

## DEVELOPMENT OF A COLORIMETRIC METHOD FOR THE DOSAGE OF $\text{OI}^-$ ANIONS AND $\text{I}_2$ IN AQUEOUS MEDIA

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### ABSTRACT

Lactoperoxidase catalyzes the oxidation of thiocyanate ( $\text{SCN}^-$ ) and iodide ( $\text{I}^-$ ) in presence of hydrogen peroxide in hypothyocyanite ( $\text{OSCN}^-$ ) ions and, depending on the pH, in hypoiodite ( $\text{OI}^-$ ) ions or in iode ( $\text{I}_2$ ). Oxidized  $\text{SCN}^-$  and  $\text{I}^-$  are part of the lactoperoxidase system, which is a natural biological protection in cow milk, and are described as having inhibitory properties against pathogenic human bacteria, fungi and viruses. We have developed an aqueous solution containing only  $\text{OSCN}^-$  and  $\text{OI}^-$  ions (without the enzyme) and we tested it successfully against plant pathogens. In order to characterize this new soft chemical control against plant pathogens we had to determine the concentration of  $\text{OSCN}^-$  and  $\text{OI}^-$  ions. The dosage of  $\text{OSCN}^-$  consists in a well referenced colorimetric method but no procedure is described for the determination of  $\text{OI}^-$  ions. We have thus developed an easy method, based on the oxidation of the amine moiety of 3,3',5,5'-tetramethylbenzidine (TMB) by  $\text{OI}^-$  or  $\text{I}_2$  in a strongly absorbing blue product for the detection and dosage of both molecules. Interestingly the  $\text{OSCN}^-$  ions are not able to oxidize TMB and render this method specific to enzymatic oxidized iodide. We have calculated its sensitivity, repeatability and linearity. This method could also be used for the determination of  $\text{OCl}^-$  and  $\text{OBr}^-$  ions produced during the enzymatic oxidation of chloride and bromide by mammalian's peroxidases.

### INTRODUCTION

Peroxidases are oxidoreductase enzymes that are part of the non-immune natural defense system described in milk and exocrine secretions such as saliva, tears, airway mucosa or intestinal secretions [1]. Peroxidases have no antibacterial activity by themselves, but exerted their activity in presence of cofactors which are hydrogen peroxide  $\text{H}_2\text{O}_2$ , and a second ion, being the pseudohalogen thiocyanate ion ( $\text{SCN}^-$ ), or a halogen chloride ion ( $\text{Cl}^-$ ), bromide ion ( $\text{Br}^-$ ), or iodide ion ( $\text{I}^-$ ) depending on the peroxidase [2]. Lactoperoxidase, present in cow milk, catalyzes the oxidation of  $\text{SCN}^-$  and  $\text{I}^-$  in presence of  $\text{H}_2\text{O}_2$  in hypothyocyanite ( $\text{OSCN}^-$ ) ions and, depending on the pH, in hypoiodite ( $\text{OI}^-$ ) ions or in iode ( $\text{I}_2$ ) [2, 3]. These molecules are described as having inhibitory properties against pathogenic human bacteria, fungi and viruses [2]. As we demonstrated the efficacy of  $\text{OSCN}^-$  and  $\text{OI}^-$  against plant bacteria and fungi, we needed to characterize the two molecules for agricultural applications and homologation procedures. The dosage method for  $\text{OSCN}^-$  is known but no one is described for the determination of  $\text{OI}^-$ .

### MATERIAL AND METHODS

3,3',5,5'-tetramethylbenzidine (TMB) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Hydrogen peroxide 35%, iode 0.5 M, sodium acetate, acetic acid, potassium thiocyanate and potassium iodide were purchased from VWR. Aluminium polychlorosulfate (Pac Sachtoklar) was purchased from Brenntag. Green clay and lactoperoxidase were provided by Taradon Laboratory. Absorbances were recorded with an UV-visible spectrophotometer SmartSpec™ 3000 from Biorad. The 5-thio-2-nitrobenzoic acid (Nbs) method was used for the determination of OSCN<sup>-</sup> concentrations [4].

#### **Enzymatic production of OI<sup>-</sup> and/or OSCN<sup>-</sup> ions**

In 1 L tap water containing lactoperoxidase (50 mg) and clay (2 g), 0.78 mM KI, 0.34 mM KSCN and 1,2 mM H<sub>2</sub>O<sub>2</sub> were added while shaking. After 1 minute of incubation, 150 µl of Pac Sachtoklar was added and shaking was stopped. After 5 minutes, the clear water was used for testing the OI<sup>-</sup> ions. Same method was followed with 0.34 mM KSCN and 0.34 mM H<sub>2</sub>O<sub>2</sub>.

#### **The TMB method**

Stock solutions of TMB at 1 mg/ml DMSO were kept at -20°C. Working solutions of TMB were freshly prepared by diluting 1 ml of stock solution in 9 ml 50 mM sodium acetate buffer, pH 5.4. In one ml of TMB at 0.1 mg/ml, 50 µl of sample is added and mixed. After 180 minutes, absorbance is read at 655 nm.

Standard curve was constructed with titred 0.5 M iode solution from diluting initial solution with distilled water to obtain a calibration curve ranging from 0.1 mM to 1.4 mM iode.

Ions solutions samples were tested undiluted and diluted with distilled water.

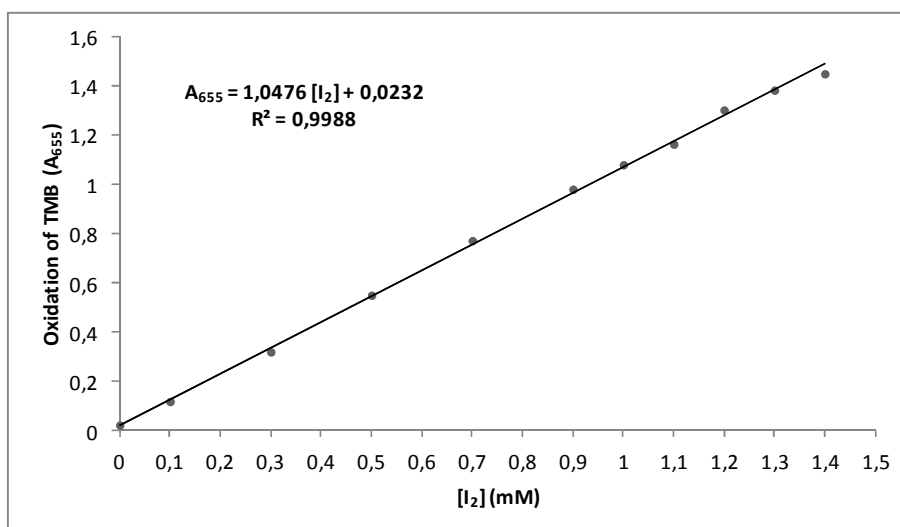
#### **Protocol for the validation of an analytical chemistry method**

The limit of detection (LD), the limit of quantification (LQ), the repeatability and the linearity of the TMB method were calculated following the protocols described in [5]. LD and LQ were calculated with the I<sub>2</sub> calibration curve. The LD is the standard deviation (s) multiply by 3. The LQ is s multiply by 10. The concentration chosen for the calculation of LD and LQ has to fit two conditions: i) to be between (LD x 5) and (LD x 7) and ii) having a ratio R (mean/LD) between 4 and 10. Repeatability was calculated with the following equation  $\frac{t_{(0,975;n-1)} \times s}{\sqrt{n}}$  where  $t_{(0,975;n-1)}$  is the T-student value with a 95% bi-interval confidence with n=9.

## **RESULTS**

#### **Calibration curve**

Absorbance is proportional to the concentration in iode. Iode oxidized -NH<sub>2</sub> groups of TMB in a blue product measured at 655 nm. Results are showed in Figure 1.



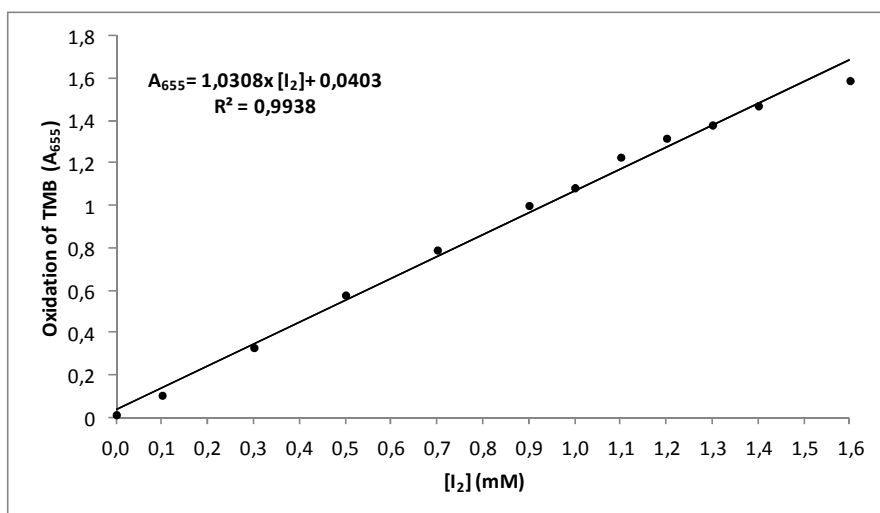
**Figure 1: Calibration curve. Linear regression and correlation coefficient were calculated on the mean of nine replicates (three replicates on three days).**

The limit of detection and quantification were calculated on a total of nine replicates (3 replicates on 3 days). They correspond respectively to 19  $\mu\text{M}$  [I<sub>2</sub>] and 65  $\mu\text{M}$  [I<sub>2</sub>]. As show in Table 1 the concentration chosen for the calculation of LD and LQ was 0.1 mM.

**Table 1: Limit of Detection (LD) and Limit of Quantification (LQ) of the TMB method for the dosage of I<sub>2</sub>.**

	I <sub>2</sub> 0,01 mM	I <sub>2</sub> 0,05 mM	I <sub>2</sub> 0,1 mM
Mean concentration	0,01582	0,05083	0,08829
Standard deviation (s)	0,00772	0,00582	0,00650
Limit of detection (LD)	0,02315	0,01746	0,01951
LD x 5	0,11577	0,08731	0,09755
LD x 7			0,13656
	0,01mM < 0,1157 Reject of concentration	0,05mM < 0,0873 Reject of concentration	0,097 < 0,1mM < 0,13 Selected concentration
Ratio R (mean/LD)			4,5254
Limit of Detection			0,019 mM
Limit of quantification			0,065 mM

The linearity is fulfilled when the coefficient of correlation is above 0.995 and the limit of linearity of the TMB method has been determined as 1.4 mM [I<sub>2</sub>] (figure 2).



**Figure 2: Linearity of the TMB method with [I<sub>2</sub>] up to 1,6 mM. Linear regression and correlation coefficient were calculated on the mean of 10 replicates.**

The repeatability was calculated on nine replicates (3 replicates on three days) with the same analyst and same spectrophotometer at several concentrations comprised between the limit of quantification and the limit of linearity (Table 2).

**Table 2: Repeatability of the TMB method.**

	Iode concentration					
	0,1 mM	0,5 mM	0,7 mM	1 mM	1,2 mM	1,4 mM
Mean concentration	0,08828662	0,502319692	0,714922641	1,010630147	1,223765137	1,364861989
Standard deviation (s)	0,00650308	0,083009658	0,090555343	0,120533456	0,112182589	0,120613746
Repeatability	0,004998701	0,063806757	0,069606874	0,09265005	0,086231017	0,092711766

#### Dosage of enzymatic production of OI<sup>-</sup> anions

Absorbance of 50 µl OI<sup>-</sup> samples from enzymatic oxidation of iodide were read after 180 minutes of incubation with TMB, which is the time needed to obtain the maximal absorbance. OI<sup>-</sup> mean concentration expressed in I<sub>2</sub> concentration through the calibration curve is 186 µM. Five times dilution of the initial solution gives non reproducible results due to a too low absorbance (Table 3).

**Table 3: Mean absorbance, standard deviation and mean difference between undiluted sample and diluted samples of 9 replicates (3 replicates/ 3 independent ions solutions).**

	(0,78 mM KI + 0,34 mM KSCN + 1,2 mM H <sub>2</sub> O <sub>2</sub> )	(0,78 mM KI + 0,34 mM KSCN + 1,2 mM H <sub>2</sub> O <sub>2</sub> ) 1/3	(0,78 mM KI + 0,34 mM KSCN + 1,2 mM H <sub>2</sub> O <sub>2</sub> ) 1/5	Difference in absorbance between undiluted sample and 1/3 diluted sample	Difference in absorbance between undiluted sample and 1/5 diluted sample
Mean Absorbance	0,218	0,084	0,062	15,20%	42,69%
Standard deviation	0,0944	0,0353	0,0264		

#### Dosage of enzymatic production of OSCN<sup>-</sup> anions

Absorbance of 50 µl OSCN<sup>-</sup> samples from enzymatic oxidation of SCN<sup>-</sup> were read after 180 minutes of incubation with TMB and concentration measured with Nbs method (Table 4).

**Table 4: Mean absorbance at 655 nm with the TMB method and mean concentration with the Nbs method (3 replicates/ 3 independent ions solutions).**

	OI <sup>-</sup> (TMB method)	OSCN <sup>-</sup> (Nbs method)
Sample n°	A <sub>655</sub> at 180 min.	μM/L
1	0,016	75
2	0,021	89
3	0,024	97

## DISCUSSIONS

The lactoperoxidase system is an antimicrobial system largely described in literature. Attention has been mainly focused on OSCN<sup>-</sup> as SCN<sup>-</sup> is the most oxidized peroxidase's substrate in *in vivo* fluids [6]. OSCN<sup>-</sup> reacts specifically through oxidation of -SH moiety and its concentration is measured by oxidation of thiol groups of Nbs [7]. HOI, the conjugate base OI<sup>-</sup> and I<sub>2</sub> are able to oxidized aromatic cycles, thiols, thioethers and very slowly amines [8, 9]. The Nbs method is not specific and in presence of OI<sup>-</sup> and OSCN<sup>-</sup> it will measure the total thiol oxidant activity [10].

TMB is a substrate for peroxidases and, in presence of H<sub>2</sub>O<sub>2</sub>, undergoes an enzymatic oxidation into a blue product largely described for the measurement of peroxidase activity [11]. Enhanced TMB activity has been recorded with added iodide and bromide but not with chloride and thiocyanate [12, 13]. It has also been described for the indirect measurement of enzymatic OCl<sup>-</sup> and OBr<sup>-</sup> production by trapping them as taurine haloamines [14, 15]. Oxidation of TMB by chemically produced OCl<sup>-</sup>, OBr<sup>-</sup>, OI<sup>-</sup> or I<sub>2</sub> has been described [13]. OSCN<sup>-</sup> has been reported as a poor TMB oxidant [13]. This was confirmed by our results.

We have developed a direct OI<sup>-</sup> or I<sub>2</sub> quantification method after retrieval of the enzyme by decantation of the clay. Alternatively, 1) the reaction can be stopped by the addition of catalase or 2) the enzyme can be removed by ultrafiltration. This latter method cannot be used when I<sub>2</sub> is formed because it is absorbed on the 30 kDa membrane (data not shown). Aqueous oxidized iodide solutions contains a mixture of several species, depending mainly on the pH and iodide concentrations, such as HOI, OI<sup>-</sup>, I<sub>2</sub>, I<sub>3</sub><sup>-</sup> [9, 16, 17]. At pH 6 to 9, which is in the pH range of our enzymatic solution, the main active oxidized I<sup>-</sup> species is believed to be HOI [9]. Indeed the enzymatic solution is colorless, not yellowish colored and ultrafiltration didn't diminish Nbs concentrations (data not shown) so we assume that the main oxidized I<sup>-</sup> species is HOI and not I<sub>2</sub>. Calibrated solutions of HOI didn't exist and calibrated I<sub>2</sub> solutions were used to construct a standard curve. We expressed the OI<sup>-</sup> concentration in I<sub>2</sub> equivalent. It is assuming that one I<sub>2</sub> or one HOI oxidized one TMB molecule [13] which make it worthwhile to divide by two concentrations calculated from the I<sub>2</sub> standard curve for the HOI solutions. The concentration of [HOI] measured in the enzymatic solution with the TMB method was 93 μM/L. This value is in accordance with the Nbs method for which the [HOI + OSCN<sup>-</sup>] measured concentration was 138 μM/L.

HOI and I<sub>2</sub> are described as poor oxidant of amine groups [9]. The long incubation needed before stabilization of absorbance could be explained by the weak oxidant capacity of HOI. Nevertheless this was not observed with I<sub>2</sub>

which oxidized TMB within 30 minutes in a stable absorbance up to 180 minutes (data not shown) [13].

This method could also be used for direct measurement, after retrieval of the enzyme, of OCL<sup>-</sup> and OBr<sup>-</sup> with probably shortening TMB-incubation time since both are more reactive oxidants than HOI.

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