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#### Analytical Methods

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## Droplet-based luminescent sensor supported onto hydrophobic cellulose substrate for assessing fish freshness following smartphone readout



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#### ABSTRACT

In this work, two sensitive droplet-based luminescent assays with smartphone readout for the determination of trimethylamine nitrogen (TMA-N) and total volatile basic nitrogen (TVB-N) are reported. Both assays exploit the luminescence quenching of copper nanoclusters (CuNCs) produced when exposed to volatile nitrogen bases. In addition, hydrophobic-based cellulose substrates demonstrated their suitability as holders for both in-drop volatile enrichment and subsequent smartphone-based digitization of the enriched colloidal solution of CuNCs. Under optimal conditions, enrichment factors of 181 and 153 were obtained with the reported assays for TMA-N and TVB-N, respectively, leading to methodological LODs of 0.11 mg/100 g and 0.27 mg/100 g for TMA-N and TVB-N, respectively. The repeatability, expressed as RSD, was 5.2% and 5.6% for TMA-N and TVB-N, respectively (N = 8). The reported luminescent assays were successfully applied to the analysis of fish samples, showing statistically comparable results to those obtained with the reference methods of analysis.

#### 1. Introduction

Food quality monitoring is of utmost relevance to maintain human health safety. Particularly, proteins and other nitrogen-containing compounds present in fish and other marine products are degraded by biochemical and microbial processes (Chan et al., 2006; Prabhakar et al., 2020), resulting in the generation of ammonia (NH<sub>3</sub>-N), dimethylamine (DMA-N) and trimethylamine (TMA-N), the total content of which is known as total volatile basic nitrogen (TVB-N), i.e. a total index widely used for fish freshness assessment. Thus, TMA-N and TVB-N are commonly monitored to assess the degree of fish freshness, since their presence is related to the quality deterioration of fish after harvesting due to off-flavor formation (Bekhit et al., 2021; Lin et al., 2021; Zhang et al., 2022).

TMA-N and TVB-N are usually determined by the corresponding AOAC official methods (AOAC, 2000b, 2000a). Particularly, TMA-N is determined by the AOAC 971.14 method, which involves homogenization of fish samples with trichloroacetic acid (TCA), followed by solvent extraction with toluene and colorimetric determination after chemical reaction with picric acid (AOAC, 2000b), whereas the AOAC 999.01 method makes use of an ammonia ion selective electrode for determination of TVB-N (AOAC, 2000a). In addition, the European Union proposed the distillation of a sample extract with subsequent titration of volatile bases absorbed by an acid receiver as the reference method for TVB-N determination in fish samples (European Commission, 1995). Several alternative methodologies have been reported in the literature for the determination of TMA-N and/or TVB-N in fish products to overcome certain drawbacks of the above mentioned methods, including optical, electrochemical and mass spectrometric detection, requiring in certain cases chromatographic or electrophoretic separations (Prabhakar et al., 2020). In spite of the improvements achieved (e.g., sensitivity and degree of automation), certain limitations remain, including extended analysis time, tediousness, relatively expensive and hardly portable instrumentation and/or the necessity of highly skilled operators (Chan et al., 2006; Lin et al., 2021). In addition, complex sample preparation processes involving non-negligible amounts of hazardous chemicals are commonplace in these methods, resulting in the generation of substantial wastes (Dehaut et al., 2015). Thus, the development of alternative strategies that enable the straightforward determination of TMA-N and TVB-N even at the early stages of storage with potential portability is highly desirable.

A cutting-edge trend in analytical chemistry concerns the development of affordable and rapid non-instrumental methods. In this vein, everyday IT and communications devices can substantially contribute to

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the development of non-instrumental detection systems, commonly in combination with paper-based analytical devices (PADs) (Capitán-Vallvey et al., 2015; Fan et al., 2021; Grudpan et al., 2015; Kalinowska et al., 2021). Some advantages of PADs include the reduction of costs, portability and ease of operation. However, PADs usually show limited sensitivity, which can be solved using a number of preconcentration strategies (Bendicho et al., 2021). In this sense, the implementation of hydrophilic PADs modified with a number of molecular and nanostructured materials in headspace microextraction systems has enabled the sensitive detection of volatile analytes (Gorbunova et al., 2020; Huang et al., 2016; Placer et al., 2021, 2022; Saraji & Bagheri, 2018). Furthermore, waterproof cellulose-based substrates have recently demonstrated their suitability and convenience as holders to perform both in-drop enrichment and plasmonic colorimetric sensing of volatiles with improved sensitivity when compared with hydrophilic PADs (Villarino et al., 2022).

Metal nanoclusters (NCs) are tiny nanomaterials that are arousing growing interest in catalysis, bioimaging or sensing due to their intriguing properties between those of larger metallic nanoparticles and molecule-like compounds (Shi et al., 2017; Yao et al., 2021; Yu et al., 2015). Particularly, CuNCs are easy to synthesize, their cost is significantly lower than that of NCs derived from noble metals and, more importantly, CuNCs show unique photoluminescence, thus attracting considerable interest as luminescent nanoprobes for sensing purposes (An et al., 2020; Pena-Pereira et al., 2019; Zhang et al., 2018). However, as far as we know, CuNCs have not been evaluated yet as luminescent probes for determination of TMA-N and TVB-N.

For this purpose, a sensor was built upon confinement of a luminescent probe (i.e. CuNCs) into a droplet which was supported onto a hydrophobic cellulose substrate and subsequently exposed to the headspace above fish extracts. With this configuration, the application of different experimental conditions (i.e. extracts with and without formaldehyde) enabled both the enrichment and sensing of relevant volatile nitrogen-containing bases such as TMA-N and TVB-N for fish freshness assessment.

#### 2. Experimental

#### 2.1. Reagents and materials

All reagents were of analytical reagent grade. High-purity deionized water produced from a Millipore Sigma Simplicity ultrapure water system (Millipore Iberian S.A., Madrid, Spain) was used. Polyvinylpyrrolidone (PVP) from Fluka (Buchs, Switzerland), copper sulfate pentahydrate from Probus (Badalona, Spain), 1-ascorbic acid from Sigma-Aldrich (St. Louis, MO, USA) and sodium hydroxide from AnalaR Normapur, VWR (Leuven, Belgium) were used to synthesize CuNCs. The following reagents were used for the preparation of standard solutions: ammonium chloride (Sigma-Aldrich), dimethylamine (DMA-N) and trimethylamine (TMA-N) as hydrochlorides (Sigma-Aldrich). Additionally, 25% (w/w) NH<sub>3</sub>-N solution, 40% (w/w) DMA-N solution and 40% (w/ w) TMA-N solution (Sigma-Aldrich) were used to estimate the enrichment factors achieved by the proposed in-drop luminescent assays. In addition, ethanol and toluene from AnalaR Normapur; picric acid, phenolphthalein, methylene blue and methyl red from Sigma-Aldrich and boric acid and sodium carbonate from Panreac (Barcelona, Spain) were used to apply the reference methods for TMA-N and TVB-N determination (European Commission, 1995; AOAC Official Method 971.14, 1995). Furthermore, histamine (Fluka), acetaldehyde (Merck), benzaldehyde (Acros Organics, Geel, Belgium) and ethyl acetate (Sigma Aldrich) were used to evaluate the selectivity of the assays. Trichloroacetic acid from Panreac, 37% (w/w) hydrochloric acid and 39% (w/v) formaldehyde from AnalaR Normapur were also employed.

Whatman 1PS Phase Separator (Maidstone, Kent, UK) consisting in filter paper impregnated with a stabilized silicone, was used as hydrophobic cellulose-based substrate, whereas the hydrophilic Whatman No. 1 filter paper (Whatman) was used for comparison purposes.

#### 2.2. Apparatus

A Samsung Galaxy A70 smartphone (Samsung, Seoul, South Korea) and a C-10 Chromato-Vue mini-UV viewing cabinet (Analytik Jena, Jena, Germany) with a 254/365 nm UV lamp (The Science Company, Lakewood, CO, USA) were used for digitization of CuNCs-containing microdrops.

A Horiba Fluoromax-4 spectrofluorometer (Horiba Scientific, Edinson NJ, Tokio, Japan), an Agilent Cary 300 UV–vis spectrophotometer (Agilent Technologies, Palo Alto, USA), and a JEOL JSM-2010 transmission electronic microscope (Tokyo, Japan) were used for the characterization of CuNCs. In the latter case, electron energy loss spectroscopy (EELS) was performed in the scanning transmission electron microscopy (STEM) mode.

A Xerox ColorQube 8580 printer (Rochester, New York, USA) and a Phoenix instrument RSM-02HP+ magnetic stirring hot plate (Garbsen, Germany) were employed to pattern hydrophobic barriers on Whatman No. 1 cellulose substrates by wax printing as described elsewhere (Carrilho et al., 2009).

A VELP Scientifica UDK126A Distillation Unit (New Jersey, USA) was used to carry out analyses of fish samples according to the reference methods. Microextraction experiments were carried out employing 15 mL amber vials sealed with PTFE-faced silicone rubber septa.

#### 2.3. Data processing

RGB Color Detector App (The Programmer, Google Play Store) and ImageJ, a free image analysis program (Schneider et al., 2012), were used for data acquisition. Specifically, ImageJ was employed for obtaining the analytical results included in the manuscript, whereas the RGB Color Detector App has been complementarily used during method development. Statgraphics Centurion XVI.I (StatPoint Technologies, Warrengton, VA, USA) software was used for the optimization of experimental parameters.

#### 2.4. Preparation of CuNCs

CuNCs were prepared following a template-assisted procedure as described elsewhere (Pena-Pereira et al., 2019; Shi et al., 2017). Briefly, 1 mL of 10 mM Cu(II) and 1 mL of 100 mM ascorbic acid were added sequentially to an 8 mL volume of 6.2% (w/v) PVP aqueous solution. After adjusting the pH to 4.0 by drop wise addition of 0.1 M NaOH, the solution was left for 3 h at 75 °C. Finally, the solution was centrifuged at 6000 rpm during 10 min and the supernatant was kept in amber vials at 4 °C until use.

#### 2.5. Fish sample pretreatment

Six fish samples were analyzed in this work to evaluate the applicability of the developed assays. European sea bass (*Dicentrarchus labrax*), Gilthead seabream (*Sparus aurata*) and Atlantic pomfret (*Brama brama*) were procured deceased from a local market in Vigo, Spain, where they were stored on flake ice. In addition, commercial frozen fish fillets of European hake (*Merluccius merluccius*), Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) were purchased from a local supermarket in Vigo, Spain, where they were stored at -20 °C. All samples were transported on ice and analyzed the same day of purchase.

Fish samples were pretreated following the procedure described in the AOAC Official Method 971.14 before analysis (AOAC Official Method 971.14, 1995). In brief, fish samples were thawed (during one hour in the refrigerator) if needed, then minced and 10 g of sample was homogenized with 20 mL of 7.5% (w/v) TCA. The blended solution was then centrifuged for 10 min at 2500 rpm and the supernatant was collected and transferred to a 25 mL volumetric flask for further

#### processing.

#### 2.6. Experimental procedure for TMA-N and TVB-N determination

For TMA-N determination, a 5 mL volume of aqueous solution (blank, standard solution of TMA-N or sample extract obtained as described in section 2.5) was placed in a 15 mL amber vial with a stir bar and 500 µL of 39% (w/v) formaldehyde solution was added. A 2.0 cmcircular piece of a hydrophobic cellulose-based substrate, namely Whatman 1PS, was placed inside the screw cap over a PTFE-faced septum, and a drop of 5  $\mu$ L of CuNCs was deposited onto the substrate before the vial was sealed. Then, 1.0 mL of a 3 M NaOH solution was externally added, and the aqueous solution was stirred at 1200 rpm for 25 min. Finally, the screw cap containing the CuNCs microdrop was placed in a dark viewing cabinet and exposed to UV irradiation (365 nm) for digitization of the drop using a smartphone, with the ISO and the exposure value (EV) set at 200 and +1.5, respectively (Fig. S1 and Video S1). The analytical response (mean color intensity difference between the standard or sample and a blank,  $\Delta Ic$ ) was measured in the B channel of the RGB color space.

The above procedure was followed for TVB-N determination, except that i)  $NH_3$ -N standards were used for calibration and ii) the addition of formaldehyde to the aqueous solution was omitted.

#### 3. Reference method for TMA-N determination in fish samples

TMA-N was also determined in fish samples by the AOAC Official Method 971.14 (AOAC, 2000b). In brief, blanks, standards or sample extracts (obtained as described in *Section 2.5*) were placed in a tube and the volume was adjusted to 4 mL with ultrapure water. Then, 1 mL of 39% (w/v) formaldehyde, 10 mL of toluene and 3 mL of sodium carbonate were added to the solution. The mixture was shaken vigorously by hand and 7 mL of the toluene phase was transferred to another tube containing 0.2 g of anhydrous sodium sulfate. Finally, 5 mL of a 0.02% (w/v) picric acid solution was added to 5 mL of the organic phase to form a colored complex whose absorbance was measured at 410 nm.

#### 4. Reference method for TVB-N determination in fish samples

The determination of TVB-N in fish samples was carried out on the basis of the reference method recommended by the European Commission (European Commission, 1995). Thus, 10 mL of fish extract, obtained as described in *Section 2.5*, was placed into a distillation tube together with 3 drops of 0.5% (w/v) phenolphthalein. A volume of 40% (w/v) NaOH solution was then added for alkalinization of the solution, and the volatile bases were absorbed by a 20 mL volume of a 4% (w/v) aqueous boric acid solution placed in an Erlenmeyer flask together with 4 drops of Tashiro indicator (methylene blue: methyl red, 1:2). The amount of TVB-N present in the receiver solution was finally determined by titration against a standardized 0.01 M HCl solution.

#### 5. Results and discussion

#### 5.1. Characterization of CuNCs in the absence and presence of bases

CuNCs were firstly characterized by UV–vis spectrophotometry, fluorescence spectroscopy and STEM-EELS (Fig. 1). Thus, the UV–vis spectrum of CuNCs exhibited bands at *ca.* 230, 330 and 380 nm (Fig. 1A), being presumably associated with inter-band electronic transitions (Wang et al., 2017), whereas the lack of bands appearing around 500–600 nm evidenced the absence of large copper nanoparticles (Wang et al., 2016). In addition, the fluorescence spectra of CuNCs displayed maximum excitation and emission wavelengths located at 378 and 424 nm, respectively (Fig. 1B). CuNCs with a size diameter of  $2.8 \pm 0.8$  nm were obtained, as shown in the STEM image and the corresponding size distribution histogram (Fig. 1C and D).

Furthermore, the Cu- $M_{2,3}$  edge observed at 74 eV in the low loss electron energy loss spectrum acquired in a specific CuNC clearly corresponds to Cu (Fig. 1E).

Significant (and reversible) effects on both the UV–vis and fluorescence spectra of CuNCs were noticeable at acidic and alkaline pH values (Fig. 2). Thus, a UV band located at *ca*. 295 nm increased on increasing pH of CuNCs solution, whereas the appearance of an additional UV band at *ca*. 232 nm with gradual decrease in the absorbance at *ca*. 295 nm occurred at acidic pH (Fig. 2A). In addition, a significant quenching of the fluorescence of CuNCs occurred at increasing pH values (Fig. 2B). The reversibility of the above effects was verified by successive adjustments of the pH value of CuNCs solutions to 2 and 12 with 0.1 M HCl and NaOH solutions, respectively (Fig. 2C and D). The slight decrease in the analytical response observed in the study could be attributed to the required dilution.

Based on the potential of CuNCs for pH sensing, additional studies were carried out with volatile *N*-containing basic volatiles of relevance for fish freshness assessment, namely NH<sub>3</sub>-N, DMA-N and TMA-N. As expected, the UV–vis and fluorescence spectra of CuNCs were modified when treated with the volatile nitrogen bases in an analogous way to that observed in alkaline media (Fig. 3A and B). Thus, the luminescence of CuNCs was significantly quenched in the presence of all evaluated *N*-containing bases, and optical reversibility was also observed. The STEM images of CuNCs exposed to NH<sub>3</sub>-N, DMA-N and TMA-N, shown in Fig. 3C-E, are characterized by particle agglomeration in contrast to those obtained in the absence of bases (Fig. 1C).

A sensing mechanism based on pH-induced agglomeration of CuNCs is proposed herein. The non-covalent interactions that maintain CuNCs dispersed at acidic pH values are presumably affected by the deprotonation of capping ligands in the presence of the volatile bases, thus leading to unstable colloidal solutions that result in the agglomeration of CuNCs (Dong et al., 2016, 2017; Wang et al., 2015), which further influences the optical properties of CuNCs.

# 5.2. Optimization of parameters for the determination of TMA-N and TVB-N $% \mathcal{T}_{\mathrm{TVB}}$

Two analytical strategies involving CuNCs have been devised for the non-instrumental determination of two chemical parameters that can be related to fish freshness, namely TMA-N and TVB-N. The stability of CuNCs on the hydrophobized cellulose substrate (i.e., Whatman 1PS), was evaluated in preliminary experiments. Thus, CuNCs drops of increasing volumes were deposited onto Whatman 1PS substrates and exposed to the headspace above aqueous solutions (blanks) for 30 min to evaluate their spreading and thus defining their potential applicability in microextraction experiments. As can be deduced from Fig. S2, CuNCs drop volumes of even 35 µL were hanged from Whatman 1PS substrates, even though with a non-negligible frequency of drop detachment. Drop volumes of up to 25 µL showed non-significant droplet spreading and remarkable stability, especially when drop volumes of up to 15 µL were used. The mean color intensity of CuNCs drops increased slightly with the drop volume, reaching a plateau when the drop volumes started to spread significantly (i.e., 25 µL).

The effect of experimental variables that affect both digitization and trapping /interaction of the volatile bases with the CuNCs-containing microdrop hanged from Whatman 1PS substrates was then evaluated and the obtained results are discussed in the sections below.

#### 5.2.1. Digitization and image processing conditions

The selection of appropriate digitization and image processing conditions is of paramount relevance for achieving optimal sensitivity and selectivity in smartphone-based detection methods. Thus, individual channels in the color modes RGB, CMYK and grayscale (GS) were firstly evaluated for acquisition of the analytical response. As shown in Fig. S3, the highest analytical responses were achieved with the blue (B) and cyan (C) channels, as could be expected considering the emission



Fig. 1. UV-visible spectrum (A), excitation/emission contour plot (B), STEM image (C) and size distribution histogram (D) of CuNCs. EELS graph of CuNCs (E).



Fig. 2. UV-visible spectra (A) and fluorescence emission spectra (B) of CuNCs adjusted to pH 2 and 12. Reversibility of the UV-vis spectrophotometric response (C) and fluorescence response (D) of CuNCs with pH.



Fig. 3. UV–visible spectra (A) and fluorescence emission (solid line, excitation wavelength: 378 nm) and excitation (dash line, emission wavelength: 424 nm) spectra (B) of CuNCs exposed to N-containing bases. STEM images of CuNCs exposed to NH<sub>3</sub>-N (C), DMA-N (D) and TMA-N (E).

wavelength of blue-emitting CuNCs (424 nm) (Pena-Pereira et al., 2019).

Digitization conditions were also assessed bearing in mind their key role on the light that can be detected by the camera sensor of the smartphone and, therefore, on the analytical response. Thus, two digitization parameters, namely EV, which in turn considers aperture and shutter speed values, and ISO, which reflects the sensitivity of the camera's sensor to light, were considered. Fig. S4 shows the effect of digitization variables on the analytical response. As can be deduced from the Figure, the analytical response was found to be highly dependent on the digitization conditions selected. An ISO of 200 and an EV of +1.5 were selected for subsequent studies since they provided the maximum analytical signal.

#### 5.2.2. Screening and optimization of experimental parameters

To assess the relevance of experimental parameters on the process, a Plackett-Burman (PB) design was used for screening purposes, followed by a Central Composite Design (CCD) for optimization of the most relevant experimental variables. Thus, a saturated PB design was firstly employed to evaluate the effect of seven experimental parameters, namely extraction time, temperature, sample volume, NaOH concentration, NaCl concentration, stirring rate and CuNCs volume, and four dummy variables were also included in the design to assess the experimental error (Table S1) (Anastácio & Carvalho, 2013; Borges et al., 2016). As can be deduced from the Pareto chart shown in Fig. S5, only two factors, namely extraction time and temperature, showed a significant effect on the analytical response with a 95% probability. As expected, the extraction time was found to be the most important experimental variable showing a significant positive effect associated to the key importance of this parameter in mass transfer processes. Temperature showed a significant negative effect on the analytical response (i.e., the analytical signal obtained at 40 °C was significantly lower than obtained at 20 °C with a 95% probability). The effect of this experimental parameter on the analytical response was evaluated in detail, and the obtained results are shown in Fig. S6. As can be deduced from the Figure, the analytical response increases up to ca. 15  $^\circ \mathrm{C}$  and decreases at temperature values above 25 °C. Thus, the highest analytical response was obtained in the range 15–25 °C and, accordingly, magnetic stirring with temperature control is recommended, especially outside this temperature range. The effect of temperature on the analytical response can be attributed, on the one hand, to the increased mass transfer of basic volatiles from the aqueous solution to the headspace and, on the other hand, to the detrimental effect of temperature on the fluorescence intensity of CuNCs, as reported in the literature (Shi et al., 2017) and experimentally observed (Fig. S6). Based on the results depicted in Fig. S5, two variables that showed a positive effect on the analytical response, namely extraction time and sample volume, were selected for subsequent optimization by CCD (Table S2), and the levels of the rest of experimental parameters that yielded non-significant effects on the analytical response were selected considering operational convenience. As can be inferred from the Pareto chart of the main effects obtained from the CCD (Fig. S7A), both extraction time and sample volume were found to be statistically significant at the 95% confidence level, whereas the interaction between variables and quadratic effects was found to be no significant. According to the response surface for the CCD (Fig. S7B), an extraction time of 25 min and a sample volume of 5 mL were selected as a compromise solution between analysis time, sensitivity and sample consumption. To sum up, the selected conditions were as follows: extraction time, 25 min; temperature, 20 °C; sample volume, 5 mL; NaOH concentration, 0.5 M; NaCl concentration, 0% (w/ v); stirring rate, 1200 rpm; and CuNCs volume, 5 µL.

#### 5.2.3. Selectivity studies

The selective determination of TMA-N in the presence of other volatile amines was finally verified. Formaldehyde is commonly used for sequestering non-TMA-N volatile bases *via* formation of non-volatile imines (AOAC Official Method 971.14, 1995). Thus, extraction studies were carried out under optimal conditions after the addition of 500  $\mu$ L of 39% (w/v) formaldehyde to aqueous solutions of NH<sub>3</sub>-N and DMA-N in the absence and presence of TMA-N. Remarkably, non-TMA-N volatile bases yielded analytical responses below the limit of quantification (LOQ) of the assay for TMA-N determination even at 50 mM concentration levels, i.e., ca. 5-fold higher than the maximum allowable TVB-N level (Prabhakar et al., 2020). It can be inferred from the above results that the luminescence quenching of CuNCs-containing microdrops exposed to fish extracts in the presence and absence of formaldehyde could be exploited for determination of TMA-N and TVB-N, respectively (Ruiz-Capillas et al., 2000). In addition, the effect of acetaldehyde, benzaldehyde, histamine and ethyl acetate on the determination of TMA-N and TVB-N was evaluated at a concentration level of 50 mM of each potential interferent, with no significant effects on the analytical signal.

#### 5.3. Analytical characteristics

The analytical characteristics of the droplet-based luminescent assays were obtained under optimal conditions. As in other works published in the literature (Dehaut et al., 2015; Pena-Pereira et al., 2019), a non-linear relationship between the analytical response and the concentration of the *N*-containing volatile bases was observed in this work (Fig. 4). Accordingly, a rectangular hyperbolic relationship between the analytical response and the concentration of bases was assessed, showing good agreement with the experimental data (Fig. 4). Thus, the concentration of TMA-N and TVB-N can be obtained by the rearranged equation (1) (Chaplan et al., 2014):

$$C = \frac{K}{\left(\frac{\Delta L cmax}{\Delta IC} - 1\right)} \tag{1}$$

where *C* is the concentration of the analyte,  $\Delta I_c$  is the analytical response,  $\Delta I_{cmax}$  is the maximum achievable analytical response and *K* is the concentration of analyte corresponding to half of the  $\Delta I_{max}$ . The values of  $\Delta I_{max}$  and *K*, calculated by the Excels Solver tool (Microsoft 365) (Chaplan et al., 2014; Pena-Pereira et al., 2016), were found to be 58.3 and 0.41 mM for TMA-N and 50.2 and 0.35 mM for TVB-N (as NH<sub>3</sub>-N), respectively.

The limits of detection (LOD) and quantification (LOQ), calculated in accordance with the  $3\sigma$  and  $10\sigma$  criteria, were 0.040 and 0.13 mM for TMA-N and 0.096 and 0.32 mM TVB-N (as NH<sub>3</sub>-N), respectively. The



**Fig. 4.** Calibration curves of *N*-containing basic volatiles using hydrophobic substrates (Whatman 1PS). The inset shows the images of CuNCs drops after exposure to increasing concentrations of the basic volatiles.

methodological LODs and LOQs were found to be 0.11 and 0.36 mg/100 g for TMA-N and 0.27 and 0.89 mg/100 g TVB-N, respectively. The repeatability, expressed as relative standard deviation (RSD), was evaluated at a 2 mM level and found to be 5.2% and 5.6% for TMA-N and TVB-N, respectively (N = 8). The enrichment factors were also estimated as the ratio of the K values of the calibration curves obtained by i) indrop enrichment of basic volatiles and ii) direct addition of aqueous solutions of the bases to CuNCs solutions and found to be 181 and 153 for TMA-N and TVB-N, respectively.

For comparison purposes, calibration curves were also obtained by using hydrophilic PADs containing CuNCs (Fig. S8). Detection areas with an analogous diameter to the one of a 5  $\mu$ L CuNCs microdrop (i.e. 1.87  $\pm$  0.07 mm) were created in Whatman No. 1 hydrophilic substrates by wax printing (initial internal diameter: 2.3 mm) and, after deposition of 5  $\mu$ L of the solution containing the luminescent probe, PADs were exposed to the volatiles under the experimental conditions described in *section 2.6*. The calibration curves obtained with CuNCs-modified hydrophilic PADs are shown in Fig. S8. The LODs and LOQs obtained under these conditions were 0.11 and 0.35 mM for TMA-N and 0.29 and 0.97 mM for TVB-N (as NH<sub>3</sub>-N), respectively, which are 3-fold worse than those of the drop-based assays involving hydrophobic substrates. The repeatability was also evaluated under these conditions at a 2 mM level, and the values were 6.9% for TMA-N and 6.1% for TVB-N (as NH<sub>3</sub>-N), respectively (N = 8).

In comparison with previously reported methods, the proposed assays are cost-efficient and straightforward, and show remarkable sensitivity and precision. Specifically, the LOQs of the luminescent assays are *ca*. one order of magnitude better than those of the reference methods for TMA-N and TVB-N determination (European Commission, 1995; AOAC, 2000b), while comparable to other methodologies involving conventional analytical instrumentation (Carrillo-Carrión et al., 2012; Chan et al., 2006; Veciana-Nogues et al., 1996). This aspect is particularly relevant since it enables the determination of TMA-N and TVB-N in fish samples at early stages of storage. Furthermore, the assays do not require conventional analytical instrumentation thus favoring their portability, and the use of harmful chemicals and the subsequent generation of wastes is significantly minimized, especially when compared with reference methods (European Commission, 1995; AOAC, 2000b).

#### 5.4. Validation of the luminescent assays and monitoring of fish spoilage

The proposed droplet-based luminescent sensors for the determination of TMA-N and TVB-N in fish samples were validated by comparison with the corresponding reference methods (sections 2.7 and 2.8). Three of the fish samples analyzed, namely European hake (Merluccius merluccius), Atlantic cod (Gadus morhua) and Atlantic salmon (Salmo salar), belong to a category of fishery products whose TVB-N levels are regulated by the Commission regulation (EC) 1022/2008 that amended Regulation 2074/2005 at a 35 mg N/100 g level (European Commission, 2008, 2005). There is no regulation for TMA-N in fish, and no regulatory TVB-N limits are available for European sea bass (Dicentrarchus labrax), Gilthead seabream (Sparus aurata) and Atlantic pomfret (Brama brama). Thus, maximum acceptable TMA-N and TVB-N levels proposed in the literature for fish samples have been considered for comparison purposes (Dehaut et al., 2015; Prabhakar et al., 2020). The results obtained by the analysis of six different fish samples (Table 1) were statistically compared by a paired t-test at a 95% confidence level. No significant differences were observed between the proposed and reference methods since the paired t-values for TMA-N ( $t_{exp}$  1.33) and TVB-N ( $t_{exp}$  0.96) were lower than the critical *t*-value at this level of significance ( $t_{exp}$  2.57) when TMA-N and NH3-N standards were used for calibration, respectively. Thus, no significant differences in the accuracy of the proposed and reference methods were identified. The concentration levels of TMA-N and TVB-N in the analyzed samples were in the range 0.71-0.97 mg N/100 g and 7.3-10.9 mg N/100 g, respectively. These levels are

Table 1

Analytical results obtained by the determination of TMA-N and TVB-N in fish samples by the proposed and reference methods.

Sample	TMA-N (mg/100 g) <sup>a</sup>		TVB-N $(mg/100 g)^{b}$	
	Proposed method	Reference method	Proposed method	Reference method
Hake	$\begin{array}{c} \textbf{0.88} \pm \\ \textbf{0.06} \end{array}$	$\textbf{0.82}\pm\textbf{0.08}$	$\textbf{7.80} \pm \textbf{0.21}$	$8.2\pm0.3$
Cod	$\begin{array}{c} \textbf{0.71} \pm \\ \textbf{0.04} \end{array}$	$\textbf{0.76} \pm \textbf{0.03}$	$\textbf{8.9}\pm\textbf{0.6}$	$9.3\pm0.5$
Salmon	$\begin{array}{c} \textbf{0.76} \pm \\ \textbf{0.05} \end{array}$	$\textbf{0.69} \pm \textbf{0.04}$	$\textbf{7.4} \pm \textbf{0.4}$	$\textbf{8.0} \pm \textbf{0.5}$
Sea bass	$\begin{array}{c} 0.77 \pm \\ 0.03 \end{array}$	$\textbf{0.78} \pm \textbf{0.04}$	$\textbf{7.3}\pm\textbf{0.4}$	$6.97 \pm 0.20$
Sea bream	$\begin{array}{c} \textbf{0.86} \pm \\ \textbf{0.05} \end{array}$	$0.84\pm0.04$	$9.7\pm0.3$	$10.2\pm0.3$
Pomfret	$\begin{array}{c}\textbf{0.97} \pm \\ \textbf{0.06} \end{array}$	$0.92\pm0.04$	$10.9\pm0.7$	$10.5\pm0.5$

<sup>a</sup> Paired t value is 1.33 and  $t_{crit}$  is 2.57 ( $\alpha$  0.05, 2 tails).

<sup>b</sup> Paired t value is 0.96 and  $t_{crit}$  is 2.57 ( $\alpha$  0.05, 2 tails).

well below the regulatory TVB-N limits for hake, cod and salmon (European Commission, 2008, 2005), and below the maximum acceptable levels for TMA-N and TVB-N recommended in the literature for fish samples (Prabhakar et al., 2020).

Finally, the evolution of TMA-N and TVB-N levels of sea bass was monitored under three different storage conditions, namely at room temperature (ca. 20  $^{\circ}$ C), stored in the refrigerator at 4  $^{\circ}$ C and stored in the refrigerator in flake ice. Particularly, farmed sea bass was selected in this study due to its wide availability in the market and the fact that a minimum time between harvest and processing occurred. Storage temperature and time show a clear influence on fish spoilage. Enzymatic and microbial degradation of proteins and other nitrogen-containing compounds increases with temperature, which significantly increases the rate of TMA-N and TVB-N generation. As shown in Fig. 5A, the TMA-N levels dramatically increased at short storage times when sea bass was stored at room temperature, whereas a slight increase of TMA-N levels was produced during the first days of storage at the remaining conditions. Thus, the concentration levels recommended in the literature as maximum acceptable levels (i.e. 10-15 mg N/100 g (Dehaut et al., 2015; Prabhakar et al., 2020)) were reached after only three days when stored at room temperature, whereas the spoilage process was much slower for samples preserved at lower temperatures, reaching TMA-N levels close to 10 mg N/100 g after one week of storage. The evolution of TVB-N levels in sea bass showed a similar trend (Fig. 5B). Thus, the sample stored at room temperature rapidly increased its TVB-N levels, reaching the maximum acceptable level (i.e. 30 mg N/100 g) after two days of storage (Dehaut et al., 2015; Prabhakar et al., 2020).

#### 6. Conclusions

Two novel smartphone-based luminescent assays have been developed in this work for the determination of TMA-N and TVB-N in fish samples. A microvolume of CuNCs enabled the simultaneous enrichment and sensing of nitrogen-containing volatile bases on the basis of the luminescence quenching of the probe. Hydrophobic cellulose substrates were found to be highly convenient as drop holders in both the enrichment step and image acquisition. The proposed assays, characterized by their affordability and sensitivity, yielded comparable results to those obtained with reference methods for determination of TMA-N and TVB-N, and show much promise as alternatives for fish freshness monitoring.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence



**Fig. 5.** Evolution of TMA-N (A) and TVB-N (B) concentration levels present in sea bass stored at 20  $^{\circ}$ C, 4  $^{\circ}$ C and 4  $^{\circ}$ C in flake ice. The recommended limits for TMA-N (10–15 mg/100 g) and TVB-N (30 mg/100 g) in fish samples (Dehaut et al., 2015; Prabhakar et al., 2020) are represented in the figure for reference purposes.

the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136475.

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