Chapter

Role of Biogenic Amines in Protein Foods Sensing: Myths and Evidence

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Abstract

Myriads of sensors have been proposed to signal the spoilage of a piece of meat. It is assumed and taken for granted that biogenic amines, BAs, harmful by-product indicating the last phase of degradation, must be present in the volatilome developed over the decaying meat. This chapter aims to clearly explain BAs' role in protein food spoilage: undoubtedly produced inside the meat, never present in the headspace, where sensors are displayed. The BAs presence in the headspace represents a sort of myth. It is plenty of evidence that BAs cannot be present in the volatilome over the meat. The BAs' volatility is pH-dependent. As shown by their protonation constants, the strongly buffered pH of proteinaceous food prevents their vapour phase transition. The chemical analyses made at the same degradation time, on the meat and the headspace above the meat, corroborate the real composition of the volatilome, demonstrating the BAs absence. The sensors here described, designed on volatilome evidence, succeed to follow the entire process, from the SAFE condition to the WARNING and the HAZARD. The final prototype works reliably on real protein foods (i.e. chicken, beef pork and fish), not enriched and stored at the home condition.

Keywords: smart labels, entire meat spoilage detection, naked-eye devices, BAs volatility, pH indicators, intelligent packaging

1. Introduction

In recent decades, the interest in systems able to detect food degradation, giving a quick response, simple to be read, and suitable for implementation in control systems and smart labels has increased continuously. [1–6] This interest is more valid in foods such as meat and fish. Despite the spread of vegan and vegetarian diets, their consumption has been steadily increasing worldwide for decades, also caused by the dramatic economic development of Eastern Country of the last forty years. The impressive trend is visibly depicted in the two graphs of **Figures 1** and **2**. [7] Unfortunately, the common feature of meat food is its intrinsically high perishability. All protein foods require proper processing, highly controlled environments, and storage in chilled conditions (cold rooms, refrigerators, and freezers), being very sensitive to interruptions in the cold chain. Furthermore, consumers pay increasing attention to their food quality and claim to have direct control over freshness.

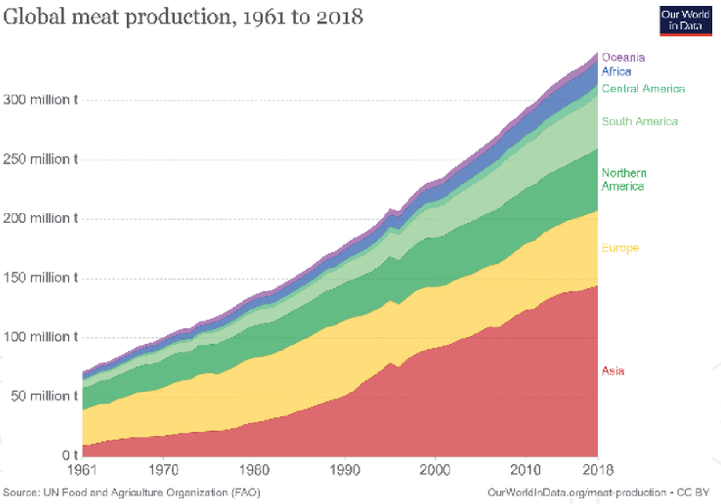

Figure 1.
Worldwide meat production from 1961 to 2018, as reported in reference [7].

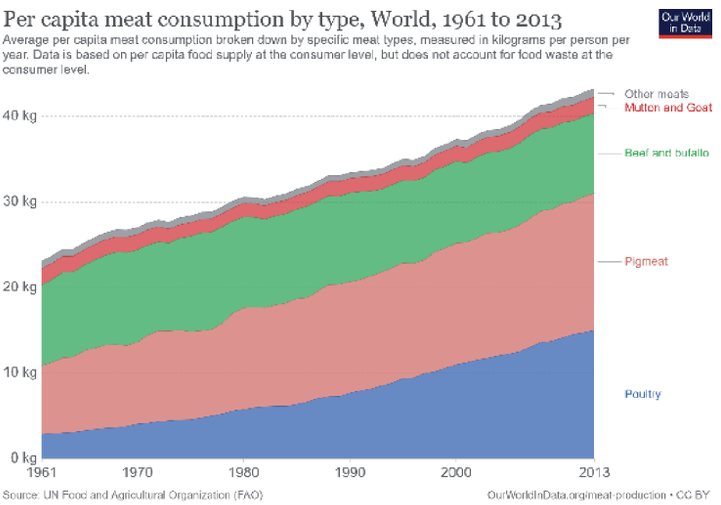

Figure 2.
Worldwide capita meat consumption from 1961 to 2013, as reported in reference [7].

Establish the quality of protein-based foods is anything but trivial. The first concern that makes the freshness assessment paramount is to reduce the risk of food poisoning, which is classified according to the responsible microorganism (bacteria, viruses, or parasites). The foodborne diseases could be the consequence of the consumption that has exceeded the expiration date. Significant contributions also come from lousy storage, adverse events along the supply chain, or the consumer's improper manipulation. Food poisoning remains a public health issue and still affects 23 million people a year only in Europe. [8] Secondly, but no less critical, freshness assessment is mandatory to reduce unjustified food waste. The expiration date is established conservatively, as a consequence of what assessed above. The ambiguity of food labels (*use by* date, *best before* date, *expiry* date, *sell by* date), is sometimes misleading. Consequently, too many people throw away food only because it has passed the expiration date, often sacrificing food that is still perfectly safe. According to FAO, the social cost of food waste is around $ 882 billion. Food waste is worth € 15.5 billion per year in Italy, about 1% of Italian GDP; € 3 billion comes from waste during the food chain, between production and distribution. [9] In this scenario, the foods' quality control systems include specific analytical measures that provide reliable and irrefutable data but are limited for control agencies, requiring long-time analyses, equipped laboratories, and trained personnel.

Instead, the possibility of knowing the quality of the single piece of meat/fish that was purchased in the local supermarket and forgotten in the bottom of the fridge must be based on simple systems of low cost and easy to understand. Consequently, there has been a great effort in developing devices to this purpose. [2] The largest category of prototypes, also in an advanced commercial step, is focused on collateral properties, indirectly related to the food freshness. Devices aimed to control the cold chain's maintenance, [3, 10] or the modified atmosphere's integrity for the food packed under CO_2 [11] are clear examples. Sophisticated control of the meat's colour and the atmosphere required apps or trained people, and are far to give instrumentless and straightforward responses. It is also true for all electronic noses or tongues. [12–14], or devices that require a sophisticated algorithm. [15] Undoubtedly, the colourimetric sensors are the best candidates to develop a device to assess the freshness. These sensors, placed in the tray over the meat, revel the volatilome change from safe to hazard conditions through a simple colour change. These devices are based on a colour code, developed as an on/off strategy or a change of colour; they intrinsically offer an easy interface to the consumer or untrained people, as supermarket staff. The literature is plenty of proposals exploited on this principle, mainly based on pH indicators. [16–25].

In principle, for each degradation step, the volatile by-products exhibit different acid–base behaviour, resulting in changes in the headspace's acidity over meat samples. For instance, in beef meat, the early post-mortem (within one hour of slaughter) pH is between 6.7-7.1 [26] which does not differ from that of other dead animals. Indeed, when any muscle is converted into meat, the glycogen is hydrolysed by anaerobic glycolysis into lactic acid, manifested by a pH decrease to values between 5-6. Immediately after, bacteria start decomposition of the most straightforward consumable substances. At this step, despite the wide variety of microorganisms and substrates, the spoilage process of all proteinaceous foods is similar, being mainly related to the classes of precursors commonly present in these matrices. Firstly, the degradation of sugars and fats by bacteria produces molecules able to migrate from the meat to the headspace. In this phase, the volatile organic compounds, VOCs, are dominated by EtOH, 3-methyl-1-butanol and free fatty acids, mainly acetic acid. Consequently, the acidity in the headspace remains at a level around 5. Until this phase lasts, protein foods are safe products. A proper sensing device would exhibit the ability to recognise this stage, and, oddly, no attention was deserved to signal it. Only when glucose and its direct metabolites are depleted, the catabolism of proteins starts, producing an assortment of amines, known as the biogenic amines, BAs, sulphur compounds and thiols. As the spoilage proceeds, the discolouration and the production of off-odours make this stage manifested. By now, the by-products are toxic, and meat consumption at this stage could be a severe hazard. A sensing system must be able to recognise this step visibly.

The overwhelming majority of papers on this topic have stubbornly focused on sensing BAs in the headspace. Undoubtedly, BAs are present within the meat. They can be quantified by instrumental methods [27, 28] and titrated indirectly through methods that define the total volatile basic nitrogen, TVBN. The standard method is based on the digestion of meat in perchloric acid or trichloroacetic acid to extract the basic substances, transforming them into ammonium salt. A distillation in strong basic medium allows quantifying all the basic nitrogen as ammonia. [29].

Nevertheless, their existence in the solid does not imply finding them into the headspace, and BAs' fate is less dynamic than expected. This assumption has been proven when the volatile composition is determined. [30–32] If BAs were present in the volatile mixture, the pH would increase at definitively basic values. However, it is not the case. The golden point is: BAs are weak bases, many of them involved in one or more protonation equilibria. During degradation, only neutral

molecules can pass into the headspace. In the case of meat and other biological matrices, the pH is buffered at a value around seven. It is evident that, at these pH values, all the amines present a positively charged protonated form and cannot pass into the vapour phase. Also in advanced degradation, the acidity of meat slightly decreases, never exceeding neutrality, preventing amines occurrence in the headspace. When receptors' choice is based on synthetic or enriched samples tests, indicators with colour change at basic pH are often selected [15, 24]. However, this strategy fails when applied to real sample at home conditions (meat kept for a limited number of days in the refrigerator). It must be underlined that the fish represents the only exception: some amines are present also in the headspace due to different nitrogen excretion mechanism. Possibly for this reason, in the literature, many papers have focused on sensing the fish spoilage, [14, 18, 19, 33]. Nevertheless, also for fish, neither the pH reaches basic values, nor BAs different from trimethylamine can be detected in the headspace. The following will explain why the assumption of BAs sensing in volatilome has developed throughout the last twenty years of literature. This myth also persists when the devised solution would refuse the assumption.

2. The creation of a myth

The idea of the possible control of food quality based on sensors started to be discussed in the literature from the late nineties. Yano and coworkers presented in 1996 a biosensor based on electrochemical detection of putrescine and xanthine. [34] Another enzyme sensor array was proposed for the determination of BAs inside the meat. [35] The possibility to apply molecular recognition also for volatile amines detection was explored by [36]. These papers intended to underline the signal's versatility based on a development, or a change, of a colour. As long as real samples analysis is concerned, BAs' detection was mainly dedicated, as already underlined, to fish meat. [18, 19, 33] For instance, Pacquit and collaborators [33] developed a colourimetric sensor based on bromocresol green as a receptor. The sensor is responsive to the headspace composition and changes its colour from yellow to green, as the spoilage proceeds. The authors assessed that BAs' production causes pH change shown by the sensor, but they did not explain why they selected an indicator with $\log K_a$ less than five. They correlated the colour change with the bacterial growth patterns in cod and whiting fish samples, which is indirectly true, but they did not care to attest the presence of amines in the headspace. Electronic tongues for freshness analyses appeared in those years and the contribution of Gills and coworkers, who presented an array of sensors [14] for fish freshness assessment, is an example. This paper allows to point out another issue often encountered in the attempt to propose practical applications. The research is well organised, the array made of sixteen electrodes is well presented, the validation analyses performed. Nevertheless, it does not make sense to have a device able to assess spoilage to start from the 6th- 8th days of sea bream fillet chilled stored. After one-week chilled storage, there is no need for sixteen sensors, not even one, to assess the fish freshness. The point deserves a comment; there is much room between a successful experiment and a possible workable application based on that device, that could operate under real-life conditions. It is also the case where a chameleon probe to assess the biogenic ammines was presented. [37] The successful recognition was not performed in the vapour phase, but in solution with synthetic samples spiked with known BAs amounts. Also, Soga in 2013 [38] proposed an inkjet-printed paper-based colourimetric array to discriminate volatile ammines, but it was tested on vapours of seven primary amines. Solinas and collaborators [16] presented a colourimetric sensors array to check the meat freshness

from the real samples' VOCs analyses. However, the final two sensors array could discriminate chicken spoilage after 0-3 days from that after 5-7 and 10-12 days, stored in chilled conditions and modified atmosphere containers (30%CO_2 and 70%N_2). They applied the same device also to pork sausages. [39] The authors demonstrated that the change of colour is correlated with the microbial analysis. They clearly stated that one sensor specifically reacts with biogenic amines and aminated compounds present in the headspace. In [40] the researchers set up a portable optoelectronic nose applied to beef, chicken, pork, fish and shrimps samples, calibrated with H_2S, $(CH_3)_2S$, trimethylamine and cadaverine, but not proving all these analytes being present in the headspace of the investigated samples. The optical sensor design to detect amines during food spoilage, based on the colourimetric recognition of ammonia and biogenic amines, [41] works with all the most common BAs coming from a pH 8.4 buffer solution turning from green to red. The authors claim that the colour change on real meat samples is not equally good because the receptor's logK_a is 6.1, not enough to detect BAs. As will be argued, the reason is that in the headspace there are not BAs at all. They declare to look for a receptor with a lower logK_a. That device will work, not because of interaction with BAs, but because acid development ends, as highlighted below. A sensor designed explicitly for colourimetric detection of thiols and biogenic amines was developed by some of the same authors but tested only in solution on spiked samples. [42] The misleading idea is to deal with biogenic amines that can turn an indicator in its acid form into the basic one. In the paper of Kuswandi, two sensors based on methyl red, logK_a around 4.8, and bromocresol purple logK_a around 6.3, both placed in their acid form [20] are indeed able to sense the spoilage. They proved that the meat's pH, neither after 14 days of storage in a fridge, exceeds 7.30. If this receptor work, it is because it is not sensing amines, as authors claim. The sensor reveals that acid substances are no longer produced. Indeed, even if basic substances were now released, they are immediately neutralised by the buffer systems, preventing further pH increase and BAs volatility. The beef of the example is declared eatable after seven days of storage in the fridge. One week storage seems definitive too long, even if the meat, according to their description, does not come from a maturation process. Such a process is mandatory to reach the desired tenderness, especially with beef meat. As a comment, it is difficult comparing the performance of devices designed to respect different domestic regulatory regimes. As it happens in many fields, the acceptance of common regulation will improve the comparability and the reciprocal improvement in the research.

More recently in many colourimetric sensors, the conviction of sensing volatile amines persists, even if Chen, for instance, developed a device based on a selection of dyes that can assess the change of the pH, in the pork meat. The change the authors claim is correlated to BAs inside the meat since they estimated the TVNB. To be underlined that the pH ranged from 5.8 to 6.7 at the 8th degradation day. After one week in the fridge, it is hard to imagine the meat as eatable [17].

In a more recent paper, the authors synthesised pH indicators specifically for sensing ammonia vapours. They claim that the sensors react to ammonia produced over crabs, and one of them changes its colour between 7 and 12. How it could turn its colour over shrimps remains unclear. [24] A chlorophenol red-based sensor is also successfully employed to sense the spoilage but once more not because of amines in the headspace, as claimed [25].

3. Acid–base equilibria of BAs

A reliable picture of what develops over a piece of meat is acquired when the BAs' acid–base properties are carefully analysed. It suddenly becomes clear why

BAs cannot be found in the headspace over the piece of meat, if not at a miserable amount, at least under the average storage period, as it could happen to meat/fish in a domestic fridge. In **Figure 3**, the acid–base speciation schemes of the most common BAs are shown. They were calculated based on equilibrium constants reported in the literature.

Specifically see, for spermidine and putrescine, [43] cadaverine, [44] histamine, [45] spermine, [46] tyramine, [47] 2 ph-ethyl amine, [48] tryptamine, [49] trimethyl-amine. [50] In **Figure 3**, the vertical dotted lines are drawn in correspondence of the pH values where the fraction of L is equal to 0.1. It is worth to note that for spermidine, cadaverine, spermine, tyramine, the volatile L form reaches 10% only at pH higher than 10, putrescine at pH = 9.80, 2 ph-ethylamine and tryptamine at pH = 9.20. So, even if the catabolism of protein produces BAs into the meat, they cannot be part of VOCs if not in an insignificant amount. Trimethyl-amine, which is typically a by-product of the fish meat, is the only one that has a fraction of L equal to 10% at pH = 8.80, but this fraction represents 1% of the total at pH = 7.4. As underlined elsewhere, the fish metabolism differs from that land vertebrates, having the extraction system mainly through gills and skin. That is why the fresh fish has a fishy odour, and it explains why BA was found in the fresh cod fillets headspace. It is the only case where a headspace device could sense a BA.

The conclusions driven by these calculations, clearly highlighted by the graphs of **Figure 3**, are nothing but corroboration of the experimental evidence reported in papers dealing with real meat samples analyses, both in the solid and in the headspace previously discussed [27, 28, 30–32, 51, 52]. They are presented to make more explicit the real role of BAs in meat spoilage detection, performed by headspace sensors.

Nevertheless, insisting on the idea of looking for BAs in the headspace of spoiled meat can lead to devices that are not correctly centred on the target analytes. The comparison between the by-products dominating the two separated bodies, i.e. the

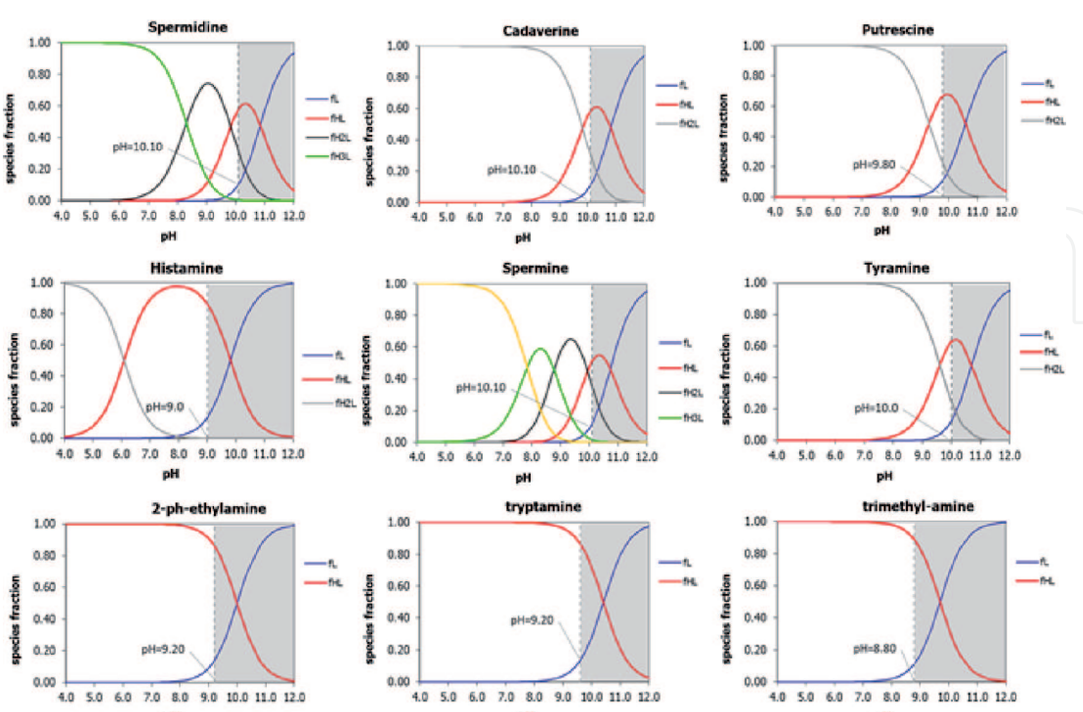

Figure 3.
Acid–base speciation scheme of some BAs calculated from logK_a values found in the literature. [43–50] the vertical line corresponds with the pH values at which the fraction of the fully deprotonated form (L) is 10%. The shadow area represents the L domain, where L fraction is above 0.1.

meat and the VOCs over it, analysed at the same degradation step, offers the best strategy to design a sensor and understand that misleading unambiguously. Here the analysis of the two samples of protein food, such as chicken and cod fillet, at different degradation steps is shown. These were the results of a validation procedure for assessing an array of colourimetric sensors' correct behaviour. The array's performances were explored to develop an intelligent label to signal chicken, beef, pork, and fish spoilage. See reference [4–6]. The device will be described below, and two patents have been deposited to protect the idea. [53, 54] The meat samples were analysed by HPLC-ESI/MS analysis, while the headspace HSSPME/GC–MS analysis. Details of the experimental procedures and the analytical methods can be found in reference [5, 6]. There are many papers where the composition of volatile substances [29–32] and the by-products developed inside the meat [27, 28] are presented. The findings of the experiments reported here are in agreement with this literature. The aim was not to fully characterise the substances produced during the spoilage but to underline the different main classes of substances developed in a meat sample and the atmosphere over it, at the same degradation level.

The results of the analyses are shown in **Table 1** for the chicken meat samples, in **Table 2** for the cod fillets. In the chicken meat case, the qualitative identification of BAs present in the solid was clear enough to distinguish the samples belonging to different degradation steps. In an early stage (analysis done within the first day after purchase), samples were classified as SAFE, and no amines were detected. For samples at the third-day chilled storage, where a slight discolouration and off-odour are perceived, classified as a WARNING zone, 4 out of 7 amines were found. All the BAs under investigation were detected in samples analysed after 4-5 days of storage, classified as HAZARD, when a strong off-odour was also perceived. Opposite, in the case of cod fillets, almost all the BAs can be detected, even in the samples analysed just after purchase. It is well known that fish samples contain amines, even when fresh since the fishy odour is due to volatile amines, in particular trimethylamine.

It must be considered that, due to the high perishability of this food, the production of BAs is very fast and significant even in the time required for the analysis, being the manipulation performed at room temperature and lasting some hours. This drawback originated with the continuous changes in meat composition during analysis was also underlined by Marta Mikš-Krajnik [31].

Nevertheless, a substantial difference can be observed between the samples analysed immediately (SAFE samples) and the ones after three-day storage (HAZARD samples) in the amount of BAs detected. Except for 2-phenylethylamine

BA	Ion(P)	S	Area S	W	Area W	H	Area H
Spermidine	146	—	—	✓	573 723	✓	558 980
Cadaverine	103	—	—	—	—	✓	199 409
Putrescine	89	—	—	—	—	✓	13 577
Histamine	112	—	—	✓	38 082	✓	37 320
Spermine	203	—	—	✓	55 085	✓	45 372
Tyramine	138	—	—	—	—	✓	25 4478
2-ph-ethylamine	122	—	—	✓	32 923	✓	30 311

Adapted with permission from reference [5].

Table 1.
The BAs detected in chicken meat, performed through HPLC-ESI/MS analysis, in correspondence with the three degradation steps, S (safe), W (warning) and H (hazard).

and tryptamine, the pick areas of amines almost double from SAFE samples to the HAZARD ones.

At the same time, the headspace was analysed, too. The results are reported below, in **Tables 3** and **4** for both foods.

In the case of chicken meat, the interpretation is straightforward. Initially, in the samples analysed within the first-day storage (SAFE), very few classes of compounds were detected, mainly acid compounds. For the samples analysed in at the intermediate spoilage, ketones and esters were found, released after the bacterial catabolism of sugars and their derivatives. However, no amines were detected, even if, at this stage, they were present in the meat, see **Table 1**. Nevertheless, more surprisingly, neither in the meat classified as HAZARD, when all the BAs were detected in large amount in the solid, see **Table 1**, amines were found in the headspace, confirming our previous assumption on this topic. [5, 6] Deserve note the presence of indole, in the last samples belonging to the HAZARD stage, originated by bacteria degradation of tryptophan.

In the case of cod fillets, the composition of the headspace in the two samples was very similar, and trimethylamine was present in both samples, nevertheless

BA	Ion(P)	S	Area S	H	Area H
Spermidine	146	✓	13 079	✓	28 800
Cadaverine	103	✓	31 739	✓	62 863
Putrescine	89	—	—	—	—
Histamine	112	✓	8 993	✓	40 463
Spermine	203	✓	12 111	✓	34 185
Tyramine	138	—	—	✓	19 399
2-ph-ethylamine	122	✓	53 750	✓	68 334
Tryptamine	161	✓	52 519	✓	63 562

Adapted with permission from reference [5].

Table 2.
The BAs detected in cod fillets through HPLC-ESI/MS analysis, at two degradation steps, S (safe) and H (hazard).

	S	W	H
Alcohols	✓	✓	✓
Aldehydes	✓	✓	✓
Ethanol	✓	✓	✓
Acids	✓	✓	✓
Ketones	—	✓	✓
Esters	—	✓	✓
Thiols	—	✓	✓
Biogenic amines	—	—	—
Indole	—	—	✓

Adapted with permission from reference [5].

Table 3.
The class of substances detected in the headspace of chicken meat, performed through HSSPME/GC–MS analysis, in correspondence with the three degradation steps S (safe), W (warning) and H (hazard).

	S	H
Alcohols	✓	✓
Aldehydes	✓	✓
Ethanol	✓	✓
Acids	✓	✓
Ketones	✓	✓
Esters	✓	✓
Thiols	✓	✓
Trimethylamine	✓	✓
Light volatile amines	—	✓
Biogenic amines	—	—
Indole	—	✓

Adapted with permission from reference [5].

Table 4.
Class of substances detected in the headspace of cod fillets, performed through HSSPME/GC–MS analysis, at two degradation steps, S (safe) and H (hazard).

crucial details were observed. Volatile amines (here defined as ammonia, mono and dimethyl amines) were present in the HAZARD step, but again BAs were not detected; secondly, indole was revealed in the second part of the degradation process, similarly to what observed in chicken meat samples.

The instrumental analysis performed simultaneously, confirmed that BAs were produced, even in large quantity, in the solid when food is no longer eatable. Conversely, BAs are not detected in the headspace at any step. This distribution is caused by the buffers present in the food that leave BAs in their protonated form, preventing their possibility to pass into the vapour phase. As a result, the pH in the headspace shows only a very slight increase and consequently only the receptor with a very low log K_a turned out to be informative to detect the beginning of the final spoilage stage value, showing a complete and glaring conversion to the basic form. Only for fish samples, some small volatile amines are present but at such a low concentration as not to require a different sensing approach from that one proven to be suitable for chicken meat.

4. Monitoring spoilage from a new perspective

Based on the evidence that BAs cannot be present in the headspace, and the protein catabolism is associated with a small increase of the pH, the idea of controlling the entire spoilage can be faced differently. The two separate steps of spoilage can be identified through indicators that change their colour to sense slight pH modification, placing them into the array in the most convenient form.

A clear signal must help to identify the persistence of the safety zone when protein catabolism is still far away. Depending on the type of food, the beginning of the release of dangerous metabolites could arise in a short time, as it happens for fish, or long enough to allow to detect a transition in between. These behaviours reflect a common situation in cooking: the fish turns quickly from being judged eatable, to an undesirable aspect, a discolouration and a strong unpleasant smell. Opposite we all experienced how other meats take longer time to evolve to a final state and, consequently, to widen our indecision about their fate. At least another sensor, or two, must detect this further step or the transition to the final one, well different from the first.

On this knowledge, a first array was tested. It was a sort of "proof of concept" to assess the feasibility to employ the sensor for naked-eye detection of the spoilage. The indicators were here fixed by ion exchange on a cellulose-based sheet. Details can be found in ref. [5, 6, 53, 54].

A fundamental aspect of the strategy deserves a particular focus. The devices were tested on the same plastic trays usually employed in the supermarket to sell meat, keeping food in the domestic refrigerator for a time lasting just a couple of days after the expiration day, never more than 5-6 days, even less in case of fish. This type of meat as prepared in the supermarket, not in modified atmosphere, is supposed to be consumed within three days after purchase, thus much longer monitoring time lengths sounds useless. As already commented, very commonly, in the literature, the observation times are extended to weeks.

To detect the different stages of meat degradation, the receptors must be selected carefully. In **Table 5**, the most common pH indicators are reported. Receptors from one to four, with higher $\log K_{a1}$, can signal the phase characterised by the acid development. If placed in their basic form over the meat, they change in colour of their protonated form, because of the reaction with the acidic substance typical of the first degradation step. It is worth to note that none of them come back to the basic colour when the proteins catabolism starts, neither bromothymol blue. It is another proof that the pH of the headspace is below seven, as already argued. Conversely, the following dangerous transition can be signalled by an indicator with lower $\log K_{a1}$, put in its acid form. The sensor selected was that containing chlorophenol red indicator. It remains unchanged during the first step, but it turns into its basic colour as soon as acids are not produced anymore.

This strategy is presented in the array of sensors reported in [4–6].

The five indicators of **Table 5**, plus another one able to sense sulphur and thiols, based on Ellman's reagent, was all tested, to select a reasoned final triplet.

An example of the pilot experiment is reported in **Figure 4**. More details can be found in the original papers. It was chosen to operate in conditions like that encountered in real life, to obtain a tailored product, that works under condition that a consumer experienced. Most of the meat and fish are sold in supermarkets prepared by the central slaughterhouse into plastic trays (PP) and covered with low permeability polyethene plastic film. The ratio between the amount of meat and the volume of the headspace, the optimum amount of dye able to combine sensitivity and naked-eye detection and the most reliable and efficient sensors preparation procedure were all taken into account.

A naked eyes evaluation of the evolution of the colours identifies the most informative sensors. The development of a glaring colour among different degradation steps is mandatory for a practical application, and some sensors resulted in practice better than others. Nevertheless, the photos of the sensors colour' changes

	$\log K_{a1}$	$\log K_{a2}$
m-Cresol purple	8.32	1.57
o-Cresol red	8.20	1.11
Thymol blue	8.9	1.5
Bromothymol blue	7.1	
Chlorophenol red	6.0	

Table 5.
The selection of golden pH indicators for spoilage meat sensing based on their protonation constants, as found in reference [55, 56].

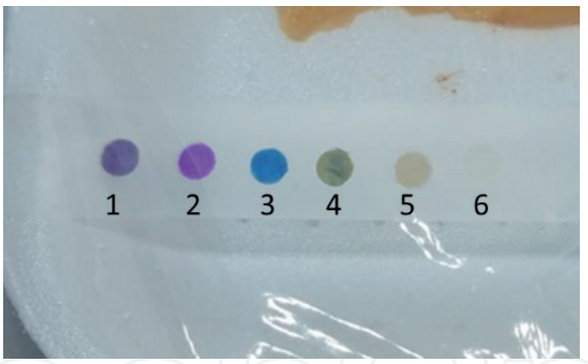

Figure 4.
*An example of the array placed over the tray containing the poultry meat, with sensors from one to six based on m-cresol purple (**1**), o-cresol red (**2**), bromothymol blue (**3**), thymol blue (**4**), chlorophenol red (**5**), and the Ellman's reagent (**6**).*

were acquired, transformed into RGB triplets, for each sensor at any registered time. The PCA, Principal Component Analysis, well known chemometric tool, was selected to represent the data. Indeed, PCA allows summarising in a few graphs all the information contained in a wide dataset. It is the case of the RGB triplets data collected during the entire degradation. After performing PCA, the most important variables, i.e. the most significant sensors that describe the spoilage, are identified in the so-called loadings plot. In the scores plot, samples with similar properties result close to each other, i.e. the samples at the same degradation step are grouped. Details about PCA could be found elsewhere, but the PCA applied to the present dataset, confirms the qualitative findings. In the case of chicken breast, beef and pork meat, a stage between the safe step and the hazard one can be detected, not for fish. [6] In **Figure 5**, as an example, the PCA models on the first two components for chilled stored chicken samples are shown. Only the three most significative sensors are included. In the loadings plot, the different contribution of the three sensors arises. In the scores plot, the sensors' evolution, from colours describing safe samples placed on the left, toward hazard condition to the right emerges clearly. The model was validated; it is not an artefact. Indeed, external samples are correctly projected into the model, see the blue samples in **Figure 5**. Chemical analyses of samples assigned to the three steps effectively belong to different spoilage stage.

The results confirm that the sensors' change of colours follows the real evolution of volatilome. The array allows a naked eye evaluation, feasible also for a consumer. It means that it is possible to assign a precise colour to each step: these reference colours can be printed on a label. The array inserted into the package containing the meat samples will change its colour according to the spoilage evolution. Comparing the colour of the array with the reference, anyone can decide at home the fate of the meat in the fridge, without any doubt.

As a further evolution of the array, the solid support was changed. [57] In the first cellulose-based array, the dyes were fixed by ion exchange. This easy and cheap construction was chosen for its versatility, low cost, and the possibility to obtain a large number of testing materials was a necessary proof of concept.

The derivatisation on EVOH copolymer, intensely employed by the food packaging industry as an oxygen barrier film, was the choice to covalently bind the receptor on the support. [53, 54] These derivatives are stable, still very cheap and prevent any leaching of the indicator. The final product, obtained in grains, was filmed by a mechanic press under controlled temperature and pressure. From the foils, sensors of 0.5 cm of diameter were obtained using an office puncher. [57] An example of the materials is presented in **Figure 6**. The reference [58] reports the synthesis and the characterisation of the material.

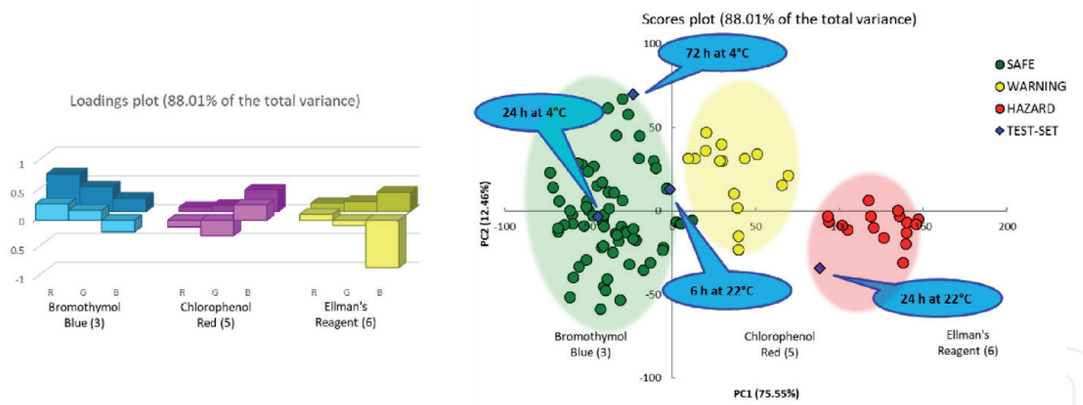

Figure 5.
PCA model on the data set of the RGB data of three indicators, based on the first two components that explain 88.1% of the total variance. On the left, the loadings plot, in foreground values on the PCA1, in the background those on PCA2. On the right, the scores plot. The blue bubbles identified the projection of external samples. The green, yellow, and red shadow areas collect most of the samples defined as safe, warning, and hazard. Adapted with permission from reference [5].

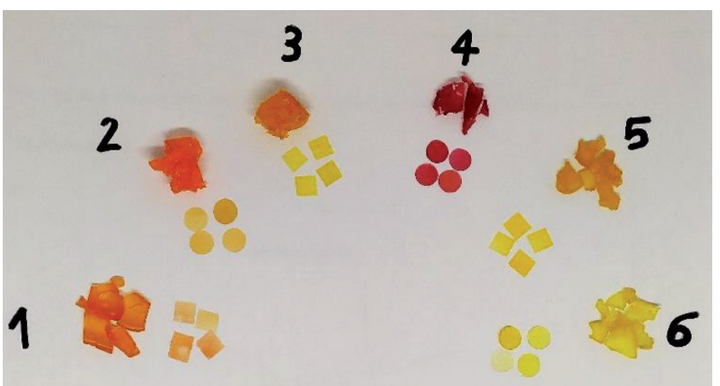

Figure 6.
Grains and miniaturised sensors obtained after functionalisation of EVOH with different sulphonftaleins, from left to right m-cresol purple (1), o-cresol red (2), bromothymol blue (3), thymol blue (4), chlorophenol red (5) and bromophenol blue (6).

These sensors exhibit glaring, highly reproducible colours. As seen in **Figure 6**, the materials are suitable to be tested as meat spoilage sensors. The receptors' encapsulation into the polymeric mainframe, makes indicators fixed into the mainframe weaker acid and their protonation constants moved to higher values, at least one order of magnitude. [57, 58]. This evidence was taken into account and the array modified. The acids developed over the tray can still be revealed by any receptor with logK_{a1} higher than seven, see **Table 5**. The first four indicators, placed in their basic form, are still, or even more, suitable candidates. Indeed, they very quickly react with the acidic volatilome, typical of the first save degradation step. The chlorophenol red-base sensor, previously employed to detect the transition toward spoiled meat does not work anymore. In the EVOH derivative, it changes the colour at too high pH. Solution tests demonstrated it very clearly, as shown in **Figure 7** At pH equal to seven, the dye in solution is definitely in its basic colour, while when inserted in the solid, equilibrated at the same pH, it is only in the transition from its acidic to its basic colour.

The solution that accounts for the sensors' acid–base properties changes is to develop a dual-sensor device.

The acid–base equilibria of *o*-cresol and the colours associated with the three different species are reported in the upper part of **Figure 8**. The advantage is that in the EVOH derivative, being the protonation constant higher, the fully protonated form is obtained in less extreme pH values and once obtained is stable at the air.

The idea of the dual-sensor based on EVOH derivatised with *o*-cresol is conceptualised in the lower part of **Figure 8**.

Suppose the *o*-cresol red-base sensor is placed in its fully protonated and deprotonated forms, as reported in the last picture on the right of **Figure 8**. Once the sensor is filmed, the spot at the two extreme acidic and basic forms exhibits almost the same violet colour. On the contrary, the yellow colour of the mono-protonated form almost disappears. The double *o*-cresol based sensor works as an on/off device. The first transition is signalled by the basic form of the indicator, which turns off. It means that the fully deprotonated form reacts with acid becoming the pale yellow of the monoprotonated form. As long as acids developed in the headspace, during the first phase, the other spot placed in fully protonated form, remains violet; in the on/off terms, switched on. The final stage is signalled by the last spot shutdown, due to the slight increase of the pH environment.

The first tests on real chicken samples seem very promising. This device's advantages will be evident when moving to the lab product to a market validation prototype. There is not any need to carefully print the reference colours and for the consumer to compare three different colours. The intelligent label can be read as binary on/off signal. Other protein foods must be tested, and further experiments have been already untaken. Presently, some features, such as low cost, easy implementation, absence of leaching, have been achieved.

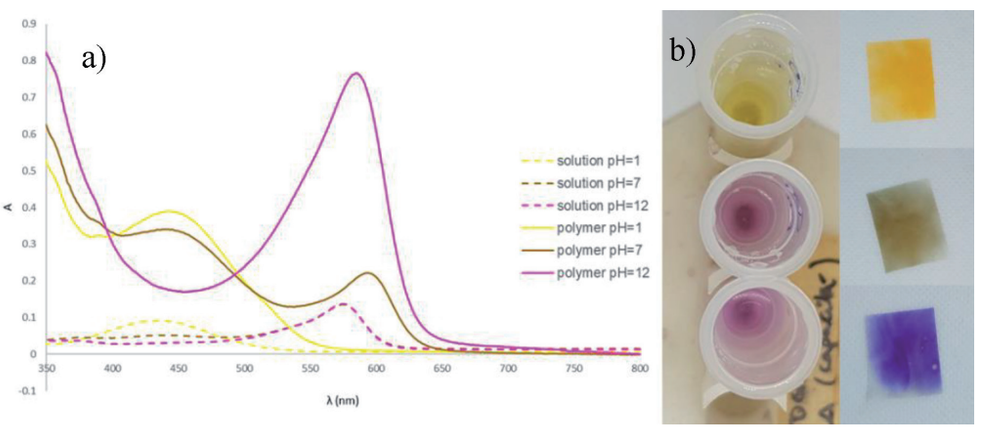

Figure 7.
UV–vis spectra (a) and corresponding photographs (b) of chlorophenol red-solution 11 μM (dashed lines) and the corresponding colour of the chlorophenol red-EVOH@ (solid lines) after equilibration at pH 1 (up), 7 (Centre) and 12 (down).

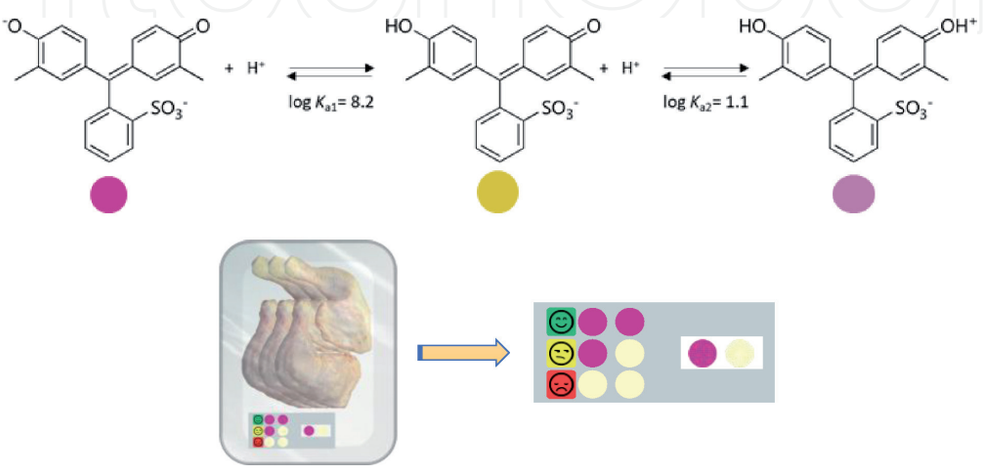

Figure 8.
In the upper part, the different acid–base forms of the o-cresol red and their colours. In the lower part, the new prototype of the intelligent label inserted in the tray with its magnification.

The device illustrated here is at an advanced level of development compared to most of the ideas proposed in the last twenty years' literature, aimed at developing smart labels to be inserted in food packaging. In the case studies presented, the authors expect the idea to develop into a concrete product. The mission is to answer two main issues behind the continuous development and increase of the proteinaceous foods market. Food poisoning is still a public health concern: such labels could substantially reduce the consumption of failed food, especially when it origins at home, after purchase. A not less critical point, clear indication of the spoilage level could limit food waste and allow food consumption beyond the expiry date. Indeed, when the food is stored in optimal conditions, the expiry date induces to dismiss food still edible since the date is established conservatively. In our economy, the waste of still eatable food represents a substantial not admissible social cost.

5. Conclusion

The misleading idea to find BAs in the headspace was born and persist in the literature that deals with the colourimetric sensors designed to follow the protein food spoilage. Often the researchers assess to detect biogenic amines. However, they selected receptors with $\log K_a$ values low enough to sense the slight increase in pH, not caused by the presence of BAs in the volatilome. Indeed, even in an advanced decomposition state, the meat pH values never exceed neutrality, preventing BAs from being present in their volatile form.

There is a great demand to have optodes for degradation monitoring. The fundamental issues in the design of these sensors are summarised below.

First start point should be an accurate description of the problem, which, on the contrary, has often been rough. In this field, maybe better results in less time would have reached. Nowadays, many optodes, present in the literature, are close to the objective, but often unknowingly, as underlined above.

The second point that deserves attention is that candidates for intelligent labels must be developed under conditions close to that a consumer faced real life. The research must go on with proof of concept, but dealing with applied science, greater attention to boundary conditions is desirable.

The device here presented fulfils all the issues and is an advanced development phase, even if preindustrial. For instance, EFSA authorisation to declare the labels suitable for food packaging is pending; the pilot production in high quantity will start only within this year to deeply evaluate the industrial scalability. The research is presently focused on compostable or eatable materials, to be used as solid support. The aim is to offer a green alternative to plastic based EVOH derivatives. The environmental impact must become a pressing concern when new products move from the lab into the market, being the footprint into the world always as light as possible.

There is common awareness about the social and economic impact of the intelligent systems in the market. The authors hope to see reliable smart devices into the market that give food quality granted and have made a contribution in this sense.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

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