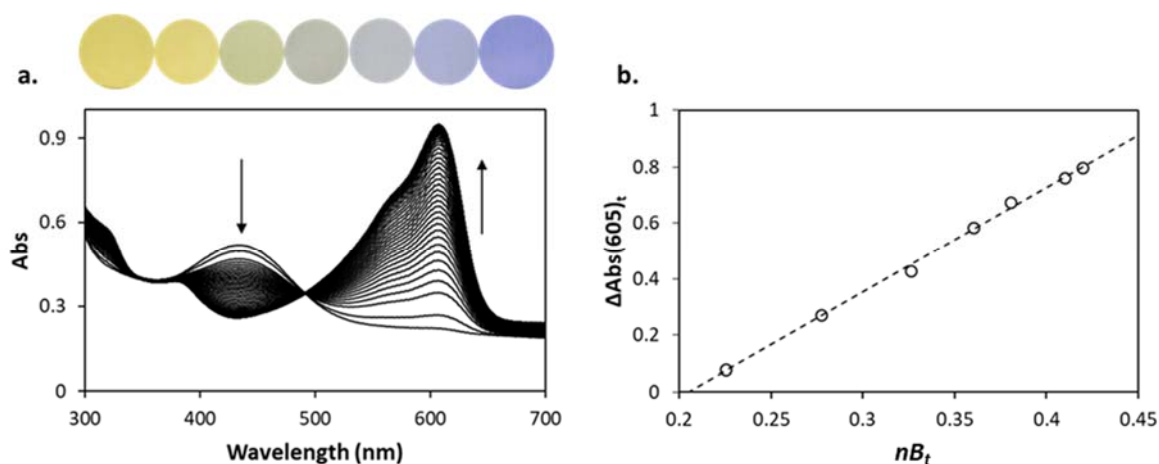


Figure 1 - (a.) Absorbance spectra of a typical TVB-N plastic indicator film (ca. $42 \mu\text{m}$), showing the conversion of the original yellow-coloured film to blue (also shown as a series of digital images above the graph) upon exposed to a 0.125 mM TMA vapour; the spectra were recorded every minute; (b) plot of the change in the absorbance at 605 nm , $\Delta\text{Abs}(605)_t$, calculated using the absorbance data in (a) as a function the normalised RGB blue parameter, nB_t , values for which were calculated using the R, G and B values extracted from the digital images of the TVB-N indicator illustrated in (a).

The spectral changes illustrated in figure 1(a) allow the change in absorbance at 605 nm , i.e. $\Delta\text{Abs}(605)_t$, to be determined as a function of TMA vapour exposure time, t , where $\Delta\text{Abs}(605)_t$ is the difference in absorbance at 605 nm at time, t , and that at $t < 0$, i.e. before exposure, when no BPB^- is present. Note: from Beer's law, $\Delta\text{Abs}(605)_t$ is proportional to $[\text{BPB}^-]$, or more precisely, $[\text{N}(\text{CH}_3)_3\text{HN}^+\text{BPB}^-]$, in reaction (3). Similarly, RGB colour analysis of the digital photographs illustrated in figure 1(a) allow the value of nB_t to be determined and the plot of $\Delta\text{Abs}(605)_t$ vs. nB_t , illustrated in figure 1(b), reveals a simple, direct relationship between these two optical parameters, indicating that the value of nB_t is a direct measure of $[\text{N}(\text{CH}_3)_3\text{HN}^+\text{BPB}^-]$. This feature is invaluable when it comes to using the TVB-N indicator in food packaging, where recording the UV-vis absorption spectrum is no longer practical, but where a quantitative method for determining the concentration of $[\text{N}(\text{CH}_3)_3\text{HN}^+\text{BPB}^-]$ is required.

3.2 TVB-N indicator calibration

The optical response of the TVB-N indicator plastic film was then probed as function the level of TMA in the gas phase, $[\text{TMA}]_{\text{vap}}$. The latter parameter was varied by filling a Drechsel bottle with different wt.% dilute TMA solutions ($0 - 4 \text{ wt}\%$), and bubbling a continuous stream of nitrogen (100 mL min^{-1}) through each solution. Each gas stream, produced in this manner, with its different level of TMA in the vapour phase, i.e. $[\text{TMA}]_{\text{vap}}$, was then flowed through an optical gas cell, placed in a UV-vis spectrophotometer containing the TVB-N indicator. Figure 2(a) illustrates the resulting $\text{Abs}(605)_t$ vs

time plots determined using the TVB-N indicator when exposed to the different TMA levels in the vapour phase. These results show that within 2 h of exposure to each TMA vapour, the colour (and so absorbance) of the indicator attains a steady state value, a measure of which is $Abs(605)_{eq}$ and a feature that is consistent with the equilibrium nature of reaction (3).

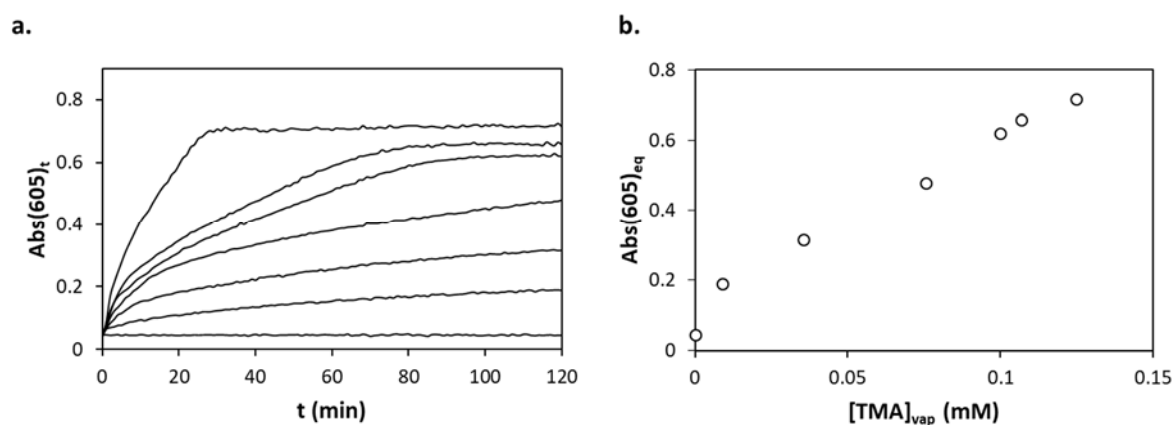


Figure 2 - (a.) Colour change profiles at 605 nm for TVB-N plastic film after exposure to trimethyl amine vapour of different concentrations; which were (from bottom to top: 0, 0.01, 0.036, 0.076, 0.1, 0.107 and 0.125 mM,, respectively, as determined by acidimetric titration, and (b.) a plot of $Abs(605)$ vs. $[TMA]_{vap}$ (mM).

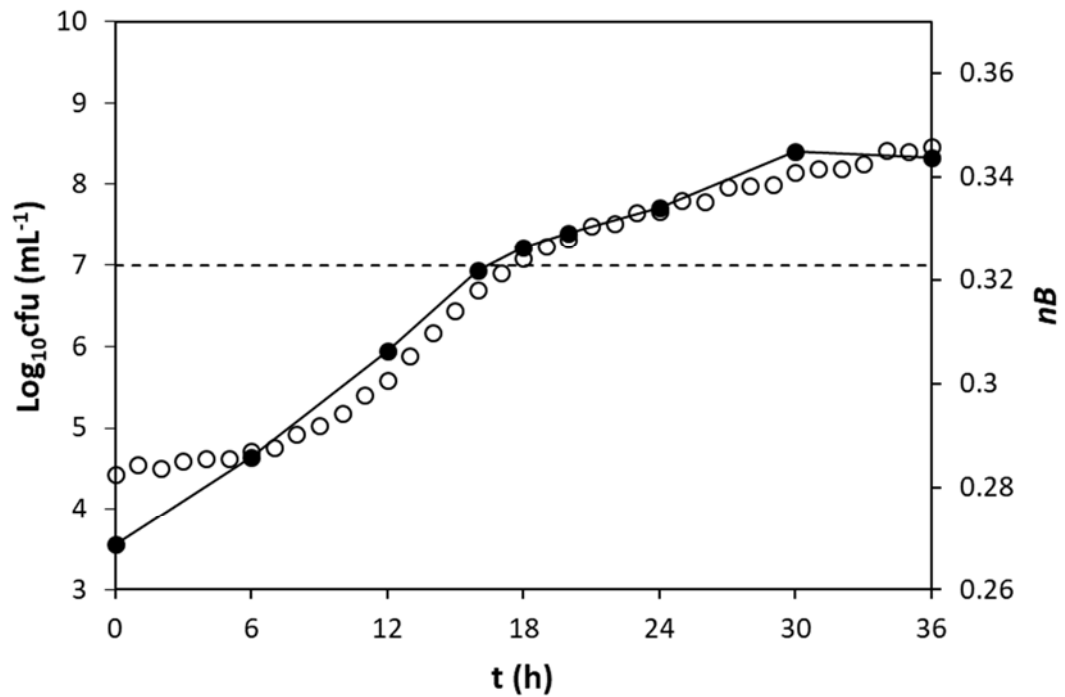
As outlined in the Experimental section 2.5, other experiments were carried out to measure $[TMA]_{vap}$, i.e. the different levels of TMA in the vapour phase, when N_2 was bubbled through the Drechsel bottle with the different wt.% dilute TMA solutions (0 – 4 wt%). These two data sets were then combined to generate the plot of $Abs(605)_{eq}$ vs $[TMA]_{vap}$, illustrated in figure 2(b), which yielded a straight line, thereby indicating that $Abs(605)_{eq}$, and therefore $[N(CH_3)_3HN^+BPB^-]$, is directly proportional to $[TMA]_{vap}$. It follows from the work in section 3.1 that nB will also be proportional to $[TMA]_{eq}$ and suggests that the more convenient and simple to measure colour analysis parameter, nB , can be used as a measure of fish freshness for packaged fish, since others have shown that *there is a direct correlation between fish freshness, as measured by TVB-N in fish, or spoilage bacterial levels and the level of TVB-N in the package headspace* [36, 37]. This latter finding is not too surprising given that fish freshness is already routinely linked to the level of TVB-N in the fish itself [14, 17, 25].

3.3 TVB-N indicator and fish spoilage

In order to test how well the TVB-N indicator responds to packaged fish spoiling at room temperature, i.e. 22°C, a 25 mm diameter disc of the plastic film was stuck to the inside of the lidding of a package containing 100 g of fresh cod, which was then sealed. Photographs of the indicator were taken immediately after packaging, and then every hour for 36 hours. The photographs illustrated in figure 3 show that a clear colour change, from yellow to blue, is observed in the TVB-N indicator as the fish spoils and takes on a slightly waxy appearance.



(a)



(b)

Figure 3 (a) Digital images of the indicator film packaged above cod at (left) 0, (centre) 24, and (right) 48 hours at room temperature (22°C) and (b) plot of the measured (closed circles) cfu levels in cod flesh and (open circles) concomitant colour change of the indicator, i.e. nB , as a function of storage package time at 22 °C.

RGB Colour analysis of the photographs taken of the indicator as a function of storage time generated a series of nB values for the different package times at 22 °C, and a plot of this data is illustrated in

figure 3(b). This colour change is due to the indicator responding to the increase in TVB-N concentration in the headspace, which others [16,25,36] have shown to be correlated directly to fish freshness based on the TVB-N level in fish and the TVC. However, in the past, the quantitative assessment of [TVB-N] in the vapour phase has required an involved analytic procedure, such as the laborious traditional TVB-N titration method [15,36], or expensive GC analysis [38], whereas here we use a simple, inexpensive plastic film TVB-N indicator, with an easily measured optical response, nB , which is proportional to $[TVB-N]_{vap}$, and so the level of TVB-N in fish.

As noted previously, the TVB-N levels detected in the headspace of the package by the indicator are due to the TVB-N in the fish which in turn is due to the growth of fish spoilage bacteria. Others have reported that $[TVB-N]_{vap}$ and the fish spoilage bacterial population, as measured by $\log(cfu)$, are directly correlated [16, 25]. In order to demonstrate this relationship in this work, a microbiological study was carried out under the same conditions as in figure 3(a), in which the bacterial levels, in units of $cfu\ mL^{-1}$, in the flesh of the fish were monitored over the same 36-hour period. The results of this study are also illustrated in figure 3(b) and reveal the expected strong correlation in shape between nB (which provides a direct measure of $[TVB-N]_{vap}$, see figure 2(b), and $\log(cfu)$).

The same set of experiments as outlined above were also conducted at 4 °C and the results of this work are illustrated in figure 4. Interestingly, the major difference between the two data sets illustrated in figure 3(a) and figure 4, is time scale, in that, at 22°C, it takes about 18 h for the bacterial levels in the fish to exceed $10^7\ cfu\ mL^{-1}$, indicated by the horizontal broken line, whereas at 4 °C, it takes nearer 4.5 days. Strikingly, in both cases the nB and $\log(cfu)$ time profiles are strongly correlated. Indeed, an nB value of ca. 0.32, which is associated with a particular green colouration of the TVB-N indicator, can in both cases be used as an indication that the fish is spoiled and no longer safe to eat. In practice, the identification of this point might be made using a digital mobile photo camera and colour analysis App or, more simply, using a colour matching card.

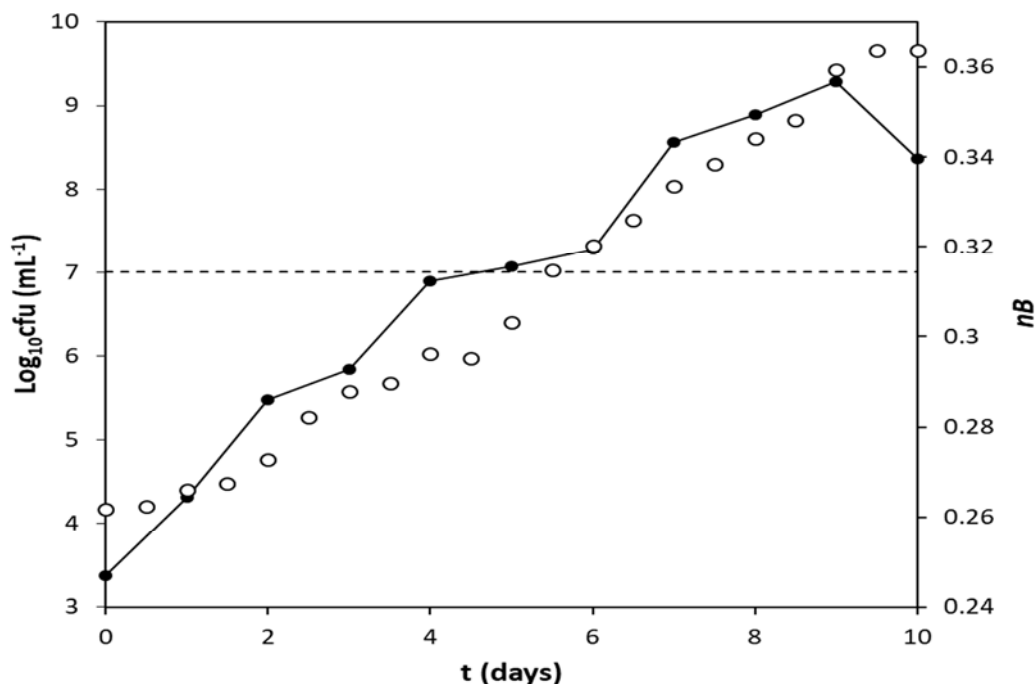
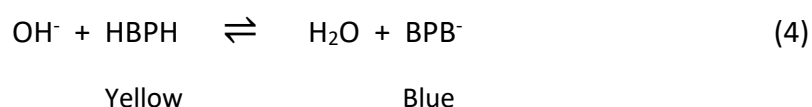


Figure 4 Plot of the measured (closed circles) cfu levels in cod flesh and (open circles) concomitant colour change of the indicator, i.e. nB , as a function of storage package time 4 °C.

3.4 Indicator stability in aqueous solution

As noted earlier, a key feature of this new plastic TVB-N indicator is that the LDPE should act as a GPM, and protect it from dye leaching and also prevent ingress of hydroxyl ions into the indicator film which would give a false positive response to TVB-N. Given the high water content of fresh fish the presence of water in any package is high and so too, therefore, is the likelihood of water contacting the indicator; thus, the stability of the TVB-N indicator in water is essential. In order to demonstrate this feature the indicator was stored in water for many weeks and exhibited no loss of dye or change in response. In a more demanding test, the indicator was also placed in a highly alkaline solution (0.1 M NaOH) and showed no change in colour even after 48 h. In contrast, a traditional ink-based ammonia indicator [19], similar to those reported in Table 1, changed colour (yellow to blue) instantly due to the simple deprotonation reaction:



When a drop of TMA solution is added to the TVB-N plastic film indicator in either aqueous solution

or the highly alkaline solution, the indicator turned blue immediately due to the TMA vapour diffusing through the gas permeable, but not ion permeable, LDPE GPM. Illustrative photographs of the film in some of these experiments are given in the Supplementary information, figure S3.

4. Conclusions

A colourimetric volatile amine sensor which undergoes a colour change from yellow to blue in response to TVB-N in the vapour phase and can be used to indicate the spoilage of fish has been developed. The indicator comprises a pH indicator, BPB, coated onto fumed silica to create a pigment that can be readily dispersed through a thin film of LDPE *via* extrusion. The colour change, as measured by absorbance or the normalised colour parameter, nB , exhibited by the TVB-N indicator is proportional to the concentration of TVB-N (in the form of TMA) in the headspace. In a study of packaged fresh cod, at room temperature (22 °C) and 4 °C, the colour response of the TVB-N indicator recorded as a function of time, correlates very closely to that the \log_{10} of the measured bacterial colony forming units on the cod. The plastic film TVB-N indicator appears impervious to dye leaching or ion migration, both of which can cause similar ink-based films to fail. These results suggest that the TVB-N plastic indicator film may find application in the fish packaging industry as a food spoilage indicator, especially if combined with a simple colour measuring technique, such as colour analysis coupled to digital photography or, more simply, colour-matching.

5. References

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