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Eliminating Absorbing Interference Using The H-point Standard Addition Method: Case of Griess Assay in The Presence of Interferent Heme Enzymes Such As NOS

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Eliminating absorbing interference using the H-point standard addition method: case of Griess assay in the presence of interferent heme enzymes such as NOS

Abstract Standard calibration methods used to determine trace analytes usually yield significant deviations from the actual analyte value in the presence of interferents in the assay media. These deviations become of particular concern when the concentration of the analyte is low, and when the results are used to draw mechanistic or kinetic conclusions, for instance in enzyme structure-function studies. In these circumstances, the H-point standard addition method (HPSAM) provides superior precision and accuracy. This method is developed here for the case of the spectrophotometric Griess assay used to determine nitrite in various enzymology investigations, such as nitrite determination in studies of nitrite reductases (NiR), or when determining nitrite as a breakdown product of nitric oxide synthesized by NOS enzymes. The results obtained by HPSAM are contrasted with those of the traditional calibration method.

Keywords Nitrite · Determination · NOS · Enzymes · Griess · Assay

Introduction

Determination of trace amounts of nitrite (NO_2^-) is crucial to a number of mechanistic and kinetic investigations addressing the structure-functions of enzymes. Examples include investigations that address the mechanisms and kinetics of the molecular functions of enzymes such as nitrite reductases (NiR) [1], and nitric oxide synthases (NOS) [2]. In the latter case, accurate determination of (NO_2^-) as a breakdown product of nitric oxide (NO) is of particular importance to current investigations, which aim to understand the mechanism of function of NOS enzymes. NOS is a class of heme enzymes which catalyze the in vivo synthesis of the effector molecule NO [3]. NO is synthesized through the oxidation of the amino acid L-arginine, and is used in vasodilation, neurotransmission, cytotoxicity, and numerous physiological processes; it is also involved in the development of a host of pathological states [4, 5].

In NOS enzymology, NO is often quantified in the form of NO_2^-, a stable breakdown product of NO in aerobic reaction media, by using a standard calibration method that uses the Griess assay. This method is one of the most popular and simplest used to spectrophotometrically detect NO_2^- concentration [6, 7]. This assay is based on a two-step chemical derivatization reaction, using sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED), and yields an azo-compound with λ_{max} at 540 nm; the latter is used to quantify the NO_2^- originally present. The Griess assay is widely used to determine NO_2^- in a variety of biological and experimental matrices, such as plasma, serum, urine and tissue culture media [8, 9]. However, serious accuracy problems may arise in the presence of absorbing interferences, especially if these are not taken into account during the calibration step.

For instance, an issue of major concern to those using the Griess assay to determine N_2O^- in NOS enzymology using standard calibration curves is that the absorption band of NOS-heme as well as that of the flavins around 520–540 nm in full-length NOS may positively interfere with the absorption reading of the azo-compound, especially when the enzyme is not separated by size exclusion prior to the Griess assay. The same is true for any heme-protein with heme Q-band absorption overlapping with that of the target azo-compound [10, 11]. Even if the enzyme is separated by size exclusion prior to conducting the assay, trace amounts of protein may remain in the solution and may significantly interfere with the final reading. This is especially critical in the case of

NOS, since the molar extinction coefficient (at 540 nm) of NOS is quite large. Therefore, the traditional standard calibration curve may lead to erroneous quantitation.

An alternative method called standard addition can be used instead. However this method cannot remove the constant error resulting from other absorbing components in the system, although it may remove the error resulting from the sample matrix. To overcome this obstacle a new procedure called the H-point standard addition method (HPSAM) was introduced in 1988 by Bosch-Reig et al [12, 13]. The method is mainly based on the principle of the dual wavelength spectrophotometer coupled with the standard addition method. Among other advantages of this method is the fact that it eliminates interferences caused by absorbing components intrinsic to the system being analyzed, as well as interference from the blank matrix solution [14, 15, 16]. The method is growing in popularity, and continues to find new applications in a number of fields [17, 18, 19, 20, 21]. A modified version of HPSAM, called generalized HPSAM, can be used to quantify the analyte concentration even when the nature of the interferent is not known [22, 23].

The objective of the present work is to explore the relative error (due to interference) associated with NO_2^- determination when performing the traditional standard calibration curve rather than the HPSAM. The performance of the new method is contrasted with that of the traditional calibration, especially when dealing with the very low NO_2^- concentrations usually involved in enzymatic initial rate determinations or under single turnover conditions.

The method is first developed and validated with myoglobin, a cheap commercially available heme-protein that gives interfering absorption similar to NOS in the 520–540 nm region. Then, determinations of NO_2^- in the presence of mouse inducible NOS (iNOS) are conducted to prove the applicability of the new method to the NOS case.

Experimental section

Reagents

All the chemicals used were of analytical grade. Nanopure deionized water (specific resistance $>18.2\ \Omega$cm) used throughout the experiment was supplied by a Barnstead water purification system. All working solutions of NO_2^- were prepared using 0.1 M standard NO_2^- solution. Heme-proteins, Horse Heart myoglobin and mouse inducible NOS, iNOS, were purchased from Sigma-Aldrich. Original concentrations of hemeproteins were quantified using their molar extinction coefficients (163.9 $mM^{-1}\ cm^{-1}$ at 410 nm for myoglobin [24], and 71 $mM^{-1}\ cm^{-1}$ at 414 nm for iNOS [25]). The Griess reagent kit was purchased from Promega and was used according to the technical instructions of this supplier.

Apparatus

UV-visible absorbance spectra were recorded on an Agilent 8453 spectrophotometer using 1-cm path UV-Vis cells. Spectra were collected between 400 and 650 nm. Measurements of pH were made with an Accumet AB15 pH-meter using a combined glass electrode.

Procedure

Myoglobin was dissolved in pH 7.6 phosphate buffer (10 mM). This solution was purified by filtration on YM30 membranes (Amicon); its concentration was quantified by measuring its absorbance at 410 nm. Spectra were recorded separately for the azo-compound, for myoglobin, and for iNOS in the region of 450–620 nm (Fig. 1) to obtain the best working wavelengths for HPSAM (see Sect. 3.1, "Selection of wavelengths"). Standard samples containing four different concentrations of NO_2^- (10, 7.5, 5.0 and 2.5 μM) in the presence of three different concentrations of myoglobin (10, 15, and 20 μM) were prepared, giving a total of 12 working standards. The total volume of each sample analyzed was 6.00 ml. The range of nitrite concentration in these working samples was selected based on actual ranges used in enzymology studies. Each standard was divided into 6×1.00 ml aliquots. Then known amounts of NO_2^- standard were successively added into each vial followed by appropriate amounts of the Griess reagent system (sulfanilamide and NED solutions). The mixtures were incubated for 10 min. The resulting solutions were then diluted twice (to 2 ml) with deionized water. The NO_2^- concentration in each standard was then quantified by performing HPSAM (see Sects. 3.2 and 3.4) by measuring the absorbance at two pre-selected wavelengths. Each data point on the graphs is

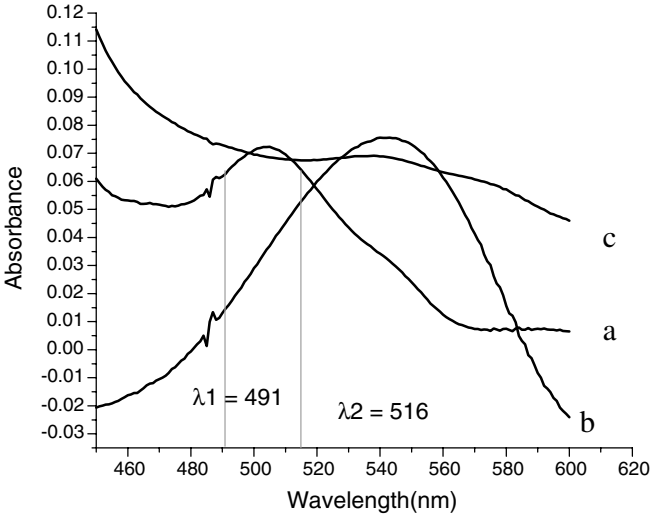

Fig. 1a c Absorbance spectra of **a** 10 μM myoglobin, **b** azo compound of Griess assay and **c** 10 μM iNOS

the mean value of results of two separate experiments. For comparison purposes, the NO_2^- concentration in each sample analyzed by the HPSAM was also quantified using a standard calibration curve constructed at 540 nm. The same procedure was performed with 10 μM iNOS as an interferent for three different NO_2^- concentrations. Determinations of NO_2^- generated in situ (through the breakdown of NO synthesized in the medium by iNOS enzymes), were also conducted as described above using the two methods.

The procedure for the NOS assay was as follows: 100 μl of iNOS (8 μM) were added to 1.00 ml of 50 mM HEPES buffer at pH 7.4 containing 0.1 mM NADPH, 1 mM arginine, 12 μM tetrahydrobiopterin and 170 μM DTT, all pre-warmed at 37 °C for 15 mins. The NOS reaction was then allowed to proceed for 12 min at 37 °C. The reaction was quenched after 12 min by adding 100 μl of ice-cold-deionized water into the reaction vessel and then stored in ice for the assay. The content of the reaction vessel was divided into six aliquots of 200 μl on which standard addition and calibration methods were carried out as described in the text.

Results and discussion

This study explores the impact of absorbing interferences on the determination of small amounts of NO_2^- using the Griess assay in the presence of interfering heme proteins, and develops a method based on HPSAM to eliminate the effect of absorbing interference(s) (see requirements in electronic supplementary material, Appendix 1 [12]). Figure 1 shows absorbance spectra for the terminal azo-compound used to determine NO_2^-, in addition to the spectra of the hemeproteins myoglobin and iNOS in the same region.

One can clearly see that when the hemeproteins myoglobin and iNOS are used in a concentration range similar to that used in actual determinations, they give significant overlapping absorbance in the 520–540 nm range, which is the most desired range of wavelength used to quantify NO_2^- in the Griess assay. It is therefore expected that the standard calibration method in the presence of these interferences will lead to significant errors.

Optimization of the system

Selection of pH

The influence of pH value on the absorbance of the azo-compound in the presence of constant interferent concentration was studied. The absorbance was found to be the highest with an assay solution at pH 7.4. This pH was then selected as the optimum pH for all assays, which also turns out to be the pH value most often used in enzymatic studies.

Selection of wavelengths

Two wavelengths for each interferent (myoglobin and iNOS) were selected based on their absorbance in order to fulfill the HPSAM requirement. At these selected wavelengths, the absorbance must be linear with the concentrations and the interferent absorbance must remain equal (electronic supplementary material, Appendix 1). Also, the analytical signal obtained from a mixture containing both the analyte and interferent should be equal to the sum of the individual signals of the two components. In addition, good accuracy and precision can be achieved when the difference in the slopes of the two straight lines measured at the two preselected wavelengths (λ_1 and λ_2) are large. Taking all of these criteria into account, the wavelengths 491 and 516 nm were found to be the best pair of wavelengths for myoglobin, while for iNOS, 544 and 554 nm were selected as the working wavelengths [26]. However, it is important to note that, although very rare, cases may exist where the selection of the working wavelengths may be prohibited by either the complexity of the sample or by the interferent not necessarily being known.

The HPSAM with myoglobin as interferent

Several working standard samples containing different concentrations of myoglobin were analyzed using the proposed HPSAM. Figure 2 was obtained by plotting the standard addition curves at 516 and 491 nm for different nitrite concentrations in the presence of 10 μM concentration of myoglobin. As described in the experimental section, the standards are diluted two times, and therefore the value at the crossing point of the two lines of each graph (which corresponds to the concentration of NO_2^- in the standard) should be multiplied by a factor of two to reflect the original concentration in the analyzed sample. As can be seen from Fig. 2, the concentrations found by the HPSAM satisfactorily reproduce the concentrations in the working samples analyzed. It is worth mentioning that these results are obtained using the samples as prepared in the presence of the interferent without pre-separation steps by size-exclusion membranes. The observed deviations from actual values in the presence of 10 μM of myoglobin as interferent are all very small compared to the deviations encountered when using the traditional calibration curves (see Sect. 3.3).

The recovery data in Table 1 shows that, except for the lowest analyte concentration (2.5 μM), and only at high interferent concentrations (15 and 20 μM), the HPSAM performs relatively well given the presence of high interferent concentration (10–20 μM). Despite the deviations from the values of the standards, the performance of the HPSAM is still superior to the traditional calibration in the same conditions (see Sect. 3.3). While myoglobin is a good mimic for developing and validating the HPSAM for the Griess assay in the presence of

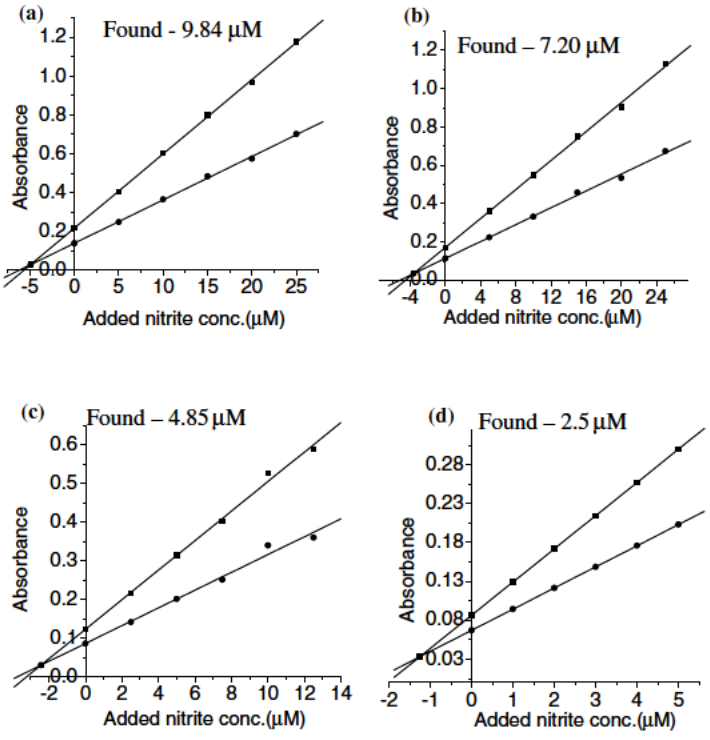

Fig. 2a d Standard addition curves obtained for **a** 10 µM, **b** 7.5 µM, **c** 5.0 µM, and **d** 2.5 µM concentrations of NO^-_2 in the presence of 10 µM myoglobin as interferent. *Top line* (*filled squares*) in each plot corresponds to the absorbance at 516 nm; *bottom line* (*filled triangles*) corresponds to the absorbance at 491 nm. Dilution factor for each standard is 2; the concentration found for each case is given at the top of the graph

interfering heme-enzymes, its specific chemistry with NO_2^- ions may interfere. The observed deviations between found and actual values may be due in part to this possible chemical interaction, which may lead to changes in the absorbance at 491 and 516 nm for myoglobin as interferent; as a requirement for the HPSAM, the absorbance at these pre-selected working wavelengths should be the same as described in the Appendix (supporting info). Even though the concentrations of interferent used in these validation assays are high, and far beyond possible interferent levels present in actual experiments in the presence of heme enzymes, the HPSAM still seems to be much more reliable than the conventional calibration method often used in enzymology, for instance in NO quantification in the form of NO_2^- in the presence of NOS heme-enzymes.

Comparison of HPSAM results with those obtained with calibration

To appreciate the accuracy of the HPSAM we compared the performance of this method with that of the traditional calibration in the same conditions. Figure 3a shows the deviation of obtained NO_2^- concentrations from actual values using the calibration method in the 2.5–10 µM range of NO_2^- concentration in the presence of different levels of interfering myoglobin concentrations. A significant discrepancy between obtained and actual nitrite concentration values is clearly observed, especially for higher interferent concentrations and/or when the concentration of NO_2^- to be determined is low. For instance, as much as 244% recovery is observed for a 2.5 µM NO_2^- standard in the presence of just 15 µM interferent concentration. Divergence from the standard is even worse at higher interferent concentration, see Table 2. While an exhaustive study of the effect of the presence of interferent on the performance of the calibration was not conducted, it is clear that deviations from actual values will be significant for samples with very low NO_2^- concentration.

The performance of the HPSAM has been tested in the same conditions used for the calibration method. NO_2^- concentrations obtained by the HPSAM are plotted as a function of actual values in Fig. 3b.

The relative accuracy of the HPSAM over calibration in reproducing NO_2^- concentrations is remarkable, particularly, if one considers the level of the hemeprotein

Table 1 Summary of recovery data by HPSAM as applied in the Griess assay in the presence of myoglobin as the hemeprotein interferent (at 10, 15 and 20 µM)

	Recovery data (µM (%))			
Nitrite standard (µM)	10	7.5	5.0	2.5
Myogobin interferent at 10 µM	9.84 (98.4)	7.20 (96)	4.85 (97)	2.5 (100)
Myogobin interferent at 15 µM	10.3 (103)	7.64 (102)	5.10 (102)	3.18 (127)
Myogobin interferent at 20 µM	9.20 (92)	7.72 (103)	5.25 (105)	3.46 (138)

Table 2 Summary of recovery data by the conventional calibration as applied in the Griess assay in the presence of myoglobin interferent (at 10, 15 and 20 μM)

	Recovery Data (μM (%))			
Nitrite standard (μM)	10	7.5	5.0	2.5
Myogobin interferent at 10 μM	9.54 (95.4)	7.71 (103)	5.40 (108)	3.73 (149)
Myogobin interferent at 15 μM	12.0 (120)	9.5 (127)	7.28 (146)	6.11 (244)
Myogobin interferent at 20 μM	12.6 (126)	10.4 (139)	8.8 (176)	6.69 (268)

interferent present in the assay. Compare for instance the 100% recovery for a 2.5-μM standard in the presence of 10 μM heme protein interferent, to 149% recovery for the same sample using the traditional calibration curve. The HPSAM eliminates or significantly minimizes the effect of absorbing interferent. Although there are some slight deviations (like 127% recovery for 2.5 μM standard in the presence of 15 μM interferent) observed with the HPSAM determinations at low NO_2^- concentrations (2.5 μM), the method is still far more reliable than a *blind* calibration in the presence of absorbing interference.

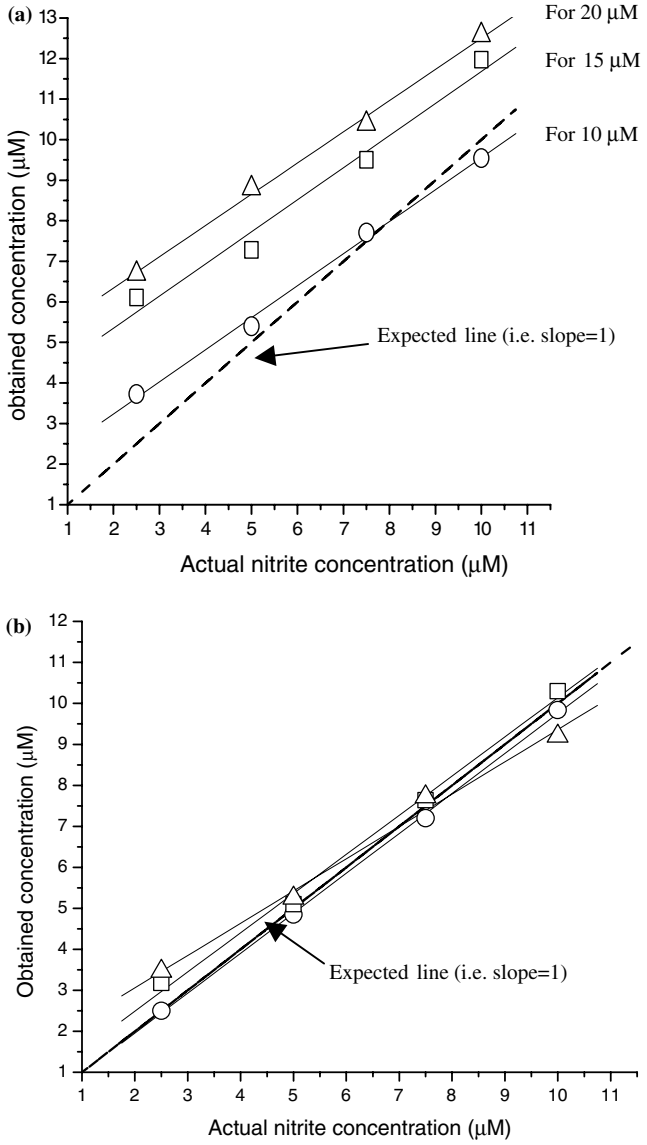

Fig. 3a, b Comparison of obtained and actual nitrite concentration values using **a** the traditional calibration method at 540 nm and **b** the new HPSAM. Three different (interferent) myoglobin concen trations were used: (*open circles*) 10 μM, (*open squares*) 15 μM, and (*open triangles*) 20 μM. The *dashed line* has a slope of unity and represents the target expected values

The HPSAM in the case of iNOS as interferent

The proposed method was tested with samples containing different NO_2^- concentrations in the presence of 10 μM iNOS as the absorbing interferent. Again, Figure 4a shows that the traditional calibration method cannot be used in this assay to accurately determine the concentration of NO_2^-, particularly, when using this information to derive mechanistic and/or kinetic conclusions (like in mechanistic/kinetic studies of the molecular function of NOS enzymes, where the end product, NO, is determined in the form of NO_2^- ions using the Griess assay). As shown previously, the deviation from actual values increases as NO_2^- concentration decreases; in fact, a deviation of 101% from the actual value was recorded with the calibration curve when the NO_2^- concentration is 2.5 μM in the presence of 10 μM iNOS interferent. This discrepancy between measured and actual concentrations values in the presence of iNOS interferent observed in the calibration almost disappears when using the HPSAM. Figure 4b shows NO_2^- concentration values obtained by the proposed HPSAM as a function of actual values in the presence of 10 μM iNOS as interferent.

Except for the low end of the nitrite (2.5 μM) where a deviation is observed, NO_2^- concentration values measured in these conditions using the HPSAM are very close to the actual values. Even for the low range of NO_2^- concentration (2.5 μM), the performance of the HPSAM (24% deviation) is still superior to that of the traditional calibration method (101% deviation).

Comparison of the performance of HPSAM and standard calibration in actual NOS reactions

Assays for mouse inducible NOS were experimentally performed for 12 min under the conditions given in the enzyme kit. NO (in the form of NO_2^-) generated after the catalytic oxidation of the substrate arginine was then quantified using the proposed HPSAM and standard calibration methods. The values of NO_2^- concentration

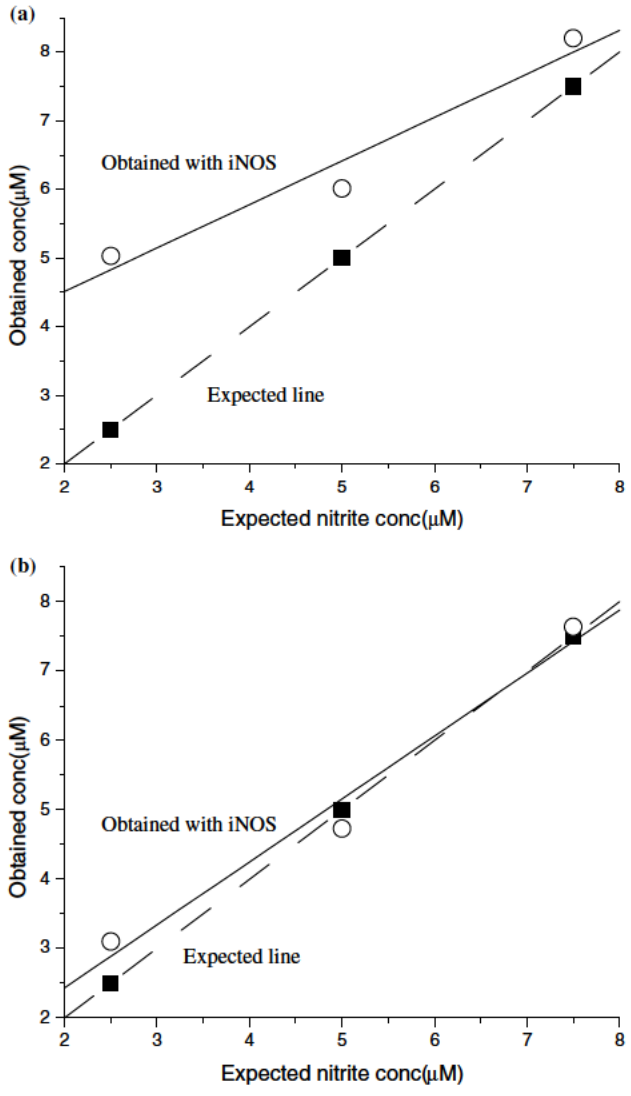

Fig. 4 Comparison of obtained and actual concentration values for results for **a** the traditional calibration method (at 540 nm) and **b** for HPSAM, in the presence of 10 μM iNOS as interferent

for the two methods are shown in Table 3, and are to be compared with the calculated concentration value based on the number of enzyme units (activity) provided by the supplier.

The results clearly show that the performance of HPSAM in the Griess assay case in actual NOS reaction and in the presence of this heme enzyme is superior to that of the traditional calibration method in the same conditions.

Table 3 Nitrite determination by HPSAM and by calibration as applied in the Griess assay in actual NOS reactions

Nitrite concentration based on enzyme units used (μM)	HPSAM method (μM (±SD))	Standard calibration method (μM (±SD))
5	4.31 (±0.17)	7.21 (±0.22)

Table 4 Nitrite determination by HPSAM and by calibration as applied in the Griess assay in controls (in other words, zero turn over in absence of NADPH) in the presence of a known amount of NO^-_2

Added NO^-_2 concentration (μM) with zero turnover	HPSAM method (μM (±SD))	Calibration method (μM (±SD))
5.0	5.1 (±0.34)	6.95 (±0.25)
4.3	4.22	6.83

Although small, the deviation from the calculated concentration may be due to the uncertainty in the values of the enzyme unit number of the sample used (the enzyme activity as determined by the supplier prior to shipping). In this regard, control experiments were run in the absence of NADPH (source of electrons, so no enzyme turnover in its absence), but in the presence of known concentrations of nitrite standard. The Griess assay was then used to determine the NO_2^- concentration in the two methods for comparison. The results of these assays are shown in Table 4.

These control experiments clearly demonstrate that the HPSAM, as developed here for the Griess assay in conditions similar to NOS assays, satisfactorily reproduces the amount of NO_2^- present (in the 5 μM range), while the traditional calibration method performs poorly. The concentrations found by HPSAM are very close to the known added NO_2^- amount in the assay medium. This tends to confirm that the slight deviation (Table 3) from the calculated value based on the enzyme units used in the actual enzyme turnover may indeed be due to the uncertainty in the enzyme activity (unit number) of the batch used in this study.

Conclusion

In this study, the HPSAM is introduced and developed for the case of the Griess assay, used to determine NO_2^- concentration in a number of enzymology investigations. This assay may yield significant discrepancy between actual and found concentration values, particularly in the presence of absorbing interferents such as heme/flavoproteins. The HPSAM significantly minimizes, if not eliminates, the effects of this type of absorbing interference and faithfully reproduces the concentration of NO_2^- even in the presence of relatively high interferent concentrations. The method would be suitable for NO_2^- determinations in mechanistic/kinetic investigations that address the function of flavo/heme enzymes, where a pre-separation step for the enzyme/protein would not be necessary.

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26. As suggested by one of the reviewers, the method can also work as a multivariate method through the selection of several pairs of wavelengths fulfilling HPSAM requirements (see [12])