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# Raw material enzymatic activity determination: A specific case for validation and comparison of analytical methods—The example of superoxide dismutase (SOD)

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**Abstract**

Implementation of ICH guideline for validation of analytical methods was tested in the case of two enzymatic assays of determination of superoxide dismutase (SOD) activity. Analytical figures of merits of two tested methods (NBT reference method and the so called WST-1 method) demonstrate the feasibility of such approach. Nevertheless specification usually admitted for physicochemical method (e.g., HPLC), needs to be clearly enlarged, for instance, up to 10% for repeatability and 20% for reproducibility. On the other hand, the cross-validation performed between the two enzymatic activity determination techniques, though based on the same principle, clearly shows that the quality of results depends on small variation in the experimental conditions. This shows that an enzymatic activity determination should be strictly related to the technique used, especially in the pharmaceutical control quality field, and confirms that analytical figures of merits are strongly function of the technique used. Finally, it was demonstrated that both NBT reference method and WST-1 method give strictly similar results.


*Keywords:* Superoxide dismutase (SOD); Enzymatic activity; Method comparison; Quality control

## 1. Introduction

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion ($O_2^-$) into hydrogen peroxide and molecular oxygen, is one of the most important enzymes in the front line of defence against oxidative stress [1,2]. Since the discovery of SOD by McCord and Fridovich in 1969 [3], several direct and indirect methods for SOD activity measurement have been developed (e.g., absorbance [4–7], chemiluminescence [8,9], electron spin resonance spectroscopy [10]) Data comparison obtained by the use of different methods is very difficult due to the fact that even a slight variation in assay conditions leads to very different results [11]. Among enzymatic activity assay, an indirect method using nitro blue tetrazolium (NBT) is commonly used due to its convenience and ease of use [12]. Briefly, the NBT method is based on the reduction of NBT by reaction with $O_2^-$ yielding to the generation of a formazan blue dye ($\lambda_{max}$: 560 nm), which is poorly water-soluble. Consequently, a non-homogeneous suspension occurs during the analysis. This can affect the reproducibility of the determination and does not allow easy adaptation to a reliable microtiter plate assay. In order to overcome this drawback, several new tetrazolium salts which generate water-soluble formazan by reduction were proposed [13–15], and a simple microplate SOD assay kit using one of the water-soluble tetrazolium salt, i.e., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was recently developed [16] (Fig. 1).

In the present work, analytical validation of the two methods and cross-validation of NBT assay method considered as the reference method against WST-1 assay kit was performed. From our knowledge, only few data deal with the validation of enzymatic method used in quality control. This is certainly due to the sparse use of such determination assay in this field. Up to now, enzymatic determinations were mainly used in clinical chemistry, and performed mostly in biological matrices. Nevertheless, the increasing treatments based on soft medicine in developed countries yield to an increased use of enzymotherapy (antioxidant, anti-stress) which raises this question. From a pharmaceutical point of view, this raises some questions con-

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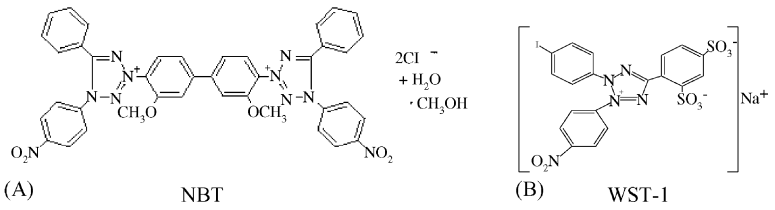


Fig. 1. Chemical structure of NBT (A) and WST-1 (B).

cerning the criteria of validation of such techniques, as this was not specifically envisaged, from our knowledge, by pharmaceutical authority so far. This work is a first attempt to evaluate the possible transposition of ICH criterions [17,18] to such determinations. In order to illustrate the problem, the case of SOD activity on raw material was detailed and discussed through two analytical methods based on the same principle described above, i.e., the reference NBT assay derived from Oberley and Spitz [12], and the WST-1 assay developed for routine analysis by the use of the microtiter plate [16].

## 2. Experimental

### 2.1. Chemicals

Bovine erythrocyte superoxide dismutase, Cu,Zn SOD (4520 units/mg of protein); xanthine (2,6-dihydroxypurine); diethylenetriaminepentaacetic acid (DETAPAC); nitro blue tetrazolium (NBT); xanthine oxidase (1.1 units/mg of protein); co-enzyme Q10 (CoQ10); catalase (2310 units/mg of protein); glutathion peroxidase (GPX) (695 units/mg of protein) were purchased from Sigma (Saint-Quentin Fallavier, France).

The WST-1 kit contained a set of 5 ml of WST-1 solution, 100 μl of enzyme solution, 100 ml of buffer solution and 50 ml of dilution buffer, and was obtained from Dojindo Laboratories Co. (Kumamoto, Japan).

All other reagents were of analytical reagent grade. All solutions were prepared with ultrapure water obtained by a Milli-Q system (Millipore, Saint Quentin en Yvelines, France).

### 2.2. Solutions

#### 2.2.1. For NBT reference method

Fifty millimolar phosphate buffer (pH 7.8); DETAPAC solution: 1.24 mM in above cited phosphate buffer (prepared every week); NBT solution: 1.96 mM in the above cited phosphate buffer (prepared daily and stored in brown glass); xanthine solution: 1.61 mM in the above cited phosphate buffer (prepared daily, adding 1 ml 0.1 M KOH solution for 50 ml xanthine solution); xanthine oxidase solution: 0.19 units/ml in the above cited phosphate buffer (prepared as requested and kept at +4 °C during the determination). Reagent mixture for assay, prepared immediately before use: 43.5 ml DETAPAC solution was mixed with 1.5 ml NBT solution and 5 ml xanthine solution.

The same batch of SOD reagent was used throughout this work. The SOD stock solution was prepared daily by addition of 3 ml of 50 mM phosphate buffer (pH 7.8) into the SOD reagent vial containing 5876 activity units (corresponding to 4520 units/mg of protein). SOD working solutions were obtained by dilution in 50 mM phosphate buffer (pH 7.8) and is prepared as needed.

#### 2.2.2. For WST-1 assay kit

The WST-1 working solution, enzyme working solution and SOD standard solution were prepared according to the technical manual kit.

### 2.3. Materials

#### 2.3.1. For NBT reference method

Spectrophotometer model Cary 100 equipped with a Peltier thermostated cell compartment (Varian, Les Ulis, France) was used. Determination was performed with 10 mm optical length Hellma® quartz cells (Fisher Scientific Labosi, Elancourt, France).

#### 2.3.2. For WST-1 assay kit

Ninety-six-well microplates Greiner Bio-one were from Fisher Scientific Labosi (Elancourt, France). Microplate reader Multiskan Ascent (450 nm filter) was from ThermoLabsystems (Thermo Electron Co., Saint-Herblain, France)

### 2.4. Methods

#### 2.4.1. Principle of the assay for both studied techniques

SOD catalyzes the dismutation of superoxide radical ($O_2^-$) to hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$). The xanthine–xanthine oxidase system generates $O_2^-$ that in turn, reduced either NBT or WST-1 to their corresponding formazan absorbing at 560 and 450 nm, respectively. In presence of SOD, the superoxide radical ($O_2^-$) undergoes a dismutation into $O_2$ and $H_2O_2$, which reduced the formazan formation. Hence, this competing assay yields to the indirect measurement of SOD activity (Fig. 2).

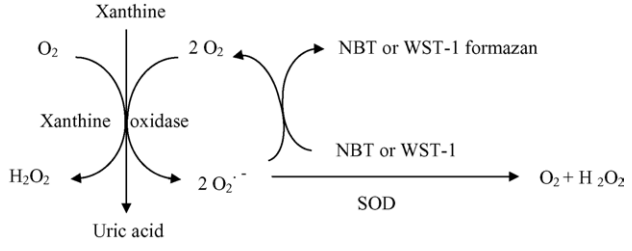


Fig. 2. Principe of SOD activity determination by NBT and WST-1 kit assay.

*2.4.2. SOD activity determination procedure*

From McCord and Fridovich [3], one unit of SOD activity was defined as the amount of the enzyme in a sample solution causing 50% inhibition ($IC_{50}$) of the rate of reduction of tetrazolium salt. The SOD activity was directly measured using a plot of the percentage inhibition versus the amount of SOD in mg/ml for NBT method and WST-1 kit assay, respectively.

*2.4.2.1. SOD assay by the NBT method.* In the NBT method, inhibition activity is determined using a kinetic mode [12]. 2.8 ml of reagent mixture containing NBT, DETAPC and xanthine as described in Section 2.2 are added to a 10 mm quartz cell equipped with a magnetic stirrer. The solution was incubated for 6 min at 25 °C in cell compartment of the spectrophotometer. Zero correction was done before addition of 100 μl of the various concentration SOD solutions under agitation. Hundred microliter of phosphate buffer (pH 7.8) was used in place of SOD test solution as zero level SOD standard for uninhibited. The reaction was then initiated by adding 100 μl of xanthine oxidase solution under agitation. One minute after addition of xanthine oxidase, the agitation is stopped and the absorbance change at 560 nm was monitored at 25 °C, against air for 5 min. The rate of change of absorbance variation $\Delta A_{560}$/min of an uninhibited assay (in absence of SOD) should be between 0.015 and 0.025, if not, the xanthine oxidase concentration is adjusted. Percentage inhibition corresponding to each standard sample was calculated as follow: $[(S_0 - S)/S_0] \times 100$, where $S_0$ and $S$ represent the slope of uninhibited (without SOD) test and with SOD, respectively.

*2.4.2.2. Microplate WST-1 assay.* After the addition of all solution in each well as described in Table 1, the microplate was stirred thoroughly and then incubated at 37 °C for 20 min. The absorbance at 450 nm of the endpoint reaction (20 min) was measured by using a microplate reader. Percentage inhibition of each sample was calculated by using following equation: $\{[(A_1 - A_3) - (A_S - A_2)]/(A_1 - A_3)\} \times 100$, where $A_1$, $A_2$, $A_3$ and $A_S$ were the absorbance at 450 nm for uninhibited test, blank sample, blank reagent and sample, respectively.

*2.4.3. Validation procedures (for NBT and WST-1 assay)*

The validation procedures were established according to the ICH [17,18]. The linearity, accuracy, precision and specificity of the two mode of determination of SOD were determined. The test was performed on five different days.

Table 1
WST-1 assay kit: schedule for samples and blanks

| | Sample or SOD standard | Blank 1 | Blank 2 | Blank 3 |
|---|---|---|---|---|
| Sample or standard solution (μl) | 20 | – | 20 | – |
| $H_2O$ (μl) | – | 20 | – | 20 |
| WST-1 working solution (μl) | 20 | 20 | 20 | 20 |
| Dilution buffer (μl) | – | – | 20 | 20 |
| Enzyme working solution (μl) | 20 | 20 | – | – |
| Absorbance at 450 nm | $A_s$ | $A_1$ | $A_2$ | $A_3$ |

*2.4.3.1. Calibration curve and linearization.* Enzymatic standard curve is inherently non-linear and, consequently, more calibration points are recommended to define the fit over the standard curve range than for chemical assays. A minimum of six non-zero calibrator concentrations is recommended [19].

The calibration curve was obtained by using six calibration SOD standards: 0.049, 0.098, 0.147, 0.196, 0.294, 0.392 and 0.490 units/ml. For NBT assay, absorbance change measured at 560 nm can be linearized by the method of Asada et al. [20]. The ratio of slope in the absence of SOD to that in the presence of the SOD ($S_0/S$) was plotted versus theoretical concentration of SOD. For WST-1 assay, the linearization of calibration curve was plotted as logarithm of ($\Delta A_0/\Delta A_S$) versus concentration of SOD, where $\Delta A_0 = A_1 - A_3$ and $\Delta A_S = A_S - A_3$. The difference of the two calculations mode is due to the first that a kinetic method is used with NBT and endpoint reading for WST-1 kit assay.

*2.4.3.2. Precision and accuracy.* The precision is the agreement within a series of individual measurements of an analyte when the analytical procedure is applied repeatedly for multiple samplings of a homogeneous population sample. The accuracy is the closeness of mean test results obtained by the analytical method to the true value.

According to the ICH [17,18], precision should be determined in three different cases: repeatability, intermediate precision and reproducibility.

In this work, the repeatability and the intermediate precision (within-lab reproducibility) were determined by the replicate analyse ($n = 5$) at three different SOD concentration levels (i.e., low/medium/high, that is: 0.098, 0.294 and 0.490 units/ml) used for calibration curves. The accuracy of the method was expressed by RE% = (mean measured SOD activity value)/(theoretical SOD activity value) × 100.

*2.4.3.3. Specificity.* The specificity is the ability of the method to determine accurately and specifically the analyte of interest in the presence of other components which can be expected to be present in the same biological matrix where SOD can be isolated (i.e., melon extract). In this work, we checked the SOD activity measurement in the presence of $O_2^-$ scavengers as catalase, glutathione peroxidase (GPX) and Co-enzyme Q 10 (CoQ 10), respectively. Inhibition curves were recorded in the absence and in the presence of the same amount (mg of protein/ml) of catalase, GPX or CoQ 10, than the SOD tested.

## 3. Results and discussion

### *3.1. Validation of NBT method and WST-1 kit Assay*

*3.1.1. Linearization*

For NBT method, the ratio of NBT reduction rate in the absence of SOD to that in the presence of the SOD ($S_0/S$) was shown to be proportional to the concentration of SOD. However, for WST-1 kit assay, the logarithm of ($\Delta A_0/\Delta A_S$) was plotted versus concentration of SOD. The calibration curve obtained by least-squares regression analysis was shown on Fig. 3.

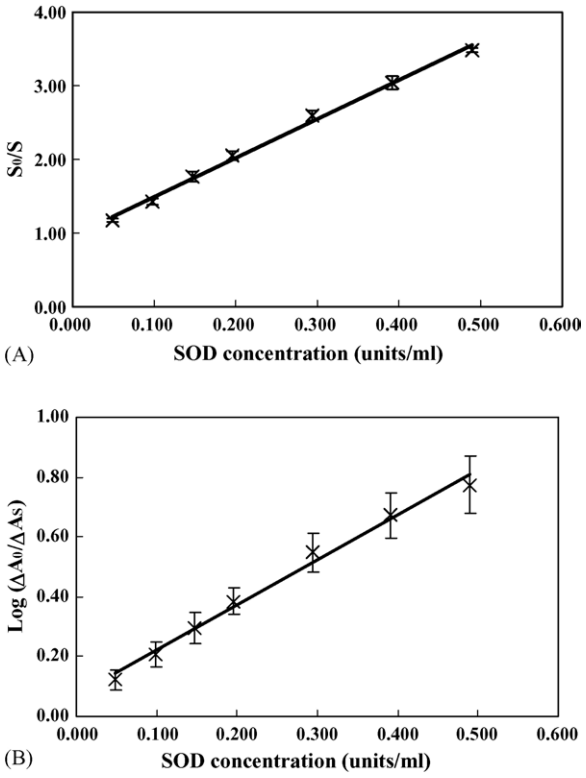


Fig. 3. Linearization of the inhibitory response curve for SOD determination. NBT method (A) and WST-1 kit (B).

The linearity of these two methods was checked by, first, the correlation coefficient which was superior to 0.99 for both (Table 2). The intercepts were statistically different from zero (*t*-test). The significant slopes with a linear relation were also calculated by the *F*-test. Consequently, direct determination of the SOD activity (concentration in units/ml) can be inferred from these two linearized inhibition curves. As seen, the precision remains constant over the whole range of SOD concentrations level tested for NBT test in contrast to WST-1 determination, for which the response uncertainty seems to be linked to the concentration as confirmed by residues plot (data not shown).

### 3.1.2. Repeatability and intermediate precision

As shown in Table 3, the precision is <7.5% whatever the method used. This result although larger than physico-chemical method (especially HPLC) demonstrated a relatively acceptable precision of the two tested methods. It is clear that in comparison to liquid chromatographic methods the recommended specification needed to be adapted, that is in the present case, enlarged.

Table 2
Parameters of linear regressions

| Equation | NBT assay ($n = 7 \times 5$) $R = aC + b$ | WST-1 kit assay ($n = 7 \times 5$) $\log R = aC + b$ |
|---|---|---|
| Slope ± S.D. | 5.289 ± 0.086 | 1.501 ± 0.089 |
| Intercept ± S.D. | 0.961 ± 0.024 | 0.072 ± 0.019 |
| Correlation ($r^2$) | 0.996 | 0.990 |
| $t$ (0.01, 1, 33)[a] comparison of intercept to 0 | 39.7 | 3.7 |
| $F$ (0.01, 1, 33)[b] signification of slope test | 3756 | 477 |

$R = S_0/S$ for NBT method and $R = \Delta A_0/\Delta A_S$ for WST-1 kit assay; $C$ = SOD concentration in units/ml.

[a] The intercepts are different from 0 according to student test, $t(0.01, 1, 30) = 2.457$.

[b] The slopes are significant according to $F$-test, $F(0.01, 1, 30) = 7.56$.

It should be pointed out that, surprisingly, the repeatability seems better for the NBT technique in comparison to the fully automated WST-1 assay whereas the NBT test involves a poorly soluble formazan dye formation which is depicted as a drawback in term of precision of measurement. One explanation should be a lesser overall precision of the optical measurement of the 96 wells micro plate reader in comparison to a classical double spectrophotometer using quartz cells and temperature controlled measurements (±0.1 °C) and a 2 nm bandwidth.

This is confirmed by the intermediate precision measurements, which exhibited an important variability especially for low concentration levels (Table 4).

In both methods lower levels are associated with high variability especially for WST-1 assay. This non-acceptable variability should make that the measurement range being further reconsidered.

### 3.1.3. Selectivity

Some well known antioxidant and $O_2^-$ scavenger species were selected to evaluate the selectivity of both studied assay of SOD activity determination. From our tests, the catalase, glutathione peroxidase and Co-enzyme Q10 did not interfere with SOD activity determination by NBT assay and WST-1 kit method as shown on Fig. 4 which plot the percent of inhibition calculated as described in Section 2.4.2 as a function of SOD concentration (mg/ml) for NBT and WST-1 method, respectively. We used classical statistical one-way analysis of variance (ANOVA) to detect any significant differences ($P = 0.05$) between the four inhibition curves obtained in absence and in presence of the same amount (mg of protein/ml) of catalase,

Table 3
Repeatability

| Sample | NBT assay ($n = 5$) | | | WST-1 kit assay ($n = 5$) | | |
|---|---|---|---|---|---|---|
| | Mean ± S.D. | CV (%) | R.E. (%) | Mean ± S.D. | CV (%) | R.E. (%) |
| Theoretical value (SOD units/ml) | | | | | | |
| 0.098 | 0.093 ± 0.007 | 7.02 | 4.55 | 0.100 ± 0.021 | 20.71 | 2.06 |
| 0.294 | 0.336 ± 0.0156 | 4.69 | 14.23 | 0.301 ± 0.018 | 6.06 | 2.57 |
| 0.490 | 0.506 ± 0.028 | 5.59 | 3.25 | 0.478 ± 0.026 | 5.34 | 2.32 |

Table 4
Intermediate precision (within-lab reproducibility)

| Sample | NBT assay ($n = 5$) | | | WST-1 kit assay ($n = 5$) | | |
|---|---|---|---|---|---|---|
| | Mean ± S.D. | CV (%) | R.E. (%) | Mean ± S.D. | CV (%) | R.E. (%) |
| Theoretical value (SOD units/ml) | | | | | | |
| 0.098 | 0.1035 ± 0.0175 | 16.90 | 5.70 | 0.078 ± 0.044 | 55.85 | 20.29 |
| 0.294 | 0.3404 ± 0.0482 | 14.15 | 15.86 | 0.316 ± 0.042 | 13.33 | 7.68 |
| 0.490 | 0.5131 ± 0.0717 | 13.97 | 4.79 | 0.468 ± 0.064 | 13.62 | 4.53 |

GPX or CoQ10, respectively [21]. So, we can reasonably conclude in the absence of interference of $O_2^-$ scavenger enzymatic system towards the SOD assay. Moreover, it can be noted that WST-1 assay although measured at a lower wavelength (e.g., $\lambda = 450$ nm) than NBT method dose not exhibit any spectroscopic interference with the risk of impairing the results.

### 3.2. Correlation of NBT method and WST-1 kit assay

The activity values of SOD were 24627 ± 1850 units/mg of protein ($n = 5$) and 28105 ± 1980 units/mg of protein ($n = 5$) by NBT method and WST-1 kit, respectively.

The SOD activity determined by NBT method was compared with that obtained by WST-1 kit (Fig. 5). Although established a limited number of samples, an excellent correlation can be observed between the two methods: $y = 1.0113 \times + 0.011$ ($r^2 = 0.997$, $n = 8$). The slope of the regression curve was approximately 1.01, underlying that both methods exhibit a similar sensitivity.

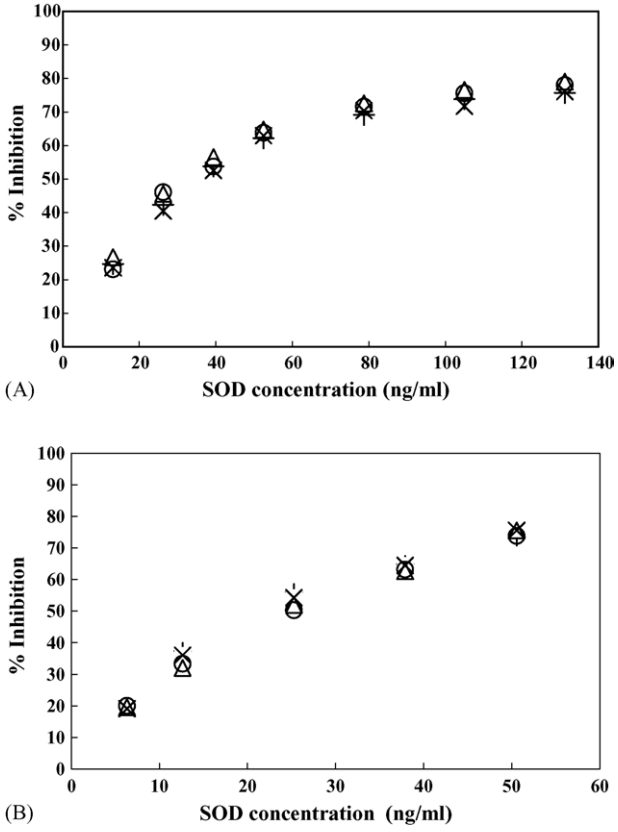


Fig. 4. Selectivity of SOD assay (A) with NBT determination; (B) with WST-1 kit. SOD (+); SOD + Catalase (×); SOD + GPX (△); SOD + CoQ10 (◯) (for experimental detail see text).

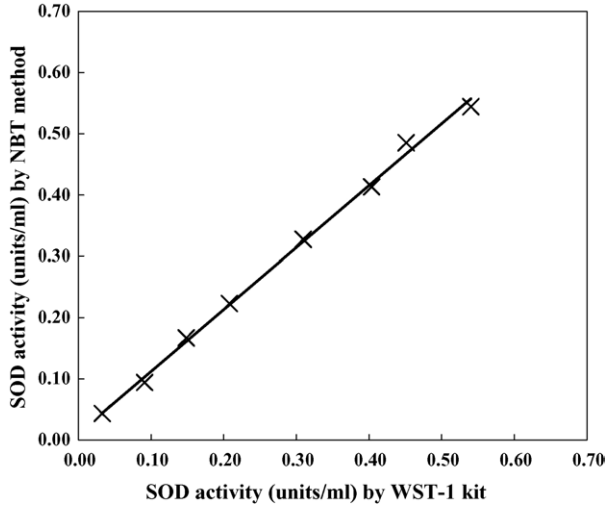


Fig. 5. Correlation between NBT and WST-1 assay determination of SOD.

## 4. Conclusion

From this work, we can draw two main comments. Firstly, it is clearly possible to validate an enzymatic assay according to ICH guideline. The usual specification dealing with physico-chemical methods need to be enlarged although the high degree of automation characterizing the two tested methods. Surprisingly, in our context, the NBT reference method exhibits very similar analytical figures of merits (i.e., precision) than the fully automated WST-1 assay. Consequently, for a routine use in quality control, the NBT reference method, although the known drawback of a relatively poor soluble formazan, can be envisaged in parallel to more automated system as illustrated by WST-1 kit assay. Secondly, this work shows that, provided a similar standard being employed, special care and strict respect of the experimental constraints taken into account, the cross-validation of enzymatic activity determination methods that appear is easily attainable. In the present study, both methods were demonstrated to show fully correlated results, which make the analyst free of choosing the more adapted technique owing to laboratory equipment.

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