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(Article begins on next page)

Immobilized bi-enzymatic system for the determination of biogenic amines in solution

Luca Lavagna^a, Maria Laura Tummino^{b,1}, Giuliana Magnacca^{b,c}, Ingrid Corazzari^{b,d}, Enzo Laurenti^{b*}

^a: Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, Torino, Italy

^b: Department of Chemistry, Via P. Giuria 7, Torino, Italy

^c: NIS Centre, Università degli Studi di Torino, Via P. Giuria 7, Torino, Italy

^d: "G. Scansetti" Interdepartmental Centre, Università degli Studi di Torino, Via P. Giuria 7, Torino, Italy

*Corresponding author: enzo.laurenti@unito.it

Abstract

Biogenic amines are a group of important organic bases involved in the metabolism of living organisms and present in biological fluids, food or beverages. Their identification and quantification represent relevant issues in the field of bio-analytical and food chemistry. Beyond the traditional detection techniques, other methods have been recently exploited, as enzymatic-based systems. Herein, diamine-oxidase (DAO) and soybean peroxidase (SBP) were immobilized onto silica spherical monoliths (prepared from biowaste-derived substances) to realize a practical and fast enzymatic system, able to quantify biogenic amines in solution. The mechanism consisted in the oxidation of amines by DAO with the formation of H₂O₂, which in turn activated the SBP reaction producing an indamine dye. The amount of amine was indirectly determined by the spectroscopic measurement of the final product. Preliminary kinetic tests on DAO/SBP catalytic system using histamine as DAO substrate showed the maximum activity at pH 8. Furthermore, the stability of the functionalized monoliths over several reaction cycles and their catalytic action towards cadaverine, tyramine, tyrosine and histamine were studied. The results showed that the immobilized DAO/SBP system was effective in the determination of different substrates for many cycles, reaching a detection limit comparable to chromatographic methods.

Keywords: Biogenic amines, Diamine oxidase, Soybean Peroxidase, Silica Monoliths, Waste-derived substances.

¹ Present Address: Institute of Intelligent Industrial Technologies and Systems for Advanced Manufacturing, Italian National Research Council, Corso G. Pella 16, Biella, Italy

1. Introduction

Many organic bases containing amino groups are naturally present in biological fluids as results of physiological activities. Biological amines include neurotransmitters as catecholamines (i.e. dopamine, epinephrine and nor-epinephrine) and indolamines (i.e. serotonin), or aliphatic amines resulting from the decarboxylation of aminoacids in living and dead organisms (i.e. putrescine and cadaverine) [1–4]. Biogenic amines detection is a valuable tool for different analytical purposes, since these compounds can be cancer markers [5–7] or indexes of freshness and quality for a wide variety of foodstuffs [8,9]. For instance, the levels of histamine, putrescine and cadaverine in foods are important indicators of bacterial spoilage [10]. Biogenic amines have been traditionally detected using gas and liquid chromatography, but other methods such as capillary electrophoresis, mass spectrometry, ion mobility spectrometry, colorimetry and sensors have also been employed [11–14]. Since these methods often require a step of sample pre-treatment and/or a long analysis time, recently some alternatives as amperometric biosensors, combining classical methods with enzymatic reactions, have been proposed [15]. The detection limit of these techniques (Limit Of Detection, LOD [16]) is in the range 10-400 nM, both depending on the matrix containing the amines and the chosen technique (see **Table S1**).

In this work, an alternative enzymatic system containing Diamine-Oxidase (DAO) and Soybean Peroxidase (SBP) was developed in order to obtain a stable and effective detector of amines in solution. DAO belongs to the class of copper-containing amine oxidases which catalyze the oxidative deamination of primary amines by dioxygen to form aldehydes, ammonia, and hydrogen peroxide [17]. Whereas, SBP is a class III secretory plant Fe(III) heme peroxidase [18] with a good thermal stability and higher resistance to deactivation with respect to other peroxidases [19,20]. The catalytic cycle of plant heme peroxidases involves the two-electron reduction of hydrogen peroxide and the one-electron oxidation of two substrate molecules. The combination of SBP and DAO in the presence of amines provides the reaction mechanism shown in **Figure 1** that involves the formation of H₂O₂ (from DAO activity), which in turn is able to oxidize organic substrates in a reaction catalyzed by SBP. As co-substrates for SBP reaction we chose 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) since they lead to the formation of a stoichiometric amount of a colored and stable product [21], that can be easily determined by a spectrophotometric method and exploitable to determine the amount of amines in solution.

DAO and SBP were immobilized together on silica monoliths. These supports were prepared following a previously reported procedure [22] inspired to the glass-frit bonding technique [23] and employing biowaste-derived substances (BBS-GC, bio-based substances derived from green compost) as binders. BBS-GC consist in a mixture of macromolecular/supramolecular aggregates of molecules, differing in molecular weight, and containing silica and metal ions [24], whose use allow to obtain stable monoliths (avoiding the

addition of more expensive reagents) and to recycle biowaste-derived substances with a glance to circular economy.

2. Materials and methods

2.1 Materials

Diamine-oxidase (DAO, E.C. 1.4.3.22) from porcine kidney was purchased by Sigma-Aldrich Italia and Soybean peroxidase (SBP, EC 1.11.1.7; RZ = 2.0) from Bioresarch Products Inc. (Iowa, USA). Both the enzymes were used without further purification. The silica powder (SIPERNAT®320) was purchased from Evonik. Other chemical reagents were of analytical grade (Sigma-Aldrich, Italy).

Bio-based substances from green compost (BBS-GC) were obtained from urban biowastes sampled from the ACEA Pinerolese Industriale S.p.A. waste treatment plant in Pinerolo (Italy) and aged for more than 180 days. The isolation of BBS-GC was performed following a previously reported procedure [25].

2.2 Sample preparation

Monoliths (SiO₂-M) were synthesized following a previously reported method by mixing BBS-GC and silica in water [22]. After drying, the mixture was manipulated to obtain spheres with a diameter of 5 mm and a final weight of approximately 50 mg, successively calcined at 500 °C for 4 hours to obtain mesoporous solids. Monoliths were activated with 3-aminopropyltriethoxysilane (APTES) by reaction in water at pH 4.0 (80 °C for 3 hours). APTES-coated monoliths were added to a glutaraldehyde solution 2.5% v/v in 0.1 M phosphate buffer at pH 7.0, reacting for 1 hour in the dark at room temperature. After the reaction, the unreacted glutaraldehyde was removed by filtration and the monoliths were washed. Then, the monoliths were added to a solution containing 15 mg of DAO and 5 mg of SBP in 0.1 M phosphate buffer at pH 7.5 and left to react at 4 °C for 20 hours. The final red products (SBP/DAO-M) were washed with the same buffer, left to air-dry overnight and stored at 4 °C until use. For comparison, monoliths functionalized with SBP only (SBP-M) were prepared by using a similar procedure. A detailed procedure for each step of sample preparation is available in the SI.

The amount of immobilized proteins was estimated by using a modified Bradford method [26] and calculated as the difference between the concentration present in the initial solution and that recovered after the reaction.

2.3 Characterization methods

Surface area and pore volumes were obtained by N₂ adsorption at 77K in an ASAP2020 gas-volumetric apparatus (Micromeritics, Norcross, GA, USA). The samples were previously outgassed at 60 °C until a standard residual pressure of 10⁻² mbar was reached. The specific surface area was calculated by the

Brunauer–Emmett–Teller (BET) method [27], whereas the porosity features were evaluated by applying the Barrett-Joyner-Halenda (BJH) model [28].

Infrared measurements were performed on self-supporting pellets of about 50 mg cm⁻¹ using the FT-IR Bruker IFS 28 spectrophotometer (Globar source, MCT detector), equipped with a vacuum line, at a resolution of 4 cm⁻¹ and accumulating 128 scans/spectrum. The spectra were carried out at room temperature after 5 minutes vacuum degassing in a home-made cell for in situ measurements.

Thermogravimetric–evolved gas analyses (TGA-EGA) were performed by employing a Pyris 1 TGA thermo-balance (PerkinElmer, Waltham, MA, USA) coupled with a temperature/time-resolved FT-IR detector (PerkinElmer, Spectrum 100) equipped with a thermostated conventional gas cell for the analysis of the evolved gaseous species. Samples (ca. 10 mg) were placed in a platinum open pan and heated from 25°C to 800°C (rate: 20°C min⁻¹) under dynamic nitrogen atmosphere. Gaseous species evolved during the heating ramp were piped to and continuously analyzed by the FTIR detector. Spectra were acquired in the 4000-600 cm⁻¹ wavenumber range and analyzed with the Spectrum software following a well-established methodology [29,30] to identify the nature of the gaseous species. Temperature-resolved FT-IR profiles of the evolved species were obtained from the intensity of a representative peak (namely, 1650 cm⁻¹ for H₂O, 965 cm⁻¹ for NH₃, 2360 cm⁻¹ for CO₂ and 3015 cm⁻¹ for CH₄).

2.4 Kinetic measurements

The catalytic activity of SBP/DAO-M was studied in different experimental conditions by using the Ngo and Lenhoff method for the determination of peroxidase activity [21]. In a typical kinetic test, 300 μL of DMAB (5.2 × 10⁻³ M) and 30 μL of MBTH (2.34 × 10⁻³ M) solutions were mixed in a cuvette with 2.2 mL of buffer, then 1 mL of amine solution at the desired concentration and one monolith, or the correspondent amount of enzymes in solution, were added to the reaction mixture. The formation of the blue-violet product was followed recording the increase of absorbance at 590 nm ($\epsilon_{590\text{nm}} = 47600 \text{ M}^{-1}\text{cm}^{-1}$ [21]) in a UNICAM UV300 (Thermospectronic, Italia) spectrophotometer, equipped with a Peltier cell for temperature control and magnetic stirring system, using 1 cm optical path quartz cuvette. In the kinetic measurements with SBP in solution or with SBP-M, 1 mL of H₂O₂ (final concentration, 5.8 × 10⁻⁴ M) was used instead of the amine solution.

Catalytic activities were evaluated at different pH values, using cadaverine (1.5 × 10⁻³ M) as a substrate for DAO: the tests were run in triplicate in a triple acetate-borate-phosphate buffer (0.05 M each), checking the pH of the mixture before and after the reaction. Enzymatic activities were calculated by the initial rate methods and expressed as mM of product obtained per minute.

Determination of the maintenance of the enzymatic activity of monoliths was made in phosphate buffer 0.1 M at pH 8. After each measurement, the monolith was separated from the reaction mixture, washed twice with 10 mL of reaction buffer and re-introduced in the cuvette with fresh reagents for a new reaction cycle.

The possible non-enzymatic formation of the reaction product was monitored in blank control with all the reagents except the monolith.

For the determination of LOD for cadaverine, tyramine, tyrosine and histamine, one SBP/DAO-M monolith was placed in the cuvette together with DMAB, MBTH and the desired concentration of amine (obtained by progressive dilution of stock solutions 1×10^{-3} M) in a phosphate buffer 0.1 M at pH 8. Then the reagents reacted under stirring for 30 min, the monolith was extracted from the cuvette and the resulting absorbance at 590 nm was measured.

3. Results and discussion

3.1 Physico-chemical characterization

The comparison between the FT-IR spectra of the pristine and functionalized monolith (**Figure S1**) highlights the modification induced by the functionalization: (i) the disappearance of the sharp ν_{OH} signal at 3750 cm^{-1} present in $\text{SiO}_2\text{-M}$ since OH groups were involved in the covalent immobilization of the enzymes; (ii) the formation of a shoulder at 1650 cm^{-1} in SBP/DAO-M attributable to the formation of the imine bond between the proteins and the monolith surface [31], (iii) the presence of the signals around 3000 cm^{-1} together with the shape variation of the large absorption at 3200 cm^{-1} , attributable to the stretching signals of C-H and N-H, related to the enzyme structure [32].

Gas-volumetric adsorption of N_2 evidences the modifications induced by the enzyme immobilization on the specific surface area and porosity of monoliths. The adsorption isotherms (**Figure 2**) are of the IV type of IUPAC classification, typical of mesoporous samples. After the functionalization, the hysteresis loop decreased in height without changing in shape, indicating the decrease of the porosity in SBP/DAO-M with respect to the pristine monoliths. The BJH model [28], applied to the desorption branch of the isotherms, shows a total pore volume in the pristine monolith of $1.61\text{ cm}^3/\text{g}$ compared to $0.6\text{ cm}^3/\text{g}$ for the functionalized one. The pore size distribution curves indicate that smaller pores in a narrower range are present in the system SBP/DAO-M (**Figure 2, inset**). At the same time, from the BET model [27] the surface area of the functionalized monolith resulted $114\text{ m}^2/\text{g}$, lower than $164\text{ m}^2/\text{g}$ of the pristine one. These results indicate that the functionalization effectively proceeded on the entire surface, including the internal surface of the pores.

These data are particularly interesting if compared with the results previously obtained for SBP-M monoliths, namely a surface area of $124\text{ m}^2/\text{g}$ and a total volume of the pores equal to $0.98\text{ cm}^3/\text{g}$ [22]. Thus, the additional decrease in both surface area and total pore volume for SBP/DAO-M can be mainly attributable to the presence of DAO.

TGA data are in agreement with the above-described results. In **Figure 3** the thermograms and their first derivatives (D-TG) are reported. In the temperature range between 30 and $180\text{ }^\circ\text{C}$, SBP/DAO-M and $\text{SiO}_2\text{-M}$ exhibited virtually the same mass loss (ca. 2%), which was attributed by FT-IR analysis (**Figure S2**) to the

release of physisorbed or chemisorbed water from the surface and the pores. At higher temperature (>230 °C), the thermal behavior of the samples diverged and SBP/DAO-M underwent a mass loss of approximately 9 % due to the evolution of NH₃, CH₄ and CO₂, as evidenced by FT-IR (**Figure S2**). The release of such species can be attributed to the thermal degradation of the organic component, which occurs via bond cleavage and rearrangement reactions: NH₃ may derive from the thermal rupture of peptidic bonds in the enzymes backbone and imine bonds introduced with the immobilization procedure, CH₄ can derive from aliphatic chains present both in the two proteins and in the spacer (glutaraldehyde), and CO₂ may be the product of decarboxylation and/or intramolecular oxidation of carbon by oxygen atoms present in the sample.

3.2 Kinetic measurements

A total amount of approximately 5 mg/g of proteins was quantified in the SBP/DAO-M samples, confirming that, in agreement with the physico-chemical characterization, the immobilization process was effective. The Bradford method is not suitable to discriminate SBP and DAO content on the monoliths, but the kinetic results confirm the presence of both of them on SBP/DAO-M monoliths.

In order to determine the best operational conditions, the catalytic properties of DAO and SBP were initially studied at different pH values by using cadaverine and DMAB-MBTH as DAO and SBP substrates, respectively. The activities were measured both in solution and with the two enzymes immobilized on the monoliths, also comparing the results obtained by using SBP only (**Figure 4**).

The pH of maximum activity for the SBP/DAO system is higher than for SBP alone and the shift is much more evident when the two enzymes are immobilized on the support. This behavior is in agreement with previous data concerning SBP immobilized on silica [31] and is probably caused by the slightly lower local pH in the close proximity of the silica surface with respect to the solution, depending on the anionic nature of the monoliths. As the maximum activity of SBP/DAO-M has reached at pH 8, this value was chosen for the subsequent activity measurements.

Recycling tests were performed on SBP/DAO-M to assess stability and reusability. Taken the initial activity as 100%, its decrease was regular, with a loss of about 3% for each reaction cycle, probably due to the non-complete stability of the Schiff base. After 15 cycles of reactions (**Figure S3**), the monolith maintained an activity of approximately 55 %, but the rate of product formation is still six times higher than the rate observed without enzymes.

On the other hand, SBP/DAO-M samples seem to be less stable than SBP-M, since in a previous work the retention of 75 % of the initial activity after 20 reaction cycles was reported [22]. This is probably a consequence of a slightly lower stability of the bond between DAO and monolith surface, as actually observed in preliminary test with monoliths functionalized with DAO only (data not shown).

The last experiments concern the determination of the LOD for four biogenic amines (tyramine, tyrosine, cadaverine, and histamine) by using SBP/DAO-M as a sensor. The results are shown in **Figure 5** in terms of

absorbance values at 590 nm (the maximum of the final blue-violet product) obtained at the end of each experiment with the corresponding concentration of amine.

The analysis of these results shows that the test is effective up to a concentration of 1×10^{-7} M for all substrates. The response seems to be highly substrate-dependent and the ratio between absorbance and concentration is not linear. This irregular distribution of results depends on the characteristics of each substrate, such as the number of amino groups (two in cadaverine and one in the others) and the different affinity for DAO [17], but it could be partially overcome by making calibration lines for each analyte. Finally, it is worth underlining that the detection limits determined in this study are consistent -and in some cases are lower- with respect to those reported in the literature (see **Table S1**) and could be further lowered using a different enzyme activity detection system or a more sensible detector.

4. Conclusion

In this work, a multi-enzymatic device with two enzymes (DAO and SBP) immobilized on silica monoliths was developed for the determination of biogenic amines. The monoliths were prepared by using low-cost green waste-derived substances which are valorized in the production of a siliceous mesoporous functionalizable platform for biotechnological applications.

The detection and quantification of amines resulted faster than those obtainable by traditional techniques, and the device was usable even at low concentrations of substrates. Although the reusability of each monolith for several measurements was limited, the device maintained high sensitivity for up to 15 cycles, therefore this study represents the proof-of-concept that the method is feasible for amine determination. Nevertheless, the system can be improved, and the measurements optimized considering the following suggestions: *(i)* the real DAO/SBP relative ratio on the support needs to be accurately quantified to reach the best value, *(ii)* the bond between enzymes and monolith needs to be further stabilized to avoid the DAO release, *(iii)* the detection method should be modified in order to enhance the sensitivity and achieve the determination of amines in concentration lower than 10^{-7} - 10^{-8} M.

Finally, an additional interesting aspect of this research is the development of a general method to realize a sensor by immobilizing different enzymes on the same support. This procedure could be extended to the determination of other type of analytes simply using the most appropriate enzymes.

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Figure captions

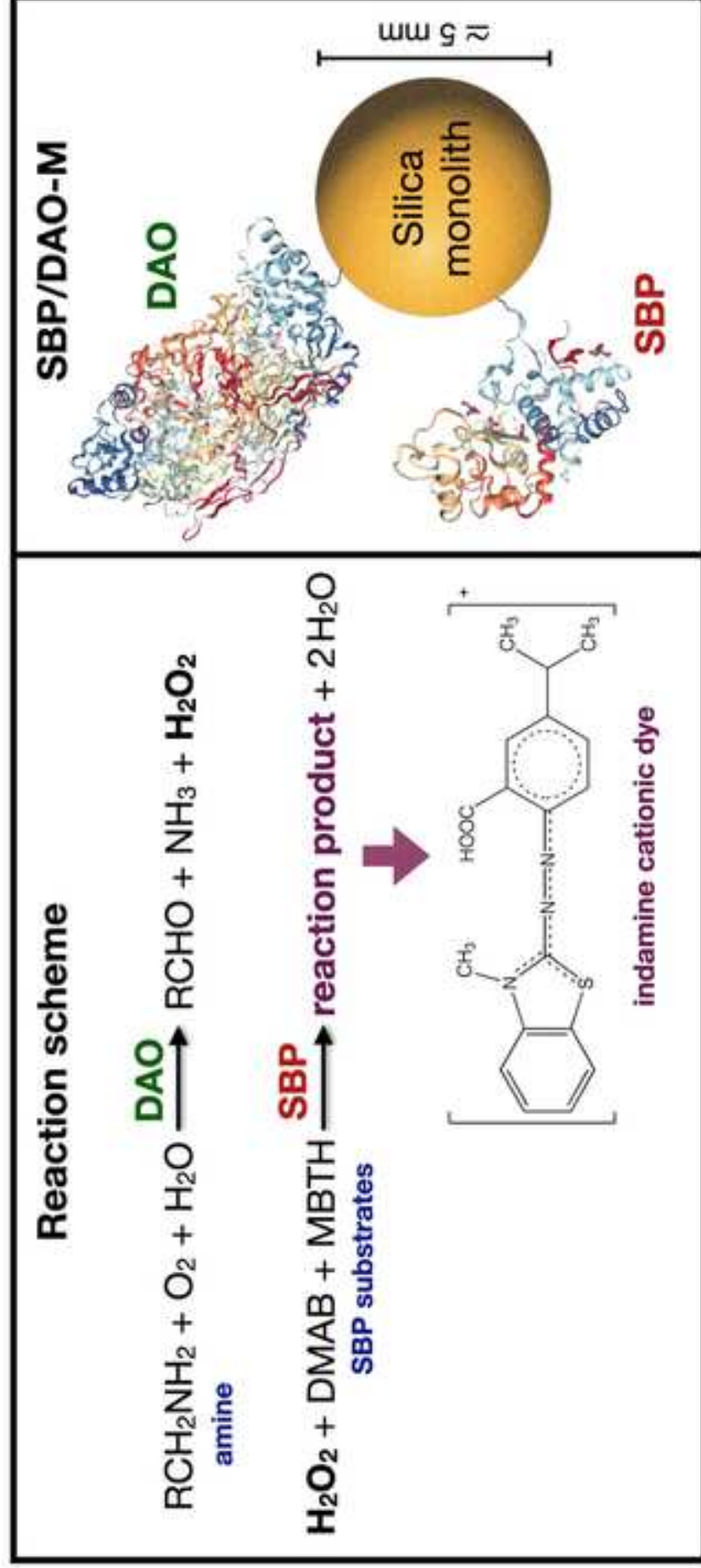
Figure 1: On the left: reaction scheme showing the role of DAO and SBP in the production of a stable and colored reaction product (the structure of the indamine cationic dye was proposed by Ngo and Lenhoff [21]). On the right: a drawing of the SBP/DAO-M silica monolith.

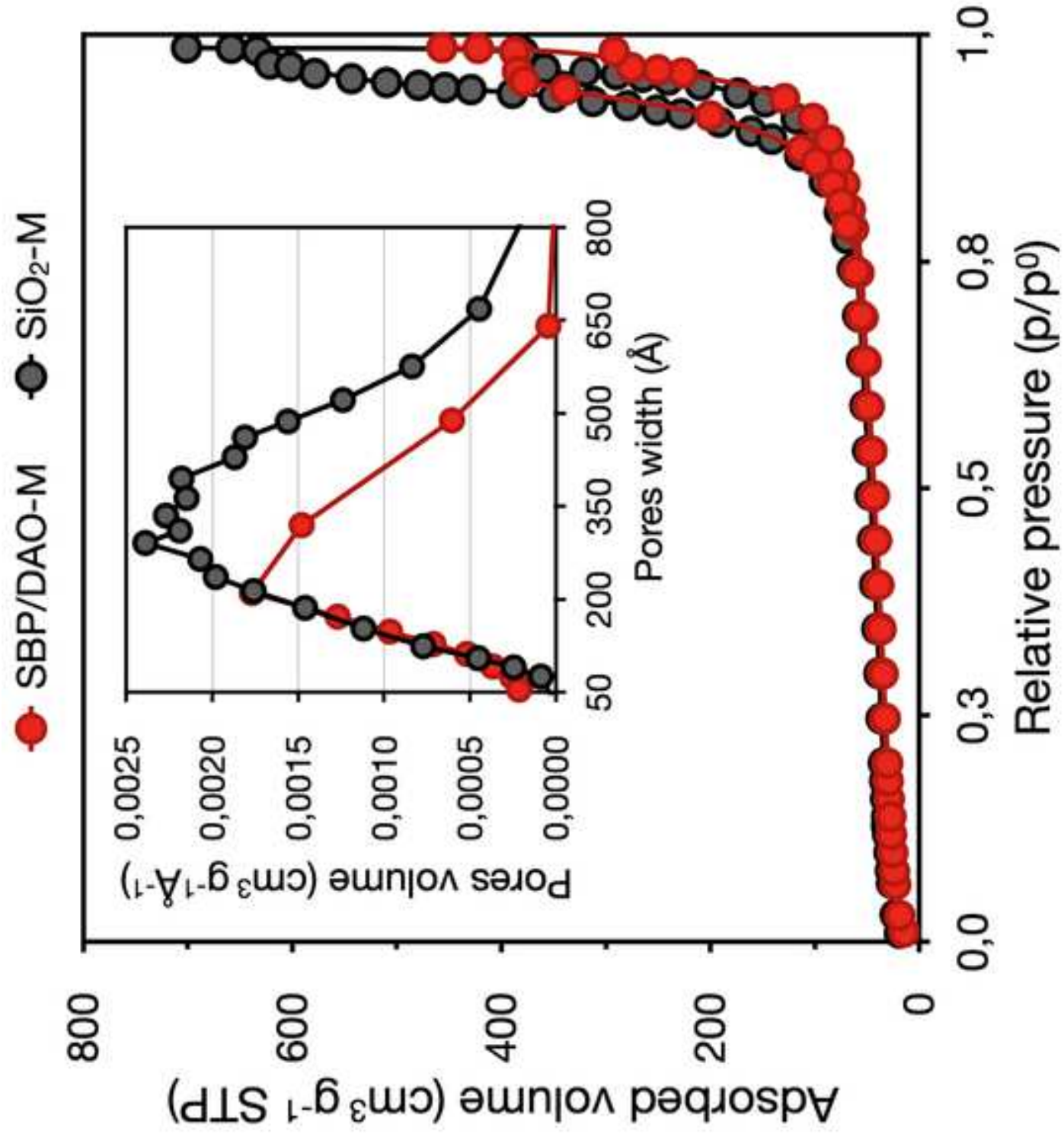
Figure 2: Adsorption-desorption isotherms of N₂ at 77K obtained for SiO₂-M (black) and SBP/DAO-M (red). In the inset, the pore size distribution obtained by means of BJH model.

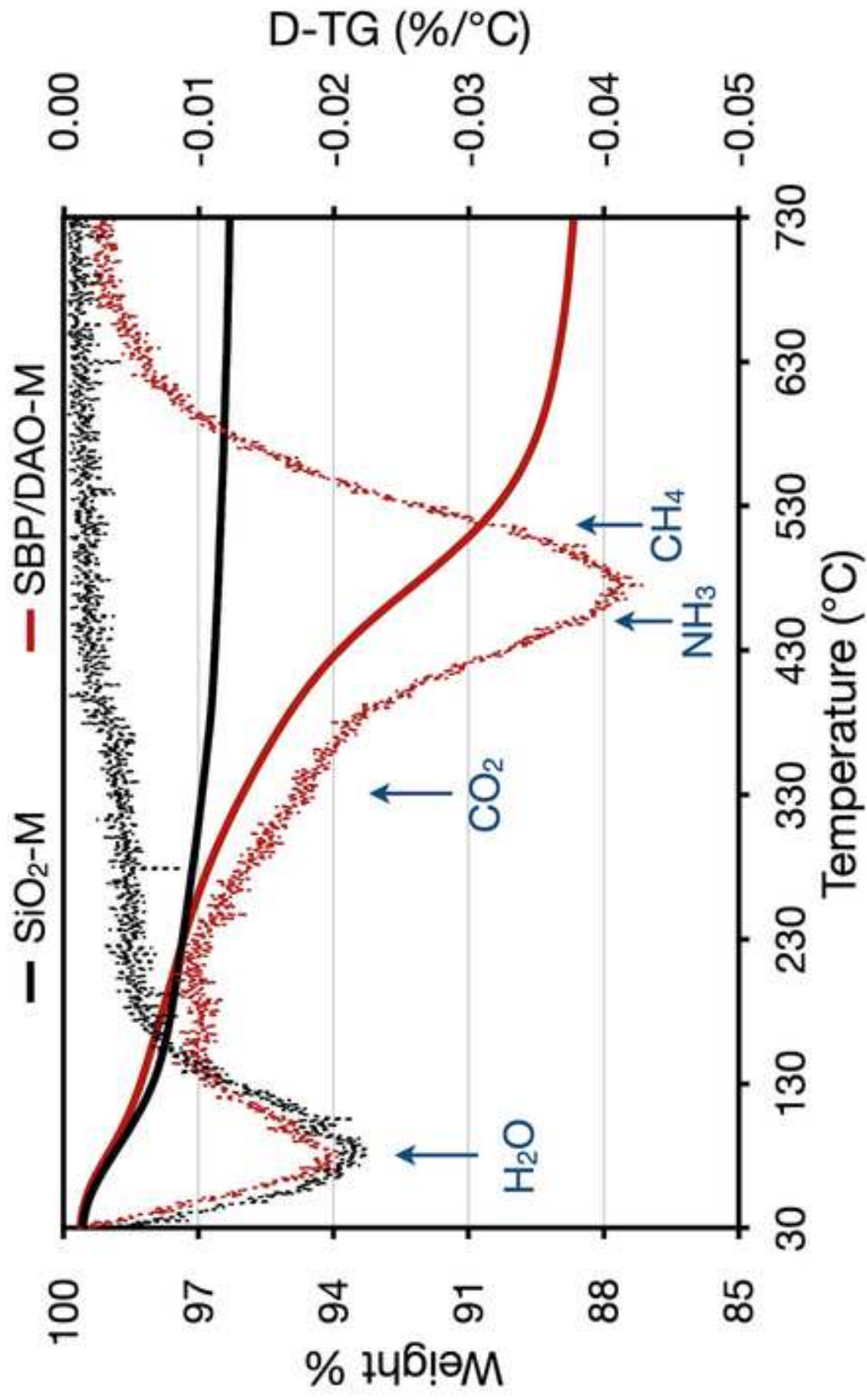
Figure 3: Results of TGA analysis of SBP/DAO-M (red) and SiO₂-M (black). Dotted line shows the corresponding first derivative of TGA data. Blue arrows indicate the approximate temperature at which each gaseous species was detected by FT-IR (see text for details and **Figure S2** for complete analysis of evolved gases).

Figure 4: Enzymatic activity vs pH profiles at 25°C with enzyme(s) in solution (top) or immobilized on monoliths (down). Data referred to SBP alone are in black, results with SBP and DAO in solution, or with SBP/DAO-M, are in red. Fitting curves were obtained by means of Henderson-Hasselbalch equation.

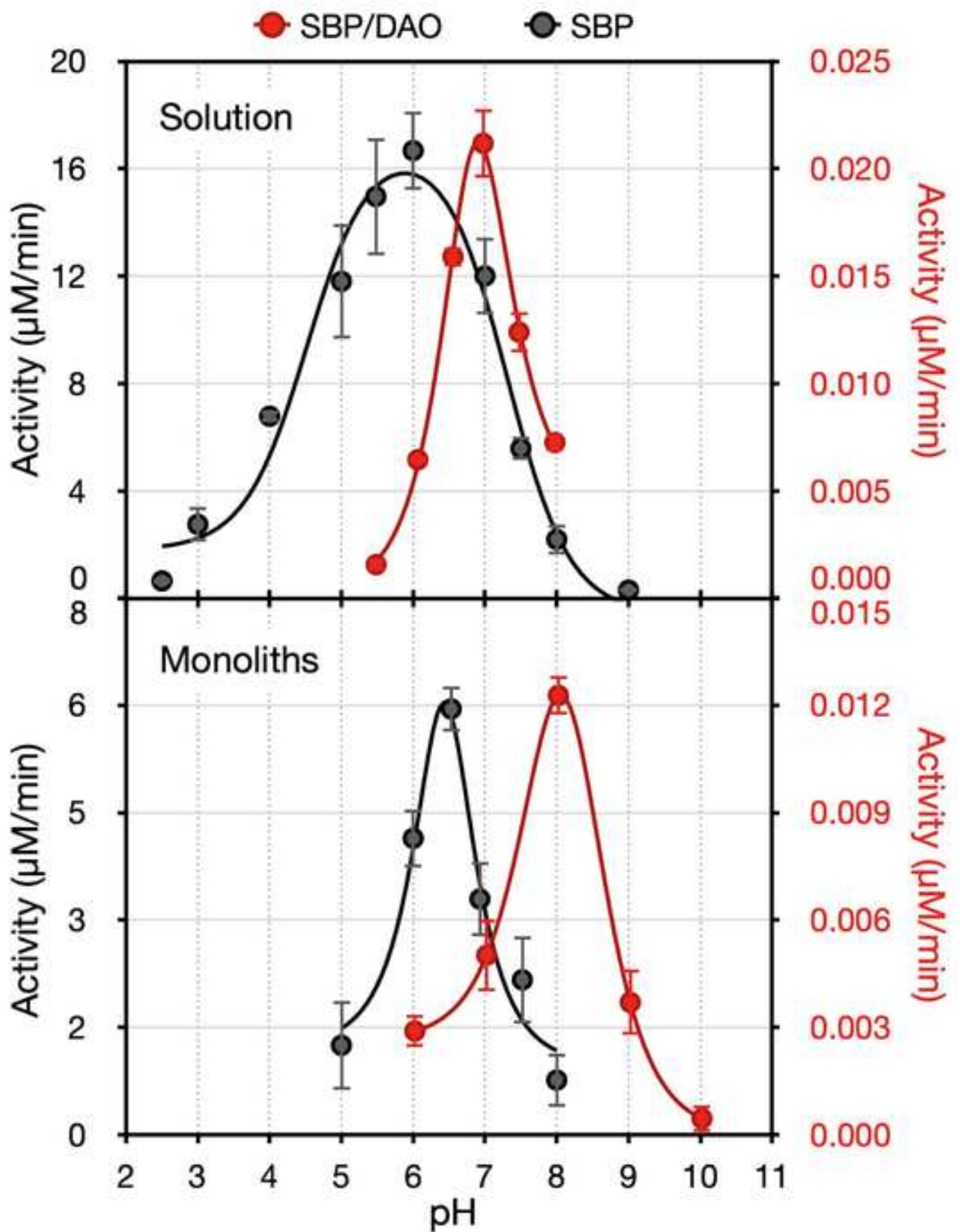
Figure 5: Test to evaluate the limit of detection (LOD) of biogenic amines by using SBP/DAO-M as catalyst. The results are reported in terms of absorbance value at 590 nm obtained at the end of each experiment by using the concentration of amine indicated in the legend.

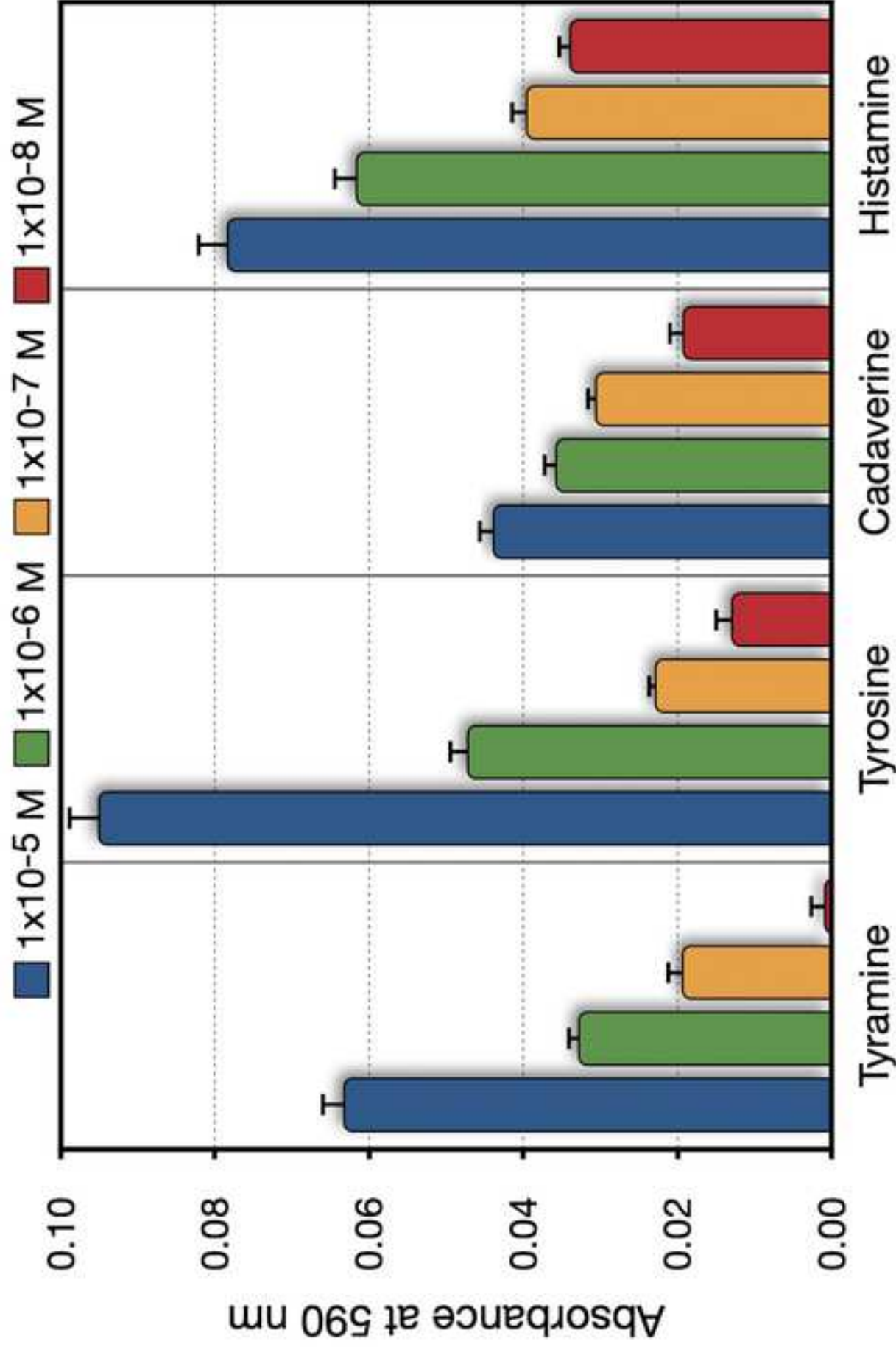






Figure_3





Immobilized bi-enzymatic system for the determination of biogenic amines in solution

Luca Lavagna^a, Maria Laura Tummino^{b,1}, Giuliana Magnacca^{b,c}, Ingrid Corazzari^{b,d}, Enzo Laurenti^{b*}

^a: Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, Torino, Italy

^b: Department of Chemistry, Via P. Giuria 7, Torino, Italy

^c: NIS Centre, Università degli Studi di Torino, Via P. Giuria 7, Torino, Italy

^d: "G. Scansetti" Interdepartmental Centre, Università degli Studi di Torino, Via P. Giuria 7, Torino, Italy

*Corresponding author: enzo.laurenti@unito.it

¹ Present Address: Institute of Intelligent Industrial Technologies and Systems for Advanced Manufacturing, Italian National Research Council, Corso G. Pella 16, Biella, Italy

Table S1: List of some techniques used for the detection of cadaverine in different matrixes and the related limit of detection (LOD).

Technique	Matrix	LOD	Reference
Fluorescence Liquid Chromatography (after derivatization)	Cadaverine standard solution	400 nM	[1]
High Performances Liquid Chromatography–MS/MS (after derivatization)	Wine	100 µM	[2]
Gas Chromatography– MS/MS (after derivatization)	Port Wine and Grape Juice	10 nM	[3]
Capillary electrophoresis – MS/MS	Beer and wine	20 nM	[4]
Amperometric sensor	Fresh chicken meat	300 nM	[5]

- [1] A. Zotou, M. Notou, Enhancing Fluorescence LC Analysis of Biogenic Amines in Fish Tissues by Precolumn Derivatization with Naphthalene-2,3-dicarboxaldehyde, *Food Anal. Methods*. 6 (2013) 89–99. doi:10.1007/s12161-012-9409-3.
- [2] K. Nalazek-Rudnicka, A. Wasik, Development and validation of an LC–MS/MS method for the determination of biogenic amines in wines and beers, *Monatshefte Fur Chemie*. 148 (2017) 1685–1696. doi:10.1007/s00706-017-1992-y.
- [3] S.C. Cunha, M.A. Faria, J.O. Fernandes, Gas chromatography-mass spectrometry assessment of amines in port wine and grape juice after fast chloroformate extraction/derivatization, *J. Agric. Food Chem*. 59 (2011) 8742–8753. doi:10.1021/jf201379x.
- [4] D. Daniel, V.B. dos Santos, D.T.R. Vidal, C.L. do Lago, Determination of biogenic amines in beer and wine by capillary electrophoresis-tandem mass spectrometry, *J. Chromatogr. A*. 1416 (2015) 121–128. doi:10.1016/j.chroma.2015.08.065.
- [5] D. Telsnig, K. Kalcher, A. Leitner, A. Ortner, Design of an Amperometric Biosensor for the Determination of Biogenic Amines Using Screen Printed Carbon Working Electrodes, *Electroanalysis*. 25 (2013) 47–50. doi:10.1002/elan.201200378.

Detailed methods used in the synthesis of monoliths

Monolith preparation

Monoliths (SiO₂-M) were synthesized following a previously reported method [6]. 10 g of BBS-GC were kept under stirring in 150 mL of double distilled water for 2 hours. Then, 40 g of silica were added to the BBS-GC solution, adding a small volume of water to facilitate the reagents mixing. The system was left under a vigorous stirring for other 2 hours. At the end of the process, the mixture was left drying until it became thicker enough to be manipulated to obtain spheres (approximately with a diameter of 5 mm and a final weight of 50 mg). Successively, the material was dried at room temperature for 2-3 days and calcined at 500 °C for 4 hours in order to remove the organic matter and obtain solid mesoporous spheres.

Enzyme immobilization

About 1 g of SiO₂-M was suspended in 75 mL of a 10% v/v solution of 3-aminopropyltriethoxysilane (APTES) in water at pH 4.0 and refluxed at 80 °C for 3 hours. Then, the monoliths were filtered on a Buchner funnel, washed with double distilled water and dried for 1 hour at 110 °C.

Successively, 0.6 g of APTES-coated monoliths were added to 50 mL of glutaraldehyde solution 2.5% v/v in 0.1 M phosphate buffer at pH 7.0 and allowed to react under stirring for 1 hour in the dark at room temperature. After the reaction, the unreacted glutaraldehyde solution was removed by filtration and the monoliths were washed four times with phosphate buffer 0.1 M at pH 7.0.

The functionalized monoliths were added to 10 mL of a solution containing 15 mg of DAO and 5 mg of SBP in 0.1 M phosphate buffer at pH 7.5 and left to react at 4 °C for 20 hours. The final red products (namely SBP/DAO-M) were washed four times with the same buffer, then left to dry in the air overnight and stored at 4 °C until use. For comparison, monoliths functionalized with SBP only (SBP-M) were prepared by using a similar procedure.

The amount of immobilized proteins was estimated by using the Bradford method appropriately modified [7] and calculated as the difference between the concentration present in the initial solution and that recovered after the reaction. UV-visible measurements were made in a double-beam spectrophotometer UNICAM UV300 (Thermospectronic), equipped with a Peltier cell for temperature control and a magnetic stirring system, using 1 cm optical path quartz cuvettes.

- [6] G. Magnacca, E. Laurenti, E. Vigna, F. Franzoso, L. Tomasso, E. Montoneri, V. Boffa, Refuse derived bio-organics and immobilized soybean peroxidase for green chemical technology, *Process Biochem.* 47 (2012) 2025–2031. doi:10.1016/j.procbio.2012.07.021.
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Figure S1: FT-IR spectra of SiO₂-M (black-line) and SBP/DAO-M (red-line) monoliths after evacuation.

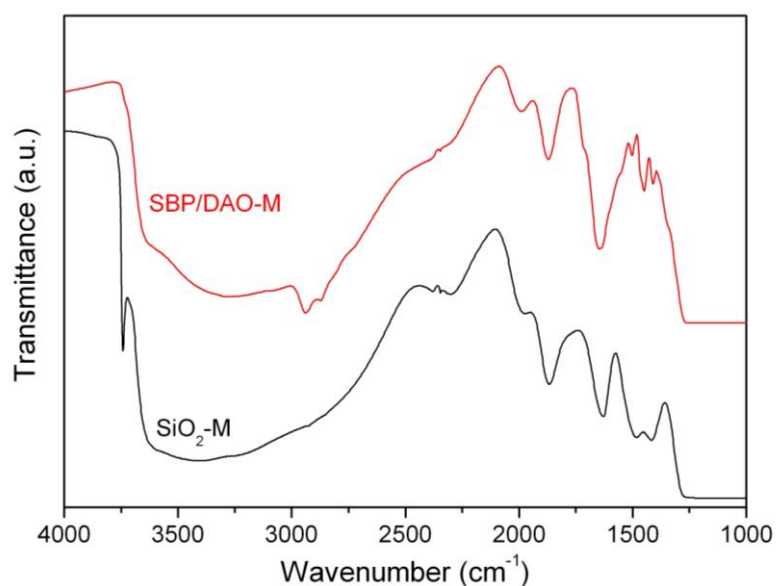


Figure S1 shows the FT-IR spectra of the pristine and functionalized monolith. Both the spectra show intense bands at frequencies below 1300 cm⁻¹ due to the bulk modes of silica, where the almost complete absorption of the incident radiation is observed. At frequencies between 2100 and 1500 cm⁻¹ three signals (harmonics and combinations) linked to the silica reticular modes are present. The pair of bands at 1500-1400 cm⁻¹ is attributable to signals related to carbonate species (of monodentate type) formed on the surface of silica as a result of the interaction of atmospheric CO₂ with the basic oxygen sites of the monolith (produced by inorganic impurities carried by the biosurfactant used in the preparation of monoliths). Around 2350 cm⁻¹ the Σ_u^+ signal of the not perfectly compensated CO₂ present in the atmosphere is visible. At frequencies greater than 2500 cm⁻¹ all the stretching signals of the O-H groups relative to the surface hydroxyls engaged in hydrogen bonds are observed and at 3750 cm⁻¹ the signal of silanol free from interactions is observed. At frequencies lower than 3000 cm⁻¹, the C-H stretching modes can be observed mostly due to hydrocarbon impurities adsorbed on the surface of the monolith.

In the spectra of the SBP/DAO-M, a very intense band at 1600 cm⁻¹ with a shoulder at 1650 cm⁻¹, due to the formation of the imine bond were observed, and some signals at about 3000 cm⁻¹ due to the stretching of the C-H groups present in the immobilized proteins. The shape of the large signal due to hydroxyls engaged in hydrogen bonds changes and suggests that at 3200 cm⁻¹ a stretching signal of N-H groups could be also present [39]. The disappearance of the sharp ν_{OH} signal at 3750 cm⁻¹ indicates that, as expected, hydroxyl groups present on the silica surface reacted during the functionalization of monoliths. The carbonate-like vibration bands at 1500-1400 cm⁻¹ are less evident in the DAO/SBP-M spectrum. Similarly, the large signal observed at about 2300 cm⁻¹ in the SiO₂-M (libration vibration of physisorbed water molecules), probably because the functionalization process allows removing most of the CO₂ adsorbed molecules and decreases the high hydrophilicity of the support. In conclusion, the recorded spectra confirm the presence of the

enzymes on the monoliths and the presence of the intense signal at 1600 cm^{-1} allows to confirm that the enzymes are covalently immobilized on the surface of the monoliths.

Figure S2: TGA/FT-IR analysis for SBP/DAO-M (left panel) and SiO_2 -M samples (right panel). Top: weight loss and corresponding first derivative obtained by TGA (corresponding to the data shown in **Figure 3**. Down: release of H_2O , CO_2 , NH_3 and CH_4 resulting by FT-IR analysis of the gases evolved by the samples.

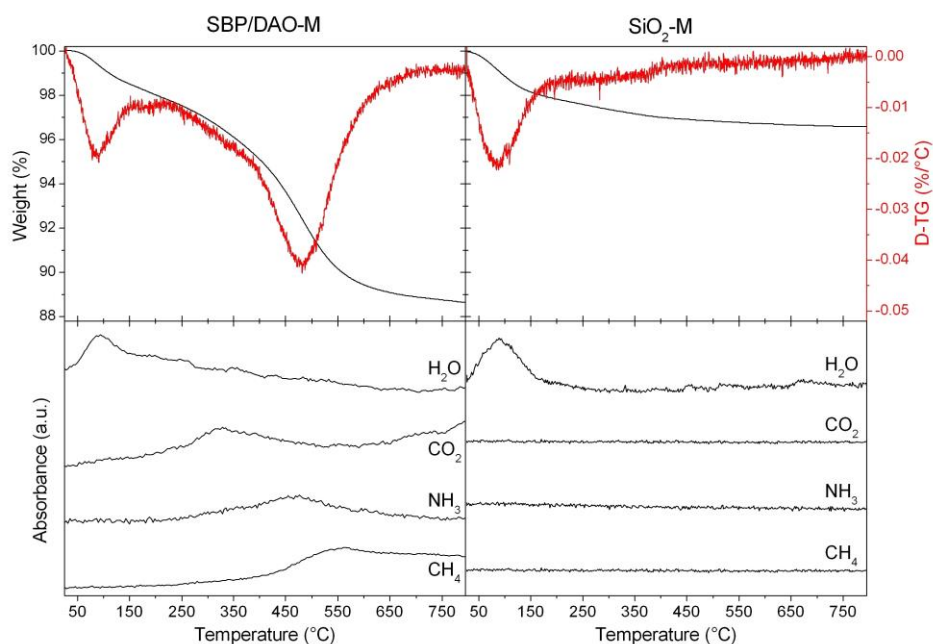
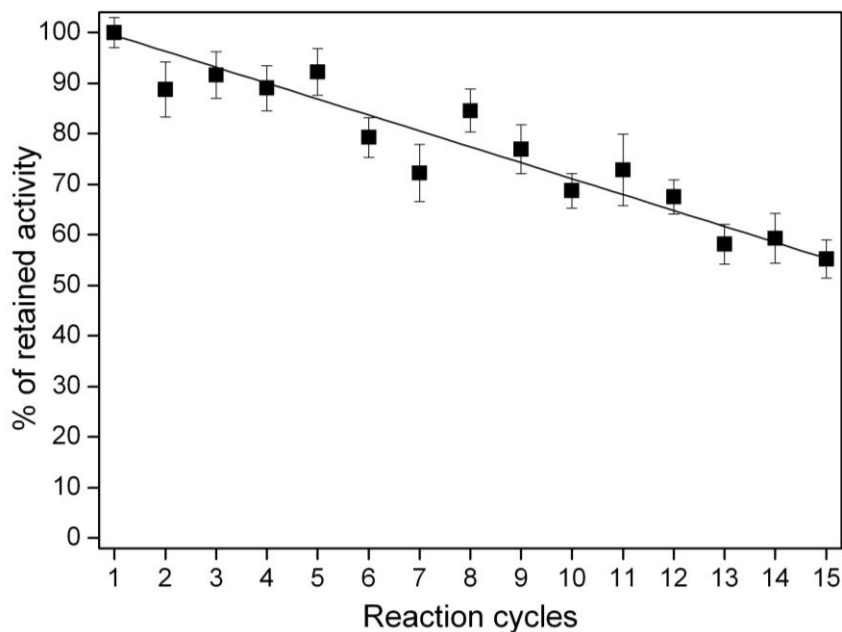


Figure S3: Retained activity of SBP/DAO-M for 15 reaction cycles.



Luca Lavagna: Investigation, Methodology, Writing - Original Draft

Maria Laura Tummino: Methodology Validation, Writing - Review & Editing

Giuliana Magnacca: Investigation, Writing - Review & Editing

Ingrid Corazzari: Investigation

Enzo Laurenti: Conceptualization, Supervision, Writing - Review & Editing