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Analytical possibilities of Putrescine and Cadaverine enzymatic colorimetric determination in tuna based on diamine oxidase: A critical study of the use of ABTS

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ABSTRACT

The joint determination of putrescine (Put) and cadaverine (Cad) in the presence of other biogenic amines is studied using their enzymatic reaction with diamine oxidase (DAO). Three alternative methods are studied based on the intrinsic colorimetric properties of DAO or horseradish peroxidase (HRP), and the use of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) colorimetric reagent, respectively. In this last case an in-depth study is carried out in order to explain and solve some drawbacks usually associated with the use of this reagent (especially interferences, interaction with enzymes and instability), and to propose new analytical methodologies which this reagent allows to achieve (transient signal and the use of the violet species). Finally, the method has been applied to Put + Cad determination in a tuna sample without interference of other biogenic amines. The result has been compared with that obtained using a method based on HPLC-MS, which has allowed the new methodology to be validated.

1. Introduction

Putrescine (Put) and Cadaverine (Cad) are biogenic polyamines (BPA), specifically alpha omega-diamines, a sub-group of the biogenic amines (BA). BPA have great importance at the physiological level [1]. Low BPA levels reduce the biogenic synthesis of nucleic acids and proteins (leading to cellular growth inhibition). Normal levels are linked to correct cellular growth, but high BPA levels may lead to apoptosis or to malignant transformation [2]. Consequently, BPA biological synthesis and degradation should be balanced in order to keep BPA concentrations within appropriate narrow margins.

Foodstuffs are an additional (and non-controlled) source of BA [3]. The BAs are incorporated during manufacturing, as occurs with fermented foods (cheeses, wines, sauerkraut, etc.), but they also appear in non-fermented foods (e.g. scombroid fish, fruit juices, pork meat) when they are beginning to lose their freshness, due to microorganism proliferation.

The most frequent BA in foods [4–7] are Histamine, Tyramine, Phenylethylamine, Tryptamime and, from the BPA sub-group, Put and Cad. The concentration differs greatly from one food to another [5].

When foods containing BA are ingested, the biological BA levels are unbalanced and consequently physiological problems could be triggered.

From the health point of view, the most extensively studied are Histamine and Tyramine because they are related to “Scombroid poisoning” and “Cheese reaction,” respectively. Currently, Histamine is the only BA for which maximum limits have been set in the EU (Commission Regulation (EC) 2073/2005). Several studies suggest that the absorption, metabolism and/or potency of one BA might be modified by the presence of another. Put and Cad have not been classified as toxic, but they are able to enhance the toxicity of low concentrations of Histamine [8] (Put and Cad inhibit the action of monoamine oxidase and other enzymes which metabolize Histamine). Furthermore, during cooking Put and Cad can give rise to nitrosamines, which are carcinogenic substances [9]. To prevent all these problems, Put and Cad need to be controlled or monitored in foods. Therefore, having rapid and easy methods for their determination is of considerable interest.

BPA or BA mixtures are currently analyzed in foodstuffs using classical methodologies based on the combination of a sample treatment with the appropriate solvent and the application of an instrumental separation technique to the extract [10]. For example, for solid samples

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a conventional procedure consists of acid treatment (using trichloroacetic, perchloric or hydrochloric acids) followed by reversed-phase (U)HPLC with molecular absorption or fluorescence detection [11–13] after derivatization, although TLC, GC (after derivatization) or capillary electrophoresis have also been proposed.

For one BA or BPA (or the total concentration of them) monitorization, the most frequently used methods found in bibliography are electroanalytical (bio)sensors, based on enzymatic reactions, and amperometric detection. For BPA, a classical sequence of two enzymatic reactions is used (Fig. 1). In the first one, the enzyme diamine oxidase (DAO) catalyzes the BPA oxidation (to the corresponding aldehyde) by O_2 (giving H_2O_2). In the second one, the enzyme peroxidase (HRP) catalyzes the oxidation of a charge transfer mediator by H_2O_2, giving a product active in amperometry [14–16]. In this reaction only one of the NH_2 groups of the BPA reacts and the product is later cycled.

Although the same scheme is frequently used for the colorimetric determination of biochemical metabolites, replacing the charge transfer mediator by a chromogen (such as 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [17,18] or 3,3′,5,5′-Tetramethylbenzidine (TMB)), for BPA determination colorimetric methods based on this scheme [18], or on enzymatic reactions [19], are scarce. These methods are usually based on using synthetic chemical receptors. In this context, interesting methods based on the volatility of the low molecular weight BA coupled with the use of an acid-based indicator, a more specific chromogen [20–22] or a chromophores array [24] have been described. These also allow this methodology to be implemented in smart packages [22,23] for the naked-eye instant control of these compounds. For methods in solution, more selective chemical and biological receptors have been proposed [25], either with intrinsic chromogenic properties which are combined with those of gold nanoparticles (AuNP) [23,24] or, more recently, only using the chromophore properties of AuNP [26]. Some nanomaterials can also act both as receptors and as chromophores, but their selectivity against BPA (or BA) need to be carefully tested [26,27] in real samples.

Many enzymes present intrinsic spectroscopic (UV–vis absorption and fluorescence) properties due to the corresponding coenzymes. In some of them, if the reaction conditions are properly chosen it is possible to observe changes in these intrinsic properties during the corresponding enzymatic reactions, so they can be used as the analytical signals in batch methods or biosensors [28]. For example, the HRP presents several absorption bands due to the heme-group, whose exact λ and ε depend on the heme-group oxidation state and on the ligands

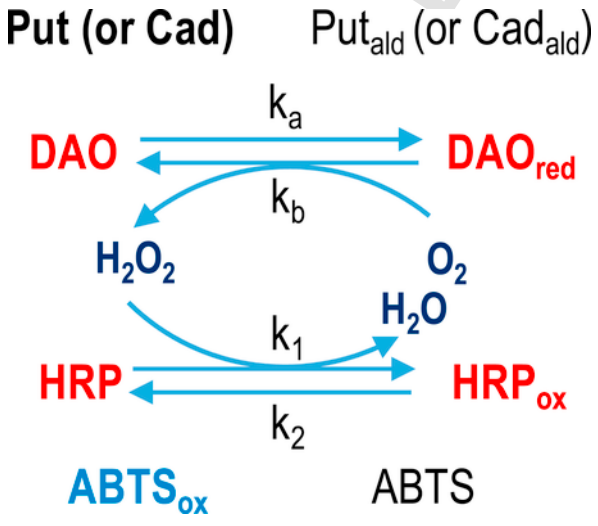

Fig. 1. Sequence of the enzymatic reactions used for BPA determination.

linked to this atom [29]. Going back to the scheme in Fig. 1, if the indicating reaction is carried out in the absence of ABTS, the HRP- H_2O_2 reaction gives an oxidized form of the (HRPII), having an absorption spectra different from that of HRP. This difference can be used as the basis of a spectrophotometric biosensor for either H_2O_2 monitorization [30] or an analyte [31,32] which produces H_2O_2 in a previous enzymatic reaction, Put or Cad in this case. DAO from *Lathirus cicera* contains a cofactor composed of a tri-copper group linked to a topa-quinone group, which absorbs in the visible spectral region [33]. As far as we know, these properties have not yet been tested for analytical purposes.

Taking Fig. 1 as a basis, the aim of this paper is to study the analytical possibilities of DAO, HRP and ABTS for Put and Cad determination. In the latter case, secondary reactions which can occur with this reagent (which are not usually taken into account) are discussed. The possible interferences caused by the rest of the BA usually present in these types of sample have also been studied. The optimized methods are used for BPA determination in a tuna extract sample (scombroid fish) with good results.

2. Material and methods

2.1. Reagents and solutions

Phosphate buffer solution (0.1 M, pH 6.0) was prepared from Na_2HPO_4 and NaH_2PO_4 solids (Sigma S9638 and S9763).

Hydrogen peroxide stock solution (33% w/v) was supplied by Panreac (131077.1211). Peroxidase from *Horseradish* (HRP EC 1.11.1.7) was obtained from Sigma (P8125 88.6 Umg^{-1}). Diamino oxidase (DAO EC 1.4.3.22) was taken from three different organisms: *Lathirus cicera* (Molirom P021, 280UmL^{-1}), *Porcine kidney* (Sigma D7876, 0.05Umg^{-1}) and Plant (Bio-Research 903X, 5000U).

Cadaverine (C8561), Putrescine (P7505), Histamine (53300), Phenylethylamine (P6513), Tyramine (T287998), Tryptamine (246557) and 2,2′-Azino-bi (3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) (A1888) were supplied by Sigma. All solutions were daily weighed and dissolved in the buffer solution; ABTS solutions were stored in darkness.

The ruthenium solution (Ru) was prepared by dissolving 1 mg of tris(2,2′-bipyridyl) dichlororuthenium (II) hexahydrate) (Sigma 544981) in 0.50 mL of dimethyl sulfoxide (Panreac131954.1611).

2.2. Equipment

The molecular absorption measurements were performed using a Hewlett-Packard model HP 8452A diode-array spectrophotometer equipped with a HP 89090A Peltier temperature and stirrer control accessory. Depending on the measurement wavelength, quartz (Hellma QS 101) or glass (Hellma Q 101) cuvettes were used of 1 cm of path length.

The fluorescence measurements were carried out with a Photon Technology International (PTI) Time Master fluorescence spectrometer working in a L-configuration (model TM-2/2003-PTI) and a quartz (Hellma QS 101) cuvette.

2.3. Procedure

The variation of the absorbance during the enzymatic reaction was measured at different wavelengths depending on whether the DAO intrinsic signal (465 nm), the HRP intrinsic signal (405 and 420 nm) or the ABTS signal (420 and 730 nm) was used.

After choosing the wavelength, the appropriate reagent concentrations were added into the cuvette with the buffer solution: a) 76 U/mL of DAO in the DAO method; b) 2 U/mL DAO and 25 U/mL HRP in the

HRP method; and c) 2 U/mL of DAO, 2 U/mL of HRP and $4*10^{-4}$ M of ABTS in the ABTS method (total volume in the cell, 2 mL). The cuvette was then introduced in the spectrometer, the stirrer was connected and the measurement was started in the kinetic mode. After 50 s (to obtain the baseline), 20 μL of the analyte (or sample) solution was injected and the variation of absorbance during the reaction was recorded over time. When ABTS was used, a filter must be placed between the lamp (D_2) and the cell to avoid the photooxidation of the dye. During the optimization studies, the concentration of the reagents and other conditions were modified depending on the parameter to be studied.

The measurements of the O_2 consumption during the enzymatic reaction of DAO and BA were made by fluorescence (457 nm excitation and 618 nm fluorescence wavelengths). For this, phosphate buffer (0.1 M, pH = 7), DAO (2U/mL) and Ru ($1.40*10^{-5}$ M) solutions were placed in the cuvette (total volume 2 mL) and the initial fluorescence value was obtained. After 90 s, 20 μL of the analyte (Cad, Put, …) solution was injected and the variation of fluorescence during the reaction was measured.

All measurements have been carried out a minimum of three times (n = 3).

2.4. Tuna sample treatment

The sample was prepared and treated by the *Laboratorio de Salud Pública de Aragón* (LSPA).

The tuna sample was submitted to acid treatment by using a previously validated method [34]. In short, 2.5 g of tuna was treated with 20 mL 5% trichloroacetic acid; the samples were shaken in a vortex for 30 s. Then, the mixture was submitted to ultracentrifugation for 10 min at 4000 rpm (4 °C). This operation was repeated twice. The filtrated was taken to 50 mL with 5% trichloroacetic acid.

One fraction of it was analyzed by HPLC-MS and another was analyzed by the colorimetric procedure previously described.

3. Results and discussion

3.1. DAO and HRP intrinsic properties

3.1.1. DAO intrinsic properties

DAO coming from three different organisms were tested (see Material and methods section), but that from *Lathirus cicera* was finally chosen because of its higher activity. This enzyme contains a 6-hydroxydopa quinone (HPQ) moiety linked to Cu(II) as the active center. The detailed mechanism through which DAO catalyzes BA oxidation by O_2 corresponds to a ping-pong type, and has been previously studied [35]. However, considering the aim of this paper, it can be simplified to a two step process, as indicated in Fig. 1.

First, a kinetic study was conducted in order to characterize the reactivity of DAO with Put, Cad and the most representative BA present in the samples (Histamine, Tyramine, Phenylethylamine and Tryptamine). To do so, the O_2 consumption during the enzymatic reaction was measured (Fig. S1) using a fluorescence oxygen sensor. The order of the reaction rate obtained was as follows

$$\text{Put} \approx \text{Cad} \gg \text{Histamine} \approx \text{Phenylethylamine} \gg \text{Tryptamine} \approx \text{Tyramine} \quad (1)$$

which agrees with the kinetic constants previously reported [36]. The DAO molecular absorption spectra variation during the reaction using Put as the substrate is given in Fig. S2. The original (DAO, oxidized) form does not show recognizable maxima. When the substrate concentration was lower than the O_2, no spectral changes were observed during the reaction (indicating that the DAO reoxidation step is very fast). Only when the substrate concentration was higher than the O_2 did a new small maximum appear at about 465 nm. According to the bibliography, this spectrum corresponds to an alternative reduced (semiquinonic) form which only appears when the whole O_2 present in the solution has been consumed (about $2.2*10^{-4}$ M at 20 °C). The absorbance changes at this wavelength can be used as the analytical signal, but this methodology has two drawbacks: 1) The amount of the enzyme necessary to observe these spectral changes is very high; 2) Even in these conditions the maximum absorbance change is low, so the expected method sensitivity is very low. The quantification limit obtained was about 0.35 mM (which corresponds to the concentration given in Fig. S2).

3.1.2. HRP intrinsic properties

As has been indicated, the HRP and HRPII forms present different absorption spectra (Fig. S3). Then, during the H_2O_2/HRP reaction, transient signals at 420 nm are observed whose height and area are related to the peroxide concentration. When this reaction is coupled to the DAO/Put system, similar absorbance changes (due to Put) are observed. It is possible to use these absorbance changes as the analytical signal avoiding the use of a chromophore (ABTS, TMB, …) and to re-use the HRP for different measurements (Fig. S4). About 15 measurements can be formed using the same HRP aliquot, although more than 5 are not recommendable if reproducible measurements are to be obtained. After the DAO concentration optimization, Fig. S5 shows the absorbance *versus* time representations obtained after submitting individual BA to the enzymatic reaction with DAO/HRP. In general, BA behave similarly to that indicated in (1). Again, Histamine and Phenylethylamine give a signal which can interfere in BPA determination, but their effect will only be important when Histamine or Phenylethylamine concentrations are about 10 times higher than those of BPAs. Particularly remarkable was Tyramine and Tryptamine behavior. As can be expected, no signal was observed with both BA; however, the signal of Cad and Put decreases in the presence of Tyramine and Tryptamine. They are able to suppress the signal given by BPA because they are able to react with HRPII and to regenerate HRP (Fig. S6A). The interference caused by Tyramine is strong (Fig. S6B). Even for concentrations lower than that of the BPAs, its interference is important. This problem could be solved if the DAO and HRP reactions are phased out and HRP is added after Tyramine has completed its reaction with DAO, but a very long reaction time would be necessary. Tryptamine causes a similar effect on HRPII but its regeneration rate is lower than that of Tyramine so its interference is quantitatively less important. Moreover, the Tryptamine concentration in most of the samples is usually very small.

The analytical figures of merit for Put and Cad determination have been obtained using the absorbance change at 420 nm as the analytical parameter. The linear response range, the sensitivity and the RSD for both BPAs are shown in Table 1. As can be seen, the sensitivity of Put is higher than that of Cad. A calibration study was carried out with

Table 1
Figures of merit for Cadaverine and Putrescine determination using HRP intrinsic properties or HRP-ABTS system.

	Calibration line ([Cad] or [Put] in M)	range (M)	RSD (%)
HRP (420 nm)	ΔAmax = −0.0004 + 28931 [Put]; fO.997	1.2*10–6 to 5.0*10-6	3.7% (n = 4)
	ΔAmax = −0.0016 + 21366 [Cad]; r = 0.999	1.2*10–6 to 7.0*10-6	4.8% (n = 4)
	ΔAmax = 0.0019 + 22960 [Cad + Put]; r = 0.999	$1.6*10^{-6}$ to $6.0*10^{-6}$	4.6% (n = 4)
HRP/ABTS (730 nm)	ΔA_{max} = 0.0005 + 26036 [Put]; r = 0.999	$5.0*10^{-7}$ to $3.0*10^{-6}$(*)	4.2% (n = 4)
	ΔA_{max} = −0.0007 + 24467 [Cad]; r = 0.998	5.0* 10–7 to $3.0*10^{-6}$(*)	5.0% (n = 4)

(*) Maximum concentrations tested.

mixtures of Put and Cad in proportions of 50%, and an intermediate slope was obtained, closer to that of Cad. The signals obtained for mixed solutions of Cad and Put in different proportions were interpolated in this calibration line. The errors obtained were between 3 and 10%, which would allow an estimation of the total concentrations of cadaverine and putrescine in real samples using the intrinsic signal of HRP.

3.2. Method based on the H_2O_2/HRP/ABTS reaction: previous considerations

Several chromophores have been proposed as colorimetric indicators in methods involving H_2O_2/HRP, TMB and ABTS being the most important. Both present similar properties, but for measurements in solution ABTS is preferred because of its solubility in water. Although the H_2O_2/HRP/ABTS system is frequently used in analytical methodologies, there are several aspects which are not appropriately described in the bibliography and could help to avoid some problems.

3.2.1. Instability of the ABTS compound

ABTS can exist in three oxidation states [37,38]:

$$ABTS\,(colorless) \underset{+1e^-}{\overset{-1e^-}{\rightleftarrows}} ABTS_{OX}\,(blue) \underset{+1e^-}{\overset{-1e^-}{\rightleftarrows}} ABTS_{OX2}\,(red/violet) \quad (2)$$

Since in ABTS/H_2O_2/HRP reactions the ABTS concentration is always in excess of that of H_2O_2, the $ABTS_{ox2}$ is not formed. The oxidation to the blue $ABTS_{ox}$ is the process of choice. This compound presents several absorption maxima. The strongest appears at 420 nm, but its blue color is due to that presents in the visible region (about 730 nm). Both spectral regions are used for quantification but the maximum at 730 presents less interference problems and this will be used throughout the rest of this paper.

It is known that ABTS forms suffer from slight instability, due to a photooxidation to form the ABTSox species. This can be avoided using an appropriate optical filter (Fig. S7). Moreover, it has been reported that ABTSox suffers disproportionation according to Refs. [37,38]:

$$2\ ABTS_{OX} \underset{k_2}{\overset{k_1}{\rightleftarrows}} ABTS + ABTS_{OX2} \quad (3)$$

This last process is slow (k_1 or k_{-1} constants have not been previously reported) and is one of the factors responsible for the loss of the blue color with time. From (3) it is clear that the higher the ABTS concentration used, the smaller the disproportionation, so it is recommendable to use high ABTS concentrations (see Fig. 2). Moreover, the higher the ABTS concentration, the larger the linear response range. However, this effect does not explain why the ABTSox absorbance continuously decreases even in very high excess concentrations of ABTS. A very interesting aspect is the HRP concentration. The kinetic of the H_2O_2/ABTS/HRP reaction is very fast, so low HRP concentrations are sufficient. However, when high HRP concentrations are used, the blue color is lost more quickly (Fig. 3). This can be explained considering the reducing properties of the protein. We have stated [30] that HRPII can be reduced to the HRP initial compound by the action of tyrosine and tryptophan moieties present in the aminoacidic envelope surrounding the protein. These amino acids may also be able to reduce $ABTS_{ox}$, according to the process:

$$ABTS_{OX} + HRP \xrightarrow{k_2} ABTS + HRP' \quad (4)$$

where HRP' represents the "oxidized" resulting HRP. In the supplementary material (Appendix S1) a detailed theoretical/experimental study is presented in order to demonstrate the process (4) and to calculate the k_1 and k_2 values, these being:

$$k_1 = 39\,(\pm 2)\,s^{-1} \quad k_2 = 480\,(\pm 480 s^{-1}) \quad (5)$$

We have obtained similar results after changing the HRP excess for albumin, which also presents several tyrosine and tryptophan moieties close to the protein surface, so any protein can potentially act as an $ABTS_{ox}$ reducer. It is important to take this effect into account when combining the ABTS/HRP with another enzymatic reaction or when the method is to be applied to a protein-containing sample. In conclusion, the HRP concentration affects the colorimetric method. First, HRP is necessary to catalyze the ABTS oxidation by H_2O_2 and, second, HRP is able to reduce the ABTSox.

3.2.2. Conditions for H_2O_2 determination in batch and continuous mode

We can conclude that the best experimental conditions for H_2O_2 determination by the blue color measurement (ABTSox) are obtained using low HRP and high ABTS concentrations. Fig. S8 shows the calibration line obtained in these conditions. Considering the stoichiometry of the H_2O_2/ABTS reaction, the maximum theoretical sensitivity for H_2O_2 determination using this method will be given by (averaged value):

$$\text{maximum sensitivity} = 2\,\varepsilon^{730}_{\text{ABTsox}} = 3.25 \times 10^4 M^{-1} cm^{-1} \quad (6)$$

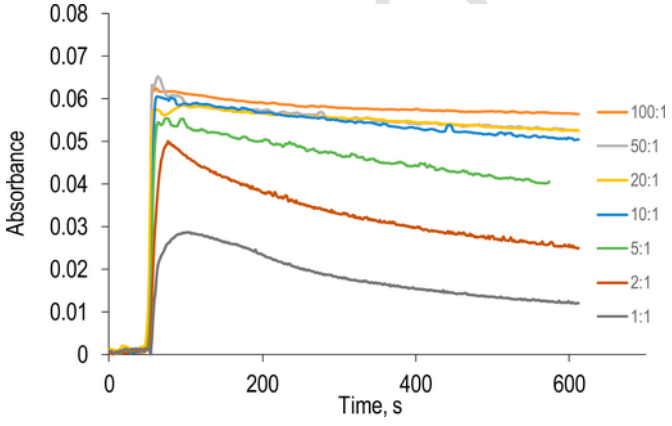

Fig. 2. ABTS concentration effect on the stabilization of the ABTSox signal. Experimental conditions: λ = 730 nm, [HRP] = 2 U/mL, [H_2O_2] = 2.2x*10^{-6} M, pH = 6.

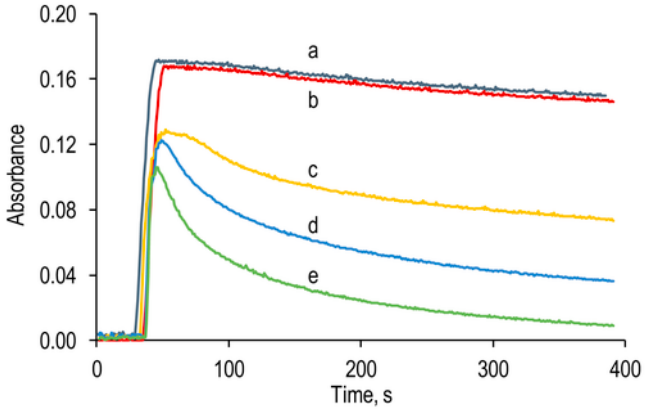

Fig. 3. HRP concentration effect on the ABTSox signal. Experimental conditions: λ = 730 nm, [ABTS] = 4.4*10^{-5} M, [H_2O_2] = 2.2*10^{-6} M, pH = 6. [HRP] concentrations used (in U/mL) were as follows: **a)** 0.8; **b)** 1.5; **c)** 5.0; **d)** 15; **e)** 30.

The sensitivity we obtained in the optimized conditions was statistically similar (*t*-test) to this value, indicating that the experimental conditions are optimized. Accordingly, the detection limit (DL) will be given by:

$$DL = \frac{3\, s_{Bl}^{730}}{2\, \epsilon_{ABTSox}^{730}} \quad (7)$$

being s_{Bl}^{730} the standard deviation of the blank values, which normally depends on the dominant noise in the spectrophotometer used. It is important to take this into account because in recent years several families of materials (nanomaterials in many cases) have appeared in the bibliography presenting so-called "peroxidase-like" activity and the quality of many of them has been tested using the H_2O_2/ABTS reaction. From equations (5) and (6) it can be deduced that these new materials are not able to improve the sensitivity and/or the DL of H_2O_2 determination using HRP.

Moreover, the effect of HRP previously shown (4) provides a different way to perform the H_2O_2 determination. If a very high HRP concentration is used, then the whole $ABTS_{ox}$ formed during the enzymatic reaction can be completely regenerated back to ABTS and a transient signal is obtained. This permits the same ABTS/HRP aliquot to be used for several H_2O_2 measurements. As an example, Fig. 4 shows the results obtained after the addition of H_2O_2 aliquots (90 μL) of the same concentration ($9.8*10^{-5}$ M). With the ABTS/HRP concentrations used in this proof of concept, up to 5 measurements can be carried out (after normalizing considering the different concentrations a RSD = 6.5% was obtained), but this can modified. These results also allow this reaction to be used as the basis for a reversible sensor.

3.3. Method based on the H_2O_2/HRP/ABTS reaction: Put and Cad determination

The very first experiments were carried out using the HRP and ABTS concentrations considered optimum in the previous section. In these conditions the Put and Cad Abs = f(t) obtained are shown in Fig. 5 A. As can be seen, both compounds give similar signals. The only difference was due to the final decrease of the absorbance. This decrease is higher than that expected just considering the comproportionation (2) and/or the reduction back by HRP (3). This has been studied in detail and we found that it was due to the later reaction of $ABTS_{ox}$ with the corresponding product (aldehyde) of the enzymatic reaction, which regenerates back the ABTS according to:

$$2\ ABTS_{OX} + AB_{al} \underset{k_{OX}}{\overset{k_{reg}}{\rightleftarrows}} AB_{acid} + ABTS \quad K = \frac{K_{reg}}{K_{OX}} \quad (8)$$

where BA_{al} and BA_{acid} represent the aldehyde and acid species of the substrate. Fig. 5A also indicates that the corresponding K is higher for Cad. This produces a different slope (sensitivity) of the calibration lines obtained for both analytes (Fig. S9), which is a drawback for the joint determination of both analytes. Moreover, the slopes of the calibration are lower than those maxima expected given by (6). According to (8) this problem could also be reduced by increasing the ABTS concentration (see Fig. 5B and also Fig. S10).

Fig. 6 also shows the ABTS absorbance profiles obtained after applying the reaction to the most common BA present in the samples. Again, the signals produced by Histamine and Tyramine are marked. In the HRP method, Tyramine interferes because it is able to regenerate HRPII, but in this method this interference is not observed. This is because the kinetic of the HRP regeneration by ABTS is faster than that by Tyramine, so the $ABTS_{ox}$ formation predominates over the interference.

At the beginning of the reaction the Histamine absorbance increases up to a maximum, which then decreases down to the initial absorbance (zero). Considering the previous results, we hypothesized that the product of the reaction with DAO/O_2 (imidazole acetaldehyde, His_{al}) behaves according to (7), the regeneration process being faster than the oxidation. Again, the addition of more ABTS provides a partial solution to this problem (Fig. 7), so Put and Cad can be determined in the presence of a 5/1 Histamine excessfig8.

The optimization studies for Put and Cad determination by this method were limited to the pH and the DAO concentration. Before the optimization, we examined whether DAO was able to regenerate $ABTS_{ox}$ in a similar way to HRP. The experimental results (Fig. S11) indicated that up to at least 6 U/mL, DAO does not affect the $ABTS_{ox}$ stability. After DAO optimization (Fig. S12), the analytical figures of merit for Put and Cad were obtained (Table 1, and Fig. S10). As can be seen, both analytes can be determined with the same sensitivity (test-t) which is also of the same order as that obtained for H_2O_2 (the maximum allowed (5)) indicating that in the experimental conditions used the whole Put and Cad is finally transformed into $ABTS_{ox}$.

In this case it is also possible to measure Put and Cad using high HRP concentrations in order to obtain $ABTS_{ox}$ transient signals similar to those described in the previous section for H_2O_2 (Fig. 4). The results are given in Fig. 8. These results indicate that the use of this methodology can be established as the basis for a partially reversible sensor for Put and Cad determination.

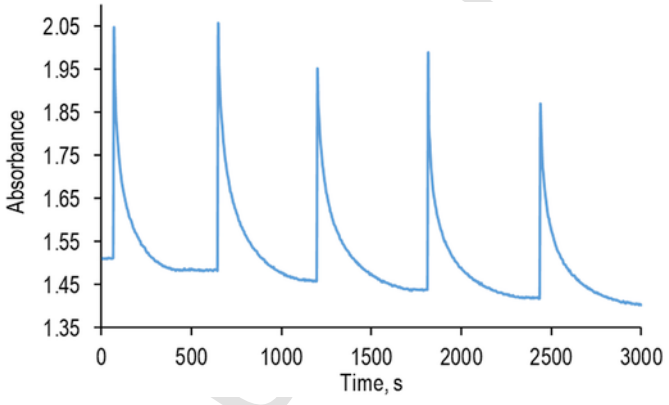

Fig. 4. Transient signal obtained using very high HRP concentration. Experimental conditions: λ = 730 nm, injection volume = 90 μL, [H_2O_2] = $9.8*10^{-5}$ M, [HRP] = 290U/mL, [ABTS] = $4.4*10^{-5}$ M, pH = 6.

3.4. Put + Cad determination in a real sample

Both methods, that based on DAO/HRP (section 3.1) and that based on DAO/HRP/ABTS (section 3.2), have advantages and drawbacks. The final selection will depend on the type of sample to be determined, i.e. the concentrations of the BA present in the type of sample. Much previous information about the relative content of different BAs and BPAs can be found in the bibliography, so it is possible to choose the most appropriate for each type of sample. In this study, we have collaborated with the LSPA (see the Material and methods section) who provided an extract of a tuna sample which they had analyzed using a previously validated HPLC-MS method [34], allowing the 6 BAs to be determined (Table S1). Considering the relative concentration of BAs, the DAO/HRP/ABTS method was applied, and the results were compared

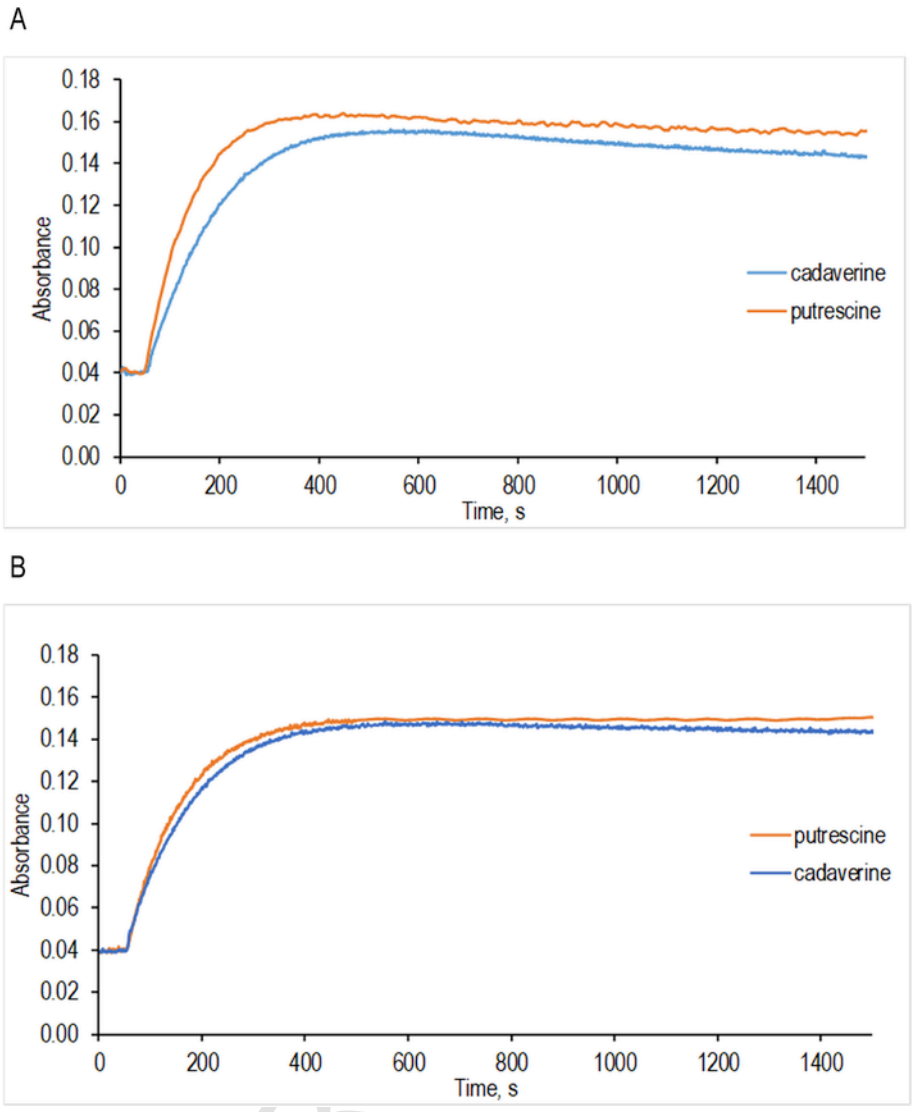

Fig. 5. Influence of ABTS concentration on the signal of Cad and Put. A) ABTS = $4.4*10^{-5}$ M B) ABTS = $4.4*10^{-4}$ M. Experimental conditions: λ = 420 nm, [Cad] = [Put] = $2.2*10^{-6}$ M, [HRP] = 2U/mL, [DAO] = 1.5 U/mL, pH = 6.

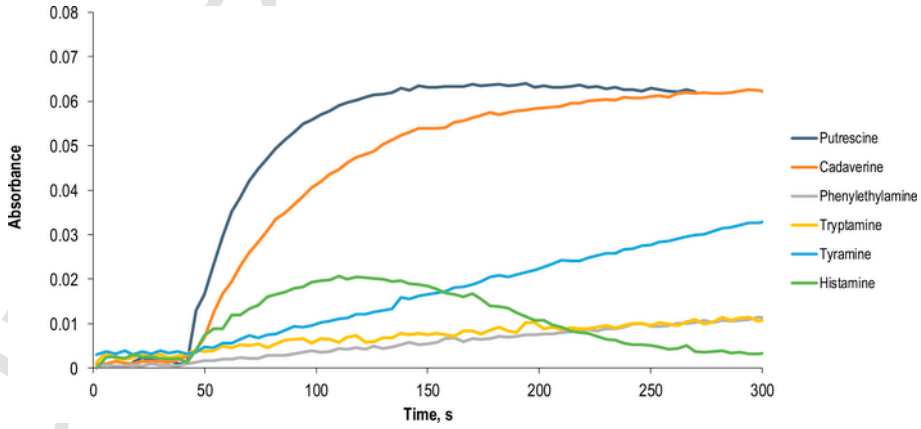

Fig. 6. Abs *versus* time recorders obtained with different BA. Experimental conditions: λ = 730 nm, [ABTS] = $4.4*10^{-5}$ M, [Cad] = [Put] = $2.4*10^{-6}$ M, [Histamine] = $2.4*10^{-5}$ M, [Phenylethylamine] = [Tyramine] = [Tryptamine] = $4.0*10^{-6}$ M [HRP] = 2 U/mL, [DAO] = 1.8 U/mL, pH = 6.

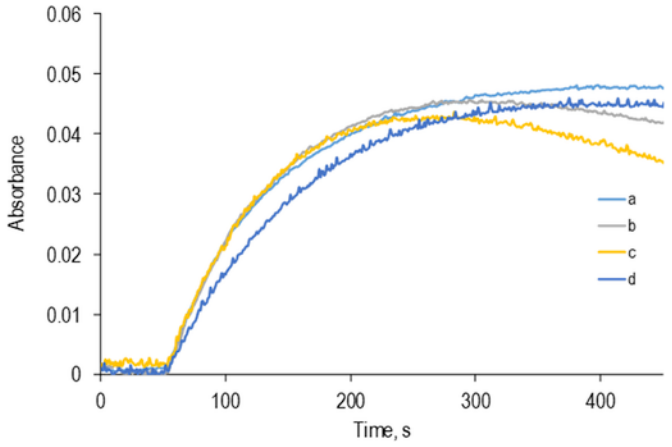

Fig. 7. ABTS effect on the Histamine effect on Put determination. Experimental conditions: [HRP] = 2 U/mL, [DAO] = 1.8 U/mL, [Put] = $2.0*10^{-6}$ M, pH = 6, λ = 730 nm [ABTS] = $4.5*10^{-5}$M **(except d).**a) no Histamine **b)** [Histamine] = $4.0*10^{-6}$M **c)** [Histamine] = $4.0*10^{-5}$M **d)** [Histamine] = $4.0*10^{-5}$ M and [ABTS] = $4.5*10^{-4}$M.

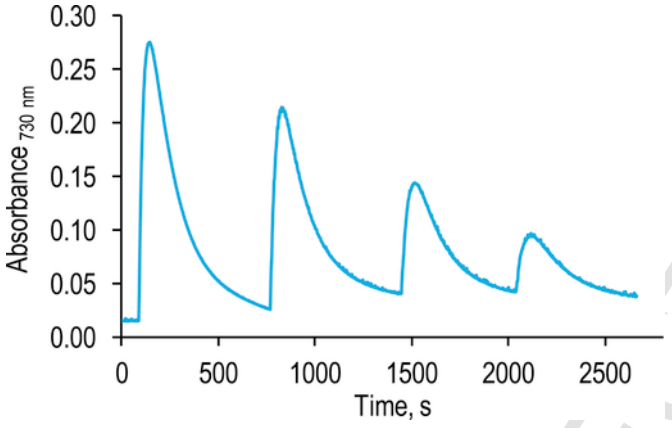

Fig. 8. Abs *versus* time transient signals (λ = 730 nm) obtained with different Put concentrations. Experimental conditions: 2 mL in the cell containing [ABTS] = $4.8*10^{-5}$ M, [HRP] = 190 U/mL and [DAO] = 2.0 U/mL, 20 μL aliquots of Put solutions are sequentially added giving the following concentrations: **a)** $3.0*10^{-5}$M; **b)** $1.5*10^{-5}$M; **c)** $7.4*10^{-6}$M; **d)** $3.7*10^{-6}$M.

(n = 3) with those obtained by the present method.

[Cad+Put], this method (n=3)	[Cad+Put], HPLC –MS method (n=3)
520 mg/Kg (±70 mg/Kg)	480 mg/Kg (±20 mg/Kg)

(

As can be seen, both results were statistically similar, according to a *t*-test (95% confidence $t_{cal} = 2.09 < t_{crit} = 2.78$) which validates this method.

Only 2 min are required for the measurement and the instrumentation is conventional and economical. The methodology therefore represents a good alternative to chromatographic methods for its speed and simplicity.

4. Conclusions

First, in this paper it has been demonstrated that the use of the intrinsic properties of enzymes is an alternative methodology which can compete in selectivity and simplicity with the most classical colorimetric methods. Second, this paper shows that the methodology based on the classical HRP/ABTS system works well when combined with DAO. However, several problems affecting the instability of the $ABTS_{ox}$ and AB_{al} species need to be previously solved, just by optimizing the ABTS concentration. Such problems could also appear when HRP/ABTS is combined with any other oxidase enzymes for the determination of other metabolites, so it is important to taken them into account. Finally, by increasing the HRP concentration, ABTSox transient signals are obtained, which allows this method to be used as the basis for reversible sensors for Put and Cad. The same idea can also be applied to other colorimetric methods based on the ABTS/HRP colorimetric reaction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120392.

References

[1] L. Persson, Polyamine homoeostasis, Essays Biochem. 46 (2009) 11–24 https://doi.org/10.1042/bse0460002.
[2] N. Seiler, R. Francis, Polyamines and apoptosis, J. Cell Mol. Med. 9 (2005) 623–642 https://doi.org/10.1111/j.1582-4934.2005.tb00493.x.
[3] M.H. Silla Santos, Biogenic amines: their importance in foods, Int. J. Food Microbiol. 29 (1996) 213–231 https://doi.org/10.1016/0168-1605(95)00032-1.
[4] N. Benkerroum, Biogenic amines in dairy products: origin, incidence and control means, Compr. Rev. Food Sci. Food Saf. 15 (2016) 801–826 https://doi.org/10.1111/1541-4337.12212.
[5] V. Ladero, M. Calles-Enríquez, M. Fernández, M.A. Álvarez, Toxicological effects of dietary biogenic amines, Curr. Nutr. Food Sci. 6 (2010) 145–156 https://doi.org/10.2174/157340110791233256.
[6] S.L. Taylor, R.R. Eitenmiller, Histamine food poisoning: toxicology and clinical aspects, CRC Crit. Rev. Toxicol. 17 (2) (1986) 91–128 https://doi.org/10.3109/10408448609023767.
[7] P. Visciano, M. Schirone, R. Tofalo, G. Suzzi, Biogenic amines in raw and processed seafood, Front. Microbiol. 3 (2012) 188 https://doi.org/10.3389/fmicb.2012.00188.
[8] G. Jairath, P.K. Singh, R.S. Dabur, M. Rani, M. Chaudhari, Biogenic amines in meat and meat products and its public health significance: a review, J. Food Sci. Technol. 52 (2015) 6835–6846 https://doi.org/10.1007/s13197-015-1860-x.
[9] A.R. Shalaby, Significance of biogenic amines to food safety and human health, Food Res. Int. 29 (7) (1996) 675–690 https://doi.org/10.1016/S0963-9969(96)00066-X.
[10] G.I. Mohammed, A.S. Bashammakh, A.A. Alsibaai, H. Alwael, M.S. El-Shahawi, A critical overview on the chemistry, clean-up and recent advances in analysis of biogenic amines in foodstuffs, TrAC Trends Anal. Chem. (Reference Ed.) 78 (2016) 84–94 https://doi.org/10.1016/j.trac.2016.02.007.
[11] F.B. Erim, Recent analytical approaches to the analysis of biogenic amines in food samples, TrAC Trends Anal. Chem. (Reference Ed.) 52 (2013) 239–247 https://doi.org/10.1016/j.trac.2013.05.018.
[12] J.L. Ordóñez, A.M. Troncoso, M.C. García-Parrilla, R.M. Callejón, Recent trends in the determination of biogenic amines in fermented beverages–A review, Anal. Chim. Acta 939 (2016) 10–25 https://doi.org/10.1016/j.aca.2016.07.045.
[13] S. Sentellas, O. Núñez, J. Saurina, Recent advances in the determination of biogenic amines in food samples by (U) HPLC, J. Agric. Food Chem. 64 (2016) 7667–7678 https://doi.org/10.1021/acs.jafc.6b02789.
[14] S. Tombelli, M. Mascini, Electrochemical biosensors for biogenic amines: a comparison between different approaches, Anal. Chim. Acta 358 (1998) 277–284 https://doi.org/10.1016/S0003-2670(97)00606-5.
[15] S. Leonardo, M. Campàs, Electrochemical enzyme sensor arrays for the detection of the biogenic amines histamine, putrescine and cadaverine using magnetic beads as immobilisation supports, Microchim. Acta (Online) 183 (6) (2016) 1881–1890, https://doi.org/10.1007/s00604-016-1821-8.
[16] X. Yang, B. Feng, X. He, F. Li, Y. Ding, J. Fei, Carbon nanomaterial based electrochemical sensors for biogenic amines, Microchim. Acta 180 (2013) 935–956 https://doi.org/10.1007/s00604-013-1015-6.
[17] M.B. Arnao, A. Cano, M. Acosta, The hydrophilic and lipophilic contribution to total antioxi-dant activity, Food Chem. 73 (2001) 239–244 https://doi.org/10.1016/S0308-8146(00)00324-1.

[18] J.I. Lee, J.H. Jang, M.J. Yu, Y.W. Kim, Construction of a bifunctional enzyme fusion for the combined determination of biogenic amines in foods, J. Agric. Food Chem. 61 (2013) 9118–9124 https://doi.org/10.1021/jf403044m.
[19] H. Usman, M.H. Abu Bakar, A.S. Hamzah, A.B. Salleh, A tapered fibre optics biosensor for histamine detection, Sens. Rev. 36 (2016) 40–47 https://doi.org/10.1108/SR-06-2015-0101.
[20] P.Q. Leng, F.L. Zhao, B.C. Yin, B.C. Ye, A novel, colorimetric method for biogenic amine detection based on arylalkylamine N-acetyltransferase, Chem. Commun. 51 (2015) 8712–8714 https://doi.org/10.1039/C5CC02370J.
[21] N. Kaur, S. Chopra, G. Singh, P. Raj, A. Bhasin, S.K. Sahoo, A. Kuwar, N. Singh, Chemosensors for biogenic amines and biothiols, J. Mater. Chem. B 6 (2018) 4872–4902 https://doi.org/10.1039/C8TB00732B.
[22] C. Schaude, C. Meindl, E. Fröhlich, J. Attard, G.J. Mohr, Developing a sensor layer for the optical detection of amines during food spoilage, Talanta 170 (2017) 481–487 https://doi.org/10.1016/j.talanta.2017.04.029.
[23] J.L. Pablos, S. Vallejos, A. Muñoz, M.J. Rojo, F. Serna, F.C. García, J.M. García, Solid polymer substrates and coated fibers containing 2,4,6-trinitrobenzene motifs as smart labels for the visual detection of biogenic amine vapors, Chem. Eur J. 21 (2015) 8733–8736 https://doi.org/10.1002/chem.201501365.
[24] N. Jornet-Martínez, M. González-Béjar, Y. Moliner-Martínez, P. Campins-Falcó, J. Pérez-Prieto, Sensitive and selective plasmonic assay for spermine as biomarker in human urine, Anal. Chem. 86 (2014) 1347–1351 https://doi.org/10.1021/ac404165j.
[25] Y. Chen, Y. Zhang, Y. Gao, J. Lee, H. Chen, Y. Yin, Visual determination of aliphatic diamines based on host–guest recognition of calix[4]arene derivatives capped gold nanoparticles, Biosens. Bioelectron. 72 (2015) 306–312 https://doi.org/10.1016/j.bios.2015.04.036.
[26] K.M.A. El-Nour, E.T.A. Salam, H.M. Soliman, A.S. Orabi, Gold nanoparticles as a direct and rapid sensor for sensitive analytical detection of biogenic amines, Nanoscale Res. Lett. 12 (1–11) (2017) 231 https://doi.org/10.1186/s11671-017-2014-z.
[27] K.A. Rawat, J.R. Bhamore, R.K. Sinhal, S.K. Kailasa, Microwave assisted synthesis of tyrosine protected gold nanoparticles for dual (colorimetric and fluorimetric) detection of spermine and spermidine in biological samples, Biosens. Bioelectron. 88 (2017) 71–77 https://doi.org/10.1016/j.bios.2016.07.069.
[28] J. Galbán, I. Sanz-Vicente, E. Ortega, M. del Barrio, S. de Marcos, Reagentless fluorescent biosensors based on proteins for continuous monitoring systems, Anal. Bioanal. Chem. 402 (10) (2012) 3039–3054 https://doi.org/10.1007/s00216-012-5715-2.
[29] D.G. Pina, A.V. Shnyrova, F. Gavilanes, A. Rodríguez, F. Leal, M.G. Roig, I.Y. Sakharov, G.G. Zhadan, E. Villar, V.I. Shnyrov, Thermally induced conformational changes in horseradish peroxidase, Eur. J. Biochem. 268 (2001) 120–126 https://doi.org/10.1046/j.1432-1033.2001.01855.x.
[30] V. Sanz, S. de Marcos, J. Galbán, Hydrogen peroxide and peracetic acid determination in waste water using a reversible reagentless biosensor, Anal. Chim. Acta 583 (2) (2007) 332–339 https://doi.org/10.1016/j.aca.2006.10.054.
[31] V. Sanz, S. de Marcos, J. Galbán, Uric acid determination using uricase and the autotransducer molecular absorption properties of peroxidase, Anal. Chim. Acta 607 (2008) 211–218 https://doi.org/10.1016/j.aca.2007.11.051.
[32] V. Sanz, S. de Marcos, J. Galbán, Direct glucose determination in blood using a reagentless optical biosensor, Biosens. Bioelectron. 22 (2007) 2876–2883 https://doi.org/10.1016/j.aca.2007.11.051.
[33] P. Pietrangeli, S. Nocera, R. Federico, B. Mondov, L. Morpungo, Inactivation of copper-containing amine oxidases by turnover products, Eur. J. Biochem. 271 (2004) 146–152 https://doi.org/10.1046/j.1432-1033.2003.03913.x.
[34] ITE-FQ080-02: determination of biogenic amines by HPLC-MS/MS. Public health laboratory, Government of Aragón (Spain). HYPERLINK https://gobierno.aragon.es/estaticos/GobiernoAragon/Departamentos/SanidadBienestarSocialFamilia/Sanidad/Profesionales/13_SaludPublica/Cartera_Servicios_Laboratorio_20190507.pdf https://gobierno.aragon.es/estaticos/GobiernoAragon/Departamentos/SanidadBienestarSocialFamilia/Sanidad/Profesionales/13_SaludPublica/Cartera_Servicios_Laboratorio_20190507.pdf https://gobierno.aragon.es/estaticos/GobiernoAragon/Departamentos/SanidadBienestarSocialFamilia/Sanidad/Profesionales/13_SaludPublica/Cartera_Servicios_Laboratorio_20190507.pdf.
[35] S. Hirota, T. Iwamoto, S. Kishishita, T. Okajima, O. Yamauchi, K. Tanizawa, Spectroscopic observation of intermediates formed during the oxidative half-reaction of copper/topa quinone-containing phenylethylamine oxidase, Biochemistry 40 (2001) 15789–15796 https://doi.org/10.1021/bi011631o.
[36] P. Pietrangeli, R. Federico, B. Mondoví, L. Morpurgo, Substrate specificity of copper-containing plant amine oxidases, J. Inorg. Biochem. 101 (2007) 997–1004 https://doi.org/10.1016/j.jinorgbio.2007.03.014.
[37] S.L. Scott, W. Chen, A. Bakac, J.H. Espenson, Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rates of the 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) radicals and ions, J. Phys. Chem. 97 (1993) 6710–6714 https://doi.org/10.1021/j100127a022.
[38] G. Gramss, Formation of the azodication ($ABTS^{2+}$) from ABTS [2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonate)] in sterile plant cultures: root–exuded oxidoreductases contribute to rhizosphere priming, Soil Syst. 2 (1–16) (2018) 26 https://doi.org/10.3390/soilsystems2020026.