

Fish Freshness Monitoring Using an E-Tongue Based on Polypyrrole Modified Screen-Printed Electrodes

Irina Mirela Apetrei, Maria Luz Rodriguez-Mendez, Constantin Apetrei, and Jose A. de Saja

Abstract—In this paper, a novel e-tongue for evaluating biogenic amine compounds is reported. The method uses an array of voltammetric electrodes chemically modified based on screen-printed electrodes. The electrochemical signals toward amines consist in complex voltammetric curves. Cyclic voltammograms show redox processes related to the electrochemical activity of the amine under study, and redox peaks associated with the electrochemical activity of the electroactive material. Additionally, the electroactivity and basic character of amines influences considerably the electrochemical behavior of the electroactive material. The viability of the method is tested for the fish freshness monitoring. The samples are Pontic shad (*Alosa Pontica*), a fish living in the northwestern part of the Black Sea. Pontic shad migrates in the Danube River for spawning. The pattern of responses provided by the array can be used to discriminate and evaluate the fish freshness state. Principal component analysis confirmed the capability of the sensors array to fish freshness monitoring. Partial least squares–discriminant analysis showed that this method could be used for the analysis and determination of fish freshness as well as determination the postmortem time elapsed.

Index Terms—Biogenic amines, e-tongue, fish freshness, polypyrrole.

I. INTRODUCTION

BIOGENIC amines are basic nitrogenous compounds with aliphatic (putrescine, cadaverine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) structures that can be found in several foods, in which they are principally produced by microbial decarboxylation of amino acids, with the exception of physiological polyamines [1], [2].

Biogenic amines (BA) may be of endogenous origin at low concentrations in food such as vegetables, fruits, meat, fish

and milk. High concentrations have been found as result of microbial decarboxylation of the corresponding amino acids. Many of BA have important physiological effects (e.g., histamine, serotonin, dopamine, tyramine) and have an significant biological activity [3], [4]. Furthermore, secondary amines such as putrescine and cadaverine play an important role in food poisoning as they can potentiate the toxicity of histamine [5], [6]. The quantity of biogenic amines could be considered a marker of the level of microbiological contamination in food [7]. Therefore, it is important to monitor BAs levels in food.

BAs in food are widely studied; numerous information on formation and occurrence of the BAs in foods is given in recent reviews [8], [9].

Different methods have been published to isolate and estimate BAs, but there is no single quantitative analytical method for the determination of biogenic amines in all foods. The complex sample matrix, the presence of interfering compounds and the existence of several biogenic amines simultaneously in the same sample are typical problems encountered in the analysis of food for BAs.

Paper chromatography, thin layer chromatography, gas chromatography, and high performance liquid chromatography have all been used for the separation and identification of amines. Fluorometric methods have been also used for the determination of amines individually [10]–[12].

Different electrochemical sensors or biosensors for the detection of biogenic amines based on different materials and enzymes have been presented in the literature [13], [14].

Biosensors, which combine biological recognition through enzyme specificity with construction simplicity, have been reported as a good and cheap alternative for the traditional ones. In this way, amine oxidases based electrodes, which catalyze the oxidative deamination of amines to produce aldehyde, ammonia and hydrogen peroxide, have been reported [14]. For detection of catecholamines biosensors based on enzyme tyrosinase have been reported [15].

Electrochemical sensors have also demonstrated to be useful to detect biogenic amines by voltammetric methods using bare diamond, metal electrodes and chemically modified electrodes [13], [16].

Although the measurement of a single amine can provide important information about fish freshness [17], the assessment of fish spoilage requires the use of technologies capable to provide global information about the biogenic amines and other spoilage sub-products present in the sample.

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I. M. Apetrei is with the Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmacy, “Dunărea de Jos” University of Galați, Galați 800008, Romania (e-mail: irina.apetrei@ugal.ro).

M. L. Rodriguez-Mendez is with the Department of Physical Chemistry and Inorganic Chemistry, Industrials Engineering School, University of Valladolid, Valladolid 47011, Spain (e-mail: mluz@eii.uva.es).

C. Apetrei is with the Department of Chemistry, Physics and Environment, Faculty of Sciences and Environment, “Dunărea de Jos” University of Galați, Galați 800008, Romania (e-mail: apetreic@ugal.ro).

J. A. de Saja is with the Department of Condensed Matter Physics, Faculty of Sciences, University of Valladolid, Valladolid 47011, Spain (e-mail: sajasaez@fmc.uva.es).

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Electronic tongues have rarely been used for detection of amines or for fish quality monitoring [13], [18], [19]. Usually electronic tongues are constructed using potentiometric sensors that measure the potential created across a membrane produced by the diffusion of ions. Arrays of metallic voltammetric sensors can detect electroactive species [20]. The sensors chemically modified with electroactive materials can provide a certain advantage because they are sensitive to both the presence of ions and of electroactive substances [21]–[23].

In this work, disposable electrodes have been developed where polypyrrole doped with different anions have been deposited onto miniaturized screen-printed electrodes (SPE). The electrochemical behavior of these new sensors has been analyzed. The capability of the array to detect biogenic amines (including ammonia (NH₃), dimethylamine (DMA), trimethylamine (TMA), cadaverine (CAD) and histamine (HIS)) usually formed during fish spoilage processes has been evaluated using multivariate data analysis. The capability of the system to monitor the spoilage of real fish samples has also been analyzed by means of Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA).

II. METHODOLOGY

A. Apparatus

Voltammetric measurements were performed on an Biologic Science Instruments SP 150 potentiostat/galvanostat using the EC-Lab Express software. An Elmasonic S10H ultrasonic bath was used for dissolving and homogenization of solutions. For pH measurements an Inolab pH 7310 was used.

B. Electrodes and Electrochemical Cell

Screen-printed carbon electrodes (4 mm diameter, $S = 12.56 \text{ mm}^2$) purchased from Dropsens (www.dropsens.com, model SP 110) were used as working electrode for polypyrrole deposition. A three-electrode configuration was used in all cases, a Princeton Applied Research Ag|AgCl/KCl $3 \text{ mol} \cdot \text{L}^{-1}$ and a Pt plate being used as reference electrode and counter electrode, respectively. After modification, polypyrrole modified SPE (Ppy-SPE) was used for amines determination. In these experiments the reference and the counter electrode integrated in the device were used (counter electrode - carbon, reference electrode - silver).

Cyclic voltammograms were registered from -1.1 to $+0.5\text{V}$ (the scan started at 0V) at a sweep rate of $0.05\text{V} \times \text{s}^{-1}$ (except otherwise indicated).

C. Reagents and Solutions

All reagents were of high purity and used without further purification (Sigma-Aldrich).

Biogenic amine solutions were prepared by solving ammonia (NH₃), dimethylamine (DMA), trimethylamine (TMA), cadaverine (pentane-1, 5-diamine) (CAD) and histamine (2-(3H-imidazol-4-yl)ethanamine) (HIS) in ultrapure water ($10^{-2} \text{ mol} \cdot \text{L}^{-1}$).

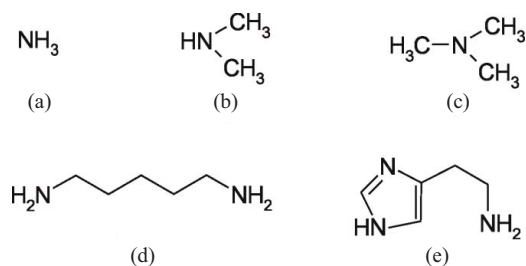


Fig. 1. Chemical structure. (a) Ammonia. (b) Dimethylamine. (c) Trimethylamine. (d) Cadaverine. (e) Histamine.

TABLE I
CONDITIONS OF ELECTROPOLYMERIZATION USED IN DEVELOPMENT OF
POLYPYRROLE SENSORS

Doping Agent	Concentration ($\text{mol} \cdot \text{L}^{-1}$)	Electrochemical Technique	Conditions
FCN	0.1	Chronoamperometry	0.8 V; 240 s
NP	0.1		0.8 V; 360 s
PWA	0.01		0.8 V; 720 s
H ₂ SO ₄	0.1		0.8 V; 240s
Mo	0.1		0.8 V; 480 s
AQS	0.01		0.8 V; 720 s

The chemical structures of amine compounds under study are presented in Figure 1.

To improve the conductivity and stability of Ppy sensors, phosphate buffer solution of pH 7 was added to amine solutions (final concentration $10^{-1} \text{ mol} \cdot \text{L}^{-1}$). All solutions were prepared with deionized water ($18.3 \text{ M}\Omega$ resistivity, Milli-Q, Millipore).

The polymeric sensors used in this study, and the optimized electrosynthesis conditions, are collected in Table I.

Stock $0.1 \text{ mol} \cdot \text{L}^{-1}$ pyrrole (Aldrich) solutions in water, containing doping agent were used in the electropolymerization process. The doping agents employed for the fabrication of polypyrrole sensors were potassium hexacyanoferrate(II) (FCN), potassium nitroprusside (NP), phosphotungstic acid (PWA), sulfuric acid (H₂SO₄), sodium molybdate (MO), and 9, 10-anthraquinone-2-sulfonic acid sodium salt (AQS).

Once prepared, the polymeric sensors were taken out from the synthesis solution and rinsed thoroughly.

D. Fish Samples

The spoilage study was carried out using Pontic shad (*Alosa Pontica*), a fish living in the northwestern part of the Black Sea. Pontic shad migrates in the Danube River for spawning [24]. Pontic shads were purchased from a local market. Fresh fishes were eviscerated and washed carefully. Four fishes of the comparable weight were stored inside a plastic box at $4 \text{ }^\circ\text{C}$ during 10 days. Every day, 1 g of the muscle of each fish was cut and chopped in small pieces. 25mL of a $0.1 \text{ mol} \cdot \text{L}^{-1}$ phosphate buffer solution pH 7 was added to fish sample and the mixture was sonicated for 5 minutes. Then, the liquid phase was separated by filtration and used in voltammetric and pH measurements.

E. Data Analysis

A non-supervised multivariate method such as Principal Component Analysis was used for the analysis of the voltammetric signals. Voltammograms were mathematically pre-processed and used as data source for multivariate data analysis according to a previously published method [21]–[23]. Using kernel method, the CV curve (i vs. E) is divided in anodic and cathodic part. Then, the anodic curve is multiplied by a number of 10 smooth, bell-shaped windowing functions, and integrated with respect to potential. By this pre-processing technique the information throughout the global response is reduced to 10 representative parameters per curve. A supervised method, the Partial Least Squares-Discriminant Analysis (PLS-DA) was used to evaluate the classification capability of the system.

All computations and chemometric analysis were carried out using the software Matlab v5.3 (The Mathworks Inc., Natick, MA, USA) and The Unscrambler 9.1 (Camo, Norway).

III. RESULTS AND DISCUSSION

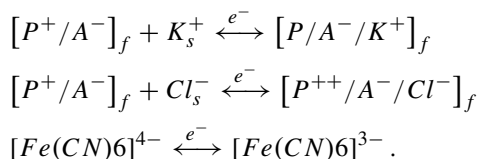
The voltammetric behavior of Ppy based sensors was tested towards a 0.1 mol L^{-1} KCl solution and towards solutions containing biogenic amines. The electrochemical behavior in KCl of sensors is shown in Figure 2.

It must be mentioned that during the first cycles, the voltammograms varied, but, succeeding scans were highly reproducible and after five scans the only evident change was a small and gradual decrease of the intensity of the peaks. Consequently, the cyclic voltammograms showed in the following figures correspond to the fifth cycle unless otherwise specified. Sensors based on Ppy were used as the working electrode in cyclic voltammetry experiments from -1.1 to $+0.5 \text{ V}$ and at a scan rate of $0.05 \text{ V} \cdot \text{s}^{-1}$ [21].

The cyclic voltammograms presented in Figure 2 indicates that the shape and position of the peaks depend on the nature of the doping anion. As can be observed in Figure 2, the doping agent affects the electrochemical behavior of polypyrrole and, in the cases of FCN, NP and PWA are observed peaks related to redox processes of doping agent within polypyrrole matrix.

Polypyrrole-based sensors immersed in $0.1 \text{ mol} \cdot \text{L}^{-1}$ KCl solution showed a redox couple related to the oxidation-reduction of the polypyrrole chain; in some cases a second peak related with the redox activity of the dopant inside of polymer matrix could also be observed (FCN and NP) [25].

The schemes of processes are:



From the cyclic voltammograms it can be concluded that a high variety of sensor responses was achieved by changing the doping agent entrapped within the polypyrrole films. The capacity to incorporate and expel ionic species during the redox processes for preserving the macroscopic electroneutrality of the electrode can be used to produce analytically useful signals.

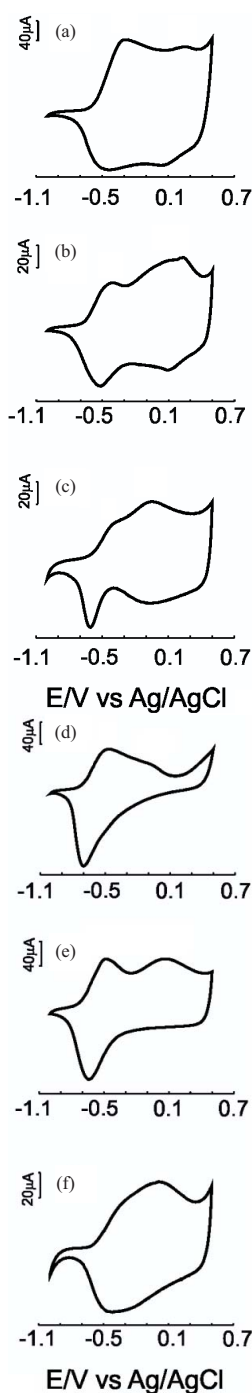


Fig. 2. Cyclic voltammograms of SPE sensors. (a) Ppy/FCN. (b) Ppy/NP. (c) Ppy/ H_2SO_4 . (d) Ppy/MO. (e) Ppy/AQS. (f) Ppy/PWA toward $0.1\text{-mol} \times \text{L}^{-1}$ KCl solution.

When the Ppy/PWA sensor is immersed in $\text{DMA } 10^{-2} \text{ mol} \cdot \text{L}^{-1}$ the signal is not stable (Figure 3a). For increasing of sensor stability the measurements must be carried out in a buffered solution. As observed in Figure 3b a stable response is obtained in these conditions after few cycles.

The influence of pH in the sensor responses was studied. The most intense response of the sensor detecting DMA was observed at pH 7.0. Therefore, a pH of 7.0 for the phosphate buffer solution was selected for the following studies.

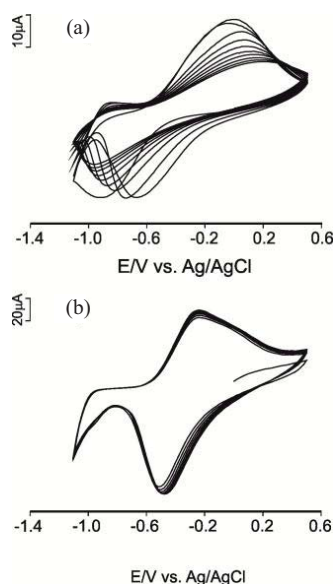


Fig. 3. Cyclic voltammograms of sensor based on Ppy/PWA. (a) 10^{-2} - $\text{mol}\cdot\text{L}^{-1}$ DMA solution. (b) 10^{-2} - $\text{mol}\cdot\text{L}^{-1}$ DMA, 10^{-1} - $\text{mol}\cdot\text{L}^{-1}$ PBS of pH 7.

The cyclic voltammogram presents two well defined peaks, one anodic at -0.25V and one cathodic at -0.50V . As can be seen, the electroactivity and basic character of DMA influences significantly the electrochemical behavior of the sensitive material.

The responses obtained when using Ppy based sensors showed a high degree of complexity (Figure 4).

The transient responses observed in the voltammograms are related to the electrode material and to the nature and concentration of the amine molecules present in the solutions (and to the interactions electrode-solution).

A. Influence of Scan Rates in Sensor Response

The dynamic character of the electrode process was further examined. For this purpose, the effect of the scan rate on the performance of Ppy based sensors was studied.

Figure 5 shows the cyclic voltammograms of Ppy/FeCN at various scan rates, ($100\text{--}600\text{ mV}\cdot\text{s}^{-1}$) in DMA solution.

The current of the peaks were proportional to scan rates (v), indicating a charge transfer limited process. All Ppy sensors have the same type of behavior.

From the slope of I - v dependence and using the Laviron equation:

$$I = \frac{n^2 F^2 v A \Gamma}{4RT}$$

where I is the peak current (Ampere), n is the number of electrons involved in the redox process, F is the Faraday constant ($F = 96,485\text{ C}\cdot\text{mol}^{-1}$), v is the potential scan rate ($\text{V}\cdot\text{s}^{-1}$), A is the electrode area (cm^2), Γ is the surface coverage ($\text{mol}\cdot\text{cm}^{-2}$), R is the ideal gas constant ($8.314\text{ J}\cdot\text{K}^{-1}\times\text{mol}^{-1}$) and T is the temperature (K) [26], the total surface coverage could be calculated.

The total surface coverage calculated was $4.34\cdot 10^{-12}\text{ mol}\times\text{cm}^{-2}$. The surface coverages obtained with all other

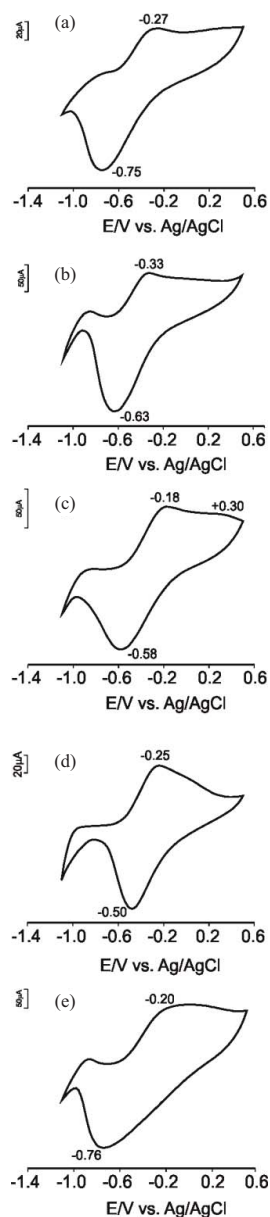


Fig. 4. Cyclic voltammograms of sensors. (a) Ppy/FCN. (b) Ppy/NP. (c) Ppy/ H_2SO_4 . (d) Ppy/MO. (e) Ppy/AQS in 10^{-2} - $\text{mol}\times\text{L}^{-1}$ -DMA, 10^{-1} - $\text{mol}\times\text{L}^{-1}$ PBS solution of pH 7.

sensors in DMA solutions are in the range $1.25\cdot 10^{-12}\text{--}6.48\cdot 10^{-12}\text{ mol}\times\text{cm}^{-2}$.

B. Detection Limits

Electrodes modified with polypyrrole doped with different doping agents were used to evaluate the detection limit by registering voltammetric measurements in solutions containing increasing amounts of amine concentrations in order to obtain a calibration curve.

Figure 6 illustrates calibration curve of the Ppy/NP towards increasing concentrations of TMA. In this example, the response of a Ppy/NP immersed in TMA solved in PBS medium is shown.

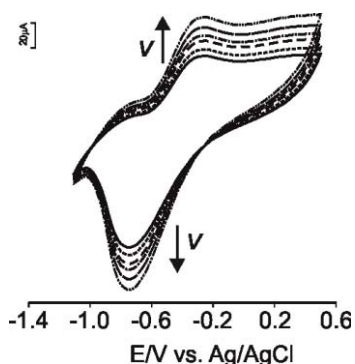


Fig. 5. Cyclic voltammograms of Ppy/FCN in $10^{-2}\text{-mol}\cdot\text{L}^{-1}$ DMA, $10^{-1}\text{-mol}\cdot\text{L}^{-1}$ PBS solution of pH 7 registered at different scan rates ($100\text{--}600\text{ mV}\cdot\text{s}^{-1}$).

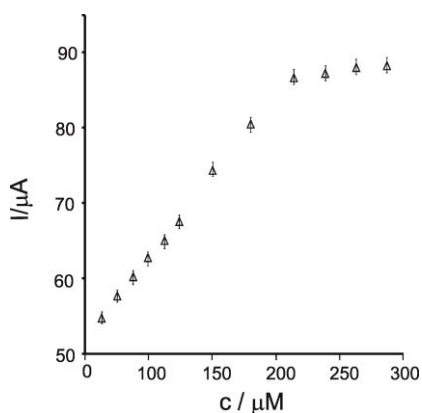


Fig. 6. Calibration curve between anodic current and concentration of TMA in 0.1-M PBS solution (pH 7.0) of Ppy/NP sensor.

As observed in the figure, an increase of the TMA concentration produced a linear increase of the intensity of the peak until a plateau is reached. In Ppy/NP, the anodic peak was linearly dependent on the TMA concentration in the range from $20\text{--}200\ \mu\text{mol}\cdot\text{L}^{-1}$ in PBS medium. In the case of TMA the detection limit was $1.58 \times 10^{-5}\ \text{mol}\cdot\text{L}^{-1}$ in PBS medium. The limits of detection and linear ranges for all sensors detecting TMA are presented in Table II.

These results are in agreement with previously results reported that obtain similar detection limit for TMA [27].

C. Reproducibility and Stability of the Ppy Sensors Immersed in Biogenic Amines

Ppy sensors were highly reproducible under successive cycling in the amine compound solutions. Therefore, after 100 scans the only noticeable change was a small and gradual decrease of the intensity of the peaks (RSD 2–6% signal drop-off).

In order to establish if the sensor signals could be recovered after registration of the cyclic voltammograms in biogenic amines the following experiment was carried out. The CVs of sensor were recorded in $0.1\ \text{mol}\cdot\text{L}^{-1}$ KCl solution. After that, the sensor was immersed in amine solutions and the CVs were recorded. Finally, the CVs were recorded again in $0.1\ \text{mol}\cdot\text{L}^{-1}$ KCl solution. The original signal of the sensor could not be

TABLE II
LIMITS OF DETECTION FOR TMA DETECTION WITH Ppy-BASED SENSORS

Sensor	Linear Range (μM)	Limit of Detection (M)
Ppy/FCN	10–180	2.32×10^{-5}
Ppy/NP	20–200	1.58×10^{-5}
Ppy/PWA	40–160	8.44×10^{-5}
Ppy/H ₂ SO ₄	20–180	4.57×10^{-5}
Ppy/Mo	20–180	6.33×10^{-5}
Ppy/AQS	40–160	9.21×10^{-5}

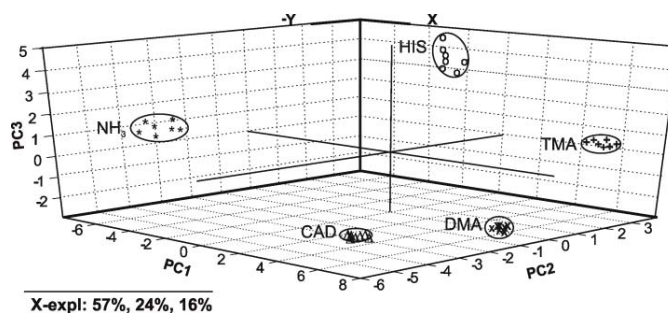


Fig. 7. PCA score plot of cyclic voltammograms of amine solutions with Ppy-based sensor array.

recovered after immersion in the amine solution. This result could be related with the irreversible processes that take place at the electrode surface. In order to evaluate the reproducibility of the electropolymerization method, seven replicates of each sensor were prepared and the polymerization charge density was calculated. In all cases, the relative standard deviation (RSD) was below 5% indicating the high reproducibility of the method.

D. Array of Sensors Based on Ppy. Discrimination of Biogenic Amines

Discrimination of model solutions containing biogenic amines could be carried out by means of multivariate data analysis.

As stated before, the responses obtained when using SPE modified with polypyrrole showed a high degree of complexity, since transient responses observed in the voltammograms are related to the electrode material and to the nature and concentration of the amine molecules present in the solutions (and to the interactions electrode-solution).

This makes possible to use the sensors in an array configuration. The pattern of responses generated by the array is a fingerprint of the sample studied. This pattern can be related with certain features or characteristics of the samples by means of chemometrics.

In order to evaluate the discrimination capabilities of the array of voltammetric sensors, Principal Component Analysis was conducted using the information obtained from the array formed by Ppy modified SPE sensors. Figure 7 shows the PCA results as a three-dimensional scores plot of principal components that allow obtaining well-defined and separated clusters.

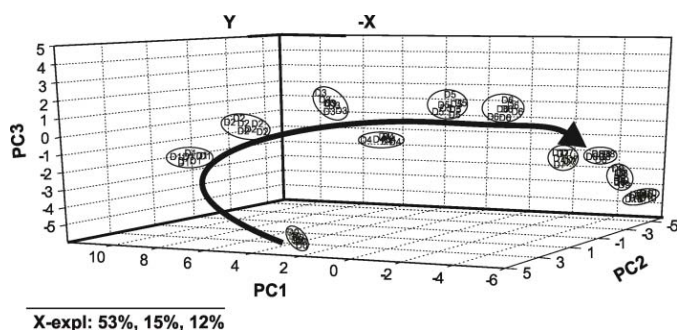


Fig. 8. PCA score plot of fish freshness monitoring with Ppy-based sensor array.

PCA has been validated by full cross validation method and an optimal number of 5 principal components have been used. The first three principal components explain the 97% of the information ($PC1 = 57\%$; $PC2 = 24\%$; $PC3 = 16\%$).

The separated clusters indicate that the five solutions could be clearly discriminated from each other. In addition, the positions of the clusters are related to the electrochemical properties of the tested solutions. It has to be noticed that the cluster corresponding to the ammonia, appears in the left side of the diagram, far apart from the rest of the amines. Aliphatic amines appear in the right side of the diagram. A clear discrimination between primary amine (CAD), secondary amine (DMA) and tertiary amine (TMA) is observed, also. The heterocyclic amine, HIS has a particular electrochemical behavior that permits to discriminate it from aliphatic amines and ammonia.

E. Fish Freshness Monitoring

Fish freshness has been monitored through the global assessment of spoilage products (including biogenic amines) using a multisensor array based in Ppy. For this purpose, fishes were eviscerated, washed and stored at 4 °C during 10 days in a closed box. Every day, muscle samples were prepared and measured with Ppy based sensors.

A characteristic pattern of the deterioration of fish stored in ice can be divided into four phases: 1) fish is fresh and has a sweet, seaweed and delicate taste (highly fresh); 2) there is a loss of the characteristic odor and taste. The flesh becomes neutral but has no off-flavors (fresh); 3) there is sign of spoilage and a range of volatile, unpleasant-smelling substances are produced; 4) fish is spoiled and has a putrid odor [28].

Principal Component Analysis was used to analyze the degradation process measured with the array of sensors. Figure 8 shows the PCA obtained using the electrochemical signals registered every day using Ppy-based sensors.

The PCA score plot of the three first principal components accounts for 79% of variance. Clearly discriminated clusters can be observed. The first cluster, that appears in the left side of the figure corresponds to samples analyzed days 1 and 2 and correspond to a highly fresh product. Samples analyzed in days 3 and 4 did not show any off odor and could be classified as fresh product. The clusters appear in the central part of

TABLE III
RESULTS OF CALIBRATION AND VALIDATION OF PLS-DA

Group	Correlation Coefficients		Root Mean Square Error	
	Calibration	Validation	Calibration	Validation
D0	0.956	0.953	0.057	0.068
D1 – D2	0.974	0.971	0.062	0.076
D3 – D4	0.954	0.948	0.087	0.095
D5 – D6	0.974	0.969	0.083	0.092
D7 – D10	0.956	0.952	0.098	0.105

the figure. Samples collected days 5 and 6 showed off odors (degraded product). The last clusters that appears on the right side of the figure corresponds to samples collected on days 7 to 10 (spoiled fish).

PLS-DA was used to classify the day of fish degradation from the sensor array response.

As presented in Table III, the fully cross-validated PLS-DA model (using an optimal number of 6 latent variables), revealed a clear identification of the fish degradation phases.

Table III collects the quantitative data derived from the PLS-DA regression model.

As observed, both the calibration and the validation values involved a good-quality model performance are achieved (large correlations between sensors and categorized variables, and low root mean square errors of calibration and validation). These results indicate that this methodology is able to real time monitor the fish freshness during storage.

IV. CONCLUSION

A multisensory systems based on carbon screen printed electrodes modified with polypyrrole doped with different doping agents has been developed and applied to the analysis of biogenic amines. The system has been used to monitor fish (Pontic shad) spoilage. An increase of the signal currents associated to biogenic amines was observed with increasing storage days. PCA and PLS-DA have been successfully used to monitor the fish freshness and to classify the day of fish spoilage. The screen-printing technology allowed the preparation of miniaturized electrodes, which are promising for the mass production of low-cost and single-use sensors, with significant sensibility and using a very small quantity of sample.

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Irina Mirela Apetrei was born in Iasi, Romania, in 1975. She received the B.Sc. degree in chemistry from the University of Iasi, Iasi, Romania, in 1997, and the Ph.D. degree from the University of Galați, Galați, Romania, in 2009.

She is currently the Head of Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmacy, "Dunărea de Jos" University of Galați, Galați, Romania. She has authored several publications in peer-reviewed journals. Her current research interests include development of electrochemical sensors and biosensors for the pharmaceutical analysis.

Maria Luz Rodriguez-Mendez received the Ph.D. degree in chemistry from the University of Valladolid, Valladolid, Spain, in 1990.

She has been the Inorganic Chemistry Chair with the Industrial Engineers School, University of Valladolid, Valladolid, Spain, since 2010. She has been involved in research on the preparation and the structural characterization of thin films of phthalocyanines and their potential applications. She is the author or co-author of over 120 publications and seven books. Her current research interests include development of sensors based on phthalocyanines.

Constantin Apetrei was born in Falticeni, Romania, in 1975. He received the B.Sc. degree in chemistry from the University of Iasi, Iasi, Romania, in 1997, and the Ph.D. degree from the University of Galați, Galați, Romania, in 2007.

He is currently a Senior Lecturer with the Department of Chemistry, Physics and Environment, Faculty of Sciences and Environment, "Dunărea de Jos, University of Galați, Galați, Romania. He is the co-author of over 30 papers. His current research interests include the development of electrochemical sensors and biosensors for the analysis of foods and beverages.

Jose A. de Saja was born in Miranda de Ebro, Spain, in 1940.

He is a professor of the Department of Condensed Matter Physics, University of Valladolid, Valladolid, Spain. He is the author or co-author of over 300 publications and has edited 11 books. His current research interests include intersection of materials science, physics, physical chemistry and device engineering, and focus on novel nanostructured materials (mainly from LB monolayers).