Development and validation of a colorimetric sensor array for fish spoilage monitoring

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ABSTRACT

Given the need for non-destructive methods and sensors for food spoilage monitoring, we have evaluated sixteen chemo-sensitive compounds incorporated in an array for colorimetric detection of typical spoilage compounds (trimethylamine, dimethylamine, cadaverine, putrescine) and characterized their color changes in response to compounds present in fresh products (hexanal, 1-octane-3-ol) used as negative controls. The colorimetric sensor array was used to follow fish spoilage over time at room temperature for up to 24 h as well as at 4 °C for 9 days. Additionally, fish decay was monitored using traditional assays measuring the quantity of thiobarbituric acid, total volatile basic nitrogen, changes in pH, O2 level, as well as following bacterial growth. We found a linear correlation between changes in pH, thiobarbituric acid content and the signal intensity recorded with the colorimetric array over time. During spoilage, the increase in signal intensity of the chemo-sensitive compounds showed a similar trend as the increase in microbial growth. We observed that the sensitivity of the chemo-sensitive compounds depends on the spoilage conditions (room temperature vs. 4 °C), highlighting the importance of the application of an array instead of single chemo-sensitive compounds when following complex changes during food spoilage.

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1. Introduction

Freshness is one of the main quality attributes for fish and meat processing, marketing and consumption. Fish and meat are gradually becoming the favored food of people in many countries as they are rich in proteins; however, the disadvantage is the comparatively short shelf-life of the products (Grau et al., 2011). Spoilage is a metabolic process that makes the products undesirable or unacceptable for human consumption due to changes in sensory characteristics (Ocaño-Higuera, Maeda-Martínez, Marquez-Ríos, & Canizales-Rodriguez, 2011). Aside from spoilage induced changes in the sensory characteristics, contamination of food products with foodborne pathogens due to malpractice in handling, processing or post process storage of the products remains an important public health issue leading to illness (Tassew, Abdisa, Beyene, & Ebre-Selassie, 2010).

Monitoring of changes in the quality of foodstuffs is a major challenge, especially in the case of fast degrading fish-based products. Odor is one of the most important parameters when evaluating the freshness of food. Each product has a characteristic profile of volatile compounds and, therefore, its own characteristic odor. Likewise, spoilage results in a different, but nevertheless, characteristic profile of volatile compounds in the same product. The odor caused by spoilage has been attributed to amines (e.g., trimethylamine (TMA), dimethylamine (DMA), ammonia, histamine, putrescine (Put), cadaverine (Cad)), short-chain carboxylic acids, sulfur compounds, N-cyclic compounds as well as unsaturated aldehydes, which may be used for monitoring fish spoilage (Ólafsdóttir & Kristbergsson, 2006). Increase in total volatile basic...
nitr 02 (TVB-N), primarily composed of ammonia, TMA and DMA, as well as the degree of secondary lipid oxidation indicated by thiobarbituric acid (TBA) level, as a result of microbial degradation are widely used to evaluate fish freshness (Egan, 1987). However, most of these approaches are time and resource consuming as well as invasive and destructive methods. The demand for new, non-invasive, cost- and time-effective sensing technologies for detection of bacterial contamination and spoilage has significantly increased in the recent years (Carey et al., 2011; Pacquit et al., 2006; Puligundla, Jung, & Ko, 2012). During the last decade, the application of chemo-sensitive compounds for the development of colorimetric sensors for food safety and quality control has become popular, since they are relatively inexpensive and simple to use, facilitating detection of volatile compounds in the headspace of food packaging without destroying the product (Pacquit et al., 2006). Chemo-sensitive compounds have been used for evaluation of food freshness (Chen, Hui, Zhao, & Ouyang, 2014; Huang, Xin, & Zhao, 2011; Huang et al., 2014; Pacquit et al., 2006; Salinas et al., 2014) with the capability to differentiate between bacterial contamination (Carey et al., 2011). In the case of fish spoilage evaluation, colorimetric sensors have been devised based on either a single chemo-sensitive compound (Kuswandi, Restyana, Abdullah, Hong, & Ahmad, 2012; Pacquit et al., 2007; Pacquit et al., 2006) or an array of such compounds (Huang et al., 2011; Zaragozá et al., 2012). Given the technological development in the field of smartphone based detection/readout (Hong & Chang, 2014; Salles, Meloni, de Araujo, & Paixão, 2014), application of colorimetric sensor arrays for monitoring food freshness and quality can in the future significantly benefit from such development applicable for intelligent food packaging (Puligundla et al., 2012).

Here, we present an evaluation of an array of 16 chemo-sensitive compounds in terms of their response to standard volatile compounds produced during fish spoilage over time as well as those characteristic for fresh fish. The array was used to follow fish spoilage over time at room temperature (r.t.) and at 4°C. The obtained results have been compared and correlated with changes in O2 level and pH, TVB-N and TBA contents, as well as microbial growth over time. Considering the complexity of food spoilage over time, we propose for monitoring of the process the application of a sensor array composed of multiple chemo-sensitive compounds rather than single or a few compounds.

2. Materials and methods

2.1. Chemicals

The spoilage indicators (trimethylamine (TMA), dimethylamine (DMA), cadaverine (Cad), and putrescine (Put)), the compounds present in fresh fish, (hexanal and 1-octane-3-ol), the chemo-sensitive compounds (Alizarin, Bromocresol Green, Bromocresol Purple, Bromothymol Blue sodium salt, Bromophenol Blue, Xylenol Blue, Chlorophenol Red, Cresol Red, Crystal Violet Lactone, Reichardt's dye, 2,6-dichloro-4-(2,4,6-triphenyl-1-pyridinio)phenolate, Phenol Red, Rosolic Acid, Methyl Red, Curcumin and Carminic acid), as well as thiobarbituric acid (TBA), magnesium oxide, sulfuric acid, sodium hydroxide, formaldehyde, methanol, boric acid, hydrochloric acid and acetic acid used for TVB-N and TBA analysis were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Tryptic glucose yeast agar (CM32S) was obtained from Oxoid Ltd. (Hampshire, United Kingdom) and peptone from Becton Dickinson and Co. (Le Pont de Claix, France). Ethanol (96%) was purchased from Kemetyl A/S (Koge, Denmark). All chemicals were of analytical grade and used without further purification. Stock solutions of the chemo-sensitive compounds (4 mM) were freshly prepared in ethanol or ultrapure water (Millipore Corporation, Billerica, MA, USA) and stored in a lightproof flask before use.

2.2. Sensor array preparation and experimental procedure

2 μL of the fresh stock solution of each chemo-sensitive compound were applied on silica gel Kieselgel 60F254 plates (2.5 x 4 cm) from Merck KGaA (Darmstadt, Germany) in duplicate (Fig. 1) using a micromilled poly(methyl metacrylate) mask to define the spot area (Ø 3 mm) (Kostesha et al., 2010). Immediately after preparation, each sensor array was fixed on the inner side of the lid of a glass jar using a double-sided tape (Fig. 1), the spotted dyes facing the headspace. Each sensor array in a closed jar was exposed for 1 h at r.t. to one of the volatile analytes dissolved in water, i.e. spoilage compounds (200 ppm TMA, 20 ppm DMA, 200 ppm Cad, and 200 ppm Put), and freshness markers (10 ppb 1-octen-3-ol and 10 ppb hexanal). The concentrations of freshness markers and spoilage indicators were defined based on values reported for the fresh (Olafsdottir & Kristbergsson, 2006) and spoiled (Etienne, 2005; Macé et al., 2012; Visciano, Schi rone, Tonolo, & Suzuki, 2012) fish products.

2.3. Sample preparation

Fresh Atlantic salmon (Salmo salar) fillets were purchased from a local market and transported in an isothermal ice box to the laboratory. The fillets were cut in a fume hood using a sterile scalpel to obtain uniform cubical samples. All the cut samples were used immediately for spoilage monitoring in closed jars performed in triplicate at r.t. and 4°C. Spoilage of beef and chicken samples, also purchased from a local market, was determined at 4°C (see Supplementary data). Each sensor array was kept in the closed jar at r.t. (up to 24 h) or 4°C (up to 9 days) until it was removed at a defined time point during time dependent spoilage monitoring. After removal from the jar, each colorimetric sensor array was vacuum packed in order to avoid any possible interference from the environment, and the color changes were recorded using a flatbed scanner (Epson V750-M Pro Perfection scanner). The vacuum packed sensor proved to be stable for at least nine days week stored at 4°C.

2.4. Physicochemical analysis

The contents of TVB-N were determined by steam distillation (Egan, 1987). Minced samples (50 g each) were soaked in PBS for 24 h at 4°C followed by a filtration step. The filtrate (400 mL) was mixed with 30 mL of 96% ethanol and 2 g of magnesium oxide, followed by distillation of the mixture and collection in 25 mL of 0.1 M sulfuric acid. The collected distillates were boiled for 10–15 min to remove carbon dioxide. After cooling to r.t., 0.2 mL of 0.2% rosolic acid indicator was added and the excess of sulfuric acid was immediately titrated back with 0.1 M NaOH. The TVB-N was expressed as mg N/100 g of sample. TBA was determined using a previously published spectrophotometric method (Vyncke, 1970). Briefly, 10 mg of sample was mixed with 50 mL distilled water and 2.5 mL of 4 M HCl and distilled for 10 min. The distillate (5 mL) was mixed with 5 mL of TBA solution (0.28 mg TBA/100 mL of 90% glacial acetic acid), after which the solution was heated in boiling water bath for 35 min and then cooled to r.t. The absorbance at 538 nm was measured using a digital spectrophotometer model 590 (Thermo Scientific, MA, USA). TBA values were expressed in mg malonaldehyde (MDA)/kg sample. pH measurements in fish samples (10 g), homogenized in 100 mL of MQ water, were carried out using a digital pH-meter (model MA 5736, Metrel, Iskra, Slovenia). Oxygen levels were measured with Optech™ Platinum O2 sensor device using disposable O2 sensor stickers from Mocon.
Sent in fresh and Put), while the response to compounds that are typically pre-
sponses when exposed to the spoilage indicators (TMA, DMA, Cad markers

3.1. Sensor array response to spoilage indicators and freshness
determination of the total viable count (TVC) of bacteria. Petri 
dishes with the plate count agar (Oxoid, CM325, Hampshire United
Kingdom) were incubated at 4 °C for 9 days, colony growth was 

2.6. Data analysis

Acquired images were digitalized using a flatbed scanner (Epson 
V750-M Pro Perfection scanner) and the color change in each spot 
was analyzed after the exposure to analytes (Songjaroen, Dungchai, 
Chailapakul, Henry, & Laiwattanapaisal, 2012) using Corel Photo-
Paint X3. Each spot in the array was analyzed manually conver-
ting the obtained color response to gray scale and determining its
intensity. The results obtained from the colorimetric sensor arrays are 
presented as relative response, the signal from the unexposed 
spot (control) representing 100% response.

The response of a whole sensor array, comprising the contri-
bution of all the chemo-sensitive compounds, was determined 
based on a data extraction method described by Alstrøm et al.
(2011). The location of each color spots was identified and the 
mean color value from the inner 75% of the spot (in terms of the 
radius) was calculated and used as the sensor response and 
expressed as signal intensity. In addition, linear data fits using least 
quares regression were calculated in order to identify correlations.
The coefficient of determination denoted by $R^2$ was used as an 
indication of correlation.

3. Results and discussion

3.1. Sensor array response to spoilage indicators and freshness
markers

The individual chemo-sensitive compounds gave different re-
sponses when exposed to the spoilage indicators (TMA, DMA, Cad 
and Put), while the response to compounds that are typically pre-
sent in fresh fish (1-octen-3-ol and hexanal) was very low (Fig. 2).
Among the sixteen chemo-sensitive compounds, Methyl Red 
(189 ± 3%), Bromphenol Blue (170 ± 2%), Bromocresol Green 
(156 ± 10%), Crystal Violet Lactone (152 ± 2%) and Alizarin 
(150 ± 2%) demonstrated the highest signal when exposed to TMA.
The colorimetric response towards DMA was lower than for TMA.

The chemo-sensitive compounds Rosolic acid (136 ± 1%), Cresol 
Red (128.0 ± 1%), Chlorophenol Red (125 ± 1%), Methyl Red 
(125 ± 1%), Xylenol Blue (116 ± 5%), on the other hand, gave the 
highest response to DMA (Fig. 2). Methyl Red, Bromphenol Blue, 
Bromocresol Green and Alizarin gave the highest response when 
exposed to TMA, Cad and Put. The relative standard deviation (RSD) 
between the sensor arrays, prior to exposure to spoilage com-
pounds, was on average below 11% RSD.

Even though Curcumin, Bromothymol Blue sodium salt and 
Bromocresol Purple showed to be the least sensitive towards the 
tested spoilage indicators, they were included in the sensor array, 
taken into consideration that in the case of real samples, given the 
complexity of food spoilage, they might be responsive to other 
compounds than the ones tested here. Moreover, given the large 
variation between the intensity of the responses of individual 
chemo-sensitive compounds towards the tested analytes, we 
propose the application of the whole array for evaluating fish 
spoilage.

3.2. Fish spoilage monitoring at room temperature using the 
colorimetric sensor array

Spoilage of salmon was followed for 24 h at r.t. in closed jars by 
evaluating the changes in the colorimetric sensor array at 1, 2, 4, 8, 
12 and 24 h Fig. 3A shows the sensor array at time zero while Fig. 3B 
presents the array after exposure to spoiled fish sample kept at r.t. 
for 24 h, illustrating that the changes in the color are related to 
compounds released during fish decomposition.

Based on the intensity of the responses of the chemo-sensitive 
compounds to fish spoilage, these were divided into four groups 
(Fig. 3C). The compounds in Group 4 (Methyl Red, Xylenol Blue, 
Crystal Violet Lactone) and Group 3 (Bromophenol Blue, Cresol Red, 
Bromocresol Green) showed a substantial increase in relative signal 
intensity from 2 h (130 ± 11% and 133 ± 30%) up to 6 h (251 ± 17% 
and 187 ± 8%), followed by a slow increase up to 24 h (288 ± 11% 
and 202 ± 10%). On the other hand, the chemo-sensitive com-
pounds in Group 2 (Bromocresol Purple, Bromothymol Blue sodium 
salt, Alizarin, Phenol Red, Chlorophenol Red, Rosolic acid) showed 
only 30 ± 7% relative increase at 24 h. 2,6-Dichloro-4-(2,4,6-
triphenyl-1-pyridinio)pheno|lurate, Curcumin and Carminic acid in 
Group 1, showed only (105 ± 8% at 24 h) relative response, while 
the control array, unexposed to fish spoilage had a small (118 ± 7% 
at 24 h) relative signal intensity increase.

The changes in the signal intensity could indicate the presence 
of volatile organic components, such as amines, which lead to pH 
increase in the headspace, produced as a result of enzymatic 
activities taking place after the postmortem stage. Amines, such as 
TMA, are produced from the decomposition of proteins, in the 
presence of microorganisms causing spoilage, and are increasing 
with the increase in TVC (Kuswandi et al., 2012; Pacquit et al. 
2007). On the other hand, the change in signal intensity could

<table>
<thead>
<tr>
<th>Methyl Red</th>
<th>Rosolic acid</th>
<th>Phenol Red</th>
<th>Reichardt’s dye</th>
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<tbody>
<tr>
<td>Crystal Violet Lactone</td>
<td>Cresol Red</td>
<td>Chlorophenol Red</td>
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<tr>
<td>Bromphenol Blue</td>
<td>Bromothymol Blue sodium salt</td>
<td>Bromocresol Purple</td>
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</tr>
<tr>
<td>Alizarin</td>
<td>Curcumin</td>
<td>Carminic acid</td>
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Fig. 1. Layout of the colorimetric sensor array (each dye applied in duplicate; the red spots (top left) indicate Methyl Red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
also be contributed to by decrease in pH in the headspace as a result of increased CO₂ levels due to bacterial growth occurring during spoilage (Borchert, Kerry, & Papkovsky, 2013; Puligundla et al., 2012).

3.3. Fish spoilage monitoring at room temperature using the oxygen sensor

Besides monitoring fish spoilage using the colorimetric array, we also measured levels of O₂, using disposable O₂ sensors immobilized in the interior of the jar. By following the O₂ levels during spoilage, we could gain information about the onset of spoilage, since it has been shown that decrease in O₂ correlates with bacterial growth (Borchert, Hempel, Walsh, Kerry, & Papkovsky, 2012; Hempel et al., 2011). The O₂ level in the closed jars was stable at 20.0 ± 0.3% until 6 h followed by a fast decrease in the O₂ level down to 9.28 ± 0.01% at 20 h followed by a further decrease to 4.56 ± 0.03% at 24 h. These results indicate that the colorimetric sensor array was able to detect the onset of spoilage earlier than the O₂ sensor since there were relevant changed in the relative signal intensity already between 2 and 6 h.

3.4. Fish spoilage monitoring at 4 °C using the colorimetric sensor array

Fish spoilage was also monitored for a longer period (up to 9 days) at 4 °C (Fig. 4) to mimic spoilage under optimal storage conditions.

The highest signal change over time was recorded from Group 4 (Methyl Red, Crystal Violet Lactone, Xylenol Blue, Bromphenol Blue and Bromocresol Green) (Fig. 4A), showing a fast intensity increase from day 4 (80 ± 10%) up to day 7 (288 ± 33%), followed by a slower increase possibly due to sensor saturation. On the other hand, the chemo-sensitive compounds in Group 3 (Rosolic acid, Cresol Red and Alizarin) and Group 2 (Phenol Red, Reichardt’s dye, Bromothymol Blue sodium salt and Bromocresol Purple) showed signal increase from day 3 (107 ± 18% and 100 ± 13%) up to day 5 (164 ± 23% and 127 ± 7%) prior to reaching saturation (Fig. 4A). The chemo-sensitive compounds in Group 1 (2,6-Dichloro-4-(2,4,6-triphenol-1-pyridinio)phenolate, Chlorphenol Red, Curcumin and Carminic acid) show a very low signal increase (13 ± 10%) at 24 h which is close to the standard deviation. During the 2nd and 3rd day, the signal from the compounds in Group 4 decreased (20%) which could be due to the presence of the compounds typical for fresh fish, since as we observed (Fig. 1) that Methyl Red and Crystal Violet Lactone are sensitive to freshness indicators.

In addition, the sensor response was evaluated based on the overall contribution of all chemo-sensitive compounds during fish spoilage at 4 °C (Fig. 4B). When evaluating the overall response of whole arrays, we found that the signal intensity had a linear relationship with the relative response obtained from the compounds in Group 4. When comparing the response of the compounds in Group 3 and 4, which provided the highest response during salmon spoilage at r.t. and 4 °C, we observed that compounds in the groups showed differences with a few exceptions (Table 1) possibly due to differential sensitivity of each compound to the volatile analytes released during fish spoilage. The reproducibility of the sensor

![Fig. 2. Response of the colorimetric array when exposed to spoilage and freshness indicators represented as changes relative to the control (100%). Error bars represent standard deviation, n = 4.](image)

![Fig. 3. Sensor array at 0 h (A) and after 24 h exposed to spoiling salmon (B). Relative response of the chemo-sensitive compounds (Groups 1–4) during spoilage monitoring at r.t. up to 24 h (C). Error bars represent standard deviation, n = 4.](image)
response between arrays upon exposed to spoilage fish was below 9% RSD.

3.5. TVB-N, TBA, pH changes, and microbial growth in fish at 4 °C vs. spoilage monitored using the colorimetric array

In order to evaluate the accuracy of the sensor array and validate it for detection of fish spoilage over time, several physicochemical parameters (TVB-N, TBA and pH) were also monitored using conventional methods and correlated with the signal from the colorimetric sensor arrays. By quantifying TVB-N, we could follow the production of volatile compounds, such as TMA, DMA and ammonia, produced during microbial degradation (Ritz, Fairchild, & Lacy, 2004). In addition, by determining TBA, we could assess the degree of secondary lipid oxidation, another important indicator of fish spoilage (Abou-Taleb, 2007). We observed that the TVB-N contents were gradually increasing during the storage period from the initial 8.17 mg/100 g to 35 mg/100 g on 9th day, at which fishery products are considered to be spoiled (European Commission, 1995). Changes in TBA level during storage at 4 °C showed a similar trend as TVB-N. The TBA value increased from 0.29 to 1.26 mg MDA/kg fish sample indicating the oxidative breakdown of lipids during spoilage (Fan, Chi, & Zhang, 2008). Based on the fact that most of the chemo-sensitive compounds incorporated in the sensor array are pH indicators, we evaluate the linear relation between the changes in the sample pH and the pH changes measurable in the headspace using the array. Since CO2 level increases during spoilage due to microbial growth, determination of pH changes gives additional information about the rate of spoilage (Borchert et al., 2012). The pH values of fish samples increased during the storage periods from 6.32 to 7.05 in agreement with the values previously reported by Gandotra et al. (Fan et al., 2008; Gandotra, Sharma, Koul, & Gupta, 2012).

As presented in Fig. 5, we found a strong linear relationship between time dependent changes in the TBA content (R² = 0.89) and pH (R² = 0.91) in the fish samples and the response obtained from the sensor array (presented in Fig. 4B), whereas the corresponding linear relation with TVB-N content (R² = 0.73) was weaker. The response of the chemo-sensitive compounds divided in the four groups was also individually correlated with TVB-N and TBA content and pH changes (Table 2). We found that the signal measured from compounds in Group 2, 3, 4 gave a good correlation with TBA and pH changes over time, whereas the response of the compounds in Group 1 did not correlate with any of the determined spoilage parameters. When comparing the R² values for the groups of colorimetric array compounds and the response obtained evaluating the whole array we observe that the R² values for Group 4 and whole array are similar.

In addition, the microbial growth was evaluated by monitoring TVC during the 9 days of storage at 4 °C, indicating that the TVC could be detected already after 3 days (Fig. 6), reaching the threshold of bacterial spoilage (10⁵ CFU/g) (Pacquit et al., 2007) between Day 5 and 6. In comparison when evaluating TVB-N changes the value at which the fishery products are considered spoiled are reached around Day 8. With the colorimetric array, changes could be detected already at Day 4 based on the compounds in Group 3 and 4 as well as overall response of the whole array, which indicates that changes recorded with the sensor array correlate well with the observed microbial growth. While the TVC is increasing substantially from Day 8 to Day 9, the signal intensity of the colorimetric array reaches a plateau, indicating a possible saturation of the sensor. In addition, we were able to monitor beef and chicken spoilage using the same sensor array (Supplementary data).

4. Conclusions

We presented a colorimetric sensor array based on sixteen chemo-sensitive compounds as a non-destructive, monitoring without damaging the food sample, method to monitor fish spoilage during bacterial decomposition. The selected compounds proved to have different sensitivities towards spoilage indicators and showed very low response to compounds typically present in fresh fish. The chemo-sensitive dyes in Group 4 and 3 were the most sensitive for detecting fish spoilage over time both at r.t. and at 4 °C. The colorimetric sensor response was found to correlate well with changes in pH and TBA content at 4 °C and followed a similar time dependent trend as microbial growth profile of spoilage at 4 °C, validating the array for fish spoilage monitoring. The results strongly suggest the potential of the colorimetric array for easy, rapid and effective fish freshness assessment able to inform retailers and consumers about the safety state of the
product. Moreover, the use of colorimetric arrays can be an especially interesting approach in combination with smart packaging for the monitoring food spoilage. Based on preliminary results the sensor array proved to be suitable for monitoring beef and chicken spoilage over time, which is the scope of further validation. The developed sensor array based on multiple chemo-sensitive compounds, in combination with smart readout, can be a non-invasive towards the food sample, low-cost, and effective and user friendly means for monitoring food spoilage over time, instead of endpoint sampling, usable for both consumers and retailers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2015.07.038.

References


