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Spectrophotometric FIA methods for determination of hydrogen peroxide: Application to evaluation of scavenging capacity

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ABSTRACT

The determination of hydrogen peroxide (H_2O_2) and the evaluation of scavenging capacity against this species were performed using five colorimetric reactions, which were adapted to flow injection analysis. The reactions chosen were based on the oxidation of iodide (I^- method), on the formation of titanium-peroxide complex (TiP method), on the formation of titanium-xylenol orange-peroxide complex (TiXoP method), on the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB method) and on the co-oxidation of phenol-4-sulfonic acid and 4-aminoantipyrine (PSA/4-AAP method). The operational conditions were studied in order to improve the sensitivity of each method. Concerning to the method sensitivity, the ranking order was TMB method > I^- method > TiXoP method ~PSA/4-AAP method > TiP method. All methods showed an excellent repeatability (RSD < 2%) and, except for I^- method, relative deviations from the reference method were <1.9%. The FIA manifolds were adapted to perform the determination of scavenging capacity against H_2O_2 and glutathione (GSH) was applied as model compound. TiP and TiXoP methods were not suitable as no inhibition or an increase of analytical signal was attained. PSA/4-AAP method was chosen for further application to dietary phenolics and pharmaceutical compounds, providing IC_{50} values for those compounds that are fast reacting antioxidants.

1. Introduction

Hydrogen peroxide (H_2O_2) has been used as an industrial product, presenting a wide range of applications. Owing to its oxidant power, H_2O_2 is utilized in numerous industrial processes mainly in the food, textile, cosmetic, pharmaceutical and military industries [1]. It can be also applied in effluent treatment, where it acts as a sterilizing, cleaning and oxidizing agent [2]. In addition, H_2O_2 plays an important role in biological systems [3], belonging to a group of molecules designated as reactive oxygen species (ROS). Despite its low reactivity concerning direct oxidation of DNA and lipids, H_2O_2 is capable of inactivating important cellular enzymes by oxidation of catalytic –SH groups [4]. Furthermore, H_2O_2 is an *in vivo* precursor of hydroxyl radical and it can also activate the production of superoxide anion radical. As both radicals can cause even more damage in cellular macromolecules, scavenging activity against H_2O_2 may be an important feature on treatment of pathologies where ROS generation takes place, such as inflammatory processes. Therefore, the assessment of scavenging properties against H_2O_2 is relevant, especially concerning characterization of drug effects and screening of pharmaceutical properties in plant extracts.

The determination of H_2O_2 can be performed using volumetric methods or procedures based on spectrophotometric, fluorimetric, electrochemical or chemiluminescence (CL) detection [1]. Automated methods have also been described, based on flow injection analysis (FIA). About 80 different manifolds have been proposed until now and about half of them are based on CL detection. These CL systems were applied to the determination of H_2O_2 in exhaled breath [5] and cigarette smoke condensate [6], rainwater [7–10], seawater [11,12], beer [13] and also to the determination of scavenging capacity against this species [14–17]. Nevertheless, for this particular application, CL detection is not convenient for two reasons. First, it is not possible to distinguish between scavenging of H_2O_2 and scavenging of other ROS (superoxide anion, hydroxyl radical) that may also be present in the reaction media [18]. Secondly, for CL reactions using luminol, the results may be biased due to antioxidant depletion by luminol-derived radicals [19]. Spectrophotometric FIA methods have also been proposed accounting for about 16% of manifolds described. Among the reactions employed, the formation of titanium(IV)-peroxo complexes were the most common [20–22]. Nevertheless, a systematic comparison between spectrophotometric methods has not been performed so far nor their application to the assessment of scavenging capacity against H_2O_2.

Therefore, the purpose of the present work is a comparative study of different spectrophotometric reactions for H_2O_2 determination, which were adapted to FIA including the oxidation of iodide

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(I^-) to iodine [23], the formation of $[TiO_2]^{2+}$ complex [24], the formation of [Ti-xylenol orange-O_2]$^{2+}$ complex [25], the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) [26,27] and the co-oxidation of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) [28]. The operational conditions will be studied in order to improve the sensitivity of each method and the respective figures of merit will be determined, allowing the comparison of the developed procedures. The applicability of these FIA methodologies will be assessed concerning not only real samples containing H_2O_2 (contact lens disinfecting liquids) but also the determination of scavenging capacity against this species.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade with no further purification. Hydrogen peroxide 30% (v/v), sodium thiosulfate, sodium hydroxide and phenol-4-sulfonic acid sodium salt were purchased from Fluka (Buchs, Switzerland). Hydrochloride acid, sulfuric acid, starch, sodium carbonate, sodium hydrogencarbonate, potassium dichromate, ammonium heptamolybdate (Mo(VI)), sodium acetate, calcium chloride, potassium permanganate (standard solution 0.02 M) were obtained from Merck (Darmstadt, Germany). Titanium(IV) oxysulfate solution, potassium phosphate monobasic and trizma® base were purchased from Riedel-de-Haën (Seelze, Germany). Xylenol orange (XO) tetrasodium salt, 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate (TMB) and L-glutathione reduced (GSH) were obtained from Aldrich (Milwaukee, WI, USA). Horseradish peroxidase (EC. 1.11.1.7, Type I, 442.5 mg/solid, 113 purpurogallin U/mg solid, 1.2 RZ) (HRP), 4-aminoantipyrine (4-AAP), sulindac, ketorolac Tris salt, atenolol, labetolol hydrochloride, timolol maleate, etodolac, trolox, meloxicam, gallic acid, caffeic acid, catalase (EC. 1.11.1.6, from bovine liver, 3390 U/mg solid, 3940 U/mg protein) and superoxide dismutase (EC. 1.15.1.1, from bovine erythrocytes, 4470 units/mg solid, 4470 units/mg protein) (SOD) were purchased from Sigma (St. Louis, MO, USA). Sodium chloride was obtained from Fisher Scientific (Waltham, MA, USA) and potassium iodide was purchased from Pronalab (Lisbon, Portugal).

2.2. Solutions

Water used in the preparation of the solutions and buffers was obtained from a Milli-Q system (resistivity > 18 MΩ cm). Ethanol absolute pro analysis was also used.

The stock solution of H_2O_2 (~0.1 M) was prepared by diluting 1 mL of the commercial H_2O_2 to 100 mL with water. The standardization of this solution was performed daily by an iodimetric titration procedure using standard sodium thiosulfate solution, and working standard solutions were prepared by appropriate dilution in water.

The solutions of KI, TMB and PSA/4-AAP were prepared by weighing and dissolving the respective compound in water. The solutions of Ti(IV)OSO_4 were obtained by dilution of the commercial solution with water and addition of 4.5 mL of 1 M H_2SO_4 (final pH 1.3). The solutions of Mo(VI) were prepared by weighing and dissolving the respective compound in 0.75 M H_2SO_4.

The stock solution of XO (2 mM) was prepared by weighing 152.1 mg, dissolving it in ethanol (6 mL), and completing the volume up to 100 mL with water, while working solutions were prepared by dilution in water.

Phosphate buffer (0.1 M, pH 5.0, 6.0 and 7.0) and acetate buffer (0.2 M, pH 4.0 and 5.0) solutions were used in the preparation of HRP solutions. The pH was adjusted with 1 M NaOH and 1 M HCl, respectively.

Tris buffer (50 mM, 750 mM sodium chloride, 5 mM calcium chloride, pH 7.4), was also prepared and the pH was adjusted with 1 M HCl. This buffer was employed in the preparation of GSH, sulindac, ketorolac, atenolol, timolol, gallic acid, catalase and SOD solutions and also used as carrier in the scavenging studies. Labetolol, caffeic acid, trolox and meloxicam were dissolved in ethanol solution 50% (v/v) and the working solutions were prepared in Tris buffer.

2.3. Apparatus

The FIA manifold comprised a Gilson (Villiers-le-Bel, France) Minipuls 3 peristaltic pump with four channels, a Rheodyne 5020 (Luton, Bedfordshire, UK) six-port rotary injection valve equipped with a 100 or 50 μL loop, a mixing coil (MC), a reaction coil (RC) submersed in a thermostatic bath at 25 °C (I.S. Co GTR 190, Milan, Italy), a Jenway 6300 (Essex, UK) UV–vis spectrophotometer equipped with a model 178.710 QS Hellma (Mullheim/Baden, Germany) flow-through cell (internal volume = 80 μL, optical path = 10 mm) and also a Kipp & Zonen (Delft, Netherlands) strip chart recorder. Omnifit (Cambridge, UK) PTFE tubing (0.80 mm i.d.) was used for manifold construction. Connectors and Y-joints were also used as confluence points.

2.4. Flow injection conditions

The influence in the analytical performance of the order in which reagents were added was evaluated through the application of three manifold configurations, which are shown schematically in Fig. 1. The flow rates of carrier (H_2O) and reagent solutions were fixed at 1.0 and 0.5 mL min^{-1}, respectively. In the configuration 1(a) the reagents were pre-mixed and then added to the sample plug. In the configuration 1(b), the sample was first mixed with one reagent solution (R_1), followed by the addition of the second reagent solu-

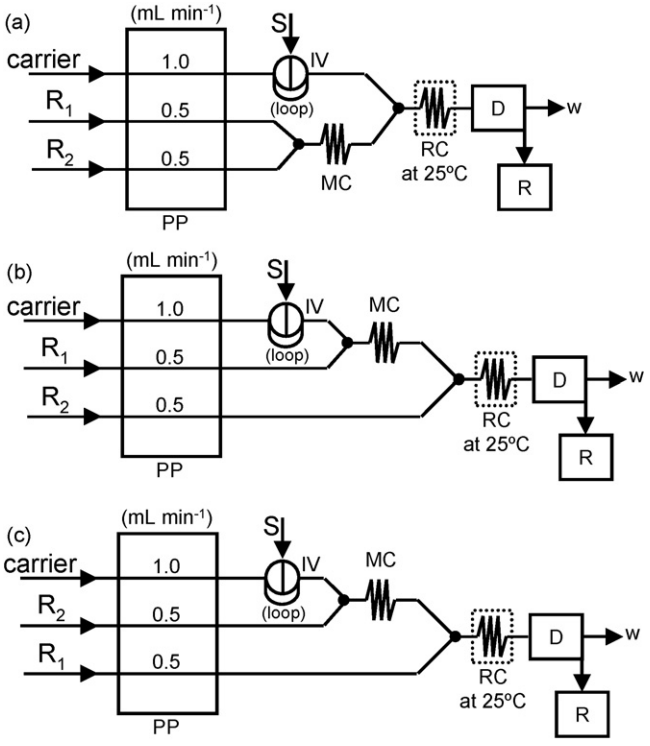

Fig. 1. FIA manifold configurations. R_1 and R_2, reagents; S, H_2O_2 standard solution or sample; PP, peristaltic pump; IV, injection valve; MC, mixing coil; RC, reaction coil; D, detector (spectrophotometer); R, recorder; w, waste.

tion (R_2). In the configuration 1(c), R_2 was added to the sample prior to R_1. For this study, the carrier was replaced by H_2O_2 solutions (continuous flow).

2.5. Analysis of samples

2.5.1. Disinfection products

Contact lens disinfecting liquids, containing H_2O_2 3% (~1.0 M), were purchased at local pharmacies. Prior to analysis by FIA system they were diluted with water. The reference method [29] consisted of a permanganometric titration: 50 mL of the diluted sample was added to 100 mL of 1.00 M H_2SO_4 solution and, then, H_2O_2 was titrated with 0.02 M potassium permanganate solution until a pink color was obtained. Samples were previously diluted 1:100 (v/v) with water. All the experiments were performed in triplicate.

2.5.2. Determination of scavenging capacity against H_2O_2

In this case, the FIA manifold was reconfigured by addition of a fourth channel as depicted in Fig. 2. One of the stream lines corresponded to the carrier. Another channel was filled by H_2O_2 solution and the last two channels contained the reagents involved in the respective colorimetric reaction. An additional coil (ScC, 50 cm) was inserted, the loop was reduced from 100 to 50 μL and the flow rates were fixed at 0.5 mL min^{-1}. Therefore, the solution containing the putative antioxidant compound was injected in the carrier stream and further mixed with H_2O_2 solution in the ScC coil. Afterwards, the remaining H_2O_2 was determined after reaction with R_1 and R_2.

Control experiments were carried out to evaluate interference from intrinsic absorption of sample compounds and also from direct reaction between sample compounds and $R_1 + R_2$. In the first case, H_2O_2 was replaced by water and both R_1 and R_2 were replaced by the respective solvents. In the second case, only H_2O_2 was replaced by water.

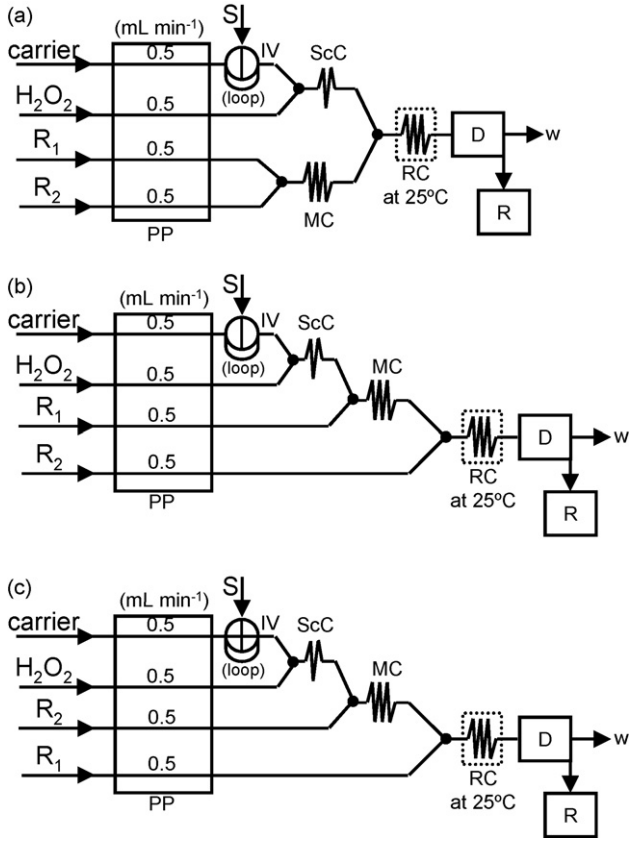

Fig. 2. FIA manifold configurations applied in scavenging studies. R_1 and R_2, reagents; S, antioxidant solution; PP, peristaltic pump; IV, injection valve; ScC, scavenging coil; MC, mixing coil; RC, reaction coil; D, detector (spectrophotometer); R, recorder; w, waste.

Table 1
Range of values used in the study of system variables and the final chosen operating conditions[a].

Method	Parameter	Range	Chosen value
I^-	Manifold configuration	a,b	a
	Length of RC (cm)	50–200	200
	KI concentration (M)	0.06–1.80	0.60
	H_2SO_4 concentration (M)	0.10–2.00	0.75
	Mo(VI) concentration (μM)	0–1000	0
TiP[b]	Manifold configuration	a,b	a
	Length of RC (cm)	50–200	50
	Ti(IV)OSO_4 concentration (mM)	6.25–125	25.0
TiXoP	Manifold configuration	a,b,c	b
	Length of MC (cm)	50 and 150	50
	Length of RC (cm)	50–200	150
	Ti(IV)OSO_4 concentration (μM)	320–960	640
	XO concentration (μM)	320–960	640
TMB	Manifold configuration	a,b,c	c
	Length of RC (cm)	50–200	50
	HRP concentration (U mL^{-1})	0–1000	1
	pH phosphate buffer 0.10 M	5.0–7.0	–
	pH acetate buffer 0.20 M	4.0–5.0	4.0
	TMB concentration (μM)	400–1600	400
PSA/4-AAP[c]	Manifold configuration	a,b,c	c
	Length of RC (cm)	50–200	50
	HRP concentration (U mL^{-1})	1–200	10
	pH phosphate buffer 0.10 M	6.0–8.0	7.0
	pH acetate buffer 0.20 M	4.0–5.0	–
	4-AAP (μM)	200–3200	800

[a] Injection volume was fixed at 100 μL for all methods.
[b] H_2SO_4 concentration was fixed at 1.00 M.
[c] PSA concentration was fixed at 100 mM.

The scavenging activity was expressed as the inhibition percentage (*I*%) of H_2O_2 and it was calculated as $I\% = (Abs_{blank} - Abs_{sample}) \times 100/Abs_{blank}$, where Abs_{blank} and Abs_{sample} corresponded to the absorbance value in the absence and in the presence of the sample compound, respectively. In order to calculate the concentration providing 50% inhibition of the blank analytical signal (IC_{50}), different concentrations were assayed and plotted against *I*%.

3. Results and discussion

3.1. Development of FIA manifolds based on spectrophotometric reactions

The operational conditions, including the mixing order of reagents, the length of RC and the concentration of reagents, were studied in order to improve the sensitivity of each methodology using a univariate approach. For some reactions, other parameters were also evaluated such as length of MC, catalytic effect of other species, pH and buffer composition. The range of values tested for each parameter and the chosen conditions for its operation are presented in Table 1. All experiments were performed at 25 °C. The injection volume was fixed at 100 μL.

3.1.1. Oxidation of I^- (I^- method)

At acidic pH, I^- is oxidized by H_2O_2 originating iodine (I_2), which is detected spectrophotometrically at 350 nm. For this reaction, the study of the mixing order of reagents was performed with H_2O_2 concentration between 100 and 1500 μM, 0.75 M H_2SO_4 solution (R_1), 0.12 M KI solution (R_2) and RC = 100 cm. When configurations (a) and (b) (Fig. 1) were applied, similar values of sensitivity were obtained: 0.403 and 0.416 mM^{-1}, respectively. Configuration (c)

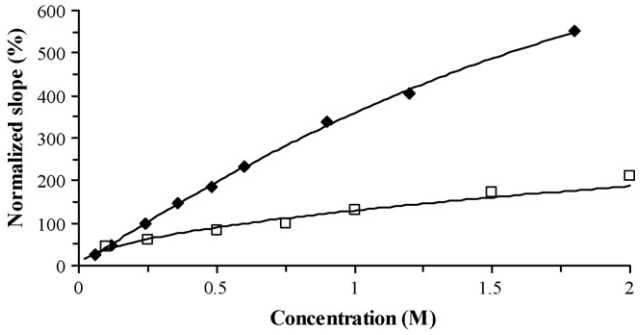

Fig. 3. Effect of the concentration of KI and H_2SO_4. (♦) and (□) represent the different concentrations of KI and H_2SO_4 tested, respectively. The values presented as 100% refer to 0.24 M KI and 0.75 M H_2SO_4, with respective sensitivity of 0.583 and 1.323 mM^{-1}.

(Fig. 1) was not tested as the mixture of H_2O_2 and KI (R_2) in a non-acidified media would not lead to the formation of I_2. Therefore, configuration (a) was selected for further experiments.

Using the same concentration of reagents, the length of RC was studied. When RC was 50 cm long, the slope of the calibration curve was 0.128 mM^{-1}. The sensitivity obtained was 1.44, 1.88 and 2.45 times higher when RC with 100, 150 and 200 cm was used, respectively. These results indicated that the sensitivity was improved with longer RC and, consequently, longer reaction time. For this reason, the RC with 200 cm was chosen.

The effect of the concentration of reagents was also investigated (Fig. 3). Keeping H_2SO_4 concentration at 0.75 M, KI concentration varied between 0.06 and 1.80 M. A linear relationship between the slope and concentration of KI was observed for concentrations lower than 0.90 M. However, a baseline drift was observed for concentrations equal or higher than 0.90 M. Consequently, the concentration of KI was selected as 0.60 M. The concentration of H_2SO_4 was tested between 0.10 and 2.00 M. A baseline drift was also observed for concentrations higher than 1.00 M. For this reason, the concentration of H_2SO_4 was chosen as 0.75 M since it corresponds to the highest sensitivity attained without this effect.

The catalytic effect of Mo(VI) in the H_2O_2/I^- reaction has been reported and it was demonstrated that Mo(VI) was able to enhance the sensitivity of the FIA method by 13 times [30]. In our experiments, the sensitivity of the developed methodology was dependent on the concentration of the catalytic agent, being improved only when the concentration of Mo(VI) was higher than 10 μM. In the presence of 10 μM of Mo(VI), it was observed an increase in sensitivity of only 15% when compared with the control experiment (without Mo(VI)). For the other two concentrations of Mo(VI) tested (100 and 1000 μM), the sensitivity was higher but the relationship between absorbance and H_2O_2 concentration was not linear. For these reasons, the Mo(VI) catalyst was not used.

3.1.2. Formation of $[TiO_2]^{2+}$ complex (TiP method)

The reaction between Ti(IV) and H_2O_2, in a strong acid medium, allows the formation of $[TiO_2]^{2+}$complex, which is characterized by an absorption maximum at 405 nm. For this reaction, the influence of the mixing order of reagents was evaluated using H_2O_2 concentration between 90 and 1800 μM, 1.00 M H_2SO_4 solution (R_1), 12.50 mM Ti(IV)OSO_4 solution (R_2) and RC = 100 cm. When configuration (a) and (b) (Fig. 1) were applied, similar slope values were obtained: 0.346 and 0.343 mM^{-1}, respectively. Therefore, configuration (a) was selected for further experiments.

Using the same concentration of reagents, the length of RC was studied. When the RC was 50 cm long, the sensitivity was 0.143 mM^{-1}. In addition, the sensitivity obtained was 97, 94 and 91% when RC with 100, 150 and 200 cm was used, respectively. Similar results were then achieved, demonstrating that the sensitivity of TiP method was independent on the length of RC. In order to decrease the analysis time, RC with 50 cm was chosen for further experiments.

As reported, this reaction only takes place in a strong acid medium [24]. Keeping H_2SO_4 concentration at 1.00 M, Ti(IV)OSO_4 concentration varied between 6.25 and 125 mM. When 6.25, 12.5, 25.0 and 125 mM of Ti(IV)OSO_4 were tested the sensitivity was 0.149, 0.159, 0.162 and 0.161 mM^{-1}, respectively. The results obtained were similar but the linear range was narrower for the two smallest concentrations of Ti(IV)OSO_4 tested. Therefore, 25.0 mM was the chosen concentration for further experiments since it was possible to achieve a larger calibration range.

3.1.3. Formation of $[Ti\text{-}XO\text{-}O_2]^{2+}$ complex (TiXoP method)

The reaction between Ti(IV), XO and H_2O_2, in strong acid medium, leads to the formation of $[Ti\text{-}XO\text{-}O_2]^{2+}$ complex, which is detected spectrophotometrically at 560 nm. In fact, the absorption spectrum of $[Ti\text{-}XO\text{-}O_2]^{2+}$ complex is greatly affected in its shape by the pH of the reaction medium, showing a local maximum at 560 nm for pH range 2.0–4.0 [25]. In order to evaluate the effect of acidity, a preliminary study was performed in batch conditions. The formation of $[Ti\text{-}XO\text{-}O_2]^{2+}$ complex was monitored from 380 to 600 nm using different pH solutions (0.9, 1.9, 2.9 and 3.5) and keeping the concentration of H_2O_2, Ti(IV)OSO_4 and XO at 1.00 mM. At 560 nm, the maximum value of absorbance (~0.300) was obtained when pH tested was 1.9 ($[H^+] = 0.013$ M). For the other situations, the analytical signal was about 50% of the mentioned. For this reason and taking into consideration the dilution effect in FIA manifold, in the further experiments, the pH of Ti(IV)OSO_4 solutions was adjusted with 1 M H_2SO_4 solution to provide $[H^+] = 0.050$ M or pH 1.3.

The study of mixing order of reagents was carried out using H_2O_2 concentration between 43 and 172 μM, 160 μM Ti(IV)OSO_4 solution (R_1), 160 μM XO solution (R_2) and RC = 200 cm. The sensitivity obtained with configurations (a) and (c) (Fig. 1) were quite similar, respectively, 0.511 and 0.587 mM^{-1}, while with configuration (b) (Fig. 1) the sensitivity was 0.736 mM^{-1}. Therefore, this configuration was selected for further experiments.

The study of the concentration of reagents was performed at the same time, being the [Ti(IV)OSO_4]/[XO] ratio fixed to 1. The concentrations tested were 320, 640, 800 and 960 μM of each reagent and RC = 200 cm. When 320 μM of each reagent was tested, the slope of calibration curve was 0.590 mM^{-1}. An increase of 62 and 75% was obtained, respectively, when 640 and 800 μM of each reagent was used. As similar results were achieved for these solutions, the lower concentration (640 μM) of Ti(IV)OSO_4 and XO was chosen for further experiments. In addition, using 960 μM solution, a reddish precipitate was formed and a baseline drift was observed.

Using the chosen concentration of reagents, the effect of length of RC was evaluated. The sensitivity was 0.702, 0.786, 0.864 and 0.899 mM^{-1} when RC was 50, 100, 150 and 200 cm long, respectively. Therefore, the sensitivity was enhanced with the increase of the length of RC. The difference between the 150 and the 200 cm was only 4%. For this reason, RC = 150 cm was selected.

The effect of length of MC was also carried out. Keeping the chosen working conditions, 50 cm of length was tested. Similar slope values were obtained when 150 and 50 cm were applied, 0.864 and 0.841 mM^{-1}, respectively, showing that the sensitivity was not affected. Consequently, for further experiments the length of MC was selected as 50 cm.

3.1.4. Oxidation of TMB (TMB method)

The oxidation of TMB with H_2O_2 in the presence of HRP leads to the formation of free cation-radicals, which show an absorption maximum at 650 nm. The effect of the mixing order of reagents

Table 2
Comparison of developed FIA methodologies.

Method	Linear range of $[H_2O_2]$ (μM)	Calibration curve ($y = mx + b$)[a]			Detection limit (μM)	RSD (%)	Determination frequency (h^{-1})
		Slope (m)	Intercept (b)	Correlation coefficient			
I^-	45–610	1.3120 (±0.0007)	−0.002 (±0.002)	0.9996	28	1.9	26
TiP	100–4500	0.1450 (±0.0001)	0.004 (±0.002)	0.9998	80	1.0	40
TiXoP	50–500	0.7970 (±0.0006)	−0.009 (±0.002)	0.9992	30	1.5	22
TMB	9–130	4.0870 (±0.0025)	0.003 (±0.002)	0.9995	3.0	1.0	23
PSA/4-AAP	30–615	0.8900 (±0.0004)	−0.008(± 0.002)	0.9997	22	1.0	26

[a] y and x represent absorbance and $[H_2O_2]$ (mM^{-1}), respectively. The values in parentheses correspond to standard deviations ($n = 15$).

was investigated with H_2O_2 concentration between 22 and 66 μM, 40 U mL^{-1} HRP solution prepared in 0.1 M phosphate buffer, pH 6.0 (R_1), 800 μM TMB solution (R_2) and RC = 100 cm. When configuration (a) (Fig. 1) was applied, the previous mixture between TMB and HRP led to the formation of a white precipitate. On the other hand, when configuration (b) (Fig. 1) was used, the analytical performance was negatively affected because the previous mixture between H_2O_2 and HRP led to the consumption of H_2O_2 before the colorimetric reaction. Enzyme solution was so introduced directly in RC and, consequently, configuration (c) (Fig. 1) was chosen. The MC was reduced from 150 to 50 cm.

Using the same conditions, the study of the concentration of HRP between 0 and 1000 U mL^{-1} was performed. When 0 and 1000 U mL^{-1} were used no analytical signal was observed. The results obtained with 1, 2.5, 5, 10, 20, 30, 40, 70 and 100 U mL^{-1} were very similar, showing a very low difference (<3%). As equivalent results were obtained, the concentration of enzyme chosen for determination of H_2O_2 was 1 U mL^{-1}.

The effect of HRP solvent was performed with 800 μM TMB. Two buffers – 0.1 M phosphate buffer and 0.2 M acetate buffer – were tested and relative sensitivity was evaluated regarding 0.1 M phosphate buffer, pH 6.0. For phosphate buffer, sensitivity decreased with the pH increase, with pH 5.0 an increase of 19% was observed while with pH 7.0 a decrease of 16% was noted. When acetate buffer, pH 4.0 and 5.0, was used, better results were achieved and the sensitivity was improved 21 and 12%, respectively. As the best result was obtained with the use of acetate buffer pH 4.0, this was chosen as solvent of HRP to further experiments.

The concentration of TMB was then studied between 400 and 1600 μM. When the lowest concentration was tested, the sensitivity was 5.170 mM^{-1}. Moreover, the sensitivity obtained was 103 and 106% when 800 and 1600 μM of TMB was used, respectively. These results demonstrated that the sensitivity was similar independently of the concentration of TMB. Consequently, the concentration of TMB was selected as 400 μM.

The effect of length of RC was also studied. When RC was 50 cm long the slope of calibration curve was 5.137 mM^{-1}. In addition, the sensitivity obtained was 99, 101 and 102% when RC was 100, 150 and 200 cm long, respectively. Results showed that the sensitivity was similar independently of the length of RC. As it allows to the decrease of analysis time, RC with 50 cm was chosen.

3.1.5. Co-oxidation of PSA and 4-AAP (PSA/4-AAP method)

In PSA/4-AAP colorimetric reaction, PSA is enzymatically oxidized in the presence of H_2O_2 to produce a phenyl radical. This last one reacts with 4-AAP and then originates a colored quinone-imine, which is detected spectrophotometrically at 490 nm. The influence of mixing order of reagents was evaluated with H_2O_2 concentration between 25 and 300 μM, 4 U mL^{-1} HRP solution prepared in 0.1 M phosphate buffer, pH 6.0 (R_1), 100 mM PSA/1600 μM 4-AAP solution (R_2) and RC = 100 cm. When configuration (a) (Fig. 1) was applied, the previous mixture between HRP and PSA/4-AAP led to the formation of a white precipitate. On the other hand, when configuration (b) (Fig. 1) was used, the analytical performance was negatively affected because the previous mixture between H_2O_2 and HRP led to the consumption of H_2O_2, as also verified for the TMB method. Therefore, enzyme solution was introduced directly in RC and configuration (c) (Fig. 1) was chosen to further experiments and MC was reduced from 150 to 50 cm.

Using the same concentration of reagents, the effect of HRP solvent was performed. Two buffers – 0.1 M phosphate buffer and 0.2 M acetate buffer – were tested. When phosphate buffer, pH 6.0, 7.0 and 8.0 were used, the sensitivity was 0.702, 0.908 and 0.972 mM^{-1}, respectively. The effect of acetate buffer, pH 4.0 was not quantifiable as very low analytical signals were attained. When compared to phosphate buffer, pH 6.0, the sensitivity obtained with acetate buffer, pH 5.0 was only 55%. Taking into account the slight difference between phosphate buffer, pH 7.0 and 8.0 (7%) and the pK_a value of $H_2PO_4^-$, pH 7.0 was chosen as solvent of HRP for further experiments.

The study of [PSA]/[4-AAP] ratio was performed, keeping HRP concentration at 4 U mL^{-1}. PSA concentration was fixed at 100 mM and 4-AAP concentration varied between 200 and 3200 μM. The slope of calibration curve was 0.714, 0.924, 1.180, 1.110 and 1.140 when 31.25, 62.5, 125, 250 and 500 ratio was tested, respectively. Therefore, the sensitivity was enhanced with the increase of [PSA]/[4-AAP] ratio. However, when the two highest ratios were tested this effect was not so pronounced. The difference between the ratio 62.5 and its double was <10%. In order to consume less reagent, 800 μM of 4-AAP was applied in further experiments.

The study of HRP concentration was also performed varying its concentration between 1 and 200 U mL^{-1}. When the smallest HRP concentration was used, the sensitivity was 0.848 mM^{-1}. The sensitivity increased about 24% up to 10 U mL^{-1}, remaining constant from 40 to 200 U mL^{-1} (111 ± 2%). Although the difference between 2 (112%), 4 (114%) and 10 (124%) U mL^{-1} was slight, the concentration of enzyme used for further experiments was 10 U mL^{-1} because the linear calibration range for H_2O_2 was extended.

The effect of the length of RC was also investigated. When RC was 50 cm long, the slope of calibration curve was 1.062 mM^{-1}. Moreover, the sensitivity obtained was 96, 100 and 95% when RC was 100, 150 and 200 cm long, respectively. Results demonstrated that sensitivity was similar independently of the length of RC. Considering the decrease of analysis time, RC with 50 cm was chosen.

3.2. Comparison of FIA methodologies

The respective figures of merit including linear range of $[H_2O_2]$, limit of detection, repeatability and determination frequency were determined (Table 2), allowing the comparison of the developed methodologies. The limit of detection was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ [31]. The repeatability was assessed by calculating the relative standard deviation (RSD) from 10 consecutive determinations of a H_2O_2 standard solution (50 μM in TMB method, 200 μM in I^- method, TiXoP method and PSA/4-AAP method and 1500 μM in TiP method). The determination frequency was evaluated taking into consideration the time required to record a complete peak.

Table 3
Concentration of H_2O_2 found (M) and values of $t_{calculated}$ when compared to the reference method.

		FIA method					Reference method
		I^- (1:4000)	TiP (1:400)	TiXoP (1:4000)	TMB (1:12,500)	PSA/4-AAP (1:4000)	(1:100)
Sample 1[a]	Concentration	1.07 ± 0.01	1.02 ± 0.01	1.03 ± 0.02	1.01 ± 0.01	1.03 ± 0.01	1.03 ± 0.02
	$\|t_{calc}\|$	4.140	1.035	0	2.070	0	–
Sample 2[b]	Concentration	1.00 ± 0.02	1.03 ± 0.01	1.02 ± 0.02	1.01 ± 0.02	1.01 ± 0.02	1.03 ± 0.01
	$\|t_{calc}\|$	3.243	0	1.081	2.162	2.162	–

The results are expressed as the mean ± standard deviation. The values in parentheses correspond to the dilution of sample prior to analysis.
[a] FIA method (n = 5), reference method (n = 9), $t_{tab\,(\upsilon=12,\,p=0.05)}$ = 2.179.
[b] FIA method (n = 5), reference method (n = 6), $t_{tab\,(\upsilon=9,\,p=0.05)}$ = 2.262.

These figures of merit are summarized in Table 2 and a typical calibration curve for each developed methodology is reported. I^-, TiXoP and PSA/4-AAP methods showed similar analytical figures for linear range and determination rate. Although TiP method allowed the determination of a larger number of samples per hour, its sensitivity was inferior to all the others. On the other hand, the highest sensitivity was attained when applying the TMB method. All the developed methods exhibited an excellent repeatability (<2%). The calibration curves presented are representative of all the others obtained, with RSD < 5% for slope and intercept (n = 3–5).

3.3. Analysis of samples

3.3.1. Disinfection products

The applicability and accuracy of the developed methodologies was assessed by the determination of H_2O_2 in two contact lens disinfecting liquid samples. The samples were analyzed according to each proposed method and by the reference method [29]. The results are presented in Table 3. Relative deviations from the reference method <1.9% were found for all methods, except for I^- method. For comparison purposes, a t-test was performed (null hypothesis). For all the methods, except I^- method, the calculated t was lower than the tabulated value, indicating that there is no evidence for significant difference between the results provided by each method when compared to the reference method at a level of confidence of 95%.

3.3.2. Determination of scavenging capacity against H_2O_2

The applicability of developed FIA methodologies to the evaluation of H_2O_2 scavenging activity was assessed by adapting the manifolds developed before, as depicted in Fig. 2 (Section 2.5.2). The concentration of H_2O_2 was fixed and adjusted according to the linear range of each methodology (Table 4). According to Schröder and Eaton, under inflammatory conditions, the local concentration of H_2O_2 found varied from 10 to 1000 μM [32], which is in agreement with the tested concentrations of this ROS, except for TiP method. Glutathione (GSH) was used as model scavenging compound. Moreover, the effect of pH buffering at the scavenging reaction coil was tested by using either water or Tris buffer (pH 7.4) as carrier solution.

In TiP method, using water as carrier, no consumption of H_2O_2 was noticed, even when GSH 100 mM was tested, while with Tris buffer a low signal, corresponding to $I\%$ = 1.4%, was obtained. As this FIA method presented the lowest sensitivity, the concentration of H_2O_2 (3000 μM) was higher than that applied on the other methods. This feature required the presence of more antioxidant compound in order to attain an inhibitory effect. In TiXoP method, although GSH did not show intrinsic absorption, an interaction between this antioxidant compound and the reagents was detected as an increase of the analytical signal directly proportional to the concentration of GSH was observed. This fact remains unclear but it may be possibly explained taking into consideration the structural similitude between GSH and xylenol orange.

For the other methods, GSH scavenged H_2O_2 in a concentration-dependent manner and the respective IC_{50} were calculated (Table 4). In TMB method, initial experiments were performed using HRP 1 U mL^{-1} prepared in 0.2 M acetate buffer pH 4.0. However, the Abs_{blank} value was quite different when water and Tris buffer were used as carrier, as a 38% lower value was attained for buffer carrier. To improve acetate buffer capacity in the presence of Tris buffer pH 7.4, its concentration was increased to 0.5 M and HRP concentration was also increased to 10 U mL^{-1}. Using this reaction conditions, the IC_{50} of GSH obtained in the presence of Tris buffer was estimated to be about 5000 μM as this value was placed in the plateau region of the inhibition curve.

In general, when water was used as carrier, the IC_{50} values of GSH were lower than those achieved with Tris buffer pH 7.4. This result showed that the pH of H_2O_2 scavenging reaction should be carefully controlled to provide *in vitro* data that would be meaningful when extrapolated to *in vivo* conditions. For this reason, in the further experiments, a buffered medium at pH 7.4 was used for the scavenging reaction.

Considering that lower IC_{50} values were attained with I^- method, this was initially chosen for further experiments. However, when testing other scavengers of H_2O_2, namely sulindac, a significant contribution of sample intrinsic absorption was observed. Considering that intrinsic absorption at the wavelength (350 nm) applied for this method is frequent and that this fact does not

Table 4
H_2O_2 scavenging capacity of glutathione assessed by the proposed FIA methods.

Method	$[H_2O_2]$ (μM)	IC_{50} (μM)[a]	
		Unbuffered medium (water)	Buffered medium (Tris buffer)
I^-	200	268 ± 11	344 ± 14
TiP	3000	NA[b]	NF[c]
TiXoP	300	NA[d]	NA[d]
TMB	30	2715 ± 120	~5000
PSA/4-AAP	200	1905 ± 139	2511 ± 177

[a] Results are expressed as the mean ± standard deviation (n = 3).
[b] NA, no activity was found within the tested concentrations ranging from 1 to 100 mM.
[c] NF, IC_{50} was not found: at the highest concentration tested (100 mM), $I\%$ = 1.4%.
[d] NA, no activity was found within the tested concentrations ranging from 50 to 100 mM.

Table 5
H_2O_2 scavenging capacity of several antioxidant compounds.

	PSA/4-AAP method	Other methods
	IC_{50} (μM)	
GSH	2511 ± 177	1384 ± 75[a]
Gallic acid	247 ± 9	7[b]
Trolox	372 ± 6	11[b]
Caffeic acid	~600	57[b]
Meloxicam	NF[c] (42 mM, *I*% = 48%)	
Etodolac	NF[c] (7.5 mM, *I*% = 7.3%)	2224 ± 28[a]
Ketolorac	NA[d] (30)	>5000[a]
Sulindac	NA[d] (2)	1476 ± 50[a]
Atenolol	NA[d] (12.5)	
Timolol	NA[d] (25)	1642 ± 145[a]
Labetolol	NA[d] (0.5)	>5000[a]
	IC_{50} (U mL^{-1})	
SOD	NA[e]	
Catalase	189 ± 14	0.050 ± 0.005[a]

Results are expressed as the mean ± standard deviation (n = 3).
[a] Values obtained using H_2O_2-induced lucigenin-CL assay [34,35].
[b] Values obtained using SIA-fluorimetric method based on homovanillic acid [33].
[c] NF, IC_{50} was not found.
[d] NA, no activity was found for the highest concentration tested, indicated in parenthesis and expressed in mM.
[e] NA, no activity was found for 447 U mL^{-1}.

allow the reliable determination of scavenging capacity, PSA/4-AAP method was chosen as an alternative as it provided the second lowest IC_{50} values for GSH and the determination was carried out at a higher wavelength (490 nm). Moreover, reactions based on oxidative coupling with formation of colored quinine-imines were seldom applied for assessment of H_2O_2 scavenging capacity.

The results obtained for all compounds tested at pH 7.4 are presented in Table 5. The values obtained by other methodologies were also included. A straightforward comparison of values is not possible, as the reactions conditions (H_2O_2 concentration, reaction time, pH) were different. However, a general comparison about relative potencies is feasible.

The results achieved demonstrated that H_2O_2 was scavenged by gallic acid and trolox. Furthermore, reaction conditions enabled the determination of the respective IC_{50} value. Using a SIA-fluorimetric assay, Pinto et al. [33] obtained lower IC_{50} values for gallic acid and trolox. However, taking into consideration the ratio $IC_{50\ trolox}/IC_{50\ gallic\ acid}$, a similar value was found for both the SIA method and the proposed FIA method: 1.57 and 1.51, respectively. Moreover, caffeic acid exhibited a maximum inhibition (55%) at 1000 μM and its IC_{50} was estimated to be about 600 μM.

Considering the results obtained for pharmaceutical compounds, meloxicam caused a inhibition of 48% at the highest concentration tested (42 mM) and only 7.3% of inhibition was attained when the highest concentration of etodolac was tested (7500 μM). For ketorolac, sulindac, atenolol, timolol and labetolol, no detectable activity against H_2O_2 was observed. This lack of scavenging capacity could be possibly explained taking into account the reaction time applied. In the present work, the colorimetric reaction was preceded by the oxidant (H_2O_2)-antioxidant reaction which occurred in 15 s instead of 5–10 min applied in the batch methods [34,35]. This feature is important in the evaluation of the putative *in vivo* effects of the antioxidant compounds since the scavenging reaction against H_2O_2 must be as fast as possible to minimize cellular damage. For this reason, the previously reported *in vitro* end-point methodologies do not reproduce *in vivo* conditions and do not allow the distinction between slow and fast reacting compounds.

Finally, in order to demonstrate the specificity of PSA/4-AAP method, catalase (which catalyzes H_2O_2 decomposition) and superoxide dismutase (SOD, which catalyzes superoxide anion decomposition) were tested (Table 5). Catalase inhibited the analytical signal in a concentration dependent manner, providing an IC_{50} value of 189 ± 14 U mL^{-1}, while SOD did not cause any signal change up to 447 U mL^{-1}. These results indicate that H_2O_2 is the species determined by the colorimetric reaction and that superoxide anion did not account for the reaction signal obtained by the FIA method. This is an important observation, considering that the major drawback pointed out to methods relying on peroxidase activity is cross-reaction with intermediate species, such as superoxide anion [36].

4. Conclusions

In the present work it was possible to establish a comparison among five colorimetric reactions for determination of H_2O_2 using similar FIA systems. Except for I^- method, all methods were accurate and, therefore, suitable for routine quality control of disinfection products containing H_2O_2.

Concerning the evaluation of scavenging capacity against H_2O_2, the FIA-PSA/4-AAP method proposed here is simple and does not rely on chemiluminescence detection, circumventing the problems presented by the low specificity of the previously described luminol based methods. Furthermore, despite the advantages inherent to computer control that are present on SIA, multicommutation and other more recent flow techniques, FIA is technically simpler and does not require computer control. This aspect is an advantage when considering implementation/operation in other fields of research, namely for screening of biological activity in plant extracts or pharmaceutical compounds.

Finally, when comparing the proposed FIA system to batch methods, the reaction conditions provided by the flow system are closer to those found *in vivo*, not only for pH, but especially when the time of reaction between oxidant (H_2O_2) and antioxidant is considered. This is undoubtedly the most important advantage over batch methods because the scavenging activity of antioxidant compounds that react fast and, therefore, closer to the time frame of generation of H_2O_2, is determined.

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