Highlights

- A freshness sensor that can be implemented for food quality assurance in the meat industry has been developed
- The freshness sensor integrated in meat packages allowed tracking the state of meat until its spoilage
- A Smartphone with a custom made App is used as detection system

Smartphone based meat freshness detection

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Abstract

In this work, a freshness colorimetric sensor has been integrated with pork meat packages. The sensor tracks rising CO_2 levels in the package associated with meat spoilage, as CO_2 levels increase with bacterial population. The color of the sensor changes depending on the quantity of bacteria present, therefore it can be correlated with the freshness of meat, in this case pork loin. Detection is achieved by a simple photograph using a smartphone, and analyzing the grey scale from the RGB space color with a custom made app. Only 2 µL of the cocktail (all components are nontoxic) is needed to prepare the sensor, which have been integrated inside meat packages using a variety of support materials prior to sealing. The Smartphone measurements have been validated using a reference method (Checkpoint Analyzer) and the results suggest it can provide the basis for a quick test of the quality of the packaged pork.

Keywords: Freshness sensor; Colorimetric CO_2 gas sensor; Pork meat; Meat spoilage; Bacterial growing; food quality

Introduction

The food industry is one of the largest and most important manufacturing sectors in Europe. Investing in food quality is vitally important in order to gain future market competitiveness. One of the aspects that companies could use to secure a strong market position is to invest in ways to demonstrate the quality of their products through the inclusion of freshness sensors.

Freshness sensors or indicators have become very popular in recent years; they give information about the quality of the product. Freshness sensors can be defined as sensors able to respond to changes produced in the food being monitored because of bacterial growth [1]. Freshness sensors can be sensitive to any characteristics that change during degradation of the product being monitored, such as changes in bacterial count [2] , gases that can be correlated with spoilage [3, 4], inherent color change [5], etc. Studies have addressed various food products such as fruit [6], vegetables [7], meat [8] and fish [9].

Freshness indicators for meat products are not greatly developed; it is a challenge for researchers to get freshness indicators that could alert customers of the state of meat at any time without the necessity of special training. In literature can be found some examples of these kind of indicators but the reality is that none can be found at supermarkets, they have not been implemented in the companies yet. A "chemical barcode" integrated in skinless breast chicken fillets packages used the CO_2 concentration to estimate bacterial count via color changes measured with a spectrophotometer [10]. Likewise, a radio frequency identification tag (RFID) was used to monitor the freshness of pork meat. Ammonia, humidity and temperature sensors were used, and the tag and sensors were attached inside the package to monitor the inner environment. This prototype was unable to be commercialized for a number of reasons, including dimensions and difficulty of data treatment [11]. Bromophenol blue was used as indicator of freshness in buffalo meat stored at room temperature. Color changes were related to freshness as the dye turned from yellow when fresh to bluish yellow when spoiled. However, the change in color occurred only on parts of the sensor rather than uniformly, and it was difficult to know accurately when the sensor crossed the threshold from fresh to spoiled [12]. In another study, myoglobin-based indicators were used for the determination of freshness in poultry products through the detection of hydrogen sulphide. While promising results were reported, further optimization of this

method is required in order to get more sensitive and fast indicators [13]. A colorimetric indicator was reported by Dudnyk *et al.* based on the change in color of the film containing anthocyanins coming from extract of red cabbage that was sensitive to amines. The analytical parameter used was the absorbance measured spectrophotometrically or the color observed by naked eye. Results were promising, but a spoilage threshold was not developed [14]. An oxygen sensitive membrane was also reported for monitoring meat quality. Samples were tracked for a week and showed how the oxygen decreased over time due to increase of bacterial respiration. However, no other measurements were carried out to correlate the microbiological state of meat with the measurements carried out [15]. Korean kimchi was also studied based on the transparency levels of a chitosan-based CO_2 indicator during storage. In this case, the freshness indicator was in liquid state, which is not desirable due to the possibility of leakage and contamination of the food [16].

Despite these attempts to develop freshness indicators that could inform consumers of food status, the reality is that these new smart labels are still not found in supermarkets. There are many reasons for this slow uptake of freshness sensing technologies, such as appropriate business model, cost of implementation, resistance to new practices, the availability of appropriate reliable sensors, and a means to provide easy access to the information.

In this work, we present the integration of freshness indicators in pork packages that could be able to alert consumers of the state of meat simply by taking a picture using their smartphone. The sensor is sensitive to CO_2, which in turn is correlated with bacterial growth and hence with degradation of meat. A water-based ink has been designed and used rather than the very well-known organic-based CO_2 sensors, due to its non-toxicity, enhanced stability and ease of use [17, 18].

Materials and methods

Reagents and materials

Meta-cresol purple sodium salt (MCP), sodium hydrogencarbonate, 2-hydroxyethyl cellulose (HEC, average MW ~90,000) and glycerin, were all sourced from Sigma–Aldrich Química S.A. (Spain). For microbiology experiments: plate count agar (PCA) LAB149, violet red bile agar (VRBA) (LAB031), maximum recovery diluent

(LAB103), buffered peptone water (LAB046), and pseudomonas agar base (LAB108) all from LAB M Heywood (Lancashire, UK) were purchased. All aqueous solutions were made using reverse-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore, conductivity 18.2 MΩ·cm).

Membrane supports used were: Biodyne B, Biodyne C, Nitrate Cellulose, Nytran N, Nytran SPC and Protran BA all from VWR (Barcelona, Spain).

Pork loin was bought from Hipercor (El Corte Ingles, Granada, Spain), special bag material Cryovac® BB3055 that is specific for fresh meat products was purchased from Sealed Air (Seville, Spain) and a heat sealer PFS-300MM Electric Impulse Sealing Machine C. was obtained from Media W.S. Trade S.L. (Barcelona, Spain).

Additionally, a laminar flow cabinet LHC-4B1 (ESCO, Germany) two incubator cameras Selecta Incubat, an autoclave Selecta Med 12, a colony counter Selecta Digital S and homogenizer Stomacher IUL, (Galileo Equipments, Madrid, Spain), were used for the microbiology experiments and the CheckPoint - Portable Gas Analyzer for quality control of modified atmosphere packages (Ametek instrumentos, S.L.U. - Dansensor Spain) was employed as reference method for CO_2 measurements inside pork packages.

The standard gas mixtures for the freshness indicator calibration and characterization were fabricated using N_2 as the inert gas by controlling the flow rates of N_2 and CO_2 using computer-controlled mass flow controllers (Iberfluid instruments, Spain) operating at a total pressure of 1 atm and a flow rate of 500 cm^3 min^{-1}.

Preparation of freshness sensor

CO_2 sensors were prepared as follows: 12.5 mg of HEC and 37.5 mg glycerol were dissolved in 1 mL of water, once dissolved 2.25 mg $NaHCO_3$ and 1.4 mg MCP were added and dissolved in the mixture using an ultrasonic bath. The sensor preparation consists of casting 2 µL of the CO_2 cocktail on one side of a nitrocellulose membrane. After that, the support was left to dry in darkness in a box for 5 min at room temperature. All components used in the freshness sensor preparation were nontoxic according to their MSDS.

In order to include the freshness sensors inside meat packages the sensors were glued to the inner part of the cover film by a double side cellopaper before sealing the pork loin fillets.

Microbiology experiments

Fresh raw pork meat was bought from a butcher, this meat was cut and packaged in 500 g quantities inside Cryovac® bags, and sealed using an impulse bag sealer. All the packages were stored at 4ºC and analyzed over time for the presence of total aerobic count and members of the families *Pseudomonadaceae* and *Enterobacteriaceae*.

The protocol for the bacteriological analysis of the samples was as follows [17], 27g of sample was weighed into a sterile petri dish using an aseptic technique, transferred into 243 mL of Buffered Peptone Water and homogenized using a stomacher for 30 seconds. Serial dilutions of 9 mL aliquots of Maximum Recovery Diluent were prepared and suitable dilutions were plated in triplicate and poured with plate count agar (PCA-Total aerobic count), Pseudomonad agar (*Pseudomonads*) and VRBA (*Enterobacteriaceae*). Plates were incubated as follows: PCA 22^{o}C for 48 hours and 37^{o}C for 24 hours, pseudomonads 30^{o}C for 24 hours and VRBA 37^{o}C for 24 hours. Following incubation plates were counted and the result reported as numbers of log cfu (colony-forming units)/g. pork

Prior opening the package a measurement of the quantity of CO_2 was taken using the Checkpoint Analyzer as reference method and a photograph of the freshness sensors was taken as well in order to analyze the color coordinates. Figure SI 1 shows a flow chart of the process.

~~**Figure 1**~~

Data analysis- color measurements in the laboratory

Membranes were first characterized using a homemade wooden enclosure designed by our group [17] illuminated with two LED-lamps (6500 K, illumination inside of the box = 9680 Lx) placed at 90◦ with respect to the camera in order to have controlled illumination. Different quantities of CO_2 gas were flushed to the array of sensors in order to perform the characterization.

The grey scale, from the RGB (Red, Green, Blue) color space of the region of interest (ROI) of the sensors were calculated using Image J software (National Institutes of Health) as the average of R, G and B coordinates.

Data analysis- color measurements in packaged meat

Color determination was carried out by photographing the membranes using a mobile phone (Samsung Galaxy S4) with no additional control of light conditions than performing the color experiment inside the laminar flow cabin with the lights on, which gives an illumination of 500 lux. Photographs were analyzed using a custom made app that automatically recognized the ROI and analysed it, giving the information of the color coordinates in terms of grey scale (Figure 1). The ROI was identified as the greatest amount of contiguous pixels, which grey scale value was included in the range of variation of the sensor. Then, an average of the grey scale coordinate from the pixels considered was calculated and used to perform the CO_2 determination.

~~**Figure 2**~~

Figure 1

Results and discussion

Sensor response to freshness marker

Different supports were used to immobilize the CO_2 sensing membrane such as Biodyne B, Biodyne C, Cellulose Nitrate, Nytran N, Nytran SPC and Protran BA. The response to different CO_2 standards was studied for each of them, and finally the ones that presented the best response were selected for further studies. Calibration functions and dynamic behavior were studied for the three optimum supports: Byodine B, Nytran N and Nytran SPC. The response to the whole range of carbon dioxide was studied obtaining the calibration function, and the limit of detection (LOD) estimated. Particular attention was placed on the sensor response at percentages below 20%, as this is the ultimate range of interest for the application. The dynamic behavior was studied in order to test the response and recovery times of the sensors. This was found to be

very fast for both response and recovery events, and no hysteresis was observed in any of the experiments performed.

The cocktail used here has been previously studied to detect CO_2 [17]. In that study, an ionic liquid was included in the composition of the cocktail in order to minimize the time of response. However, using an ionic liquid in a sensor for the food industry is not desirable due to the possibility of toxicity. Furthermore, the change in the sensor support from Mylar led to improved dynamic behavior, negating the need to use the ionic liquid, simplifying the sensor cocktail and ensuring all components employed in the fabrication of the sensor were not toxic.

Biodyne B support

Figure 2 (A,B) shows the calibration function obtained. As it can be observed in Figure 2A a response in the whole range was studied, although low CO_2 concentration was expected to be produced for spoiled meat. 20% of CO_2 was found as CO_2 threshold for pork boneless chops spoilage [17], in this case loin is used and therefore the percentage can be different, but should be close enough.

~~**Figure 3**~~

Figure 2

The calibration function obtained was $-21.95 \exp\left(\frac{x}{-12.72}\right) + 102.07, r^2 = 0.9593$. The LOD was obtained using the standard criteria: LOD = $y_b + 3s_b$, where y_b is the average blank signal and s_b is the standard deviation of the blank, using 10 replicas. An average LOD of 0.13% was obtained for this type of support.

Figure ~~3B~~ 2B shows the dynamic behavior of the CO_2 sensor on Biodyne support. In order to carry out the experiment, alternating atmospheres of pure N_2 and pure CO_2 were pumped to the system. In order to carry out the experiment, a video was recorded with a frame rate of 30 s when the membrane was exposed to alternating atmospheres of pure N_2 and pure CO_2.

The response time was calculated from between 10% and 90% of the maximum signal, returning a value of 2.5 ± 0.7 s and, the recovery time from 90% to 10% which was found to be 17.4 ± 1.5 s

Nytran N support

Figure ~~4A~~ 2C shows the calibration function for the freshness sensor on Nytran N support and Figure ~~4B~~ 2D the dynamic behavior. The calibration function obtained was $y = -21.41 \exp\left(\frac{x}{-14.33}\right) + 124.24, r^2 = 0.9670$. The LOD obtained was 1.29%. The response and recovery times found were 3.8 ± 0.2 and 25.8 ±3.7, respectively.

~~**Figure 4**~~

Nytran SPC support

The calibration function obtained was $y = -19.21 \exp\left(\frac{x}{-13.50}\right) + 109.65, r^2 = 0.9762$. Figure ~~5A~~ 2E shows the calibration function for the freshness sensor on Nytran N support and Figure ~~5B~~ 2F the dynamic behavior. The LOD obtained was 1.84 %. The response and recovery times found were 2.4 ± 0.3 and 15.2 ± 0.6, respectively.

~~**Figure 5**~~

Table 1 shows a summary of the main characteristics found for each freshness sensor using the different supports.

Support	LOD (%)	T_{res} (s)	T_{rec} (s)
Biodyne B	1.29	2.5 ± 0.7	17 ± 1.5
Nytran N	1.84	3.8 ± 0.2	26 ± 3.7
Nytran SPC	0.45	2.4 ± 0.3	15.2 ± 0.6

In all three cases, the LOD and dynamic behavior were appropriate for the intended application. All three were broadly similar in response that was highest at low percentages of CO_2, up to around 30, which correlated with the expected CO_2 range requirement for the application. Therefore, all of them could be used for their implementation in meat packages as freshness sensors, but among them the best results obtained in terms of sensitivity and dynamic behavior was using Nytran SPC, therefore from now on the support used was Nytran SPC.

Meat spoilage monitoring using freshness sensors

The bacteria chosen for the evaluation of meat deterioration over time were *Pseudomonas* spp., *Enterobacteriaceae* [10] and PCA [5]. *Pseudomonas* grow under refrigerated storage conditions and cause slime to develop on meat. *Enterobacteriaceae*, is a group of bacteria that live mostly in the intestines of animals. The majority of the animal's food-borne pathogens origin are included in this group. PCA is a general measure of the background microbiological status of meat. This includes not only bacteria from animals, but also from the slaughterhouse or meat processing environment, therefore it gives an indication of the keeping quality of the meat [19, 20]. The limit of colony forming units adopted as threshold to indicate meat spoilage is 10^7 cfu/g /mL or cm^2 [21, 22]. CO_2 concentration has been previously studied as an indicator of bacterial growth and therefore as a freshness indicator over time in different food products such as korean kimchi [16, 23], soups [2], salads [7] and meat [10]. In packaged food, the concentration of CO_2 grows over time as a consequence of bacterial growth, due to bacterial respiration [24, 25]. Therefore, both bacteriological analysis and CO_2 concentration determination were carried out to validate the functionality of the new freshness sensor. Each day of analysis, one of the stored meat packages was selected and before opening to perform the bacteriological analysis, the concentration of CO_2 was measured by the Checkpoint Analyzer and a photograph of the freshness sensor was taken using the mobile phone. There were also used two control samples that integrated the freshness sensor and a septum (to impede gas exchange), which allowed the measurement of gas concentration without breaking the inner atmosphere. These two control samples were used only for taking photographs of the freshness sensor. Experiments were performed at days 1, 2, 3, 4, 7, 9, and 10 after the meat samples were packaged.

Figure 3 shows the bacterial growth increasing over time as expected, reaching the threshold for spoilage determination (10^7 cfu/g) on day 7. (Figure SI 2 shows a photograph of a meat package with the sensors integrated)

~~**Figure 6**~~

Figure 3

The changes in the color coordinates were evaluated at days 1, 2, 3, 4, 7, 9 and 10. The measurements of CO_2 were validated using the Check Point analyzer as a reference method.

~~**Figure 7**~~

~~**Figure 4**~~

The concentration of CO_2 released over time and the color coordinate grey scale are presented in Figure 4. The sensors indicated correctly that the quantity of CO_2 grows over time as a consequence of bacterial growth. As it can be observed in ~~Figure 8~~ Figure 4 there was an increase in the color signal over time as well, the day 7 it was reached the threshold for meat spoilage at a value in the color coordinate of 98.5 ± 0.5. The results obtained with the freshness sensor were validated with the reference method.

~~**Figure 8**~~

Figure 4

According to the results, we could correlate color with bacterial count with a sigmoidal function: $Y = A3 + A4 \cdot Ln\left(\frac{A1-A2}{x-A2} - 1\right)$, (where A_1= 2.470, A_2= 7.058, A_3= 94.356, A_4= 1.162, R^2= 0.996). Therefore, just taking a photograph of the sensor we can detect the freshness of the meat (~~Figure 9~~ Figure 5). If the grey scale calculated is above 98.5 ± 0.5 means the colony of bacteria has reached the threshold for meat spoilage, and therefore should not be consumed.

~~**Figure 9**~~

Figure 5

Currently, the spoilage date is set as 'best before' date with a relatively large margin or error meaning that a lot of perfectly good food is discarded according to the date, rather than the actual condition. This sensor detects the real condition of the food, thus providing a route to reducing wastage in the food industry. In this study, the bacterial population reached the threshold for bacterial population the day 7th, but the point is that the bacterial population depends on storage conditions and initial bacterial population prior to packaging, so the threshold will be reached at a variable time, and the number of days is therefore only a rough guide. Therefore, we are proposing a revolutionary way to detect meat freshness at anytime, anywhere, just using a smartphone.

Conclusions

This work described the development of a freshness sensor that can be implemented for food quality assurance in the meat industry.

A nontoxic and low price sensing chemistry was used, based on the acidity of CO_2, being the total price for preparation less than 0.042 €.

The freshness sensor integrated in meat packages allowed tracking the state of meat until its spoilage through the acquisition of a photograph. Interestingly, the color information measured as grey scale correlated well with bacterial growth and CO_2 gas released by packaged meat. Therefore, the custom made app, if the value of the grey scale exceed the threshold found of 98.5 was able to indicate that the meat was already spoiled.

This work opens the possibility to create a new way to check the state of packaged food avoiding the need of expiration dates, the quality can be checked any moment by any person who has a smartphone.

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Figure Legends

~~Figure 1. Flow chart of experiments. A) Fresh raw pork; B) packaged pork meat with sensors integrated; C) CO_2 determination using the Checkpoint Analyzer; D) Smartphone as detection system; E, F,G) Bacteriological analysis of pork meat.~~

~~Figure 2~~. Figure 1. Flow diagram of the Android app. First the photograph of the freshness sensor: App identifying de ROI: Color coordinates calculation: Meat quality determination.

Figure 2. A) Calibration function of the CO_2 sensor on Biodyne support. Grey scale value versus CO_2 percentage; B) Dynamic behavior of freshness sensor supported on Byodine B. C) Calibration function of the CO_2 sensor. Grey scale versus CO_2 percentage; D) Dynamic behavior of freshness sensor supported on Nytran N; E) Calibration function of the CO_2 sensor. Grey scale versus CO_2 percentage; F) Dynamic behavior of freshness sensor supported on Nytran SPC.

~~Figure 3. A) Calibration function of the CO_2 sensor on Biodyne support. Grey scale value versus CO_2 percentage; B) Dynamic behavior of freshness sensor supported on Byodine B.~~

~~Figure 4. A) Calibration function of the carbon dioxide sensor. Grey scale versus CO_2 percentage; B) Dynamic behavior of freshness sensor supported on Nytran N~~

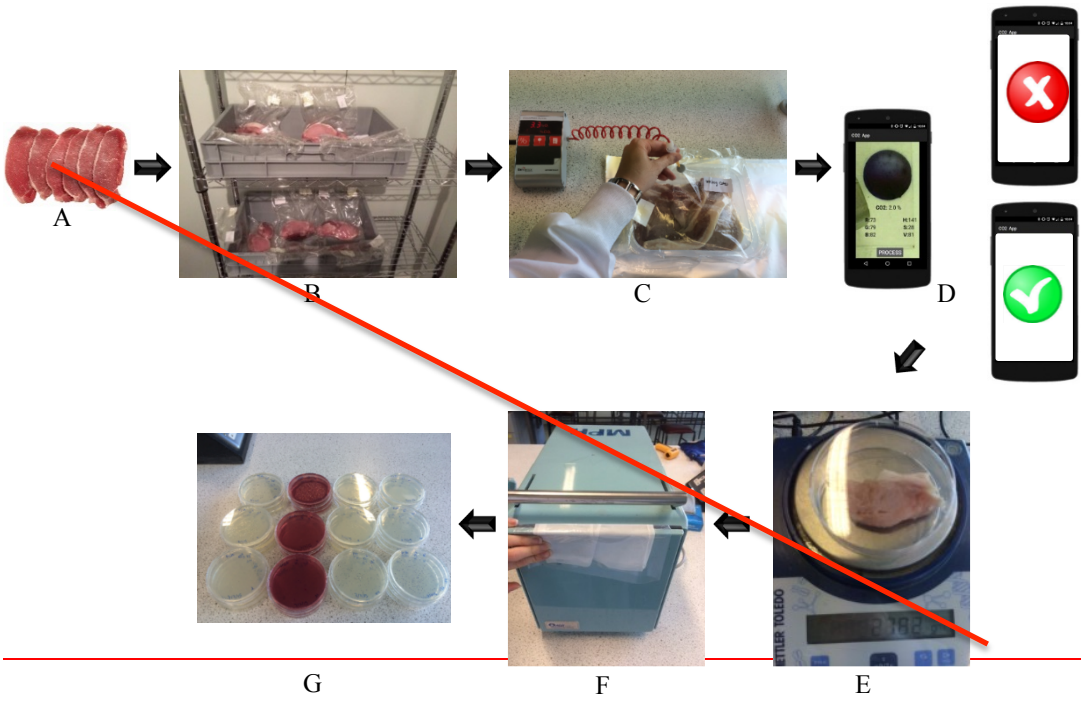
~~Figure 5. A) Calibration function of the carbon dioxide sensor. Grey scale versus CO_2 percentage; B) Dynamic behavior of freshness sensor supported on Nytran SPC~~

~~Figure 6.~~ Figure 3. Bacterial growth over time in pork meat.

~~Figure 7. Figure 4. Meat package with the sensors integrated~~

~~Figure 8.~~ Figure 4. CO_2 released over time as a consequence of bacterial growth. Grey scale is the freshness sensor response and CO_2 percentage values are obtained from the Checkpoint Analyzer. The dotted line represents the threshold related to the bacterial counts.

~~Figure 9.~~ Figure 5. Grey scale obtained from the app versus log bacterial count PCA.



~~Figure 1~~

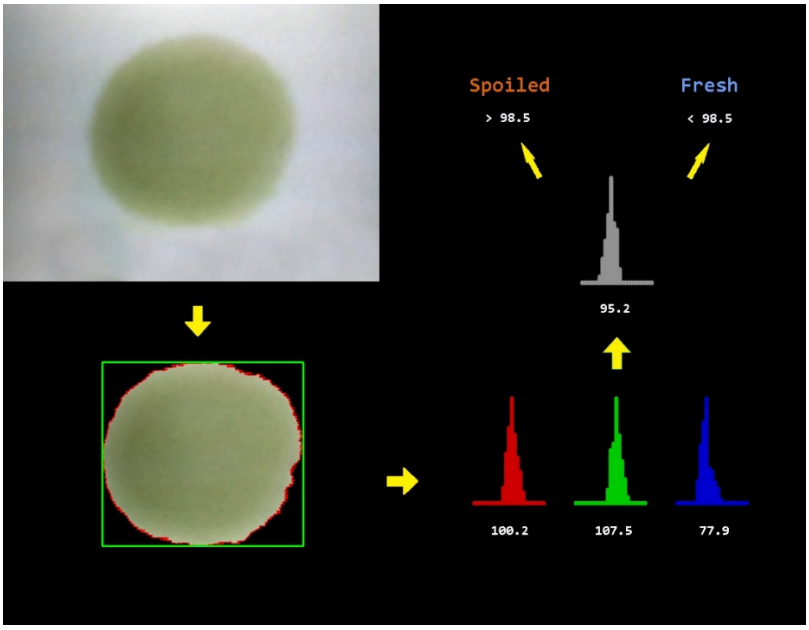


Figure 1

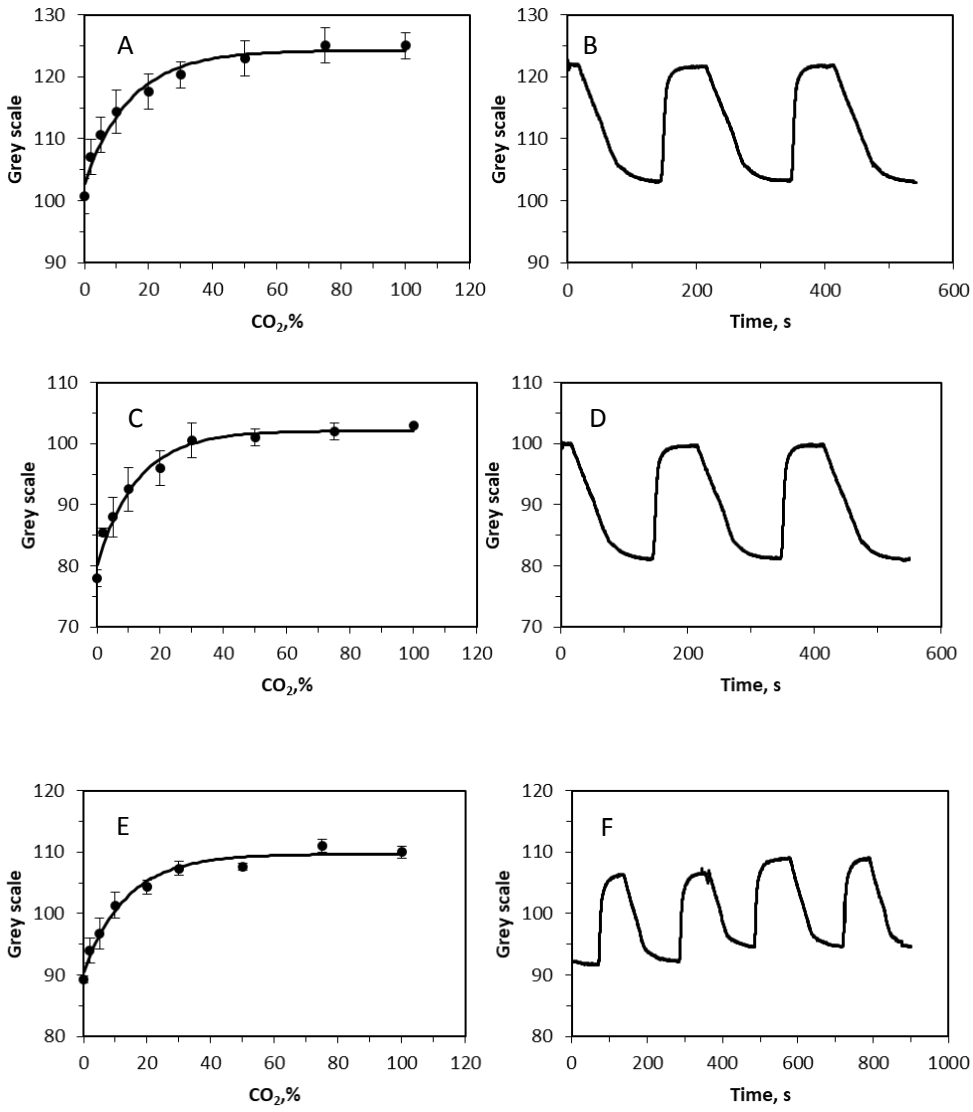
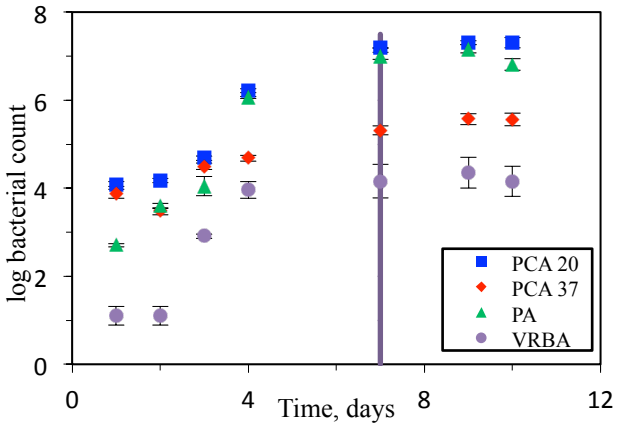


Figure 2


log bacterial count
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PA
VRBA
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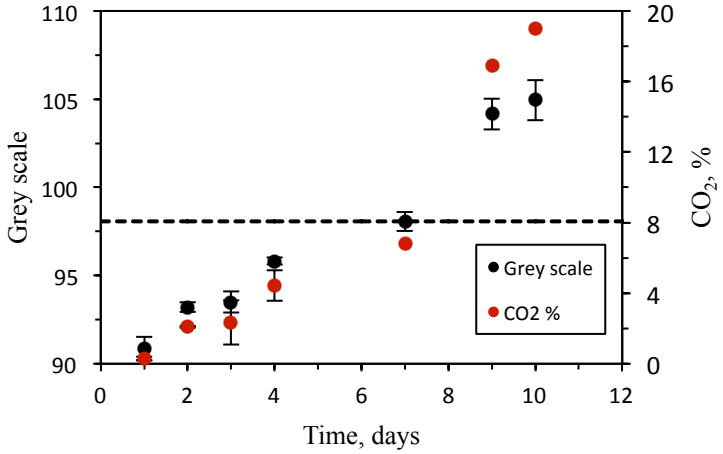
2
4
6
8
0
4
8
12

Figure 3

~~Figure 4~~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105
100
95
90
Grey scale
0
2
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Time, days
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CO_2, %
Grey scale
CO2 %

Figure 4

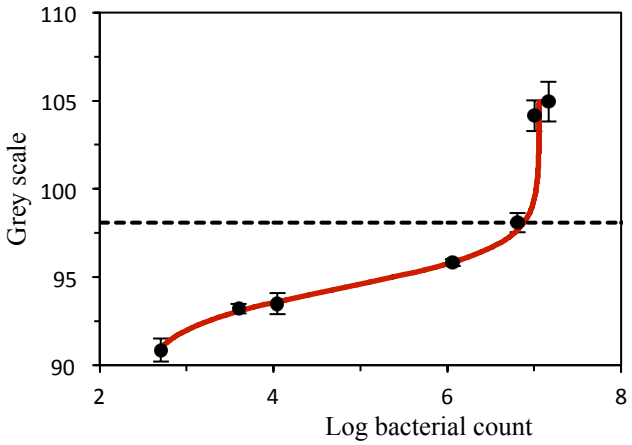

Figure 5

Smartphone based meat freshness detection

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Abstract

In this work, a freshness colorimetric sensor has been integrated with pork meat packages. The sensor tracks rising CO_2 levels in the package associated with meat spoilage, as CO_2 levels increase with bacterial population. The color of the sensor changes depending on the quantity of bacteria present, therefore it can be correlated with the freshness of meat, in this case pork loin. Detection is achieved by a simple photograph using a smartphone, and analyzing the grey scale from the RGB space color with a custom made app. Only 2 µL of the cocktail (all components are nontoxic) is needed to prepare the sensor, which have been integrated inside meat packages using a variety of support materials prior to sealing. The Smartphone measurements have been validated using a reference method (Checkpoint Analyzer) and the results suggest it can provide the basis for a quick test of the quality of the packaged pork.

Keywords: Freshness sensor; Colorimetric CO_2 gas sensor; Pork meat; Meat spoilage; Bacterial growing; food quality

Introduction

The food industry is one of the largest and most important manufacturing sectors in Europe. Investing in food quality is vitally important in order to gain future market competitiveness. One of the aspects that companies could use to secure a strong market position is to invest in ways to demonstrate the quality of their products through the inclusion of freshness sensors.

Freshness sensors or indicators have become very popular in recent years; they give information about the quality of the product. Freshness sensors can be defined as sensors able to respond to changes produced in the food being monitored because of bacterial growth [1]. Freshness sensors can be sensitive to any characteristics that change during degradation of the product being monitored, such as changes in bacterial count [2] , gases that can be correlated with spoilage [3, 4], inherent color change [5], etc. Studies have addressed various food products such as fruit [6], vegetables [7], meat [8] and fish [9].

Freshness indicators for meat products are not greatly developed; it is a challenge for researchers to get freshness indicators that could alert customers of the state of meat at any time without the necessity of special training. In literature can be found some examples of these kind of indicators but the reality is that none can be found at supermarkets, they have not been implemented in the companies yet. A "chemical barcode" integrated in skinless breast chicken fillets packages used the CO_2 concentration to estimate bacterial count via color changes measured with a spectrophotometer [10]. Likewise, a radio frequency identification tag (RFID) was used to monitor the freshness of pork meat. Ammonia, humidity and temperature sensors were used, and the tag and sensors were attached inside the package to monitor the inner environment. This prototype was unable to be commercialized for a number of reasons, including dimensions and difficulty of data treatment [11]. Bromophenol blue was used as indicator of freshness in buffalo meat stored at room temperature. Color changes were related to freshness as the dye turned from yellow when fresh to bluish yellow when spoiled. However, the change in color occurred only on parts of the sensor rather than uniformly, and it was difficult to know accurately when the sensor crossed the threshold from fresh to spoiled [12]. In another study, myoglobin-based indicators were used for the determination of freshness in poultry products through the detection of hydrogen sulphide. While promising results were reported, further optimization of this

method is required in order to get more sensitive and fast indicators [13]. A colorimetric indicator was reported by Dudnyk *et al.* based on the change in color of the film containing anthocyanins coming from extract of red cabbage that was sensitive to amines. The analytical parameter used was the absorbance measured spectrophotometrically or the color observed by naked eye. Results were promising, but a spoilage threshold was not developed [14]. An oxygen sensitive membrane was also reported for monitoring meat quality. Samples were tracked for a week and showed how the oxygen decreased over time due to increase of bacterial respiration. However, no other measurements were carried out to correlate the microbiological state of meat with the measurements carried out [15]. Korean kimchi was also studied based on the transparency levels of a chitosan-based CO_2 indicator during storage. In this case, the freshness indicator was in liquid state, which is not desirable due to the possibility of leakage and contamination of the food [16].

Despite these attempts to develop freshness indicators that could inform consumers of food status, the reality is that these new smart labels are still not found in supermarkets. There are many reasons for this slow uptake of freshness sensing technologies, such as appropriate business model, cost of implementation, resistance to new practices, the availability of appropriate reliable sensors, and a means to provide easy access to the information.

In this work, we present the integration of freshness indicators in pork packages that could be able to alert consumers of the state of meat simply by taking a picture using their smartphone. The sensor is sensitive to CO_2, which in turn is correlated with bacterial growth and hence with degradation of meat. A water-based ink has been designed and used rather than the very well-known organic-based CO_2 sensors, due to its non-toxicity, enhanced stability and ease of use [17, 18].

Materials and methods

Reagents and materials

Meta-cresol purple sodium salt (MCP), sodium hydrogencarbonate, 2-hydroxyethyl cellulose (HEC, average MW ~90,000) and glycerin, were all sourced from Sigma–Aldrich Química S.A. (Spain). For microbiology experiments: plate count agar (PCA) LAB149, violet red bile agar (VRBA) (LAB031), maximum recovery diluent

(LAB103), buffered peptone water (LAB046), and pseudomonas agar base (LAB108) all from LAB M Heywood (Lancashire, UK) were purchased. All aqueous solutions were made using reverse-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore, conductivity 18.2 MΩ·cm).

Membrane supports used were: Biodyne B, Biodyne C, Nitrate Cellulose, Nytran N, Nytran SPC and Protran BA all from VWR (Barcelona, Spain).

Pork loin was bought from Hipercor (El Corte Ingles, Granada, Spain), special bag material Cryovac® BB3055 that is specific for fresh meat products was purchased from Sealed Air (Seville, Spain) and a heat sealer PFS-300MM Electric Impulse Sealing Machine C. was obtained from Media W.S. Trade S.L. (Barcelona, Spain).

Additionally, a laminar flow cabinet LHC-4B1 (ESCO, Germany) two incubator cameras Selecta Incubat, an autoclave Selecta Med 12, a colony counter Selecta Digital S and homogenizer Stomacher IUL, (Galileo Equipments, Madrid, Spain), were used for the microbiology experiments and the CheckPoint - Portable Gas Analyzer for quality control of modified atmosphere packages (Ametek instrumentos, S.L.U. - Dansensor Spain) was employed as reference method for CO_2 measurements inside pork packages.

The standard gas mixtures for the freshness indicator calibration and characterization were fabricated using N_2 as the inert gas by controlling the flow rates of N_2 and CO_2 using computer-controlled mass flow controllers (Iberfluid instruments, Spain) operating at a total pressure of 1 atm and a flow rate of 500 cm^3 min^{-1}.

Preparation of freshness sensor

CO_2 sensors were prepared as follows: 12.5 mg of HEC and 37.5 mg glycerol were dissolved in 1 mL of water, once dissolved 2.25 mg $NaHCO_3$ and 1.4 mg MCP were added and dissolved in the mixture using an ultrasonic bath. The sensor preparation consists of casting 2 µL of the CO_2 cocktail on one side of a nitrocellulose membrane. After that, the support was left to dry in darkness in a box for 5 min at room temperature. All components used in the freshness sensor preparation were nontoxic according to their MSDS.

In order to include the freshness sensors inside meat packages the sensors were glued to the inner part of the cover film by a double side cellopaper before sealing the pork loin fillets.

Microbiology experiments

Fresh raw pork meat was bought from a butcher, this meat was cut and packaged in 500 g quantities inside Cryovac® bags, and sealed using an impulse bag sealer. All the packages were stored at 4ºC and analyzed over time for the presence of total aerobic count and members of the families *Pseudomonadaceae* and *Enterobacteriaceae*.

The protocol for the bacteriological analysis of the samples was as follows [17], 27g of sample was weighed into a sterile petri dish using an aseptic technique, transferred into 243 mL of Buffered Peptone Water and homogenized using a stomacher for 30 seconds. Serial dilutions of 9 mL aliquots of Maximum Recovery Diluent were prepared and suitable dilutions were plated in triplicate and poured with plate count agar (PCA-Total aerobic count), Pseudomonad agar (*Pseudomonads*) and VRBA (*Enterobacteriaceae*). Plates were incubated as follows: PCA 22^{o}C for 48 hours and 37^{o}C for 24 hours, pseudomonads 30^{o}C for 24 hours and VRBA 37^{o}C for 24 hours. Following incubation plates were counted and the result reported as numbers of log cfu (colony-forming units)/g. pork

Prior opening the package a measurement of the quantity of CO_2 was taken using the Checkpoint Analyzer as reference method and a photograph of the freshness sensors was taken as well in order to analyze the color coordinates. Figure SI 1 shows a flow chart of the process.

Data analysis- color measurements in the laboratory

Membranes were first characterized using a homemade wooden enclosure designed by our group [17] illuminated with two LED-lamps (6500 K, illumination inside of the box = 9680 Lx) placed at 90◦ with respect to the camera in order to have controlled illumination. Different quantities of CO_2 gas were flushed to the array of sensors in order to perform the characterization.

The grey scale, from the RGB (Red, Green, Blue) color space of the region of interest (ROI) of the sensors were calculated using Image J software (National Institutes of Health) as the average of R, G and B coordinates.

Data analysis- color measurements in packaged meat

Color determination was carried out by photographing the membranes using a mobile phone (Samsung Galaxy S4) with no additional control of light conditions than performing the color experiment inside the laminar flow cabin with the lights on, which gives an illumination of 500 lux. Photographs were analyzed using a custom made app that automatically recognized the ROI and analysed it, giving the information of the color coordinates in terms of grey scale (Figure 1). The ROI was identified as the greatest amount of contiguous pixels, which grey scale value was included in the range of variation of the sensor. Then, an average of the grey scale coordinate from the pixels considered was calculated and used to perform the CO_2 determination.

Figure 1

Results and discussion

Sensor response to freshness marker

Different supports were used to immobilize the CO_2 sensing membrane such as Biodyne B, Biodyne C, Cellulose Nitrate, Nytran N, Nytran SPC and Protran BA. The response to different CO_2 standards was studied for each of them, and finally the ones that presented the best response were selected for further studies. Calibration functions and dynamic behavior were studied for the three optimum supports: Byodine B, Nytran N and Nytran SPC. The response to the whole range of carbon dioxide was studied obtaining the calibration function, and the limit of detection (LOD) estimated. Particular attention was placed on the sensor response at percentages below 20%, as this is the ultimate range of interest for the application. The dynamic behavior was studied in order to test the response and recovery times of the sensors. This was found to be very fast for both response and recovery events, and no hysteresis was observed in any of the experiments performed.

The cocktail used here has been previously studied to detect CO_2 [17]. In that study, an ionic liquid was included in the composition of the cocktail in order to minimize the time of response. However, using an ionic liquid in a sensor for the food industry is not desirable due to the possibility of toxicity. Furthermore, the change in the sensor

support from Mylar led to improved dynamic behavior, negating the need to use the ionic liquid, simplifying the sensor cocktail and ensuring all components employed in the fabrication of the sensor were not toxic.

Biodyne B support

Figure 2 (A,B) shows the calibration function obtained. As it can be observed in Figure 2A a response in the whole range was studied, although low CO_2 concentration was expected to be produced for spoiled meat. 20% of CO_2 was found as CO_2 threshold for pork boneless chops spoilage [17], in this case loin is used and therefore the percentage can be different, but should be close enough.

Figure 2

The calibration function obtained was $-21.95 \exp\left(\frac{x}{-12.72}\right) + 102.07, r^2 = 0.9593$. The LOD was obtained using the standard criteria: LOD = $y_b + 3s_b$, where y_b is the average blank signal and s_b is the standard deviation of the blank, using 10 replicas. An average LOD of 0.13% was obtained for this type of support.

Figure 2B shows the dynamic behavior of the CO_2 sensor on Biodyne support. In order to carry out the experiment, alternating atmospheres of pure N_2 and pure CO_2 were pumped to the system. In order to carry out the experiment, a video was recorded with a frame rate of 30 s when the membrane was exposed to alternating atmospheres of pure N_2 and pure CO_2.

The response time was calculated from between 10% and 90% of the maximum signal, returning a value of 2.5 ± 0.7 s and, the recovery time from 90% to 10% which was found to be 17.4 ± 1.5 s

Nytran N support

Figure 2C shows the calibration function for the freshness sensor on Nytran N support and Figure 2D the dynamic behavior. The calibration function obtained was $y = -21.41 \exp\left(\frac{x}{-14.33}\right) + 124.24, r^2 = 0.9670$. The LOD obtained was 1.29%. The response and recovery times found were 3.8 ± 0.2 and 25.8 ±3.7, respectively.

Nytran SPC support

The calibration function obtained was $y = -19.21 \exp\left(\frac{x}{-13.50}\right) + 109.65, r^2 = 0.9762$. Figure 2E shows the calibration function for the freshness sensor on Nytran N support and Figure 2F the dynamic behavior. The LOD obtained was 1.84 %. The response and recovery times found were 2.4 ± 0.3 and 15.2 ± 0.6, respectively.

Table 1 shows a summary of the main characteristics found for each freshness sensor using the different supports.

Support	LOD (%)	T_{res} (s)	T_{rec} (s)
Biodyne B	1.29	2.5 ± 0.7	17 ± 1.5
Nytran N	1.84	3.8 ± 0.2	26 ± 3.7
Nytran SPC	0.45	2.4 ± 0.3	15.2 ± 0.6

In all three cases, the LOD and dynamic behavior were appropriate for the intended application. All three were broadly similar in response that was highest at low percentages of CO_2, up to around 30, which correlated with the expected CO_2 range requirement for the application. Therefore, all of them could be used for their implementation in meat packages as freshness sensors, but among them the best results obtained in terms of sensitivity and dynamic behavior was using Nytran SPC, therefore from now on the support used was Nytran SPC.

Meat spoilage monitoring using freshness sensors

The bacteria chosen for the evaluation of meat deterioration over time were *Pseudomonas* spp., *Enterobacteriaceae* [10] and PCA [5]. *Pseudomonas* grow under refrigerated storage conditions and cause slime to develop on meat. *Enterobacteriaceae*, is a group of bacteria that live mostly in the intestines of animals. The majority of the animal's food-borne pathogens origin are included in this group. PCA is a general measure of the background microbiological status of meat. This includes not only bacteria from animals, but also from the slaughterhouse or meat processing environment, therefore it gives an indication of the keeping quality of the meat [19, 20]. The limit of colony forming units adopted as threshold to indicate meat spoilage is 10^7 cfu/g /mL or cm^2 [21, 22]. CO_2 concentration has been previously studied as an indicator of bacterial growth and therefore as a freshness indicator over time in different food products such as korean kimchi [16, 23], soups [2], salads [7] and meat [10]. In

packaged food, the concentration of CO_2 grows over time as a consequence of bacterial growth, due to bacterial respiration [24, 25]. Therefore, both bacteriological analysis and CO_2 concentration determination were carried out to validate the functionality of the new freshness sensor. Each day of analysis, one of the stored meat packages was selected and before opening to perform the bacteriological analysis, the concentration of CO_2 was measured by the Checkpoint Analyzer and a photograph of the freshness sensor was taken using the mobile phone. There were also used two control samples that integrated the freshness sensor and a septum (to impede gas exchange), which allowed the measurement of gas concentration without breaking the inner atmosphere. These two control samples were used only for taking photographs of the freshness sensor. Experiments were performed at days 1, 2, 3, 4, 7, 9, and 10 after the meat samples were packaged.

Figure 3 shows the bacterial growth increasing over time as expected, reaching the threshold for spoilage determination (10^7 cfu/g) on day 7. (Figure SI 2 shows a photograph of a meat package with the sensors integrated)

Figure 3

The changes in the color coordinates were evaluated at days 1, 2, 3, 4, 7, 9 and 10. The measurements of CO_2 were validated using the Check Point analyzer as a reference method.

The concentration of CO_2 released over time and the color coordinate grey scale are presented in Figure 4. The sensors indicated correctly that the quantity of CO_2 grows over time as a consequence of bacterial growth. As it can be observed in Figure 4 there was an increase in the color signal over time as well, the day 7 it was reached the threshold for meat spoilage at a value in the color coordinate of 98.5 ± 0.5. The results obtained with the freshness sensor were validated with the reference method.

Figure 4

According to the results, we could correlate color with bacterial count with a sigmoidal function: $Y = A3 + A4 \cdot Ln\left(\frac{A1-A2}{x-A2} - 1\right)$, (where A_1= 2.470, A_2= 7.058, A_3= 94.356, A_4= 1.162, R^2= 0.996). Therefore, just taking a photograph of the sensor we can detect the freshness of the meat (Figure 5). If the grey scale calculated is above 98.5 ± 0.5

means the colony of bacteria has reached the threshold for meat spoilage, and therefore should not be consumed.

Figure 5

Currently, the spoilage date is set as 'best before' date with a relatively large margin or error meaning that a lot of perfectly good food is discarded according to the date, rather than the actual condition. This sensor detects the real condition of the food, thus providing a route to reducing wastage in the food industry. In this study, the bacterial population reached the threshold for bacterial population the day 7^{th}, but the point is that the bacterial population depends on storage conditions and initial bacterial population prior to packaging, so the threshold will be reached at a variable time, and the number of days is therefore only a rough guide. Therefore, we are proposing a revolutionary way to detect meat freshness at anytime, anywhere, just using a smartphone.

Conclusions

This work described the development of a freshness sensor that can be implemented for food quality assurance in the meat industry.

A nontoxic and low price sensing chemistry was used, based on the acidity of CO_2, being the total price for preparation less than 0.042 €.

The freshness sensor integrated in meat packages allowed tracking the state of meat until its spoilage through the acquisition of a photograph. Interestingly, the color information measured as grey scale correlated well with bacterial growth and CO_2 gas released by packaged meat. Therefore, the custom made app, if the value of the grey scale exceed the threshold found of 98.5 was able to indicate that the meat was already spoiled.

This work opens the possibility to create a new way to check the state of packaged food avoiding the need of expiration dates, the quality can be checked any moment by any person who has a smartphone.

Acknowledgements

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Figure Legends

Figure 1. Flow diagram of the Android app. First the photograph of the freshness sensor: App identifying de ROI: Color coordinates calculation: Meat quality determination.

Figure 2. A) Calibration function of the CO_2 sensor on Biodyne support. Grey scale value versus CO_2 percentage; B) Dynamic behavior of freshness sensor supported on Byodine B. C) Calibration function of the CO_2 sensor. Grey scale versus CO_2 percentage; D) Dynamic behavior of freshness sensor supported on Nytran N; E) Calibration function of the CO_2 sensor. Grey scale versus CO_2 percentage; F) Dynamic behavior of freshness sensor supported on Nytran SPC.

Figure 3. Bacterial growth over time in pork meat.

Figure 4. CO_2 released over time as a consequence of bacterial growth. Grey scale is the freshness sensor response and CO_2 percentage values are obtained from the Checkpoint Analyzer. The dotted line represents the threshold related to the bacterial counts.

Figure 5. Grey scale obtained from the app versus log bacterial count PCA.

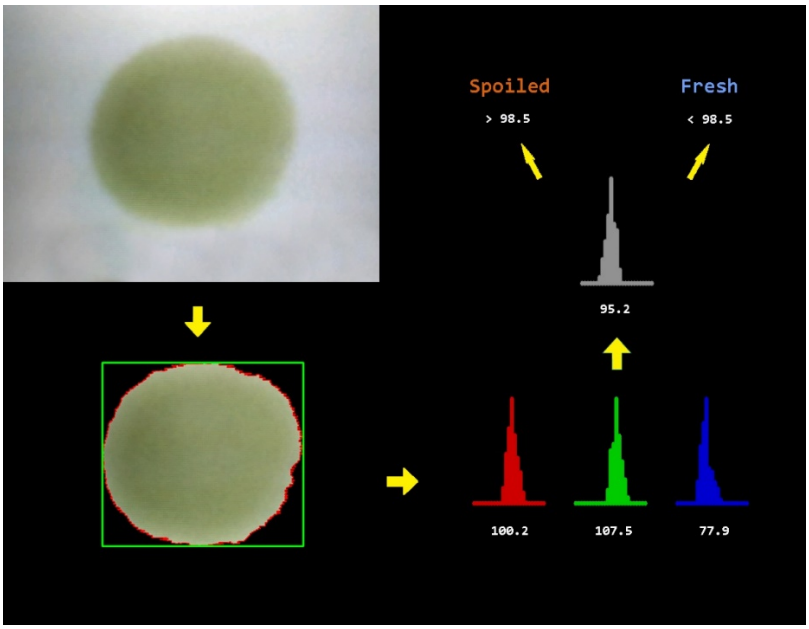

Figure 1

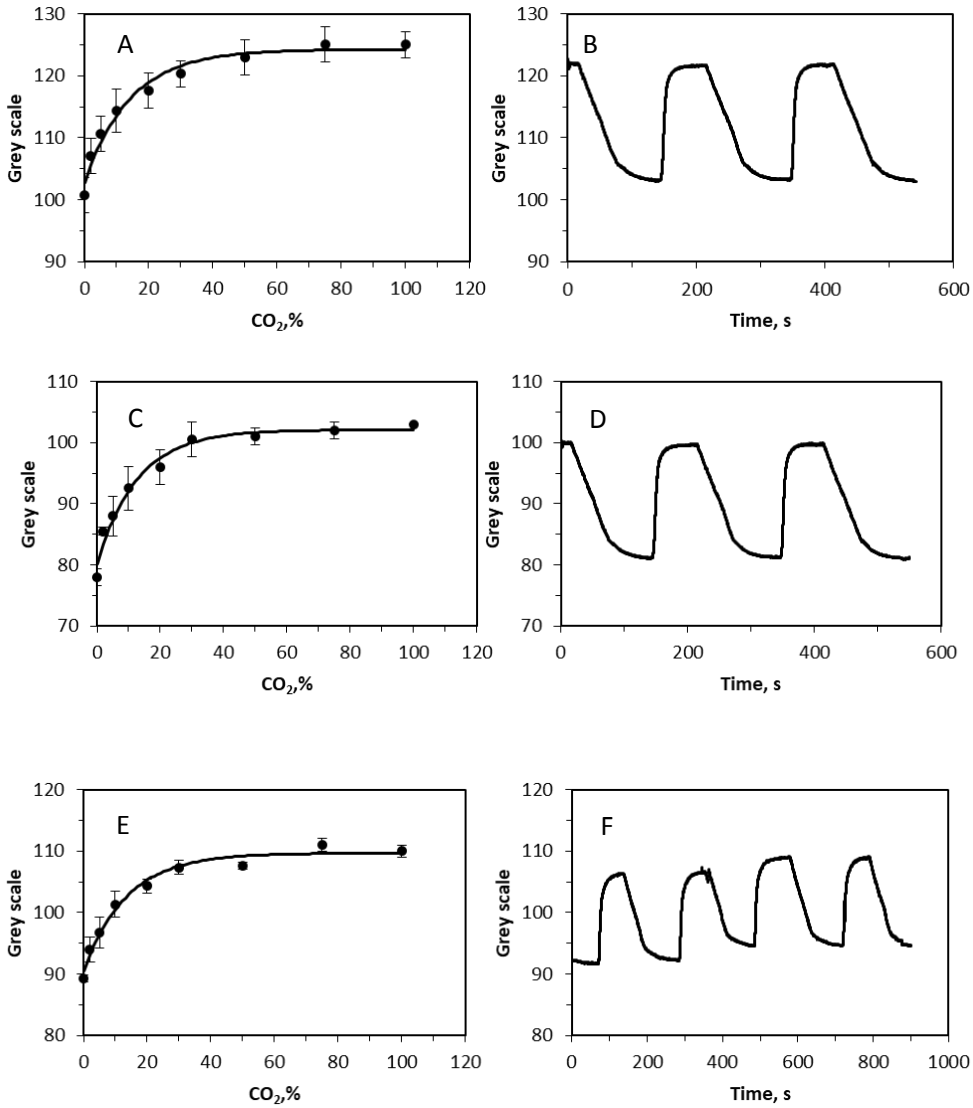

Figure 2

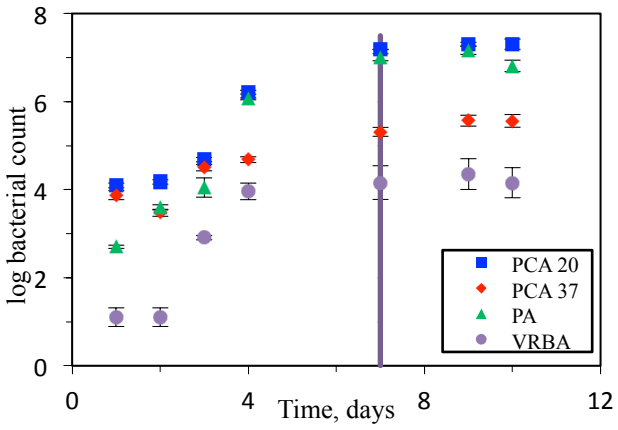
log bacterial count
Time, days
PCA 20
PCA 37
PA
VRBA
0
2
4
6
8
0
4
8
12

Figure 3

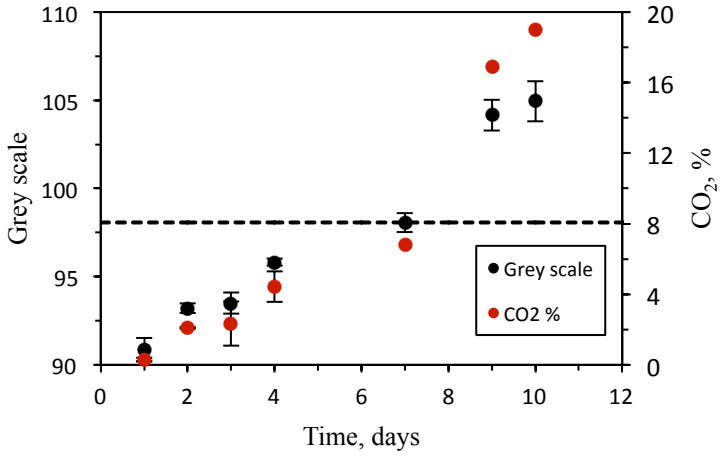
110
105
100
95
90
Grey scale
0
2
4
6
8
10
12
Time, days
20
16
12
8
4
0
CO_2, %
Grey scale
CO2 %

Figure 4

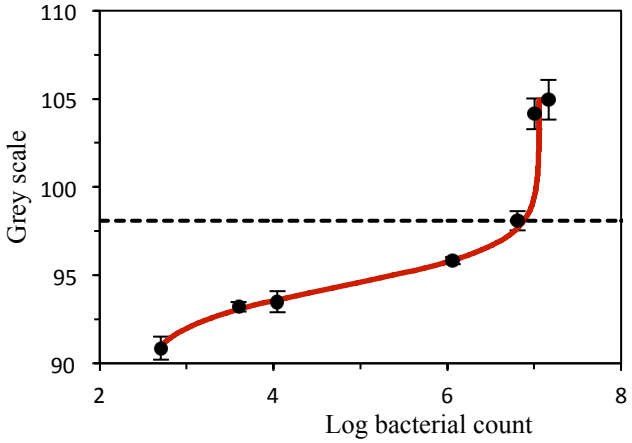

Figure 5

Supplementary Material

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*Graphical Abstract (for review)

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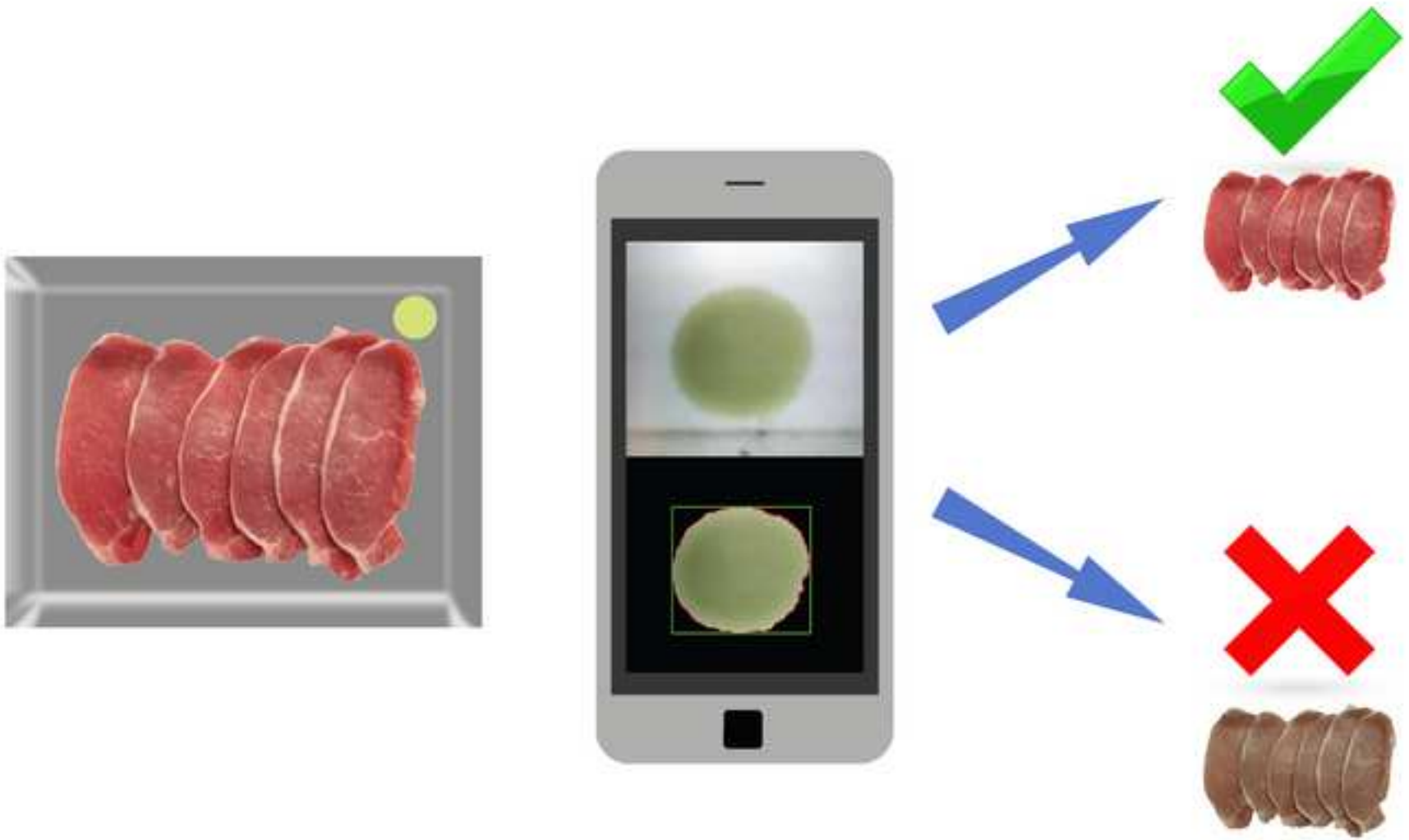

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***Credit Author Statement**

Isabel M. Perez de Vargas-Sansalvador: Conceptualization, Supervision, Writing- Reviewing and Editing, Project administration

Miguel M. Erenas: Data curation , formal analysis

Antonio Martínez-Olmos: Software, formal analysis

Fatima Mirza-Montoro: Investigation

Dermot Diamond: Writing- Reviewing and Editing

Luis Fermin Capitan-Vallvey: Conceptualization, Writing- Reviewing and Editing