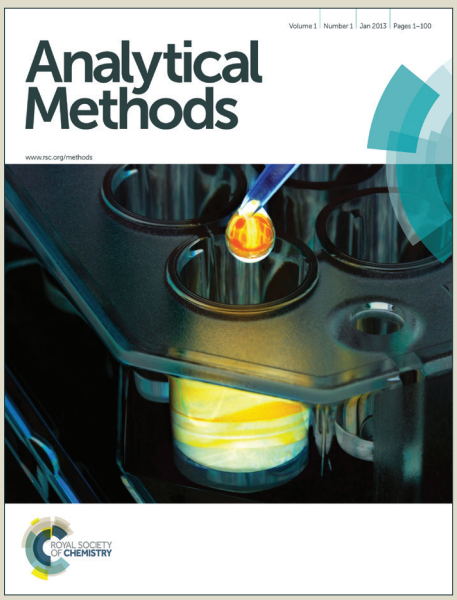
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Determination of total glutathione in earthworms by ultra-high performance liquid chromatography with fluorescence detection

Juan José Berzas Nevado[1], Rosa Carmen Rodríguez Martín-Doimeadiós[2], Francisco Javier Guzmán Bernardo[2], Carolina Rodríguez Álvarez[2]

Department of Analytical Chemistry and Food Technology

[1]*Faculty of Chemical Sciences. Av. Camilo José Cela, 9. E-13071, Ciudad Real, Spain.*

[2]*Faculty of Environmental Sciences and Biochemistry. Av. Carlos III, s/n. E-45071, Toledo, Spain.*

University of Castilla-La Mancha, Spain.

Corresponding author: Dr. Francisco Javier Guzmán Bernardo.

E-mail: fcojavier.guzman@uclm.es. Telephone: +34 925 26 88 00 (Ext. 5778). Fax: +34 925 26 88 40.

Abstract

A new, fast and simple methodology for the determination of total glutathione in earthworms, excellent bioindicators of oxidative stress, using ultra-high performance liquid chromatography with fluorescence detection is presented. Total glutathione could be a biomarker of oxidative stress caused by heavy metals such as mercury. The strategy for the analysis was to transform oxidized glutathione (GSSG) into reduced glutathione (GSH) and then to derivatize total GSH with *o*-pthalaldehyde to a fluorescent adduct.

The analysis was carried out in an Eclipse XDB-C18 (50 mm × 4.6 mm × 1.8 μm) column at 25 ºC, and using 100 mmol L^{-1} phosphate buffer at pH 7.0 and acetonitrile (phosphate buffer:acetonitrile, 85:15, v:v) as mobile phase at 1.5 mL min^{-1}. The volume injected was 5 μL and the excitation and emission wavelengths were 340 and 420 nm, respectively. Under the optimized conditions, the retention time of the derivatized analyte was as short as 0.536 min, which allows also a very short time between consecutive injections.

The instrumental limits of detection and quantification were 1.8 and 5.3 μg L^{-1} (5.7 and 17.0 nmol L^{-1}) and the calibration curve was linear up to 5 mg L^{-1} (16.3 μmol L^{-1}). The methodology was also proved to be robust and precise.

The methodology was validated using earthworms spiked with GSH, obtaining recoveries close to 100 %.

1. Introduction

Glutathione, γ-L-glutamil-L-cysteinylglycine, is present in almost all cells at millimolar concentrations. It is involved in a multitude of cellular functions, namely in protection against oxidative stress, detoxification of xenobiotics and modulation of enzyme activity by disulfide interchange [1] . Glutathione occurs mainly in the reduced form (GSH) which, in normal physiological conditions, constitutes more than 95% of total glutathione (tGSH), the rest being in the oxidized form (GSSG). When cells are exposed to increased oxidative stress, GSH is oxidized to GSSG and thus, the GSH/GSSG ratio is used to evaluate oxidative stress in biological systems [2].

The determination of tGSH, among other thiols, in biological samples is very important because it gives valuable biochemical and clinical information. GSH synthesis is intracellularly catalyzed by two enzymes, γ-glutamylcysteinyl synthetase (γ-GC syntethase) and GSH synthetase [3]. Endogenous and exogenous chemicals influence γ-GC synthetase activity and, as a consequence, tGSH levels vary as a function of the response of the cell to these perturbations. In this way, elevated thiols are associated with increased oxidative stress and suggest that increased thiols and oxidation may be causally related [4].

The determination of glutathione in biological samples, which has been reviewed recently [5], can be carried out with or without previous chromatographic separation. The most common at present is the former because of the high selectivity that can be achieved. High performance liquid chromatography (HPLC) has been the technique of choice for most authors coupled to optical [6, 7], electrochemical [8] or mass spectrometry detectors [9]. Ultraviolet-visible (UV-Vis) and fluorescence are the most widely used detectors even though there are no strong chromophores or fluorophores in GSH and GSSG structures. In order to solve this trouble, a derivatization step is necessary, preferably by the introduction of a fluorophore because it allows much lower detection limits than a chromophore [10]. The use of *o*-phthalaldehyde (OPA) is most attractive because the reaction with GSH to form an isoindole fluorescent adduct is rapid, can be carried out in mild conditions, and allows detection limits of picomols [11]. However, OPA cannot react with GSSG because thiol groups are not free. Consequently, the determination of GSSG with OPA has to be carried out after reduction to GSH, which is another key point for the determination of tGSH. Although there is a wide range of chemical reductants for this purpose, all of them show important drawbacks and a

balanced compromise between reasons for and against has to be taken. In this way, dithiothreitol (DTT) has proved to be a superior reducing agent in comparison with commonly used reducing compounds, i.e. sodium or potassium borohydride, and the reaction can be performed in a phosphate buffer of approximately neutral pH [12]. DTT is not selective for the reduction of GSSG to GSH, but it can still be used because the chromatographic separation and further fluorescence detection provides compensates for this lack of selectivity.

The introduction of ultra-high performance liquid chromatography (UHPLC) has been an important step forward in analytical and bioanalytical laboratories. UHPLC is an ideal fast-separation tool for complex mixture analysis in both isocratic and gradient modes, since this technology has been demonstrated to be capable of achieving higher peak capacity, speed, and sensitivity than conventional HPLC through the UHPLC use of sub-2-microne particles and optimised instrumentation [13]. Since biological samples can be complex and the number of samples is usually large, fast separations with high resolution and high sensitivity are often required. Nevertheless, despite the advantages of this technique, only two papers reporting the determination of tGSH or its species by UHPLC can be found in literature [14, 15]. Both have used tandem mass spectrometry (MS/MS) detection but none of them show quantitative data. Other works using HPLC-MS/MS have reported limits of detection in the low nmol L^{-1} range [16], similar to those achieved by fluorescence detection [17]. For this reason and also owing to the fact that HPLC coupled to MS detectors are more expensive and requires a tough training, fluorescence detectors are still competitive for the determination of glutathione.

The determination of tGSH in earthworms is particularly interesting because they are bioindicators of insecticide and heavy metal pollution [18, 19]. Also, acute and subchronic toxicity tests in earthworms are internationally accepted as standard. In fact, many reports have shown that insecticides and heavy metals can induce an increase in reactive oxygen species (ROS) in earthworms [20, 21]. Their defence mechanisms against ROS involve enzymes and small molecules, such as glutathione, that act as anti-oxidants. In fact, Gudbrandsen and Sverdrup [22] reported that an increased level of tGSH in *Eisenia fetida* was caused by oxidative stress and suggested that measurement of this level would be more appropriate as cellular response than the commonly used measurement of glutathione transferase activity. However, their GSH assays were based on the method of measurement of tissue sulphydryl groups described by Ellman in 1959 [23], but this method suffers from poor selectivity because it is based on a simple

spectrophotometric measurement without previous chromatographic separation, so GSH is not the only absorbing species.

The aim of this work is to develop and validate an analytical methodology for the determination of tGSH taking advantage of the analytical features of UHPLC and fluorescence detection. The methodology is going to be applied to earthworms exposed to mercury contaminated soils. Their tGSH content could be regarded as a biomarker of oxidative stress.

2. Experimental

2.1. Standards, solutions and samples

All chemicals and reagents were analytical grade or better. Solvents were HPLC grade and all solutions were prepared in ultrapure quality water (18.2 MΩ cm). GSH, GSSG, L-cysteine (CYS), DTT, OPA and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Química, S.A. (Madrid, Spain). Sodium hydroxide, borax ($Na_2B_4O_7 \cdot 10H_2O$), tri-sodium citrate 2-hydrate, sodium acetate 3-hydrate, sodium di-hydrogen phosphate 1-hydrate, tris-(hydroxymethyl)-aminomethane (TRIS), ethylene diamine tetraacetic acid disodium salt dihydrate, D(+)-sucrose, perchloric acid (70 % v:v), acetonitrile and methanol were purchased from Panreac S.A. (Barcelone, Spain). Hydrochloric acid was from Merck (Darmstadt, Germany).

Stock solutions of GSH and GSSG were prepared in 1 % HCl at 27.5 mg L^{-1} (89 and 44 μmol L^{-1} of GSH and GSSG, respectively) and stored at 4 ºC. Working standard solutions were prepared daily by exact dilution. Stock solutions of OPA were prepared at 5 mg mL^{-1} (37.3 mmol L^{-1}) dissolving 50 mg of reagent in 0.5 mL of methanol and diluted in a 0.1 M borax solution at pH 9.90 to a final volume of 10 mL. This was prepared monthly and kept at -20 ºC until used. DTT solutions were prepared daily in water at 30 mmol L^{-1} and kept at 4 ºC until used.

TRIS buffer solution was prepared so that the final concentrations were 0.1 mol L^{-1} of TRIS, 10^{-3} mol L^{-1} of EDTA, 0.25 mol L^{-1} of D(+)-sucrose and 10^{-4} mol L^{-1} of PMSF. This buffer was adjusted at pH 6.7 with HCl.

Specimens of *Lumbricus terrestris* were purchased at Poisson Fenag (Madrid, Spain) to be used as samples. This particular species has been taken as model for monitoring soil environmental pollution since 1984 [24].

2.2. Instrumentation

UHPLC analysis was performed on a Agilent 1200 SL system (Agilent Technologies Inc.) equipped with an online vacuum degasser, a high-pressure gradient binary pump, a manual sample injector (loop 5 μL), a column oven and a fluorescence detector. Data analysis was done using Agilent ChemStation software (Agilent Technologies).

A Zorbax Eclipse XDB-C18 column of 50 mm × 4.6 mm (i.d.) with 1.8 μm particle size (Agilent) was used. Mobile phase was 85% phosphate buffer solution of pH 7.0 (100 mmol L^{-1}) and 15% of acetonitrile. The mobile phase was filtered using a vacuum filtration system through 0.45 μm membrane filters. The separation was carried out at 1.5 mL min^{-1}. The column heater was set at 25 ºC and the detector was set at 340 and 420 nm as excitation and emission wavelengths, respectively [25].

A Crison 2001 pH-meter was used for pH measurements, and a T10 basic Ultra-turrax™ (IKA™ Werke GmbH & Co. KG, Staufen, Germany) was used for the homogenization of the earthworms.

A Digicen 21 R refrigerated centrifuge (Ortoalresa, Ajalvir, Madrid, Spain) was used throughout this work.

2.3. Sample preparation

The sample preparation consists of three steps and the workflow is shown as figure 1. The first step is obtaining the cytosolic fraction of the tissue. To do so, earthworms were depurated on moist filter paper for 48 hours, cut up, and kept at -80 ºC. A subsample of 3 to 5.5 g was accurately weighed and homogenized with an ultra turrax™ in the ratio 1:4 (w:v) with TRIS buffer solution and centrifuged at 9,000 × g at 4 ºC for 20 min. The resulting supernatant is the cytosolic fraction, which contains the analytes. The second step is the protein removal. For this pupose, 400 μL of ultrapure water and 200 μL of concentrated $HClO_4$ were added to an aliquot of 3 mL of the cytosolic fraction. The mixture was centrifuged at 2,100 × g for 2 min. The liquid fraction (SN1) was separated from the pellet and kept for further processing. The pellet was first added 2 mL of 50 mmol L^{-1} phosphate buffer at pH 7.0, then vortexed for 1 min in order to extract the remaining glutathione from it, and finally added 100 μL $HClO_4$ to remove the rest of proteins. This suspension was centrifuged also at 2,100 × g for 2 min. The resulting liquid fraction (SN2) was separated from the pellet, which was

discarded, and combined with SN1. All this was filtered through a 0.45 µm pore size nylon filter, adjusted to pH 7.0, and diluted to 10 mL with ultrapure water in a volumetric flask (Solution "M"). The third step is the derivatization prior to the chromatographic analysis. In this last step, 700 µL of 50 mmol L^{-1} phosphate buffer at pH 7.0, 100 µL of DTT at 30 mmol L^{-1} and 100 µL of OPA at 5 mg mL^{-1} were added to an aliquot of 200 µL of solution "M". Finally, this solution was injected in the chromatographic system for analysis.

2.4. Derivatization

As stated previously, unlike GSSG, only GSH can react with OPA to form a fluorescent adduct. The reaction of GSH with OPA is strongly pH-dependant and shows maximal fluorescent yield at pH between 9.5 and 12 [26]. Unfortunately, GSH rapidly oxidizes non-enzymatically above pH 7 [27, 28] so it was decided to carry out the derivatization at pH 7.0 with phosphate buffer as a compromise between stability of GSH, and sensitivity in the detection of the adduct. The concentration of OPA was taken from literature[11]. Thus, an aliquot of 200 µL of 0.02 mmol L^{-1} (6.14 mg/L) of GSH was transferred to an amber glass vial of 2 mL, and then 700 µL of 50 $mmol^{-1}$ phosphate buffer at pH 7.0, 100 µL of stock solution of DTT (30 mmol L^{-1}) and 100 µL of stock solution of OPA (37.3 mmol L^{-1}) were added. This solution, referred to as "S" throughout this work, was allowed to react in the dark for 5 min at room temperature to yield the fluorescent derivative.

The "S" solution was injected over two days without noticeable variation in retention time or peak area, so it was considered stable from the preparation up to 48 hours later when kept at 4 ºC.

3. Results and discussion

3.1. Optimization of the reduction

In an amber glass vial, 200 µL of GSSG at 0.02 mM (12.3 mg/L) and variable amounts of DTT so that the final concentrations were from 0.045 mM up to 3.6 mM were mixed. The solutions were derivatized as explained in section 3.1. As shown in figure 2, the peak area increased up to 1.8 mmol L^{-1} of DTT and then remained constant with increasing concentration of DTT. The peak area obtained for 2.7 mmol L^{-1} of DTT was maximal, so this concentration was selected as optimum.

3.2. Optimization of the chromatographic conditions

The optimization of the parameters affecting the analysis by UHPLC-FLD was carried out by injecting an "S" solution into the instrument. The initial chromatographic conditions were 20 mmol L^{-1} citrate buffer at pH 3.5 and acetonitrile (citrate buffer:acetonitrile, 80:20, v:v) as mobile phase, 25 ºC as column oven temperature and a flow rate of 1.0 mL min^{-1}. The detector was set at 340 nm and 420 nm as excitation and emission wavelengths, respectively.

3.2.1. Optimization of the pH of the mobile phase

For this purpose, 20 mmol L^{-1} buffer solutions at pH from 3.0 up to 7.5 were prepared either with citrate (pH 3.0 and 3.5), acetate (pH 4.0 to 5.5) or phosphate (pH 6.0 to 7.5) at intervals of 0.5 units of pH. An "S" solution was injected in the chromatograph with a mobile phase of buffer and acetonitrile (80:20, v:v) at 1.0 mL min^{-1} and 25 ºC.

The influence of the pH on the separation can be seen in figure 3. The retention time decreases slightly from pH 3.0 up to 3.5, dramatically from pH 3.5 up to pH 4.0 and then it decreases moderately up to pH 4.5. For pH above 4.5, the retention time remains stable. This behaviour can be explained by the fact that the GSH-OPA adduct contains two carboxylic groups, whose pK_a are about 3.6. Thus, at pH 3.0, the acid form of the molecule is predominant and, since it has no charge, its retention is high. Conversely, the molecule is totally ionized at pH above 4.6, so therefore the retention in a non-polar column such as C_{18} type is low. In the rest of the pH range studied, the retention time does not change because there are no more functional groups that can be affected by this factor. The pH selected as optimum was 7.0 because it provides a short retention time and also because it is the same pH as in the derivatization. Phosphate was used to obtain pH 7.0 because it provides maximum buffering capacity at this pH (pK_{a2} = 7.1).

3.2.2. Optimization of the buffer concentration

This study was carried out by injecting an "S" solution into the chromatographic system using mobile phases containing phosphate buffer solutions adjusted to pH 7.0 at concentrations ranging from nougth to 100 mmol L^{-1}.

The results showed that the shortest retention time was obtained when using water, in other words, when phosphate buffer concentration was zero. Then, the retention time increased as buffer concentration increased due to the effect of an increase in ionic strength. However, using water in the mobile phase would not buffer, which is of paramount importance in biological matrices such as the present. In this way, it was preferred to guarantee the buffering capacity of the mobile phase instead of giving priority to the shortest retention time, so buffer concentration of 100 mmol L^{-1} was selected as optimum.

3.2.3. Optimization of the composition of the mobile phase

This study was carried out by injecting an "S" solution into the chromatographic system using mobile phases containing 100 mmol L^{-1} phosphate buffer at pH 7.0 and either acetonitrile or methanol from 5% to 40% (v:v). The results showed, first, shorter retention times with acetonitrile, and, second, an exponential decrease in retention time with increasing percentage of either organic solvent in the mobile phase (Figure 4). This can be explained by the fact that the more organic solvent the more elution power of the mobile phase until the analyte is not retained at all, in other words, the analyte is eluted at t_0. This t_0 can be estimated from figure 4 as the retention time when the analyte is eluted at any percentage of organic solvent.

Therefore, acetonitrile at 15% (v:v) was selected as organic solvent in the mobile phase because it provides the shortest retention time, 0.658 min, different from t_0, 0.434 min.

3.2.4. Selection of the flow rate and temperature

The flow-rate of the mobile phase was tested from 0.5 up to 2.5 mL min^{-1}. A flow-rate of 1.5 mL min^{-1} was selected as optimum because it provided the shortest retention time, 0.536 min, without overlapping with t_0, 0.353 min, and also because the pressure was below 300 bar, which preserves column life.

Concerning temperature, 25 ºC was selected because higher temperatures can compromise the stability of the GSH-OPA adduct [29].

3.3. Validation

3.3.1. Limits of detection, quantification and linearity

Instrumental limits of detection and quantification (LOD and LOQ) were estimated in accordance to the base line noise. The base line noise was evaluated by recording the detector response over a period about 10 times the peak width. The LOD was obtained as the analyte concentration that caused a peak with a height 3-fold the base line noise level and the LOQ was calculated as 10-fold the base noise level. The figures obtained for LOD, LOQ, linearity range and calibration curve are summarized in Table 1. The instrumental LOD and LOQ obtained (5.7 nmol L^{-1} and 5.3 μg L^{-1}, respectively) are of the same order of magnitude as in Cereser et al.[17] (LOD = 2.5 nmol L^{-1}), who used fluorescence detection, and also as in Zhang et al.[16] (LOQ = 5 μg L^{-1}), who used liquid chromatography/positive electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

The linearity was checked by injecting a set of derivatized standards of GSH under the optimized conditions. The response of the detector was linear from the limit of quantification up to 20 mg L^{-1} (64.9 μmol L^{-1}), which is more than three orders of magnitude, and the intercepts was statistically not different from zero, according to Student's test "t" (p=0.05).

3.3.2. Precision

For this study, an "S" solution was injected 20 times consecutively in a day. The relative standard deviations of retention time and peak area were 0.6 % and 2.5 %, respectively. A fresh "S" solution was prepared the following day and it was also injected 20 times consecutively. The standard deviations of retention time and peak area were 0.6 % and 3.2 %. The variances of retention time and peak area in these two consecutive days were found not statistically different, according to the Snedecor´s test "F" for two tales (p=0.05).

3.3.3. Selectivity

OPA reacts with GSH through both the amino and thiol groups. This means that molecules containing either group can competitively react with OPA and thus decreasing the peak of GSH-OPA and/or overlapping in the chromatographic process. L-Cysteine (CYS) is an amino acid containing a thiol group that occurs in the cell at concentrations from one tenth to ten times the concentration of GSH [30].

In order to find out if the presence of CYS affected the formation of the GSH-OPA adduct, three standards of CYS at (4.1, 41 and 410 μmol L^{-1}) were derivatized and analyzed as described for GSH, resulting in no chromatographic peaks. Moreover, three solutions of 5 mg L^{-1} of GSH were added CYS at 0.5, 5 and 50 mg L^{-1} respectively, derivatized and analyzed. The resulting three chromatograms showed only the GSH-OPA peak, with no significant variation in peak area. Therefore, the presence of CYS was proved not to interfere in the analysis.

3.3.4. Accuracy

The accuracy was evaluated by spiking aliquots of 3 mL of cytosolic fraction (n=3) at zero (analytical blank), 23.5, and 47.0 μg mL^{-1} (75.2 and 150.4 μmol L^{-1}) of GSH. All of them were analyzed as described in figure 1 and the corresponding chromatograms are in figure 5. The quantification was carried out by injecting samples by triplicate before and after a standard. The standards were used to obtain the response factor, as follows:

$$A = k\,c$$

where A is peak area, c is concentration and k is the response factor. Then, in order to calculate the concentration in the samples, the peak area obtained was divided by the average of the response factors k of the standards run before and after each sample.

The found concentrations and recoveries obtained are summarised in table 2 and, as can be seen, they were close to 100%.

3.4. Application to real samples

A specific cleanup for precipitation of proteins of the cytosolic fraction had to be optimized (figure 1). For this purpose, perchloric acid, methanol and acetonitrile were tested and the results showed perchloric acid provided the most effective precipitation and the cleanest supernatant. Nonetheless, the recoveries of GSH after this step were always below 85%, which indicated the rest had to be extracted from the precipitate. Thus, the precipitate had to be re-suspended and precipitated again in the same conditions. Water and phosphate buffer (pH 7.0, 50 mmol L^{-1}) were tested for re-suspension and the results showed that phosphate buffer managed to extract more GSH from the precipitate than water. The supernatants were combined, filtered through a 0.45 μm pore sized filter, and diluted to a final volume of 10 mL ("M"), which enabled

the sample for its further derivatization and measurement. These two clean up steps proved to be satisfactory in terms of both selectivity and recovery.

The present methodology was applied to earthworms (*Lumbricus terrestris*) exposed to a soil containing a native mercury concentration (243.5 ± 2.7 μg g^{-1}) and to a control soil (0.272 ± 0.025 μg g^{-1}). In these experiments, mercury acts as a xenobiotic causing oxidative stress, and therefore triggers a response at cellular level. The soil moisture was maintained at approximately 50% of field capacity without need for further water additions. Then, eight earthworms were introduced in each test container, which were covered with holed parafilm and kept in the dark at 4ºC. Two earthworms were pooled after 0, 2, 14 and 28 days of exposure and analyzed for tGSH. The collected earthworms were carefully washed, placed in Petri dishes with damp filter paper for 48 h to get rid of gut contents, and sacrificed by deep freezing (-20ºC). The results indicated an increase of tGSH concentration with exposure, as shown in table 3. Although these are preliminary results, they back the fact that xenobiotics do not only affect the GSH:GSSG ratio, but also the total concentration of GSH [22].

4. Concluding remarks

We have developed, optimized and validated a methodology for analyzing tGSH in earthworm tissues using UHPLC with molecular fluorescence detection. The results show that UHPLC-FLD can be successfully used for determining tGSH, providing low detection limits (nmol L^{-1}), adequate selectivity and retention times lower than those obtained in other studies employing also UHPLC [31], thus reducing analysis times. All this, together with its limited cost, makes UHPLC-FLD competitive compared to other more sophisticated techniques, such as HPLC-MS, for the determination of tGSH.

The sample preparation has specifically been optimized for *Lumbricus terrestris*, because of their relevant role as model species for the monitoring of soil environmental pollution.

Finally, we have observed an increase in levels of tGSH in worms with increasing exposure to a Hg containing, which confirms the potential of tGSH as a biomarker of oxidative stress caused by metal exposure.

The authors are grateful to *Junta de Comunidades de Castilla-La Mancha* (PAI06-0094, PEII09-0032-5329) and the *Spanish Ministry of Science and Technology* (BQU2008-02126 and BQU2007-65991) for financial support.

References

1. A. Meister, in *Glutathione: Chemical, Biochemical and Medical Aspects, Part A*, ed. R. P. D. Dolphin, O. Avramovic, John Wiley and Sons, New York, 1989, pp. 243-279.
2. A. R. T. S. Araujo, M. L. M. F. S. Saraiva and J. L. F. C. Lima, *Talanta*, 2008, **74**, 1511-1519.
3. A. Meister, in *The enzymes*, ed. P. D. Boyer, Academic Press, New York, 3rd edn., 1974, vol. 10, pp. 671 – 697.
4. T. Wronska-Nofer, J.-R. Nofer, J. Stetkiewicz, M. Wierzbicka, H. Bolinska, M. Fobker, H. Schulte, G. Assmann and A. von Eckardstein, *Nutrition, Metabolism and Cardiovascular Diseases*, 2007, **17**, 546-553.
5. P. Monostori, G. Wittmann, E. Karg and S. Túri, *Journal of Chromatography B*, 2009, **877**, 3331-3346.
6. R. Kand'ár, P. Žáková, H. Lotková, O. Kučera and Z. Červinková, *Journal of Pharmaceutical and Biomedical Analysis*, 2007, **43**, 1382-1387.
7. D. Tsikas, J. Sandmann, D. Holzberg, P. Pantazis, M. Raida and J. C. Frölich, *Analytical Biochemistry*, 1999, **273**, 32-40.
8. R. L. Norris, G. K. Eaglesham, G. R. Shaw, M. J. Smith, R. K. Chiswell, A. A. Seawright and M. R. Moore, *Journal of Chromatography B: Biomedical Sciences and Applications*, 2001, **762**, 17-23.
9. X. Guan, B. Hoffman, C. Dwivedi and D. P. Matthees, *Journal of Pharmaceutical and Biomedical Analysis*, 2003, **31**, 251-261.
10. E. Camera and M. Picardo, *Journal of Chromatography B*, 2002, **781**, 181-206.
11. C. C. Yan and R. J. Huxtable, *Journal of Chromatography B*, 1995, **672**, 217-224.
12. C.-S. Yang, S.-T. Chou, L. Liu, P.-J. Tsai and J.-S. Kuo, *Journal of Chromatography B*, 1995, **674** 23-30.
13. N. Wu and A. M. Clausen, *Journal of Separation Science*, 2007, **30**, 1167-1182.
14. L.-S. New and E. C. Y. Chan, *Journal of Chromatographic Science*, 2008, **46**, 209-214.
15. A. D. Patterson, H. Li, G. S. Eichler, K. W. Krausz, J. N. Weinstein, A. J. F. Jr., Frank J. Gonzalez and J. R. Idle, *Analytical Chemistry*, 2008, **80**, 665-674.
16. F. Zhang, M. J. Bartels, D. R. Geter, Y.-C. Jeong, M. R. Schisler, A. J. Wood, L. Kan and B. B. Gollapudi, *Rapid Communications in Mass Spectrometry*, 2008, **22**, 3608-3614.
17. C. Cereser, J. Guicharda, J. Draia, E. Banniera, I. García, S. Bogetb, P. Parvazc and A. Revol, *Journal of Chromatography B*, 2001, **752** 123-132.
18. M. Saint-Denis, Narbonne, J.F., Arnaud, C., Ribera, D., *Soil Biology & Biochemistry*, 2001, **33**, 395-404.
19. N. Xiao, B. Jing, F. Ge and X. Liu, *Chemosphere*, 2006, **62**, 1366-1373.
20. K. J. Lenton, H. Therriault and J. R. Wagner, *Analytical Biochemistry*, 1999, **274**, 125-130.
21. P. Reddy, Sreenivasula, G., *Fresenius Environmental Bulletin*, 1997, **6**, 589 – 597.
22. M. Gudbrandsen, L. E. Sverdrup, S. Aamodt and J. Stenersen, *European Journal of Soil Biology*, 2007, **43, Supplement 1**, S261-S267.
23. G. L. Ellman, *Archives of Biochemistry and Biophysics*, 1959, **82**, 70-77.
24. OECD, 1984.
25. V. H. Cohn and J. Lyle, *Analytical Biochemistry*, 1966, **14**, 434-440.

26. C. Parmentier, P. Leroy, M. Wellman and A. Nicolas, *Journal of Chromatography B*, 1998, **719**, 37-46.
27. D. J. Anderson, B. Guo, Y. Xu, L. M. Ng, L. J. Kricka, K. J. Skogerboe, D. S. Hage, L. Schoeff, J. Wang, L. J. Sokoll, D. W. Chan, K. M. Ward and K. A. Davis, *Analytical Chemistry*, 1997, **69**, 165-230.
28. J.-L. Luo, F. Hammarqvist, I. A. Cotgreave, C. Lind, K. Andersson and J. Wernerman, *Journal of Chromatography B: Biomedical Sciences and Applications*, 1995, **670**, 29-36.
29. B. A. Newschwander-Teri and F. J. Roll, *Analytical Biochemistry*, 1989, **179**, 236-241.
30. C. K. Babu, K. M. Ansari, S. Mehrotra, R. Khanna, S. K. Khanna and M. Das, *Food and Chemical Toxicology*, 2008, **46**, 2409-2414.
31. J. T. Michaelsen, S. Dehnert, D. Giustarini, B. Beckmann and D. Tsikas, *Journal of Chromatography B*, 2009, **877**, 3405-3417.

Table 1. Instrumental limits of detection and quantification, calibration curve, and linearity range expressed in grams and in molar units.

LOD	1.8 μg L^{-1}	5.7 nmol L^{-1}
LOQ	5.3 μg L^{-1}	17.0 nmol L^{-1}
Calibration curve[a]	A = 9.9 + 0.7629 × c (μg L^{-1})	A = 9.9 + 234.22 × c (μmol L^{-1})
S.D. Intercepts	± 7.3	± 7.3
S.D. Slope	± 0.0031	± 0.97
R^2	0.9999	0.9999
Linearity range	5 – 20,000 μg L^{-1}	0.016 – 64.9 μmol L^{-1}

[a]A: peak area; c: concentration of tGSH.

Table 2. Recovery studies in spiked samples at different levels.

Concentration spiked (mg mL^{-1})	Concentration found (mg mL^{-1})	Recovery (%)
23.5	23.2 ± 0.7	98.8 ± 2.9
47.0	45.2 ± 4.5	96.3 ± 9.6

Table 3. Concentrations of tGSH in earthworms at different times of exposure to a natively mercury containing soil and to a control soil.

	tGSH (μg g^{-1})			
Time of exposure (days)	0	2	14	28
Control soil	8.60 ± 0.70	7.91 ± 0.95	8.3 ± 1.0	8.57 ± 0.12
Hg containing soil	8.60 ± 0.70	8.95 ± 0.73	9.20 ± 0.74	9.35 ± 0.76

Figure captions.

Figure 1. Workflow of the sample preparation.

Figure 2. Influence of the concentration of DTT on the reduction.

Figure 3. Influence of pH on the retention time of the adduct GSH-OPA.

Figure 4. Influence of the nature and percentage of the organic solvent in the mobile phase on the retention time.

Figure 5. Chromatograms from the analyses of 3 cytosolic fractions spiked at nought (analytical blank), 23.5, and 47.0 μg mL^{-1} (75.2 and 150.4 μmol L^{-1}) of GSH.

Figure 1

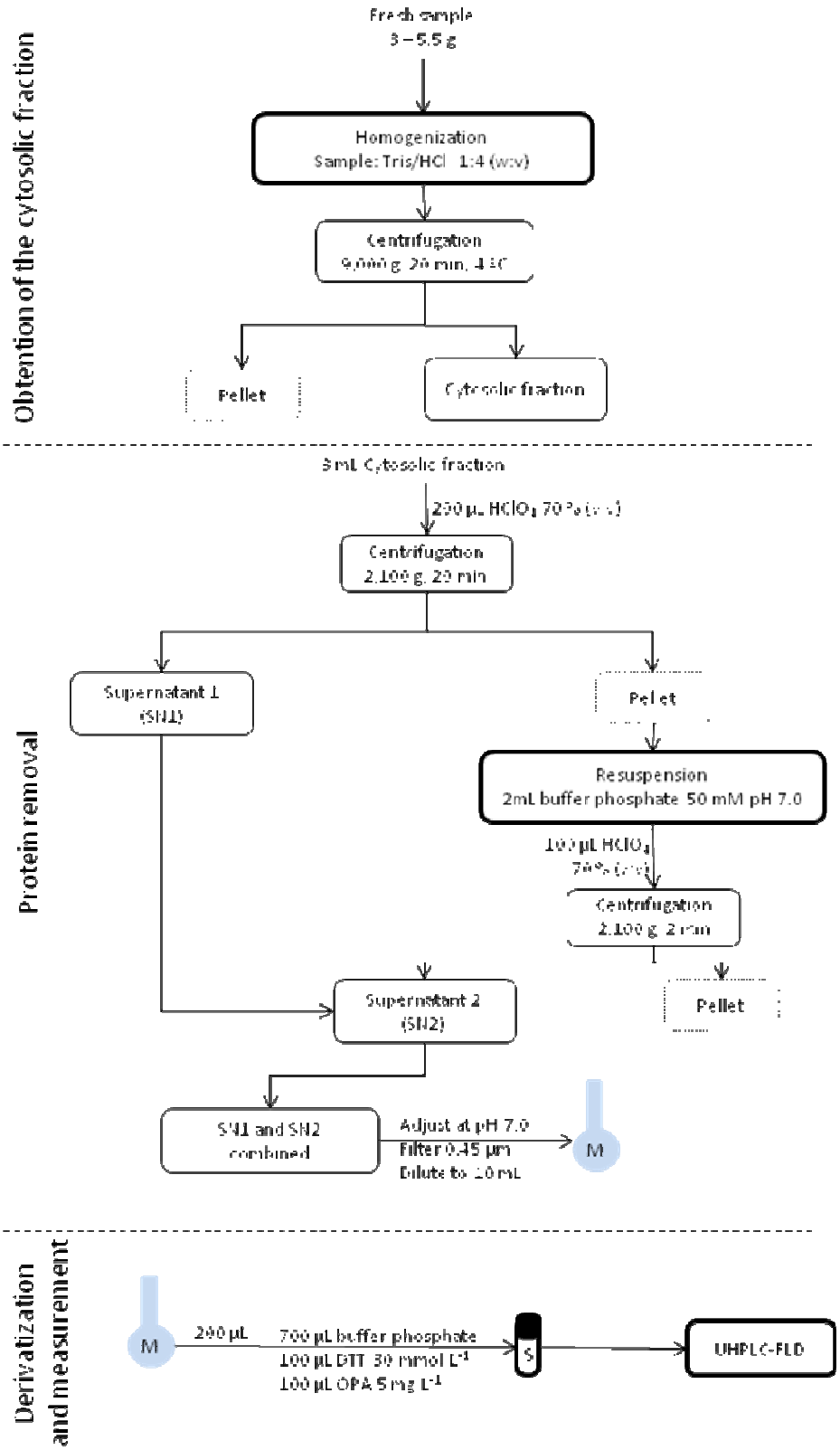


Figure 2

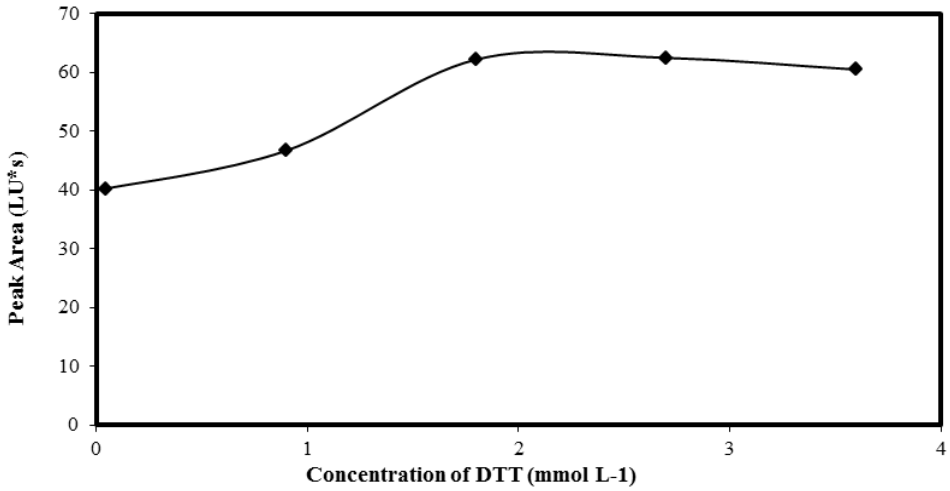


Figure 3

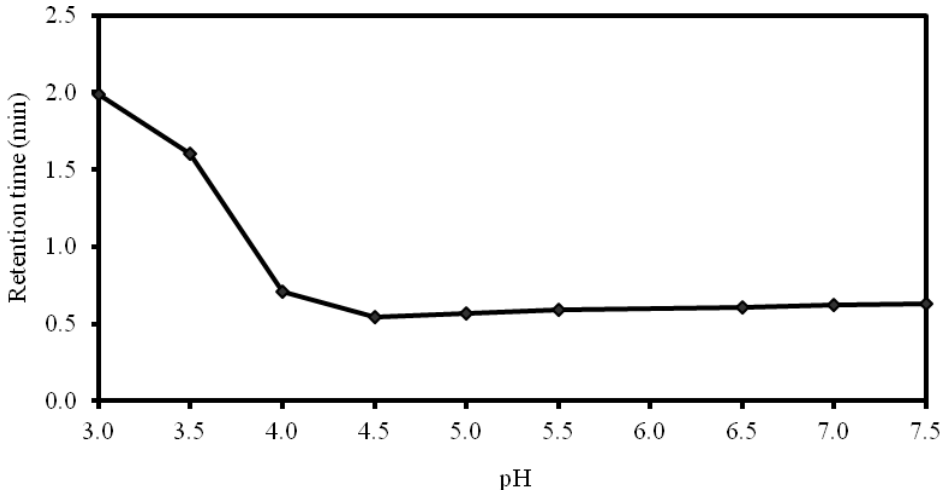


Figure 4

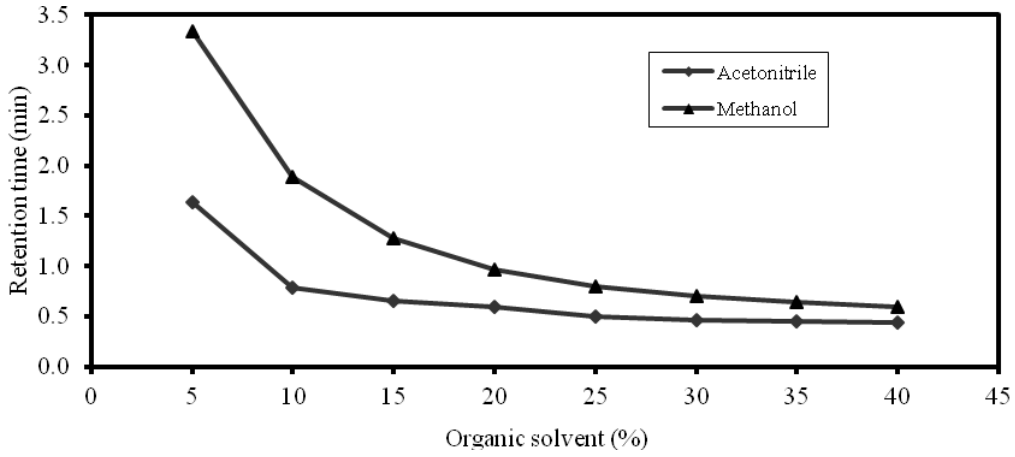


Figure 5