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Direct minimally invasive enzymatic determination of tyramine in cheese using digital imaging

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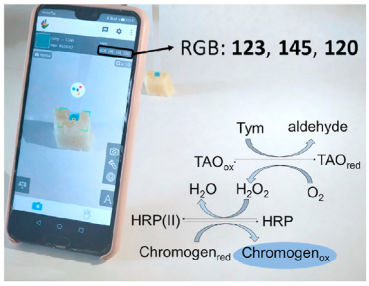
HIGHLIGHTS

- The combined absorbance of TMB at 450 and 650 increases the linear response range.
- Tyramine is minimally invasive determined in cheese by smartphone-RGB colorimetry.
- R_G gives the best analytical figures of merit, robustness and versatility.
- Tyramine test sticks have been obtained from those commercially-available for H_2O_2.
- A model allows to predict the analytical behavior of a dye different from TMB.

GRAPHICAL ABSTRACT



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ABSTRACT

An enzymatic method for the direct (without pretreatment) minimally invasive tyramine determination in cheese is proposed. Colorimetric test strips containing tyramine oxidase (TAO), peroxidase and 3,3′,5,5′-tetramethylbenzidine (Q-TAO), allow tyramine determination through the RGB chromatic coordinates of the observed blue colour (LOD = $2.6 \cdot 10^{-6}$ M, LOQ = $8.7 \cdot 10^{-6}$ M, RSD% (n = 5; $1.8 \cdot 10^{-4}$ M) = 3.2%). The strips are inserted in the sample for 2 min and then the RGB coordinates are measured using a smartphone. Previously, these Q-TAO strips have been also optimized for tyramine determination in cheese extract. To do that, a spectrophotometric method in solution for tyramine determination in cheese extracts has been developed, which included an in-depth study of the indicating reaction; this study has allowed to gain new information about the spectroscopic properties of different TMB species and, which it is more important, to detect cross-reactions between TAO and TMB species. A mathematical model has also been developed which relate the RGB signals obtained with the tyramine concentrations, the instrumental characteristics of the smartphone and the spectroscopic properties of the absorbing product of the enzymatic reaction.

1. Introduction

Biogenic amines (BAs) are low-molecular-weight nitrogenous compounds which are important nitrogen sources and precursors

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in the synthesis of hormones, nucleic acids or proteins [1]. Although these compounds occur naturally in the human body, they are also incorporated in an uncontrolled manner through diet [1–4]. A normal intake of BAs is metabolized by monoamine oxidase (MAO) and diamine oxidase (DAO) enzymes, but those patients who do not produce a sufficient amount of these enzymes, or whose activity is inhibited by the consumption of certain drugs, are liable to suffer intolerance or even poisoning [4,5].

Tyramine (Tym) is, together with histamine (His), cadaverine (Cad) and putrescine (Put), one the most frequent BAs occurring in food [4]. Tym acts as neurotransmitter [6], is vasoactive [2] and has cardiovascular and immunological effects [3,4], but at high concentrations it can trigger intolerance or intoxication processes whose clinical picture, known as the "cheese reaction", shows severe symptoms [1,4]. For these reasons, Tym is considered one of the most toxic and most dangerous for food safety according to the European Food Safety Association (EFSA) [7]; some countries have set limits for this compound in some products in their corresponding Codex Alimentarius. Tym presence in food is mainly due to the decarboxylation of free tyrosine carried out by tyrosine decarboxylase enzymes produced by certain microorganisms [4,8,9]. Therefore, products with free tyrosine and high microbial activity, such as fermented products (cheese, wine, beer, etc.), have significant Tym content [10]. Likewise, in non-fermented products, the BAs concentration increases as microbial activity increases in the course of food spoilage, so its content is used as an indicator of the quality and freshness of food products [11].

In the most commonly used methods for the determination of Tym and BAs, the first stage is the extraction and purification of the analytes (with acidic solvents such as hydrochloric or trichloroacetic acid [12]), after which they are separated and detected. The most widely used separation technique is high-performance liquid chromatography (HPLC), frequently coupled with ultraviolet or fluorescence detectors (BA derivatization is needed [13–15]), but tandem mass spectrometry (MS/MS) stands out since it eliminates the derivatization step and offers high sensitivity [16,17]. Capillary electrophoresis (CE) and thin layer chromatography (TLC) have also been proposed [18,19].

Although the aforementioned methods offer accurate and reliable results, the growing concern over food health for these and other amines [20], requires the development of faster analytical methods. Enzymatic biosensors [21], based on an amino oxidase, offer great advantages in this regard. For the specific determination of Tym, tyramine oxidase (TAO) has been successfully used [22,23]. Most of biosensors are based on electrochemical responses [22,24], Tym determination is made through the quantification of the H_2O_2 generated as a by-product in Tym oxidation (either by direct quantification or by the coupling of a second enzymatic reaction based on peroxidase -HRP-); limits of detection ranging from 0.3 to 2.4 mg/L. As for colorimetric biosensors —such as the one developed in this work (see Fig. 1), the HRP enzyme is also used together with a chromogen (such as 3,3′,5,5′-tetramethylbenzidine (TMB)) as its substrate. After reducing H_2O_2, peroxidase reverts to its initial form, oxidizing the chromogen, which goes from colourless to coloured. Colorimetric methods for the joint determination of putrescine, histamine and tyramine [23] have been proposed, and recently, our group have developed colorimetric analytical methods for the joint determination of putrescine and cadaverine both in batch and implemented in strip test [25,26]. However, to the best of our knowledge, this type of biosensor has not been proposed yet for Tym determination.

In this work, an enzymatic colorimetric analytical system for the specific, rapid and economic determination of Tym, as illustrated in Fig. 1, is developed. After an in-depth study of the indicating reaction, the method was first characterized in solution, and later implemented on test-strips (Q-TAO) prepared from commercially available H_2O_2-test strips. Both the solution and the Q-TAO methods were tested through Tym determination in cheese samples, comparing the results with each other, as well as with those obtained with a validated HPLC-MS/MS method. Later, the applicability of the method was improved by measuring the blue colour of the Q-TAO strips using a smartphone camera (RGB colour coordinates); to do that, a mathematical model which relates the RGB values (in the solid support) with Tym concentrations on the sample was deduced. Finally, the possibility of immediate and minimally invasive determination of Tym in cheese by simply placing the strip inside the sample was demonstrated. Thus, Tym determination through these strips only requires a smartphone and allows the elimination of sample treatment, which represents a time-consuming step in other existing sensors.

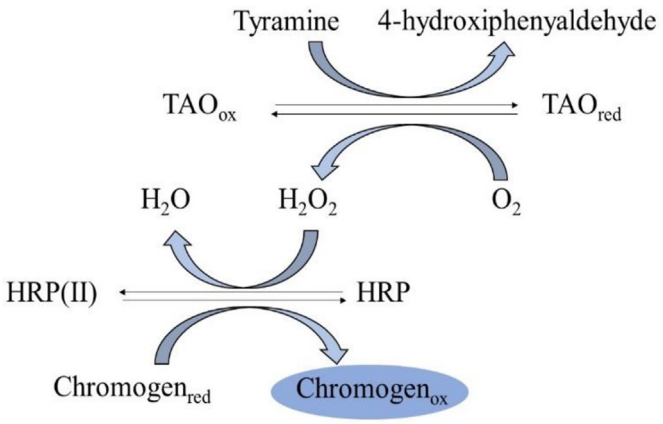

Fig. 1. Scheme of the enzymatic reaction system used in the development of the colorimetric biosensor for Tym determination.

2. Experimental

2.1. Instruments and apparatus

Molecular absorption measurements on solution were carried out in an Agilent 8453A diode array spectrophotometer, using 10 mm path length Hellma quartz cuvettes. Test strip RGB measurements were carried out with a Huawei P20 mobile phone camera and the application ColorGrab™ v. 3.6.1 (Loomatix ©). Thermo Scientific Multifuge X1R.

2.2. Reagents and solutions

Buffer solutions were prepared from Na_2HPO_4 (Panreac 131679.1211), Na_2CO_3 (Sigma EC 207-838-8) and $CH_3COONa \cdot 3H_2O$ (VWR Chemicals 27648.294). Hydrogen peroxide stock solution (33% w/v) was purchased from Panreac (131077.1211). Tyramine (Tym) (T2879), histamine (His) (53300), cadaverine (Cad) (C8561) and putrescine (Put) (P7505), as well as the dye 3,3′,5,5′-tetramethylbenzidine (TMB) (860336) were supplied by Sigma. Trichloroacetic acid was supplied by Scharlab (NS15390100).

The enzymes tyramine oxidase (TAO) (EC 1.4.3.9) from *arthrobacter sp.* (T-25) and Peroxidase (HRP) (EC 1.11.1.7) from *horseradish* (P8125) were purchased from Asahi Kasei Pharma and Sigma, respectively.

All solutions were prepared in the appropriate buffer, except TMB, which was dissolved in dimethylsulfoxide (DMSO).

2.3. Cheese sample treatment

Ten grams of cheese were subjected to leaching with 30 mL 5% trichloroacetic acid for 30 min. Then, the mixture was centrifugated (20 min, 4 °C, 5000 rpm), the solid phase was discarded and the supernatant solution was brought to neutral pH with NaOH. After that, a second centrifugation was done (20 min, 4 °C, 5000 rpm), and the supernatant was filtered through a 25 mm diameter nylon membrane filters (ALBET-NY-045-25-BL). Finally, the solution was brought to 50 mL with 0.1 M pH 6 phosphate buffer.

2.4. Test strips development (Q-TAO)

Quantofix™ Peroxid 25 test sticks (from Sigma-Aldrich) were used. To transform them into a tyramine sensor (so-called Q-TAO test sticks), 10 μL of 12.0 U·mL^{-1} TAO solution were added on the active zone of the stick and left to dry for 2 min before use.

2.5. Procedure for Tym determination

2.5.1. Cheese sample treatment

Cheese sample (2.5 g) were leached with 20 mL 5% trichloroacetic and shaken for 30 s (vortex). The mixture was ultracentrifuged (10 min, 4000 rpm, 4 °C) twice. The supernatant was taken to 50 mL with 5% trichloroacetic acid.

2.5.2. Measurements in solution

The diode-array spectrophotometer was always equipped with a yellow optical filter (which absorbs light down to 400 nm) to prevent the oxidized TMB becomes degraded by the UV-light. The absorbance at 450 nm (corresponding to yellow colour) and 650 nm (blue) were chosen for Tym determination.

In the measuring cuvette 20 μL of HRP (12 U mL^{-1}), 20 μL of TAO (50 U mL^{-1}) and 20 μL of TMB (0.01 M) are added over the corresponding buffer (generally phosphate pH = 6 0.1 M). After shaking the mixture, the kinetic register begins (with continuous stirring) and after baseline stabilization, the Tym standard or the sample solution (see 2.5.1) is spiked in the cuvette, making a total volume of 2 mL.

2.5.3. Test strips

Ten microliters of TAO solutions containing concentrations ranging from 0.6 to 48.3 U mL^{-1} were added to different test strips. After being dried (about 2 min), 10 μL of Tym was added to each of them. In this case, tyramine quantification is carried out by measuring RGB colour coordinates. The mobile phone is placed in a fixed position, held by a support and under constant illumination conditions. The analytical parameters used were R_r and R_G, defined as:

$$R_r = \frac{R_0 - R}{R_0} \quad (1a)$$

$$R_G = \frac{\frac{G}{R} - \left(\frac{G}{R}\right)_0}{\left(\frac{G}{R}\right)_0} \quad (1b)$$

R_0 and G_0 being the signals obtained in a white section of the strip.

2.6. Reference method (LSPA method)

Cheese samples were analysed by the Laboratorio de Salud Pública de Aragón (LSPA) using a previously validated method for fish samples [27]. Ten microliters of the corresponding sample solution (see 2.5.1) were analysed by HPLC-MS/MS.

3. Results and discussion

3.1. Analytical improvement of the TMB/H_2O_2 indicating reaction

3.1.1. TMB redox and pH forms

TMB is one of the most useful substrates for H_2O_2 determination using HRP, so its redox properties have been extensively studied. It is very well known that, at neutral pHs, colourless TMB (diamine form) suffers a one-electron oxidation to give TMB_{ox} (a free radical cation) and a second one-electron oxidation to give the yellow TMB_{ox2} (a diimine compound); moreover, TMB_{ox} is in equilibrium with a blue TMB:TMB_{ox2} charge transfer complex (having the same net oxidation state as TMB_{ox}) [28]. These TMB reactions are schematized in Fig. 2A. However, some aspects are still unclear, especially the pH effect on the stability of these redox forms and the molecular absorption spectra corresponding to the different species. To do that, solutions containing the adequate TMB:H_2O_2 stoichiometric relationship to obtain TMB:TMB_{ox2} (1:1) or TMB_{ox2} (1:2) and HRP were adjusted to different pHs from 2 to 11 and the corresponding molecular absorption spectra were recorder. As can be seen TMB present two different acid/base forms (pK value between 3 and 4) and TMB_{ox2} presents three different acid/base forms (with pK values of around 7 and 9 respectively). Moreover, TMB:TMB_{ox2} is stable at a pH lower than 8; this species presents a characteristic blue colour which is usually ascribed to a TMB:TMB_{ox2} charge transfer compound, however, according to the theoretical calculations TMB_{ox} is also able to give this type of spectra (Figure S1).

3.1.2. Optimisation

First, the influence of the TMB:H_2O_2 molar ratio on the redox product obtained was studied (Figure S2) by maintaining the peroxide concentration constant. With a TMB:H_2O_2 molar ratio equal to or smaller than 1, all the TMB is oxidized to TMB_{ox2}. If the concentration of TMB is increased, both oxidized forms coexist (TMB:TMB_{ox2} and the TMB_{ox2}), reaching constant values for a 1:10 M ratio. It is therefore concluded that an excess of TMB will be necessary to obtain the blue complex. The maximum possible TMB concentration ($1 \cdot 10^{-4}$ M, which is limited by its solubility in water), was used.

Second, the pH (Figure S3A) as well as the concentration of the enzyme (Figure S3B) were optimized. Despite obtaining the maximum absorbance at pH 5, pH 6 was chosen as the working pH since the optimum range for TAO, to be coupled later, is between 6 and 8 [29]. A 0.12 U·mL^{-1} HRP offers a good reaction rate (with a lower cost of enzyme).

3.1.3. Combined absorbances as the analytical parameter

Most of the analytical methods based on the H_2O_2/HRP/TMB system are designed to measure the blue colour of the TMB:TMB_{ox2} species. This methodology is very useful and sensitive, but the maximum H_2O_2 concentration which can be measured is limited (as seen in Figure S2). However, considering that the TMB_{ox2} is also coloured and its molar absorptivity is higher than that of TMB:TMB_{ox2}, it may be possible to enlarge the linear response range and the sensitivity of the method if the absorbances at both wavelengths (650 nm and 450 nm) are combined. Since the $\varepsilon_{complex}^{650\ nm} = 3.8 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ and $\varepsilon_{diimine}^{450\ nm} = 5.9 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, the combined absorbance (Abs_{comb}) was based on the ratio between these two values and responds to the formula:

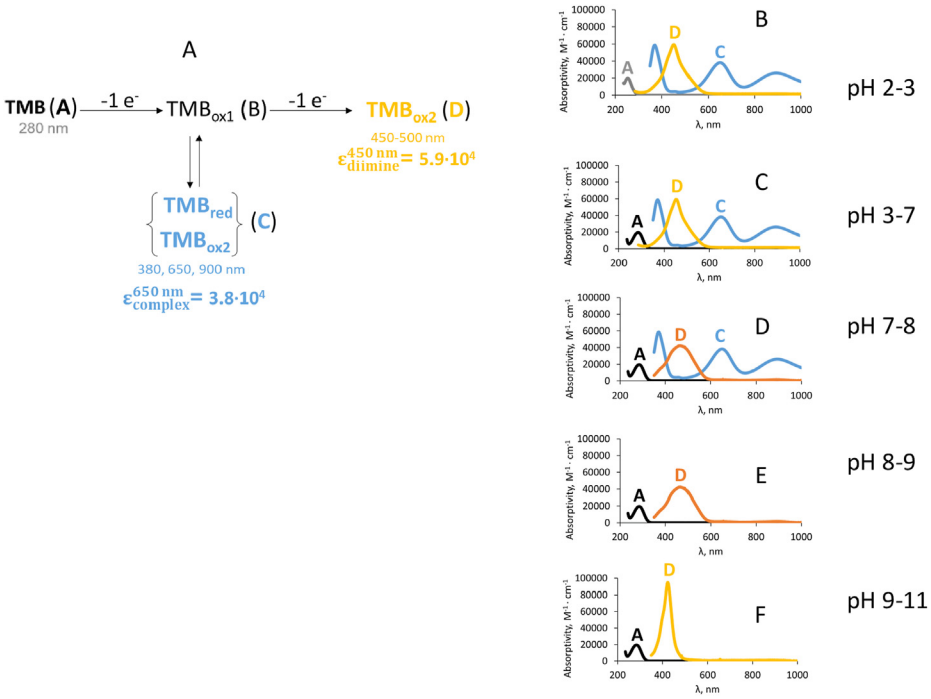

Fig. 2. Chemical and spectroscopic properties of TMB. **A)** Different oxidation states of TMB; **B)** Molecular absorption spectra of the different TMB redox forms depending on pH (conditions as indicated in section 3.1.1).

$$Abs_{comb} = 1.57 \cdot Abs_{650} + Abs_{450} \quad (2)$$

3.1.4. Analytical figures of merit for the determination of H_2O_2

Fig. 3 compares the H_2O_2 calibration lines obtained with Abs_{comb} and with absorbance at 650 nm (Abs_{650}), using the maximum TMB concentration ($1 \cdot 10^{-4}$ M). As expected, for a H_2O_2 concentration lower than $1 \cdot 10^{-5}$ M (corresponding to TMB:TMB$_{ox2}$ formation), the absorbance at 450 nm is nearly independent on the concentration, but for higher H_2O_2 concentrations, the absorbance at 650 nm begins to decrease while that at 450 nm increases. The slope of the 650 nm linear range fits with the previously given molar absorptivity indicating that all the H_2O_2 reacts with TMB. Abs_{comb} shows the linear response range in the whole H_2O_2 concentration range studied.

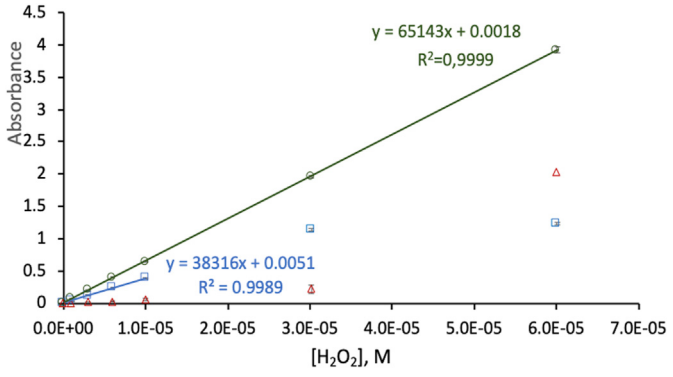

Fig. 3. H_2O_2 calibration study for the indicating reaction ([HRP] = 0.12 U mL^{-1}; [TMB] = $9.7 \cdot 10^{-5}$ M; pH 6).
□ Points and calibration line measuring at 650 nm (points that do not fit the linear range are also shown).
○ Points and calibration line obtained by combining absorbances at 450 nm and 650 nm according to (2).
△ Absorbances at 450 nm.

The analytical figures of merit of both methods (Abs_{650} and Abs_{com}) were compared. While both methods give similar precision (2.4% and 2.0% for the 650 nm and the combination method, respectively), Abs_{650} gives better limits of detection and quantification (these being $3.2 \cdot 10^{-8}$ M and $1.1 \cdot 10^{-7}$ M respectively, which are 2.3 times smaller than those of the Abs_{comb} method) and Abs_{comb} gives better sensitivity and a greater linear range; moreover, it has been statistically proven that this parameter offers a signal which is less dependent on the concentration of TMB (Figure S3 C-D).

3.2. Tyramine determination in solution

3.2.1. Optimisation of parameters

As indicated above, the determination of Tym is carried out by coupling its enzymatic oxidation (catalysed by TAO) to the indicating reaction. According to the scheme (Fig. 1), if a sufficiently high TAO concentration is added, the overall reaction will be dependent on the indicating reaction (i.e. 1 mol of Tym should give 1 mol of H_2O_2), and similar analytical figures of merit to that observed for H_2O_2 should be obtained. However, several important differences were found, which can be explained at the light of Fig. 4 and Fig. S4. Fig. 4 compares the variation of the absorbance *vs* time during Tym and H_2O_2 enzymatic reactions; the lower signal obtained for Tym indicates that the formation of H_2O_2 from Tym is not complete (probably an equilibrium is reached). From figure S4, it can be deduced that the reduced form of TAO (TAO$_{red}$) is able to react with TMB$_{ox2}$ and this yellow species cannot be formed when the oxidation and the indicating reaction are produced simultaneously. Tym quantification using equation (2), requires the two reactions (oxidation and indicating) to occur sequentially. As in test strips both reactions are carried out simultaneously, the

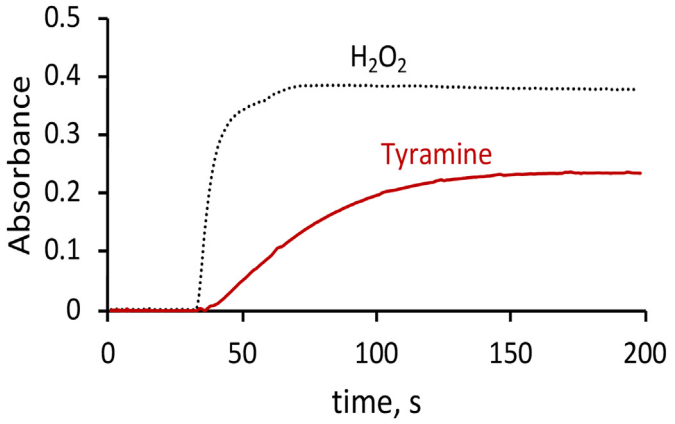

Fig. 4. Comparison of the Abs_{650} vs time representations obtained during the H_2O_2 (dashed line) and Tym (solid line) reactions. In both cases: [Analyte] = $1.0 \cdot 10^{-5}$ M, ([HRP] = 0.12 U mL^{-1}; [TMB] = $9.7 \cdot 10^{-5}$ M; pH 6.

determination of Tym was optimized based on the use of Abs_{650} as the analytical parameter.

Figure S5A shows the pH effect on the kinetic and yield of the enzymatic reaction. As can be seen, the slope of the decrease depends on the pH, demonstrating that this parameter not only affects the activity of the enzyme but also the reduction of the blue compound. The highest and the most stable signal was obtained at pH 6, so this was chosen as the optimum. The concentration of TAO was also optimized and 0.5 U mL^{-1} was chosen (Figure S5B) as the optimum since it offers an assumable reaction rate and enzyme cost.

3.2.2. Analytical figures of merit for Tym determination in solution

By measuring the absorbance at 650 nm the limits of detection and quantification of the developed method were found to be $6.9 \cdot 10^{-8}$ M and $1.9 \cdot 10^{-7}$ M respectively. These values correspond to 1.9 and 5.3 mg kg^{-1} in a cheese sample handled according to the procedure previously described (Section 2.3). The linear range goes up to $1.0 \cdot 10^{-5}$ M (274 mg kg^{-1}) with an average RSD% of 1.4% (n = 3) (Figure S5C). The sensitivity of the method, with a value of $2.3 \cdot 10^{5}$ M^{-1} cm^{-1} was, as expected (Fig. 4), 39% lower than that obtained for the HRP/TMB/H_2O_2 system. The analytical figures of merit of this method are better than those reported previously with other enzymatic biosensor for Tym, but worse than those found using immunoassays (table S1).

In order to test the potential interferences of His, Cad and Put, two different types of assays were performed. First, the three amines (in a concentration range from $1 \cdot 10^{-6}$ M to $1 \cdot 10^{-5}$ M of each BAs) were injected separately in the absence of the analyte; no signal of oxidized TMB was observed. Second, Tym was injected along with another BA (His, Cad or Put) in molar ratios 1:1, 1:2 and 1:5 of [Tym]:[BA]. No statistical differences were observed between these signals and those due to Tym alone.

When a 1:10 M ratio was assayed, small decreases in the signals (compared to that of the Tym) were observed, this effect being more critical in the case of His. This indicates that some interference is observed at this concentration level. Nevertheless, when Tym is present in cheese samples, the other BAs are usually at the same concentration levels as Tym (or lower) so no interferences are expected in this type of sample [13].

3.2.3. Tym determination in cheese

First, the reliability of the sample treatment proposed in section 2.3 was validated against the LSPA method. A cheese sample (Cheese 1) was subjected to both sample treatments and corresponding extracts were analysed by the LSPA method (reference). No significant differences between them were found, indicating that this sample treatment quantitatively extracts Tym from cheese.

Second, a blank sample was found. Six replicates of a white Gouda cheese were analysed in parallel by the LSPA method (Section 2.6) and the proposed TAO/HRP/TMB method. Concentrations lower than the corresponding quantification limits were obtained by both methods, so this sample was considered as a blank sample throughout. A recovery study was done by adding standard tyramine to a known weight of this sample. It was left overnight, obtaining a recovery of 106 ± 2% (n = 6).

Third, matrix interferences test on the Cheese 1 were carried out. These were discarded by statistically (P = 0.05) comparing the slope of a standard addition and a calibration line. Moreover, the Put, Cad, His and Tym concentrations found in this sample using the reference method (down 2 mg Kg^{-1} for Put and His, 15 mg Kg^{-1} Cad and 86 mg Kg^{-1} Tym) indicated that Biogenic Amines/Tym ratios were smaller than the corresponding interference limits.

Finally, the Cheese 1 sample was analysed in sextuplicate using both methods. The concentration obtained by our colorimetric method was 83.6 ± 3.1 mg kg^{-1} (n = 6), which was statistically similar to 86 ± 11 mg kg^{-1} (n = 3), obtained with the LSPA method. These results allow us to conclude that the new method is valid for the Tym determination in cheese and can be used for subsequent validation.

3.3. Tyramine determination using Q-TAO test strips and RGB coordinates

3.3.1. Theoretical background

In a previous paper [26] we proposed a mathematical model for quantitative determinations based on RGB colour coordinates reflectance measurements in solid media. This model was derived from the Kubelka-Munk theory and relates the brightness of any colour coordinates with the absorbing species concentration (c, in M) by a second degree polynomial function. The model was applied for R coordinate measurements. Moreover, the use of the R_r (3) minimizes the effect of the type of camera in the signal, R_0 y R being the value of the coordinate before and after the analyte addition respectively.

$$R_r = \frac{R_0 - R}{R_0} \tag{3}$$

Further studies, which are detailed in the ESM (Section S1), are now presented. First, we have simplified the terms of the polynomial function so:

$$R_r = -V\overline{\varepsilon_R}\, c + W\overline{\varepsilon_R}^2 c^2 \tag{4}$$

V and W being parameters which only depends on the average scattering coefficient ($\bar{s}$) and the optical pathlength (L) of the solid support, and ($\overline{\varepsilon_R}$) being the weighted average molar absorptivity of the chromophore into the R wavelength interval. Second, in order to try to improve the analytical quality of the method using an internal reference measurement (G coordinate), the R_G parameter is proposed:

$$R_G = \frac{\left(\frac{G}{R}\right) - \left(\frac{G}{R}\right)_0}{\left(\frac{G}{R}\right)_0} = V\left(\bar{\varepsilon}_R - \bar{\varepsilon}_G\right) c - W\left(\bar{\varepsilon}_R^2 - \bar{\varepsilon}_G^2\right) c^2 \tag{5}$$

($\overline{\varepsilon_G}$) being the weighted average molar absorptivity of the

chromophore into the G wavelength interval and $(G/R)_0$ is measured at the same time than (G/R) in a white section of the strip. Note that when the s and L values are previously obtained for a solid support, it is possible to predict the calibration line (4) or (5) for a given absorbing species (i.e., a dye) just calculating its $\overline{\varepsilon_R}$ from its spectra in solution.

In this paper, the analytical performances of both parameters will be also evaluated.

3.3.2. Optimisation and characterization of Q-TAO test strips

Q-TAO strips were prepared following the procedure described in section 2.4. Since the HRP and colorant (TMB) concentrations are fixed, the only parameter to be optimized was the TAO concentration. The evolution of the R_r coordinate over time for different TAO concentrations are shown in Fig. 5; R_G gives similar results. In the light of these results and balancing sensitivity and cost, 12 U mL^{-1} was finally chosen (i.e., 0.12 total U TAO per strip). Stabilization of R_r is achieved after 7 min.

Moreover, the stability of the strips was studied. To do this, identical strips were prepared containing the optimum concentration of TAO found, and 20 μL Tyr ($1.8 \cdot 10^{-4}$ M) were added at different times since the addition of the enzyme. As can be seen in Figure S6A, the strips maintain their maximum activity until 15 min after TAO addition.

These strips were analytically characterized for Tym determination. Figure S6B shows the resulting coloured strips together with the colours observed by the mobile application. R_r and R_G were the parameters used (Fig. 6). As can be seen both parameters follow a second-degree polynomial equation, R_G giving a higher response range (up to $7.3 \cdot 10^{-4}$ M, 500 mg kg^{-1} in cheese); moreover, for a shorter concentrations range a linear fit is obeyed. However, the limits of detection and quantification (obtained from the standard deviation of 5 blank measurements) are better for R_r ($2.6 \cdot 10^{-6}$ M and $8.7 \cdot 10^{-6}$ M, respectively, compared with $7.1 \cdot 10^{-6}$ M and $2.3 \cdot 10^{-5}$ M for R_G). The RSD% obtained with a $1.8 \cdot 10^{-4}$ M tyramine solution (n = 5) were 3.2% (R_r) and 7.7% (R_G).

3.3.3. Application to real samples with cheese extracts

These Q-TAO were used for Tym determination in cheese. Five different cheeses were purchased from a local grocery and were submitted to the sample treatment described in section 2.3. After that, 10 μL of each sample solution was added to different Q-TAO; moreover, Tym was simultaneously determined by the previously established procedure (section 3.2). R_r gave a better adjust than R_G (see Figure S6 C-D), so that parameter was finally chosen for Tym quantification in cheese. Fig. 7A compares the results obtained by both methods and table S2 gives the detailed concentration values. As can be seen, Q-TAO gives smaller concentration values than the method in solution; however, the differences are proportional (the slope of the regression line is about 0.4) indicating both that the matrix effect exists but this does not depend on the type of cheese. The RSD values (table S2) obtained with Q-TAO are higher than that obtained with the method in solution, but they are below 7%.

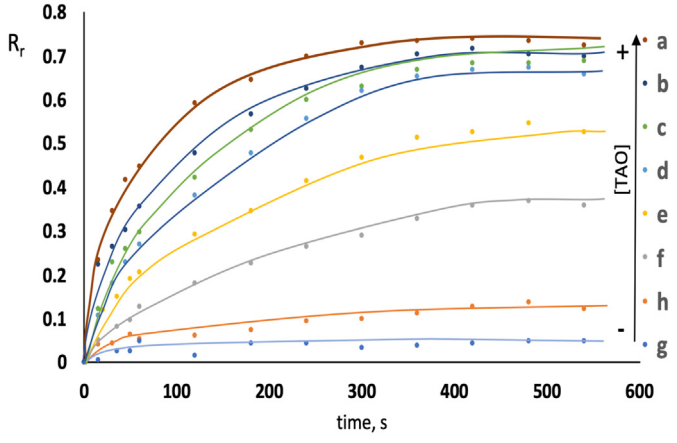

Fig. 5. R_r values obtained using Q-TAO containing different TAO units (pH = 6). **a)** 0.48; **b)** 0.24; **c)** 0.12; **d)** 0.088; **e)** 0.060; **f)** 0.030; **g)** 0.010; **h)** 0.006.

3.4. Minimally invasive direct determination of tyramine in cheese

One of the most interesting possibilities that Q-TAO offers is minimally invasive direct tyramine determination, without sample treatment, by inserting the strip in the cheese sample (Fig. 8). Tym diffuses from the cheese matrix to the strip and reacts with the TAO/HRP/TMB system.

After inserting the Q-TAO strip into the cheese, about 2 min are necessary for the signal to reach a plateau (see Video S1). To test this, Q-TAO were inserted in five cheese portions and extracted at different times; the R_r value, as can be seen in Figure S7A, is stabilized after only 2 min. Moreover, the influence of humidity on Tym diffusion was studied but no significant differences were observed.

The five cheese samples were analysed by this method (Fig. 8). The R_r signals obtained were plotted versus the concentrations obtained in solution (Fig. 7B). As can be seen, there is a second-degree polynomial relationship between Rr and the Tym concentration in cheese as occurs in solution; despite the concentration found in the Gouda sample is down the detection limit (see uncertainty in table S2), it has been retained. The intercept is statistically equal to zero, so no additive interferences are present. The slope of the line shows the relationship between the signal and the Tym concentration in mg·kg^{-1} and the ability of the method for this determination. In addition, the repeatability of this minimally invasive method was evaluated by determining five replicas of each cheese. With the exception of the blue cheese, acceptable values were obtained, which go from 3.8% for Cured 2−16% for Cured 1; to explain these repeatability values it is important to take into account that the tyramine concentration in cheese could not be homogeneously distributed. These values are reflected visually in Figure S7B, as well as graphically in the error bars included in Fig. 7B.

4. Conclusions

An initial general conclusion for analytical methods involving the TMB/H_2O_2 indicating reaction can be derived: the linear response range can be enlarged when the absorbance for TMB:TMB_{ox2} and TMB_{ox2} species are combined, instead of using the absorbance of one of these compounds.

The Q-TAO strips developed in combination with RGB coordinates measurements constitute a rapid, low-cost, instrument-free and qualified personnel non-dependent method compared with the traditional instrumental methods, on which food quality control is still dependent. The direct introduction of the strips into the food is a major advance, as it eliminates the need for treatment of the sample. Although further studies should be carried out on how tyramine diffuses from the cheese to the strips, the results obtained are promising.

CRediT authorship contribution statement

Sofía Oliver: Formal analysis, Investigation, Writing − original draft, Visualization. **Susana de Marcos:** Formal analysis, Resources, Data curation, Visualization. **Isabel Sanz-Vicente:** Methodology, Investigation, Supervision. **Vicente Cebolla:** Validation, Resources,

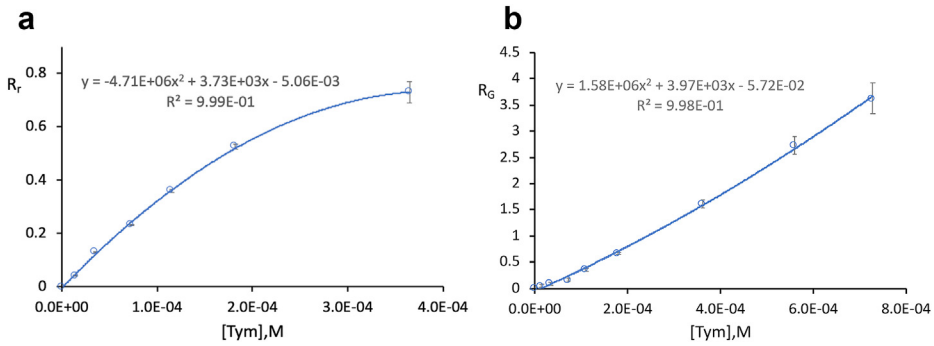

Fig. 6. Tym second order calibration lines obtained using Q-TAO (0.12 units TAO, pH = 6) and the proposed parameters:
A) R_r; **B)** R_G.

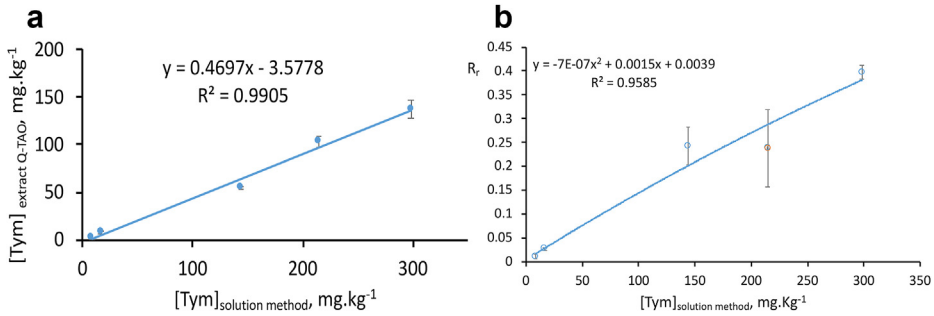

Fig. 7. Tym determination in cheeses samples.
A) Results obtained using R_r compared with those obtained using the method in solution.
B) Correlation found between the R_r values obtained after direct Q-TAO insertion in cheeses and Tym concentration measured using the method in solution.

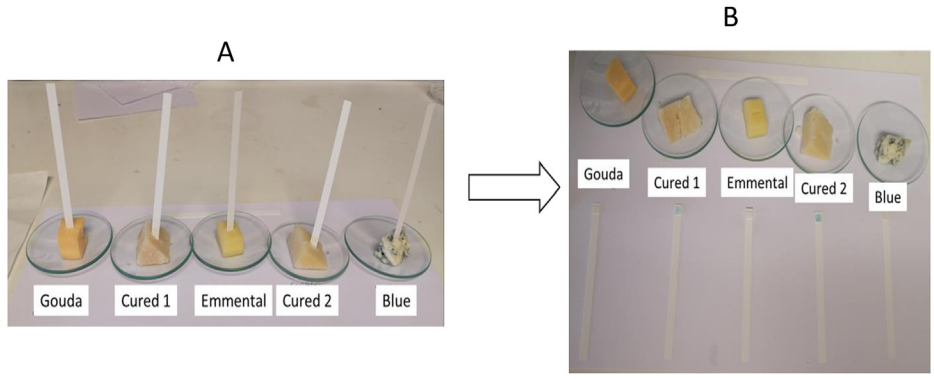

Fig. 8. Minimally invasive Tym determination in cheeses using Q-TAO.
A) The strips are inserted in the five cheeses tested (t = 0).
B) After 2 min, the strips are removed from the samples and their blue colour is proportional to the Tym concentration on cheeses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Data curation. **Javier Galbán:** Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338489.

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