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Fast microplate assay for simultaneous determination of thiols and dissolved sulfides in wastewater

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Abstract

The present paper reports the development and the application of a microplate assay for the fast and individual determination of thiols and sulfides. The method is based on the reaction of ethyl propiolate with thiols and sulfides, yielding thioacrylates absorbing in the UV range. Thioacrylate generated by derivation of sulfide with EP presents a bathochromic shift of maximal absorbance wavelength of 16 nm compared to thioacrylates formed with thiols. EP concentration, reaction time and derivatization pH were carefully optimized in order to separately quantify thiols and sulfides using a spectral deconvolution procedure in 15 minutes. The detection limits were 0.6 and 2.8 μM (63.7 and 92.4 $\mu\text{g.L}^{-1}$) for mercaptopropionic acid (used as reference thiol compound for calibration) and dissolved hydrogen sulfide respectively. Non-thiol sulfur compounds (methionine, sulfates and sulfites) or other nucleophile compound did not display any interference in the determination of MPA or sulfides. Interference from metals was suppressed with application of a reduction step with TCEP and DTPA. Finally, the method has been successfully applied to the analysis of raw and treated wastewater.

Keywords: thiols; ethyl propiolate; hydrogen sulfide; high-throughput analysis; UV spectral deconvolution.

1. Introduction

Malodorous emissions and odor control in collection and treatment of wastewater remain a major concern for operators of wastewater treatment plants (WWTP). Organic and inorganic compounds, directly or indirectly responsible for odors, are emitted by sewage or result from the anaerobic degradation of organic matter containing sulfur or nitrogen. Among these compounds, the two main inorganic odorous compounds are hydrogen sulfide and ammonia [1], but we can also mention thiols, indoles and other inorganic or organic nitrogen and sulfur compounds. Complex mixtures of sulfur compounds at low concentrations (range from $\mu\text{g}\cdot\text{m}^{-3}$ to $\text{mg}\cdot\text{m}^{-3}$) form the majority of these malodorous emissions associated with sewage treatment works. Hydrogen sulfide (H_2S), carbon disulfide (CS_2), methyl mercaptan or methanethiol (CH_3SH), dimethyl sulfide [$(\text{CH}_3)_2\text{S}$] and dimethyl disulfide [$(\text{CH}_3)_2\text{S}_2$] were the most frequently identified compounds in influent and primary effluent of a WWTP [2,3].

Thiol compounds are often quantified by separation methods like gas chromatography coupled to flame photometric detector [4] or mass spectrometer [3], or like high performance liquid chromatography (HPLC) with pre-column derivatization coupled to fluorescence [5-8], UV detection [9] or mass spectrometry [10]. Several studies have also focused on the global quantification of thiol levels mainly in biological systems by absorbance [11,12], fluorescence measurement [13,14] or enzymatic activity [15]. Many easy-to-use microplate kits, usually based on the use of the Ellman's method or fluorescent maleimide derivatives and calibrated using standard solutions of glutathione and/or cysteine, are commercialized for the measurement of free or total thiols in biological samples. Traditional Ellman's reagent can also be used for inorganic sulfides determination and numerous other analytical techniques reported

for hydrogen sulfide determination in natural waters or sewage may be mentioned including spectrophotometry [16,17], fluorimetry [18], HPLC coupled to UV-Vis detector [19], inductively coupled plasma-atomic emission spectroscopy [20] or flow systems [21,22].

Many analytical systems exist for hydrogen sulfide analysis in the gas phase. However, it is also important to monitor dissolved sulfides and thiols so as to predict H₂S emissions at facilities such as sewage treatment plants or to control the quantities of chemical inhibitors to add to a wastewater stream. Some authors have developed methodologies based on multi-wavelengths exploitation of UV-Vis spectra for the determination of sulfides in natural waters [23] or dissolved organic sulfide (DOS) in industrial wastewater [24], but these techniques require pH buffering of samples and suffer from relatively high detection limits.

Recently, Owen (2008) has described the reaction of thiol compounds with esters of propiolic acid (like ethyl propiolate, EP) under mild conditions to form stable and strongly UV absorbing thioacrylates [25]. Tzanavaris et al. (2010) have adapted this derivatization method as a sequential injection analysis system for the determination of thiols in pharmaceutical samples [26]. Even though these two studies demonstrated good selectivity of this derivation method, our preliminary tests showed that sulfides could also react with ethyl propiolate to form a stable derivative with a red shift of the maximum absorption wavelength of about twenty nanometers as compared to thiols derivatives (Fig. S1).

In the present work, we decided to optimize the derivatization properties of ethyl propiolate (EP) to develop a new and simple spectrophotometric high-throughput assay in a 96-well plate for simultaneous determination of thiols and sulfides in wastewater. The assay was validated by comparison with Ellman's and OrthoPhtalAldehyde-AminoEthanol (OPA-AE) methods on real wastewater samples.

2. Experimental

2.1. Reagents and solutions

All chemicals used in this study were of analytical reagent grade. Ethyl propiolate, sodium sulfide nonahydrate, mercaptopropionic acid, mercaptoacetic acid, dimethyldisulfide, ethanethiol, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), cysteine, glutathion, 2-mercaptoethanol, *o*-phthaldialdehyde and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were from Alfa-Aesar (Schiltigheim, France). EP and other thiol compounds stock standard solutions and working solutions were prepared in ultrapure water (Millipore, resistivity >18 MΩ cm).

2.2. Instrumentation

Microplate absorbance and fluorescence measurements were carried out on a microplate reader (Infinite M200, Tecan France SAS, Lyon, France) controlled by i-control™ software (Tecan) in clear polystyrene 96 flat-bottom well microplates for absorbance or black polystyrene 96 V-bottom wells shape microplates for fluorescence (Fisher Scientific, Illkirch, France).

2.3. Analysis of thiols and dissolved sulfides

A protocol for simultaneous determination of thiols (R-SH) and sulfides (HS⁻) has been developed at the microplate level. In addition, a reduction step by TCEP has enabled the determination of total thiols (R-SH + R-S-S-R' + R-S-Me-S-R', with Me a metal and R and R' a functional group) by measuring variation of signal after and before reduction. To compare the performance of this analytical method, thiols determination was also performed with two other methods traditionally used for this purpose and optimized in microplate format: OPA-AE derivatization method and Ellman's method. The various protocols are described below.

2.3.1. High throughput assay for reduced and total thiols, and dissolved sulfides determination by EP derivatization

The reduction of total thiols was carried out by adding 10 μ l of TCEP 20 mM in a buffer solution (0.2 M borate pH 9.5 and DTPA 2 mM) to 100 μ L of sample or standard. The plate was shaken and after 8 min 50 μ L of EP 50 mM in 0.2 M borate buffer pH 9.5 was added and absorbance spectrum was measured between 230 and 350 nm after 2 min for total thiols determination and 15 min for dissolved sulfides determination.

The same protocol can be used for reduced thiols determination by directly dispensing 100 μ L of sample into the wells of the microplate, without the reduction step by TCEP. Both protocols are summarized in Scheme 1. Quantification of total or reduced thiols and dissolved sulfides was carried out by spectral deconvolution using ACD/Spectrus Processor software (ACD/Labs 2012, Totonto, Canada) and the SIMPLISMA (SIMPLe-to-use Interactive Self-modeling Mixture Analysis) tool, based on a two-stepped regression procedure using the normalized spectral intensities [27].

2.3.2. Reduced and total thiols determination by OPA-AE method

In this paper, the OPA-AE method was adapted and optimized for microplate analysis [28] and analytical protocol was as follows: 15 μ L of sample or standard solutions was dispensed into the wells of the microplate, where 145 μ L of a reagent solution (AE 26 mM and DTPA 20 mM in 0.05 M borate buffer pH 9.9) and 130 μ L of OPA 50 μ M in ethanol were then added. Reduced thiols determination was carried out by fluorescence intensity measurement after shaking for 7 min, with wavelengths set at 330 nm and 455 nm for excitation and emission respectively.

The analytical protocol was also adapted to total thiols measurement: during the first step, 15 μ l of sample was first mixed with 15 μ l of TCEP 20 mM. After a shaking time of 6 min, the analytical protocol was continued as described in the previous paragraph.

Inorganic sulfides do not interfere in thiol compounds measurement with this OPA-AE method (Fig. S2).

2.3.3. Reduced thiols determination by Ellman's reagent

The determination of reduced thiols was also performed using Ellman's method adapted to a microplate measurement. Briefly, 100 μ l of sample or standard solution was mixed with 100 μ L of a DTNB reagent (1mg/ml in 200 mM phosphate buffer pH 8). The absorbance was measured at 412 nm after incubation for 15 minutes.

3. Results and discussion

3.1. Thiols and sulfides derivatization

As displayed in Supplementary data (Fig. S1), the maximum absorption wavelength of the products either formed between EP and a thiol compound such as mercaptopropionic acid (MPA) or with sulfide ions is found at 288 nm and 304 nm, respectively. These wavelengths have been selected for the course of this study. We deliberately chose MPA as the reference thiol compound herein because this compound has an important environmental significance [29] and possesses one of the higher pKa of thiol compounds [25], therefore requiring higher pH value than other thiols to reach optimal reaction yield (reaction scheme between MPA and EP is presented in Fig. S3).

3.1.1. pH optimization

Thiols derivatization with EP is usually carried out at basic pH to increase the reaction rate [30]. In the same time, it is well known that EP can react with other nucleophiles such as amines to yield UV absorbing adducts [25,31]. Although these interfering compounds usually react slowly with EP, they can potentially cause serious interferences in the case of environmental

1 samples with a wide range of concentrations for these compounds. Careful choice of the pH
2 value during derivation step can limit these interferences.

3 Fig. 1 displays the evolution of absorbance of EP derivatives as a function of pH value, formed
4 with i) MPA for reference thiol compound, ii) hydrogen sulfide and iii) glycine as reference
5 amine compound.

6 The absorbance of MPA derivative was stable at pH above 10, which is consistent with the
7 acidity of the thiol group for MPA ($pK_a = 10.3$) that allows reaction of MPA with EP through
8 its thiolate group. Regarding sulfides, the optimum pH for the reaction was between 9 and 9.5,
9 which suggests that EP reacts with the HS^- form of sulfides. Finally, as expected, glycine shows
10 higher response at pH around 10.5.

11 In this study, a pH value of 9.5 was thus selected as the best compromise for maximizing the
12 response of sulfides and thiols and minimizing interference of amines.

14 3.1.2. Effect of EP concentration and reaction time

15 EP concentration has obviously a significant influence on the reaction yield. The effect of EP
16 concentration was studied in the 1-75 mM range for MPA and hydrogen sulfide at pH 9.5. The
17 experimental results are shown in Fig. 2 and Fig. 3.

18 EP concentration greater than 10 mM had no significant impact on the derivatization yield of
19 MPA (Fig. 2), as demonstrated in previous studies concerning other thiols of biological or
20 environmental interest [25,26,32]. On the other hand, it can be seen that absorbance of the EP
21 derivative with HS^- is highly dependent on the concentration of EP. Hydrogen sulfide needs a
22 large excess of EP to reach significant yields, probably due to the fact that hydrogen sulfide has
23 a lower nucleophilicity than thiols. A concentration of 50 mM of EP was thus the best
24 compromise for the derivatization of MPA and sulfides and was selected for further

experiments. Glycine response was also tested at this concentration of EP and was still very low (absorbance < 0.05).

Time-dependence of derivatization has been assessed by acquiring absorbance *versus* time during the reaction between EP and MPA or hydrogen sulfide. Experiments were carried out with a 50 mM EP solution (Fig. 3). Optimal reaction times were 2 minutes for the derivatization of MPA (thereby limiting the cross interference of sulfides) and 15 minutes for the derivatization of sulfides and were therefore selected for the following experiments.

3.1.3. Oxidized thiols

It is well known that thiol group can easily complex a wide range of metals in environmental samples. Trace metals such as Cu^{2+} , Cd^{2+} , Hg^{2+} , Pb^{2+} and Zn^{2+} have particularly high affinity for SH groups, and we noticed that some complexes between thiols and metals almost completely quenched subsequent reaction with EP (Cu^{2+} and Fe^{3+} especially). Moreover, thiols can also be oxidized and form disulfide bond, which cannot be detected by direct reaction with EP. To quantify total thiols content of a sample, the analytical protocol should therefore include a reducing step to unbind metals from thiols or cleave disulfide bonds. Dithiothreitol (DTT) is one of the most used agent for reducing thiols. Its use is however limited because DTT is not stable due to air oxidation and must be kept cool and under an inert atmosphere. In recent years, TCEP was used as thiols reducing agent [32,33] and offered many advantages such as an improved stability in aqueous solution and a reactivity in a wider pH range than DTT.

Preliminary studies on quantification of MPA when bound with Cu^{2+} ions showed that TCEP alone was insufficient to correctly quantify the concentration of MPA due to 45% yield of bond cleavage (Fig. S4). Quantification could only be achieved after a reduction step by TCEP in the presence of DTPA as complexing agent with a release of 97% of free MPA (Fig. S4). In this sense, some experiments were carried out in order to optimize amount concentration of TCEP

during reduction step while a concentration of 2 mM DTPA was kept constant. The reducing efficiency of TCEP was evaluated by spiking a 50 μM MPA solution with 160 μM Cu^{2+} . Increasing concentrations of TCEP were added to the spiked MPA solution in the presence of 2 mM DTPA and left to react 6 minutes (Fig. 4.A). Experimental results showed that more than 97% of MPA was released within 6 minutes with a TCEP amount concentration between 20 and 40 mM. Reaction yield of reduction by TCEP was estimated by acquiring absorbance of EP-MPA derivative as a function of time when reduction carried out with 20 mM TCEP and 2 mM DTPA (Fig. 4.B). A reaction time of 8 minutes was sufficient to quantify total MPA content. Some experiments were also conducted to optimize the concentration of DTPA. The results showed that a concentration of 2 mM DTPA was sufficient to effectively bind Cu^{2+} and a higher DTPA concentration resulted in an increase of blank signal. A final concentration of 20 mM TCEP and 2 mM DTPA with a reaction time of 8 minutes were selected for final analytical protocol and further experiments.

3.2. Analytical parameters

The analytical parameters were defined using the optimal analytical protocols. The calibration curve obtained for MPA determination was $\text{Absorbance} = (0.006 \pm 0.0001)C + (0.0267 \pm 0.003)$, where Absorbance was measured at 288 nm and C was the MPA concentration in μM ($R^2=0.9986$; n=7 triplicates). Calibration curve was linear on the range 0-500 μM . The calibration curve obtained for sulfides determination was $\text{Absorbance} = (0.0105 \pm 0.0004)C + (0.0105 \pm 0.005)$, where Absorbance was measured at 304 nm and C was the sulfide concentration in μM ($R^2=0.9962$; n=7 triplicates). Calibration curve was linear on the range 0-250 μM . The detection limits (3σ , n=10) were 0.6 and 2.8 μM (63.7 and 92.4 $\mu\text{g.L}^{-1}$) for MPA and sulfide respectively and the quantification limits (10σ , n=10) were 2.1 μM (222.9 $\mu\text{g.L}^{-1}$) for MPA and 7.3 μM (240.9 $\mu\text{g.L}^{-1}$) for sulfide. The relative standard deviations (RSD) were

1 calculated from 10 successive standard determinations (100 μ M) and reproducibility of 1.8%
2 and 1.9% was obtained for MPA and sulfide respectively.

3 In Table 1, main analytical features of our analytical protocols and previously developed
4 methods are compared. The present work shows that the developed method has a detection limit
5 among the lowest with a 2 minutes reaction time compared to 5 to 60 minutes for other methods.
6 NAM method [13] has a similar detection limit (1 μ M) but requires a 60 minutes reaction time
7 and the response of various thiols is not uniform. The method also suffers from interference of
8 sulfites [34]. PAPAIN method [15] has the best detection limit and sensitivity but requires
9 degassing of solutions beforehand for 1 hour. The 4-DPS method displays improved reactivity
10 and sensitivity compared to the Ellman's method with a limited reaction time [12]. However, it
11 does not differentiate thiols and sulfides. The OPA-AE method adapted from the study of
12 Mopper and Delmas [8] has better analytical features but has the major drawback, as the NAM
13 method, to present a very variable reactivity for the different thiols (Fig. 5). Fig. 5 depicts the
14 relative reactivity of various thiols based on MPA as a reference. The EP method displays good
15 homogeneity in the response of the various thiols, like Ellman's method, while the OPA-AE
16 method displays a rather poor response for thiols such as GSH or MAA. The homogeneity of
17 the signals for various thiols enables general application of our methodology to quantify the
18 overall thiols.

19 For sulfides, the methods based on redox reactions are fast but suffers from interferences from
20 other reducing compounds such as nitrites [17]. Three methods compared in Table 1 present
21 fairly similar analytical performance. The advantage of the EP method developed here is to
22 separately quantify thiols and sulfides with the same derivatizing reagent in 15 minutes.

24 3.3. Interferences

1 The potential interference of other compounds on the determination of MPA and sulfide by the
2 proposed methods was studied. Series of solutions containing 100 μM of MPA or 100 μM of
3 sulfide were prepared with addition of potential interfering compounds selected for their
4 presence in environmental samples. It has been assumed that an element did not interfere when
5 the variation of resulting concentration of MPA or sulfide was lower than $\pm 10\%$ (Table 2).

6 We first evaluated the cross interferences between MPA and sulfides. Thanks to the spectral
7 deconvolution (see 2.3.1), each compound can be determined selectively in the presence of a
8 50-fold excess of the other analyte. We also evaluated the potential interferences resulting from
9 non-thiol sulfur compounds (reduced or oxidized) including methionine (in the range 0.5-3
10 mM), sulfates (20 mM) and sulfites (5 mM). Results indicate that these non-thiol compounds
11 do not lead to a significant change in the response of MPA or sulfides when they are present in
12 realistic ratios compared to targeted analytes, even for amino acids such as methionine or
13 glycine that could potentially lead to serious interference if experimental conditions were not
14 optimized. Other significant potential nucleophiles such as halides, thiocyanate or acetate (50
15 mM each) were also evaluated and displayed no interferences in the determination of MPA or
16 sulfides. Finally, interference from metals such as Cu^{2+} (160 μM), Fe^{3+} (180 μM) and Zn^{2+} (160
17 μM) was less than 6% with application of the reduction step with TCEP and DTPA.

18 19 3.4. Validation

20 Considering the possible matrix interference in real samples, determination of thiols and
21 sulfides in water samples was first validated by spiking real samples with different amounts of
22 MPA (for reduced thiols), cystine (for total thiols) and sodium sulfide (for dissolved sulfides).
23 This standard addition method has already been used in some studies about the determination
24 of thiol compounds [36,37]. The proposed method was applied to the determination of reduced
25 and total thiols and sulfides in raw and outlet wastewater spiked with 25 μM of MPA, cystine

and sodium sulfide. The recovery study of the targeted analyte was performed in triplicates. Results were compared to recoveries obtained with Ellman's method. Results indicated a good recovery of targeted analytes and an absence of matrix effect on the tested samples. The recoveries of reduced thiols were $96.4 \pm 2.1\%$ in raw wastewater and $95.1 \pm 1.3\%$ in outlet with developed method. Ellman's method gave recoveries of $96.1 \pm 1.9\%$ and $98.2 \pm 2.6\%$ for reduced thiols on the same samples. The recoveries of total thiols were $98.3 \pm 1.6\%$ and $96.6 \pm 0.9\%$ in raw wastewater and outlet respectively. The determination of sulfides showed also good recoveries too with $98.5 \pm 1.7\%$ and $102.3 \pm 0.8\%$ in raw wastewater and outlet respectively.

4. Conclusion

The development of a microplate assay for the fast and individual determination of thiols and sulfides has been successfully optimized in this study. Separated quantification of thiols and sulfides has been achieved by exploitation of the absorbance shift of the derivatives of the targeted analytes with ethyl propiolate. Total thiols can also be measured with an innovative combination of a reducing agent (TCEP) and a complexing agent (DTPA). To the best of our knowledge, this is the first method that can simultaneously quantify free thiols, total thiols (bound to metals or in the oxidized form) and sulfides in aqueous samples, with a very acceptable reaction time and low limits of detection. The method was successfully validated by application to raw and treated wastewater and proved its applicability to real environmental samples.

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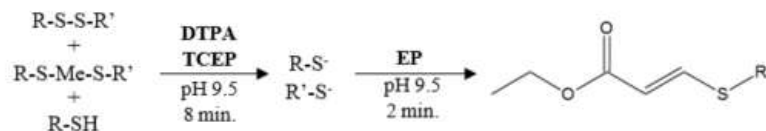
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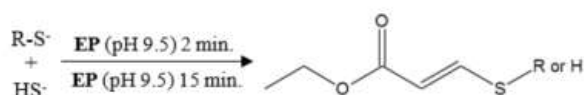
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Figures and Tables

Total thiols determination



Reduced thiols and dissolved sulfides determination



Scheme 1 Schematic depiction of the protocol for determination of total thiols, reduced thiols and dissolved sulfides.

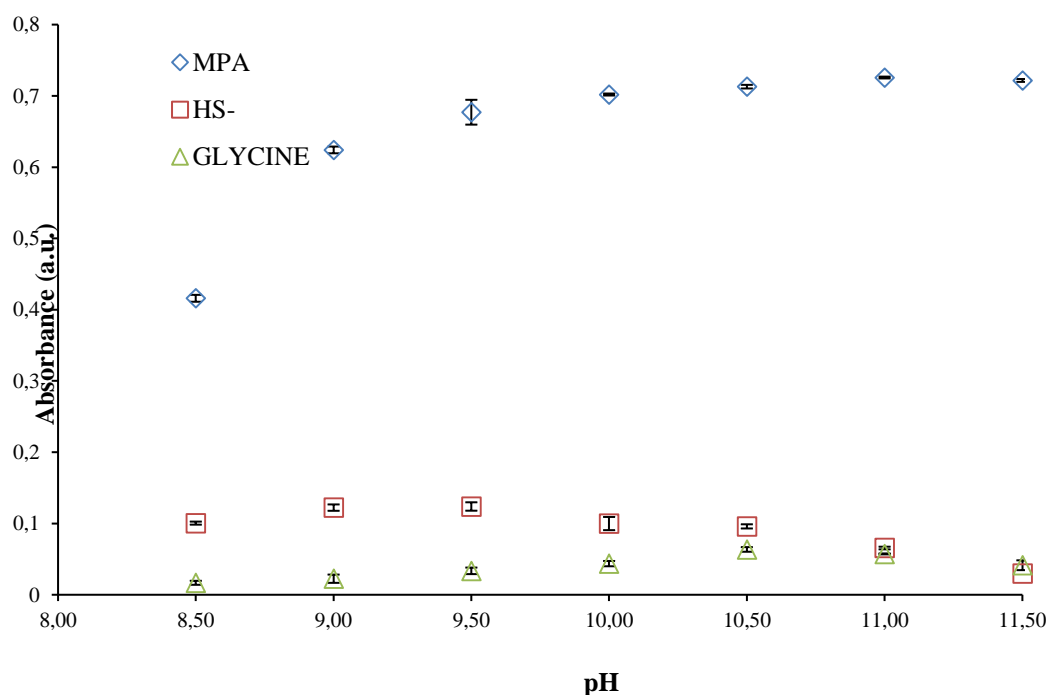


Fig. 1 Evolution of absorbance (at 288 nm for MPA (diamonds) and glycine (triangles); at 304 nm for hydrogen sulfide (squares)) as a function of pH value during derivatization step. MPA,

HS⁻ and glycine were at 100 μM each. Derivatization was carried out in duplicate with EP at 5 mM during 5 minutes, pH was buffered by borate or phosphate.

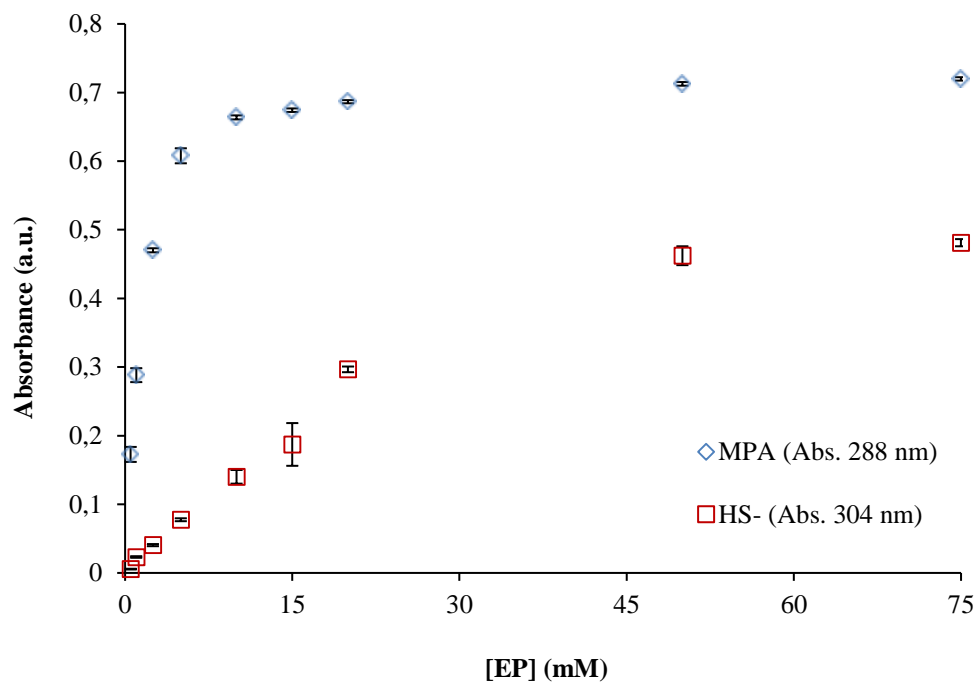


Fig. 2 Evolution of the absorbance (at 288 nm for MPA (diamonds); at 304 nm for HS⁻ (squares)) as a function of EP concentration. MPA and HS⁻ were at 100 μM each. Derivatization was carried out in duplicate during 5 minutes, pH was buffered at 9.5 by borate.

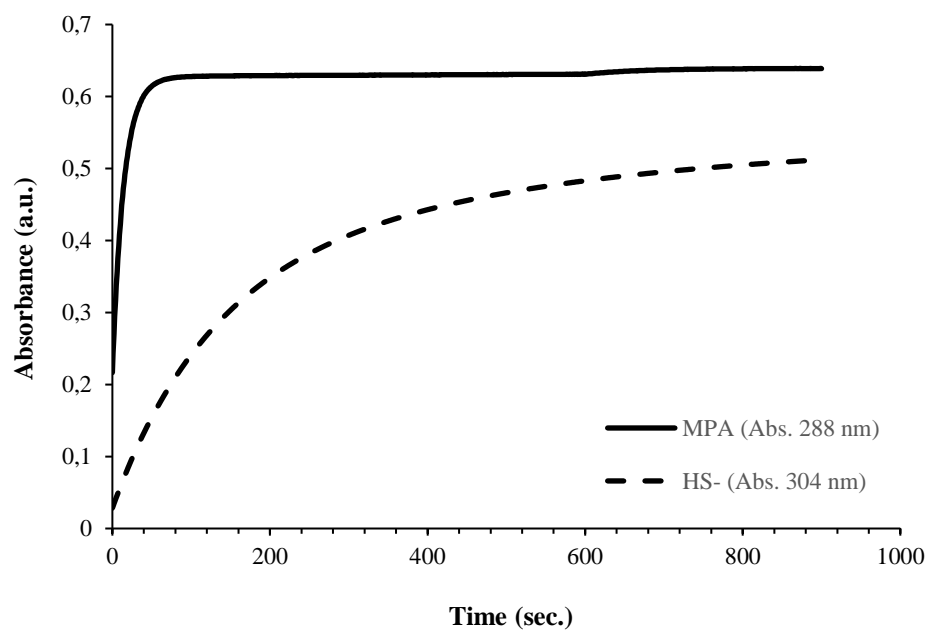


Fig. 3 Evolution of absorbance (at 288 nm for MPA (solid line); at 304 nm for hydrogen sulfide (dash line)) as a function of time. EP was at 50 mM. MPA and hydrogen sulfide were at 100 μ M each. pH was buffered at 9.5 by borate.

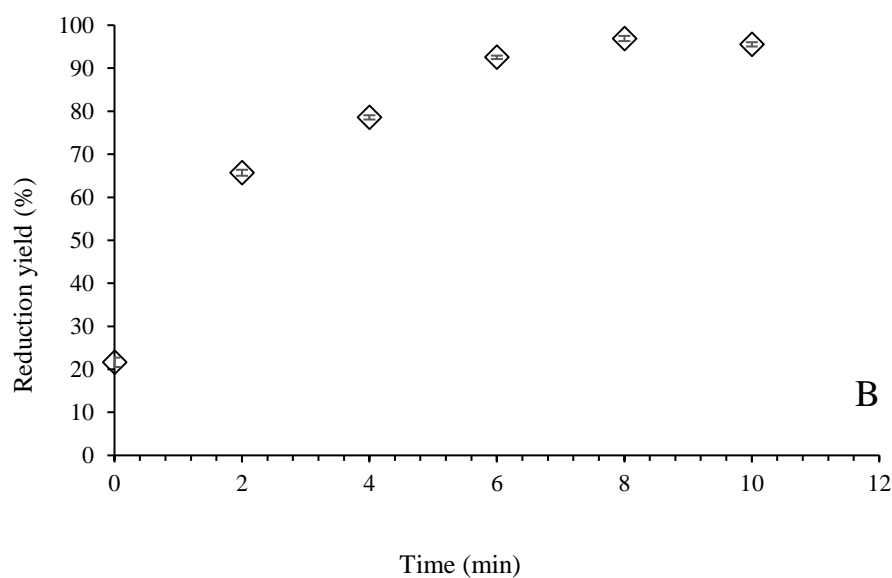
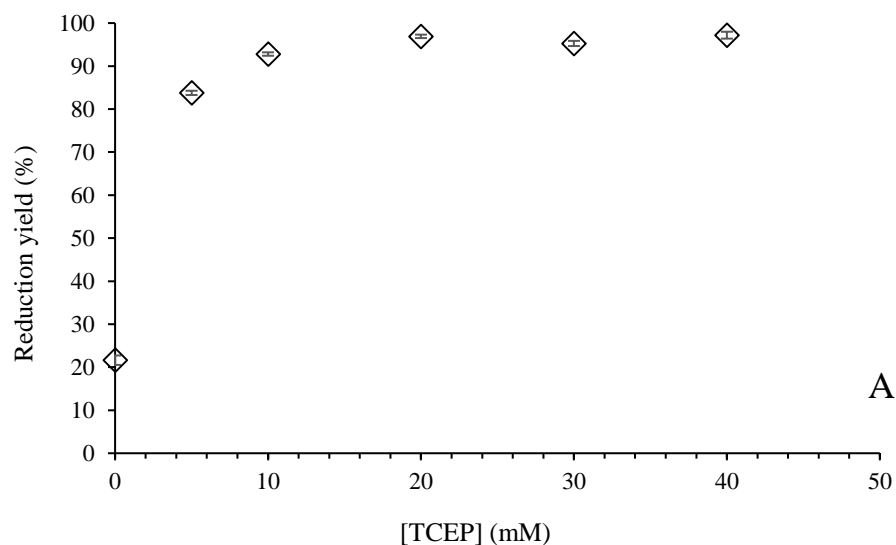


Fig. 4 Influence of various concentrations of TCEP with 6 min reduction time on reduction yield (Fig 4A); Influence of reduction time with 20 mM TCEP on reduction yield (Fig 4B). Derivatization of 50 μ M MPA in the presence of 160 μ M Cu^{2+} was carried out in duplicate with 50 mM EP during 2 minutes, pH was buffered at 9.5 by borate.

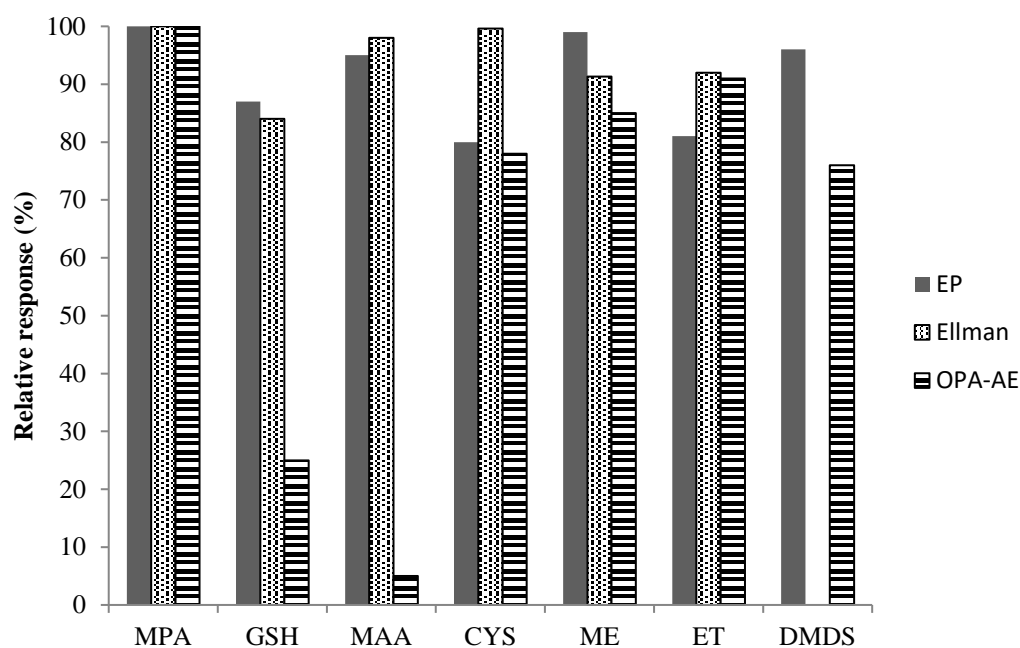


Fig. 5 Relative response of some thiol compounds compared to MPA (reference base 100) after derivatization by EP following proposed method (grey plain), by Ellman's reagent (grey 25%) or by OPA-AE method (horizontal lines). The concentration of tested thiols was 100 μ M each. GSH: glutathione - OPA: Ortho-phtalaldehyde - AE: Aminoethanol – MAA: mercaptoacetic acid – CYS: cysteine – ME: mercaptoethanol – ET: ethanethiol – DMDS: dimethyldisulfide

Table 1 Main analytical features of some studies on free thiols and sulfides determination.

NAM: N-(9-acridinyl)maleimide – 4-DPS : 4,4'-dithiodipyridine – PAPAIN: cysteine protease – 2-PDS: 2,2' dipyridil disulfide

| Analyte | Thiols | | | | | | Sulfides | | |
|---------------------------|------------|------------|--------------|------------|----------------------------------|-----------|----------------|-------------------------|----------------|
| Method (detection) | 4-DPS (UV) | NAM (fluo) | PAPAIN (Vis) | DTNB (Vis) | OPA-AE (fluo) | EP (UV) | 2-PDS (UV) | Fe(III) reduction (Vis) | EP (UV) |
| Standard | Mercaptans | GSH | Cysteine | GSH | MPA | MPA | Sodium sulfide | Sodium sulfide | Sodium sulfide |
| Range (μM) | 3-40 | 1-100 | 0.2 – 1.5 | 1-450 | 1-200 | 1-500 | 1.8-360 | 1-42 | 3-250 |
| Detection limit (μM) | 3 | 1 | 0.2 | 1.1 | 0.2 | 0.6 | 1.8 | 1 | 2.8 |
| Quantification limit (μM) | - | - | - | 3.5 | 0.6 | 2.1 | - | - | 7.3 |
| Reaction time (min.) | 5 | 60 | 60 | 15 | 1 | 2 | 5 | few min. | 15 |
| RSD (%) | - | - | - | 6.2 | 3.1 | 1.8 | - | 2.5 | 1.9 |
| Reference | [12] | [13] | [15] | [34] | Method adapted from [7] and [28] | This work | [16] | [17] | This work |

Table. 2 Recovery of MPA and sulfides in binary solution with potential interfering compounds. [EP] = 50 mM; [MPA] or [HS⁻] = 100 μM; pH = 9.5; Reaction time = 2 min. (MPA) or 15 min (HS⁻).

| MPA (100 μM) | | | Sulfides (100 μM) | | |
|----------------------|--------|----------|----------------------|--------|----------|
| Interfering compound | Amount | Recovery | Interfering compound | Amount | Recovery |
| Sulfates | 20 mM | 92.40% | Sulfates | 20 mM | 95.70% |
| Sulfites | 5 mM | 94.80% | Sulfites | 5 mM | 93.70% |
| Methionine | 0.5 mM | 95.20% | Methionine | 3 mM | 96.70% |
| Glycine | 1 mM | 93.50% | Glycine | 1 mM | 91.50% |
| Ammonium | 20 mM | 98.70% | Ammonium | 20 mM | 95.60% |
| Iodide | 50 mM | 98.30% | Iodide | 50 mM | 98.50% |
| Chloride | 50 mM | 97.90% | Chloride | 50 mM | 97.60% |
| Bromide | 50 mM | 98.90% | Bromide | 50 mM | 96.70% |
| Thiocyanate | 50 mM | 97.10% | Thiocyanate | 50 mM | 96.60% |
| Acetate | 50 mM | 98.90% | Acetate | 50 mM | 99.30% |
| Cu ²⁺ * | 160 μM | 98.10% | Cu ²⁺ * | 160 μM | 98.20% |
| Fe ³⁺ * | 180 μM | 94.30% | Fe ³⁺ * | 180 μM | 95.10% |
| Zn ²⁺ * | 160 μM | 99.60% | Zn ²⁺ * | 160 μM | 98.40% |

* with TCEP 20 mM and DTPA 2 mM