

**Comparison of analytical methods using enzymatic activity,
immunoaffinity and selenium-specific mass spectrometric detection for the
quantitation of glutathione peroxidase 1**

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Abstract

Glutathione peroxidase 1 (Gpx1) as one of the most responsive selenoproteins to selenium variation, is often used to evaluate 'selenium status' at a cellular or organismal level. The four major types of analytical methodologies to measure Gpx1 were revisited. They include an enzymatic assay, polyacrylamide gel electrophoresis (PAGE) with western blot immunodetection or detection of selenium by inductively coupled plasma mass spectrometry (ICP MS), and size-exclusion chromatography with ICP MS detection. Each of the four methods was optimized for the quantification of Gpx 1 with maximum sensitivity. The methods based on the enzymatic and immunodetection offer a much higher sensitivity but their accuracy is offset by the limited selectivity and limited dynamic range. The discussion of the advantages and drawbacks of each technique leads to the conclusion of a better understanding of the sources of error and the need for the cross-validation of the results using the different techniques to assure the quality assurance of quantitative data.

Keywords

Glutathione peroxidase 1, selenium, gel electrophoresis, chromatography ICP MS, Laser ablation ICP MS, western blot, enzymatic assay

Abbreviations

Ar, argon

GF-AAS, graphite furnace atomic absorption spectrometry

Gpx1, glutathione peroxidase 1

GR, glutathione reductase

GSH, reduced glutathione

GSSG, oxidized glutathione

H₂O₂, hydrogen hydroperoxide

HPLC, high-performance liquid chromatography

HRP, Horseradish peroxidase

ICP MS, inductively coupled plasma mass spectrometry

ICP-OES, inductively coupled plasma optical emission spectrometry

ICP-QQQ-MS, inductively coupled plasma-triple quadrupole-mass spectrometry

IEF, isoelectric focusing

IPG strips, immobilized pH gradient strips

LA-ICP MS, laser ablation-inductively coupled plasma mass spectrometry

LOD, limits of detection

MES, 2-(N-morpholino)ethanesulfonic acid

NADPH, β -nicotinamide adenine dinucleotide 2'-phosphate

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Se, selenium

Sec, selenocysteine

SEC, size exclusion chromatography

SQ-ICP MS, single quadrupole inductively coupled plasma-mass spectrometry

t-BHP, tert-butyl hydroperoxide

1. Introduction

Selenoproteins constitute an essential family of enzymes, in which selenium, in the form of selenocysteine, is critical for the catalysis of redox reactions [1, 2]. Glutathione peroxidase 1 (Gpx1) was the first selenoprotein discovered in mammals and has therefore been the most studied so far [3]. Gpx1 is the most abundant selenoprotein and is found in cytosol, mitochondria, and, in some cells, in peroxisome intracellular compartments. Gpx1 prevents the accumulation of H₂O₂ in various physiological and pathophysiological conditions, using GSH as a cofactor. This selenoenzyme is also able to reduce lipid hydroperoxides and other soluble hydroperoxides (such as t-butyl hydroperoxide or cumene hydroperoxide). Ubiquitously expressed in animals, Gpx1 has been purified from erythrocytes and is now one of the two commercially available selenoproteins together with the thioredoxin reductases. Gpx1 forms a homo-tetramer of approximately 88 kDa (22 kDa for a monomer) and has a single selenocysteine residue in the catalytic site. In mammals, Gpx1 is part of the glutathione peroxidase family composed of eight members (Gpx1-Gpx8), half of which are selenoproteins (Gpx1-Gpx4; Gpx6 is a selenoprotein in humans but not in rats or mice [4]), the others having a catalytic cysteine residue instead of a selenocysteine. Gpxs have critical antioxidant functions in different cell types, intracellular compartments or in the plasma. They also have partially overlapping substrate specificities, as reviewed in [3]. The function and substrate specificity of Gpxs are often driven by the cellular localisation.

Although essential for life, selenium must be carefully considered, since its beneficial window is narrow and follows a bell-shaped curve. The beneficial effects of selenium are mostly awarded to its insertion in the selenoproteins as a rare amino acid, the selenocysteine. To date, 25 selenoprotein genes have been identified in human genome (24 in rodents), and half of them have been functionally characterized in antioxidant defense, redox homeostasis, and redox signaling [4-7]. Selenoprotein expression is highly dependent on selenium levels

from the diet and is also affected by pathophysiological conditions [5, 8-10]. The regulation of Gpx1 expression levels in response to many physiological and pathological conditions has been thoroughly described [8, 9, 11, 12]. In fact, Gpx1 is one of the most sensitive selenoproteins to selenium level variations, and is therefore commonly used as a marker of selenium status in many epidemiological studies. Therefore, various analytical methodologies, based on the different properties of Gpx1 have been developed to quantify this selenoenzyme.

Historically (1967), it is the enzymatic activity of GPx1 that was proposed for its detection in blood samples [13]. Since Gpx1 is able to reduce a wide variety of hydroperoxides (ROOH) to corresponding alcohols (ROH) and water using cellular glutathione as the reducing reagent, several substrates have been used for *in vitro* enzymatic assays, including H₂O₂, *tert*-butyl hydroperoxide and cumene hydroperoxides. These enzymatic assays have allowed the routine quantification of Gpx1 in many biological fluids and cellular extracts [9, 11, 12, 14, 15].

Alternatively, immunodetection of Gpx1 have been widely developed mostly in biomedical research. It is based on the separation of the Gpx1 from other proteins and the blotting on the membrane containing the labelled antibodies. After the washing of the excess of the antibody the Gpx1-antibody complex is detected by chemiluminescence with a secondary antibody coupled with horseradish peroxidase (HRP) . When a luminol substrate is present, HRP produces a blue light that is quantitatively related to the amount of Gpx1-antibody complex. This strategy has allowed the relative evaluation of Gpx1 levels in many samples from various species [9, 11, 12, 14, 15].

The recognition of the importance of selenium in the activity of Gpx1 resulted in the use of an inductively coupled plasma mass spectrometric detection (ICP MS) to quantify the Gpx1 separated from other selenoproteins by gel electrophoresis (SDS or IEF) [17,18] or size-exclusion chromatography [16, 17] using the intensity of the selenium of selenium signal.

The purpose of this study was to optimize the four above methods, i.e. (i) enzymatic assay, SDS-gel electrophoresis with (ii) immunoblotting and with (iii) laser ablation – ICP MS detection, and (iv) size-exclusion chromatography – ICP MS for the quantification of selenium in the same preparation of Gpx1 with maximum sensitivity and discuss critically their advantages and limitations.

2. Materials and methods

2.1. Materials

Glutathione peroxidase (Gpx) from bovine erythrocytes, glutathione reductase (GR), L-glutathione reduced (L-GSH), tert-butyl hydroperoxide (t-BHP), and β -nicotinamide adenine dinucleotide 2-phosphate reduced (NADPH), were purchased from Sigma-Aldrich. Antibodies were purchased from Abcam (Gpx1, #ab108429) and Sigma (anti-Rabbit IgG (whole molecule) produced in goat coupled with horseradish peroxidase, #A6154); NuPAGE 4-12% bis-Tris polyacrylamide gels, MES SDS running buffer were purchased from Life Technologies.

2.1. GPx enzymatic assay

Gpx activities were measured in an enzymatic-coupled assay according to methods described previously [13]. The reaction mixture was composed of commercially available glutathione peroxidase from bovine (from 8 up to 40 mU), 0.25 mM NADPH, 2 mM reduced L-glutathione, and 1.5 U of glutathione reductase adjusted to a total volume of 250 μ L with 50 mM potassium phosphate buffer (pH 7.5). The reaction was started by the addition of 300 nM t-BHP, and consumption of NADPH was followed at 340 nm with a FLUOstar OPTIMA (BMG Labtech) microplate reader at room temperature.

2.2. SDS-PAGE for ICP MS analysis and western blot

The same lot number of dried Gpx (500 units) was used throughout the study. It had the following features: 4.72 mg of solid powder, 106 units of Gpx per mg of solid powder and 741 units per mg of protein. Gpx was resuspended in sterile H₂O, aliquoted and stored at -20°C. The precise quantities of Gpx loaded on the gels were referred to the solid powder. Different protein amounts were separated in Bis-Tris NuPAGE Novex Midi Gels with MES SDS running buffer. Gels were either stained with coomassie blue and dried or transferred onto nitrocellulose membranes using iBlot® DRY blotting System (Life Technologies). The membranes were either stained with coomassie blue and dried for LA-ICP MS analysis or directly analyzed by western immunoblotting.

2.3. Western immunoblot blots

Equal protein amounts (50 µg) were separated in Bis-Tris NuPAGE Novex Midi Gels and transferred onto nitrocellulose membranes using iBlot® DRY blotting System (Life Technologies). Membranes were probed with primary antibodies against Gpx1 at 1/5000 dilution and HRP-conjugated anti-rabbit secondary antibodies at 1:40000 dilution. The chemiluminescence signal was detected using the ECL Select Western Blotting Detection Kit (GE Healthcare) and the PXi 4 CCD camera (Ozyme) with various acquisition times (from 10 s to 5 min). Image acquisition and quantifications were performed with the Syngene softwares, GeneSys and Genetools, respectively.

2.4. Laser ablation coupled with ICP MS detection (LA-ICP MS)

A 213-nm laser (New wave research UP-213) was coupled with ICP MS (Agilent 7700) as described elsewhere [18-20]. The scanning was performed at the repetition rate of 20 Hz, fluence of 4 J.cm⁻², spot size of 250 µm, and scan speed of 100 µm.s⁻¹. Ablated material was

carried to ICP MS by 500 mL.min⁻¹ flow of He mixed just before the torch entrance with 760 mL.min⁻¹ of Ar gas. ICP MS was used in the collision cell mode using He as the collision/reaction gas. A 1.5 mm i.d. injector torch and Pt cones were used. The spectrometer was tuned off-line for the maximum Se signal-to-noise ratio. The dried SDS-PAGE or membrane was placed on glass slide with a double-sided tape. Three scans were performed for each lane. ¹³C, ⁷⁶Se, ⁷⁷Se and ⁷⁸Se signal were recorded with an integration time per element of 100 ms, except for ¹³C (10 ms). Only the sum of ⁷⁸Se signal was integrated for each peak for quantification.

2.5. Size exclusion chromatography (SEC) with ICP MS detection

An Agilent model 1200 HPLC instrument equipped with an autosampler was coupled with an Agilent 7500 ICP MS (Agilent, Hachi-oji, Japan). ICP MS was used in the collision cell mode using H₂ as the collision/reaction gas. A 1.5 mm i.d. injector torch and Pt cones were used. The spectrometer was tuned off-line for the maximum Se signal-to-noise ratio. ICP MS was set up for multi-isotopic detection (⁷⁶Se, ⁷⁷Se and ⁷⁸Se) with an integration time per isotope of 100 ms. The chromatographic column was a HiTrap 5-ml column, prepacked with Sephadex G-25 Superfine (GE Healthcare). The mobile phase was 100 mM ammonium acetate buffered with ammonia to pH 7.4, and the flow rate was 0.7 mL.min⁻¹. The GPx sequential dilutions were prepared in duplicates. A 100 µL aliquot was injected in the HPLC. ICP MS signals were exported and treated with Microsoft Excel software to represent chromatograms.

3. Results

3.1. Measurement of Gpx1 by enzymatic activity

GPx enzymes catalyze the reduction of hydrogen peroxide (H_2O_2) and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent. The intracellular Gpx activity has been measured by using a coupled enzymatic assay (see Figure 1A). Briefly, when reducing the *tert*-butyl hydroperoxide into *tert*-butanol, Gpx oxidizes two GSH to produce one molecule of GSSG. The recycling of oxidized GSSG back to reduced GSH is performed by the glutathione reductase (GR) which oxidizes NADPH to NADP. The decrease of the NADPH absorbance measured at 340 nm, is directly proportional of Gpx activity in the sample since this enzyme is the rate-limiting factor of the coupled reactions [13]. Precise quantities of Gpx1 (from 8 up to 40 mU) were mixed to the reagents listed in Table 1, and the reaction was started by the addition of the substrate, *tert*-butyl hydroperoxide, as described in Materials and Methods. The rate of NADPH decrease was measured by the slope of the curve during the linear range, when the enzymatic steady state was reached (see Figure 1B). The condition with no enzyme was used to remove background levels of naturally occurring oxidation of NADPH. Then, the rate of the NADPH decrease was plotted as a function of amount of enzymes in the reaction (Figure 1C). A linear correlation was observed between the speed of reaction (V) and enzyme amount (E_T) when the Gpx is the rate-limiting element of the enzyme-coupled reaction. This relation provided the catalytic constant, referred to as k_{cat} . It was necessary to extensively dilute the preparation of the Gpx1 stock solution to be in the linear range of the reaction, the detection limit being with 8 mU of Gpx1, which is equivalent to 75.5 pg of Gpx preparation (solid weight), 108 pg of pure Gpx and 0.477 fmol of Gpx (or Se, 1 atom of Se per molecule of Gpx).

3.2. Detection of Gpx1 by SDS – PAGE and western immunoblotting

Immunodetection of proteins immobilized on nitrocellulose membranes constitute a rather old, but worldwide used technique. It represents an adequate technique to evaluate the relative expression of a protein from one condition to another or to others. Here, we used a commercially available antibody against Gpx1 that had been previously validated for western immunoblotting detection of Gpx1 in cellular extracts [8, 9, 21]. The primary antibody was obtained by immunization of rabbits with an epitope of Gpx1. With an anti-rabbit antibody coupled with horseradish peroxidase (HRP), the presence of the Gpx1-antibody complex was revealed by the addition HRP substrates. In our case, HRP catalyzed the decomposition of luminol in the presence of peroxide to produce a blue light, which was be recorded by a CCD camera. The light produced was proportional to the HRP-coupled antibody, and therefore the presence of Gpx1. The signal acquisition by the CCD camera allowed the sequential image recording equivalent to different exposure times. Therefore, when the signal is too intense, shorter exposure times should be used to avoid saturation of the picture. On the other side, when the signal is weak, increasing acquisition times is a convenient strategy to obtain a significant signal to noise ratio for a band of weak intensity. As shown in Figure 2, the detection limit of Gpx1 depended on the exposure time, with 0.05 and 0.01 μg at either 10 s or 5 min, respectively. The signal was linear over two orders of magnitude.

3.3. Quantification of Gpx1 by LA-ICP MS in SDS-PAGE

GPx1 separated by gel electrophoresis can be quantified owing to the presence of selenium by quantifying the latter by laser ablation ICP MS (LA-ICP MS). This analytical strategy was successfully applied to the detection of selenoproteins in SDS-PAGE [20, 22] and in immobilized pH gradient (IPG) strips [18, 19]. The sensitivity depends on the amount of ablated material (laser ablation parameters) but too much ablated gel can lead to the

clogging of the torch. This is why the blotting onto a nitrocellulose membrane was also investigated. The optimum conditions (Fig. 2A), the sample was ablated by a 250 μm laser beam (213 nm), at a scan speed of 100 $\mu\text{m}\cdot\text{s}^{-1}$. Each protein lane was ablated, consecutively, three times, and the signals from ^{13}C , ^{76}Se , ^{77}Se , and ^{78}Se were recorded, as illustrated in Figure 3B. The coomassie blue staining of the GPx protein in a dried gel and in the nitrocellulose membrane, is shown in Figure 3C. Both the height and area of every ^{78}Se peak were integrated and plotted as a function of GPx amount loaded on original gels. The detection limits were 10 times lower in the case of the membrane in comparison with the gel (See Table 2). This was due to the concentration of the protein on the surface of the membrane. Also, the ablation of the membrane reduced the material load into the plasma and thus matrix suppression effect. The lanes for which no signal was detected from the background have not been represented in the histograms of Figure 3D. The signal intensity, represented in a log/log scale, was linearly dependent on the Gpx amount loaded in the gel or membrane over two orders magnitude. Our results also show that either peak height or area can be used indifferently for quantification.

3.4. Quantitative detection of Gpx1 by SEC-ICP MS

ICP MS is a convenient technique to measure the selenium on-line after the separation of selenoproteins by chromatography because of its low detection limits (in comparison with ICP emission spectrometry) and the ease of the coupling (in comparison with graphite furnace atomic absorption spectrometry (GF-AAS)). The set-up of sample injection is represented in Figure 4A. Gpx1 produces a single peak eluting at the volume corresponding to its molecular mass (Figs. 4B and C). The ^{78}Se signal integrated and plotted as a function of injected Gpx amount (in μg) becomes undetectable below 1 μg of Gpx preparation (solid powder of Gpx

vial). This corresponds to 143 ng, 6.3 pmol or 106 Units of pure Gpx, and 499 pg of Se (See Table 2).

4. Discussion

The specific quantification of the GPx1 is a challenging issue, because of its presence at low concentration levels. Therefore, the basis of our discussion is the detection limits. They were optimized for the lowest possible values to be obtained for each techniques discussed.

Sensitivity and detection limits. The method based on the measurement of the enzymatic activity is by far the most sensitive. Table 2 shows that as little as 0.5 attomole of Gpx1 can be detected. This is 100 times less than in the second most sensitive technique based on gel electrophoresis and western immunoblotting. ICP MS detection is much less sensitive. Interestingly, immunodetection of Gpx1 also relies on an enzymatic assay, since the secondary antibody is coupled to HRP. To our knowledge, the most sensitive enzymatic assay reported in the literature has a detection limit of 9 zeptomoles ($9 \cdot 10^{-21}$ mol), which is less than 6000 molecules of acetate kinase enzyme, using luciferase-luciferin coupled assay [23, 24]. Therefore, possible improvement in the detection limit of Gpx1 enzymatic assay could be achieved by coupling Gpx1 activity with an enzyme generating a luminescent product.

The detection limits of laser ablation ICP MS after electroblotting are 10 lower than in the case of western blot and chemiluminescence detection. However, there is a considerable margin here for improvement using the latest triple quadrupole detection technology (ICP-QQQ-MS). Indeed, the most abundant selenium isotope ^{80}Se is severely interfered with the argon dimer ($^{40}\text{Ar}^{40}\text{Ar}$), even when using a collision/reaction cell. Additionally, when hydrogen (H_2) is used as a collision/reaction gas to reduce argon dimer, other interferences may arise, such as $^{79}\text{Br}^1\text{H}$ and $^{81}\text{Br}^1\text{H}$, which interfere with ^{80}Se and ^{82}Se respectively. The

reduction of interferences using the triple quad technology is likely to reduce the detection limits by a factor of 10 [25, 26]. The first quadrupole (Q1) filters the mass-to-charge ratio (m/z) of selenium (^{80}Se , being the most abundant). The selected ions enter a second quadrupole (Q2) filled with O_2 to form SeO^+ (m/z 96), which is finally filtered by the third quadrupole (Q3). The use of chromatography is the least sensitive approach mostly because of the huge peak dispersion in SEC. As ICP MS is a mass sensitive detector, any reduction of the flow rate and columns of smaller diameter would not improve the detection limits. The ammonium acetate buffer used is sufficiently volatile not to affect the detection limits negatively, anyhow, the reduction of its concentration would have increased the interactions of the proteins with the stationary phase and perturb separation. A possible solution would be the use of reversed-phase chromatography allowing the preconcentration but the recoveries with the currently available stationary phases are rather low (below 10%).

Dynamic (linearity) range. The quantification of GPx1 in unknown samples requires a certain dynamic range to allow a calibration curve to be used. Our data show that the linearity of the enzymatic assay does not exceed one order of magnitude which implies the necessity of several dilutions and precise choice of calibration standards, especially when analyzing unknown samples. The linearity range of two orders of magnitude of gel electrophoresis and western blotting is by far worse of that of ICP MS detection where the linearity range of over 5 decades is readily observed in SEC-ICP MS and can be slightly limited by the electrophoresis and electroblotting conditions in gel electrophoresis – laser ablation ICP MS.

Selectivity. Another parameter to be considered during the choice of the method is the selectivity towards the other proteins and selenoproteins. The enzymatic assay is not specific since several glutathione peroxidases can use the same substrates, although with different

catalytic constants (k_{cat}) [3]. To date, there are no highly specific substrates for individual mammalian Gpxs. Therefore, this enzymatic activity reflects only the overall activity of all Gpxs in a defined cellular or tissue extract. Concerning the other selenoproteins the precise function is known for only two third of them. Enzymatic activities are only available for: glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases, methionine-R-sulfoxide reductase 1 (Msrb1) and selenophosphate synthetase 2 (Sephs2).

The western immunoblotting critically depends on the availability of the selective (specific) antibodies. In addition, the cross-reactivity of one antibody with an antigen from different species is often not straightforward, and must be systematically tested. Moreover, the titration of the antibody is highly variable from the serum of one immunized animal to another, making absolute quantification of a defined target rather arduous. In the case of Gpx1, we have tested several antibodies from various origins and came out with one offering a good ratio between high sensitivity and low non-specific binding [8, 9, 21].

The selectivity of coupled techniques towards selenoproteins is achieved by the specificity of the ICP MS detector to selenium which is very high. However, there remains the question of the selectivity within the class of the selenium-containing proteins which is conferred by the selectivity of the separation mechanism. The selectivity of the size-exclusion chromatography is by far insufficient but there is a possibility to increase it by testing the separation is denaturing (22kDa) and nondenaturing (88kDa) conditions. The selectivity of gel electrophoresis is better, especially that separations can be alternatively carried out using isoelectrofocusing strips. For example, in the case of human selenoproteome (25 selenoprotein genes), the theoretical pIs of the selenoproteins range from 4.96 and 10.54. The migration of a human protein cell extract on an immobilized pH gradient (IPG) strip 3-10 allowed the reproductive separation and detection of several selenoproteins by LA-ICP MS, including Gpx1, Gpx4, Trxrd1, Trxrd2 and SelenoF (also known as Sel15) [18, 19]. In

addition, narrower pH gradients are available to improve the resolving power of the electrophoresis and potentially separate selenoprotein isoforms, without enlarging the peak width. Concerning the human selenoproteome, the separation capacity of gel electrophoresis only based on protein size, such as SDS-PAGE, is limited since 60 % of the selenoproteins are in a range comprised between 10 and 30 kDa molecular weight range.

Note that the detection of selenoproteins by ICP MS is strictly dependent on the presence of selenium in the recovered and separated proteins. If selenium is lost during sample preparation or separation, errors occur. Therefore, the possibility of dehydroalanation (loss of Se from selenocysteine should be minimized, e.g. by derivatization).

Matrix effects and recovery. Finally, there is a question of the matrix and the recovery of GPx1 from solid samples. The size and homogeneity of the particles generated by the laser beam are critical for optimal atomization and ionization of the ablated material by the plasma torch. These generated particles depend on several parameters, several of them having significant room for improvement. The quality of the ablated material, in term of toughness, thickness and dryness, is the first parameter that has been optimized. Here, we have found that the LOD for Gpx1 was 10 times lower when a membrane was ablated instead of a gel, most likely, because the thickness of the membrane was approximately 10 times smaller. A downside is that dry nitrocellulose membranes need to be manipulated with care in contrast to dried polyacrylamide gels. Another parameter that can be improved with next generation of instrumentation is the laser itself. Potential optimizations concern energy, pulse (time and frequency), and stability of the laser beam over the scan time, which can last between several minutes to hours in case of tissue imaging. As last major parameter for solid sample detection and recovery in LA-ICP MS, the ablation chamber is crucial for optimal ablation and transfer

of ablated material into the ICP-MS. Several parameters including volume, geometry and temperature of the ablation chamber can still be improved.

5. Conclusions

The critical analysis of the four principal strategies for the quantification of GPx1 reported in the literature shows that the sensitivity of the methods such as the enzymatic assay and gel electrophoresis with immunoblotting and chemiluminescence detection is off-set by a higher selectivity of the techniques combining the separation, especially by gel electrophoresis, with Se-specific detection. The different strategies appear to be complementary. Interlaboratory studies comparing results for different matrices using different analysis principles are necessary in order to compare quantitative results obtained by different laboratories. Also, in contrast to the enzymatic assay, gel electrophoresis with immunoblotting or ICP MS detection offer the possibility of the simultaneous detection of several proteins constituting the selenoproteome. In terms of detection limits, the use of ICP triple quadrupole MS seems to be promising but the principal limitation of the Se-specific detection remains the fact that only one selenium atom is present in selenoproteins except for SelenoP that contains between 10 and 17 (depending on organism) Sec residues.

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Figure captions

Figure 1. Detection of Gpx1 by enzymatic coupled assay

A. Schematic of Gpx enzymatic assay. The limiting reaction is the catalytic reduction of the Terbutyl hydroperoxide by the Gpx. The recycling of the oxidized glutathione by the glutathione reductase is coupled to the oxidation of NADPH, which is visible at 340 nm with a spectrophotometer. The decrease in NADPH signal is directly proportional to the Gpx activity. **B.** Illustration of the kinetics obtained 40 U of Gpx (black diamonds) or none (white diamonds). The slope of the linear part of the kinetic is directly linked to the quantity of the limiting enzyme (E_T).

Figure 2. Detection of Gpx1 by western immunoblot

A. Various quantities of Gpx1 are analyzed by western immuno blots using a CCD camera. The results from different exposure time, 10sec and 5 minutes, respectively, are shown. **B.** The chemiluminescent signals are integrated and plotted as a function of Gpx amounts loaded in the original SDS-PAGE, in a logarithmic scale. The linear part is indicated by a bar.

Figure 3. Detection of Gpx1 by LA-ICP MS in SDS-PAGE

A. Schematic of the LA-ICP MS setup for the detection of Gpx1 as described in material and methods. **B.** The signal for ^{13}C (grey), ^{76}Se (red), ^{77}Se (blue) and ^{78}Se (black) are recorded with the ICP MS in a typical scan of a SDS-PAGE. The ^{13}C signal is used as an indicator of proper and constant injection of ablated material into the ICP MS. In our setup, the laser beam is shut off between each scanned line. **C.** Illustration of the migration of GPx1 in a SDS-PAGE either directly stained or transferred to a nitrocellulose membrane and then stained. Various quantities of Gpx1 were loaded as indicated above the gels. **D.** The gel or the

membrane are scanned with the LA-ICP MS, and the signal was recorded. The peak area and peak height are integrated and plotted as a function of the quantity of Gpx1 loaded in the initial SDS-PAGE. The results obtained for the SDS-PAGE and the membrane are shown in black and bleu, respectively.

Figure 4. Detection of Gpx1 by SEC-ICP MS in solution

A. Schematic of the SEC-ICP MS setup for the detection of Gpx1 as described in material and methods. Chromatograms obtained after the injection of either highest (**B.** 50, 20, 10 and 5 μg) or lowest (**C.** 2, 1, 0.5 and 0.2 μg) quantities of Gpx1. Signal for ^{76}Se , ^{77}Se and ^{78}Se are recorded, but only one replicate of ^{78}Se is shown for clarity. Raw selenium signals are shown in grey and a local average data treatments are shown in black. **D.** The ^{78}Se signal peak area is integrated and plotted as a function of the quantity of injected Gpx1, in a logarithmic scale.

Table 1. Composition of the reaction mixture for Gpx microplate enzymatic assay

Reagent	Stock conc.	Units	Working conc.	Units	Working volume	Units	Final conc.	Units
Glutathione peroxidase	1	U, μL^{-1}	2	mU, μL^{-1}	0 - 20	μL	0 - 0.04 U	mU, μL^{-1}
L-Glutathione reduced	105	mM	105	mM	5	μL	2.1	mM
β -Nicotinamide adenine dinucleotide 2'-phosphate reduced	5	mM	5	mM	25	μL	0.5	mM
Glutathione reductase	450	U, μL^{-1}	7.5	U, μL^{-1}	10	μL	0.3	U, μL^{-1}
<i>Tert</i> -butyl hydroperoxide	69.76	M	300	nM	2.5	μL	3	nM
Potassium phosphate buffer 50 mM, pH7.5					187.5 - 207.5	μL		
Final volume					250	μL		

Table 2. Comparison of the detection limits between the various methodologies.

List of the Limits of detection (LOD) for Gpx1 reached with various methodologies. The stock of 500 U of purified bovine Gpx contained 4.72 mg of solid, 106 Units.mg⁻¹ of solid, 741 Units.mg⁻¹ of protein.

Methodology	μg of Solid	ng Gpx	pmol Gpx	mU	pg Se
Enzymatic activity	0.0000755	0.0108	0.000477	0.0080	0.0377
Western blot	0.01	1.43	0.0631	1.06	4.99
LA-ICP MS					
Membrane	0.1	14.3	0.631	10.6	49.9
Gel	1	143	6.31	106	499
SEC-ICP MS	1	143	6.31	106	499

Figure 1

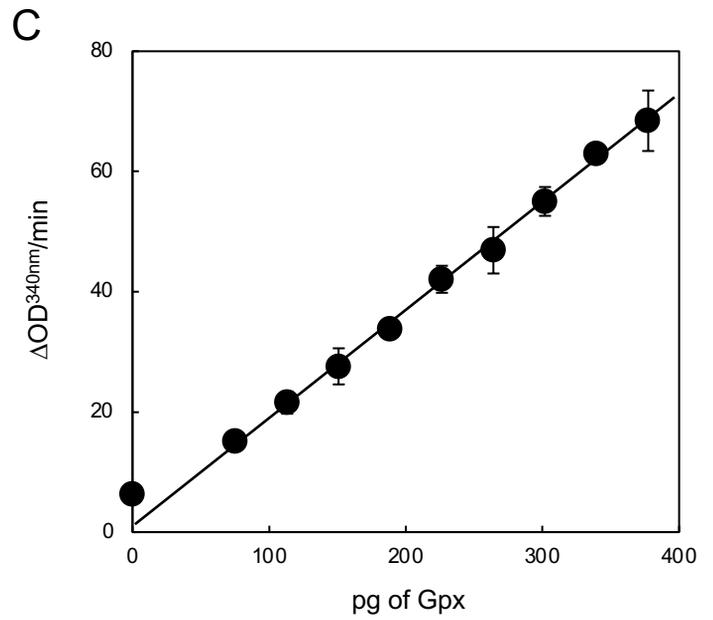
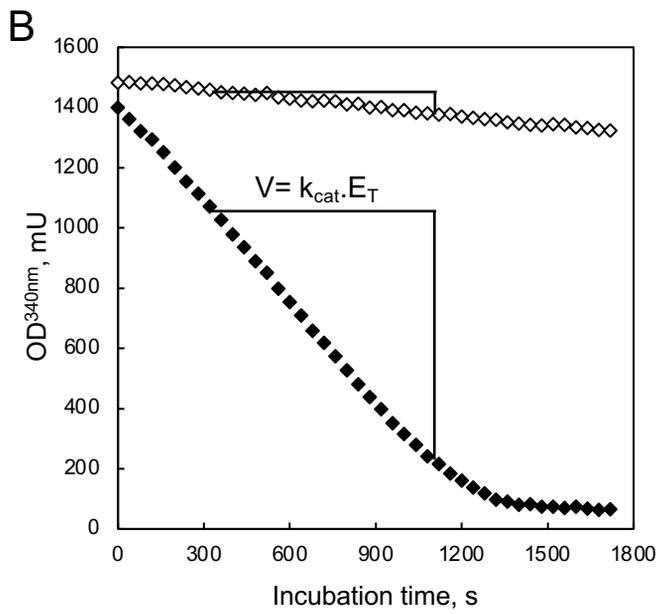
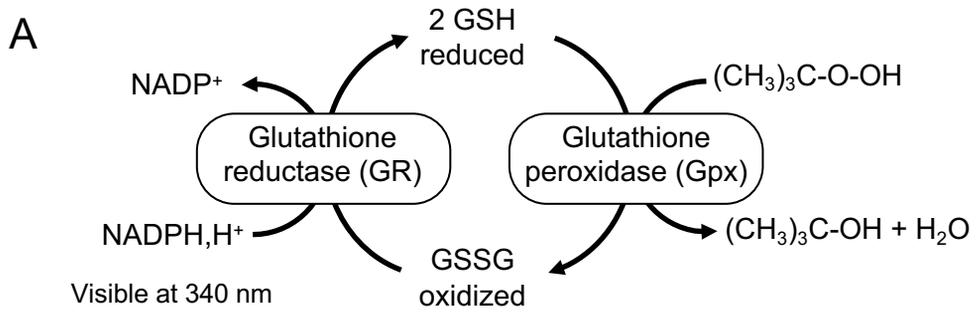


Figure 2

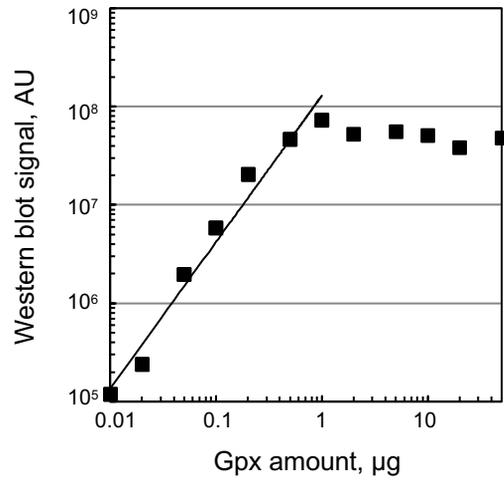
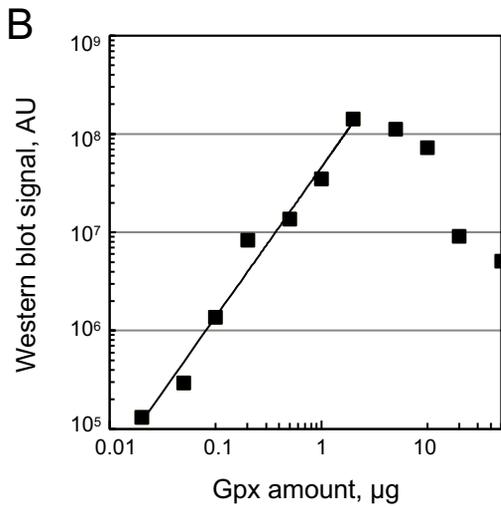
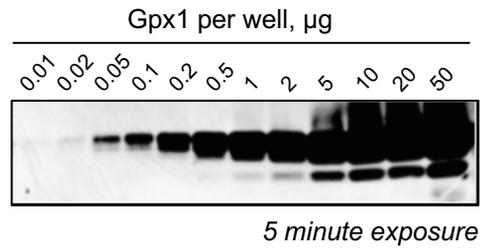
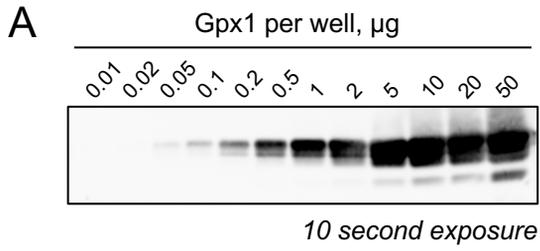


Figure 3

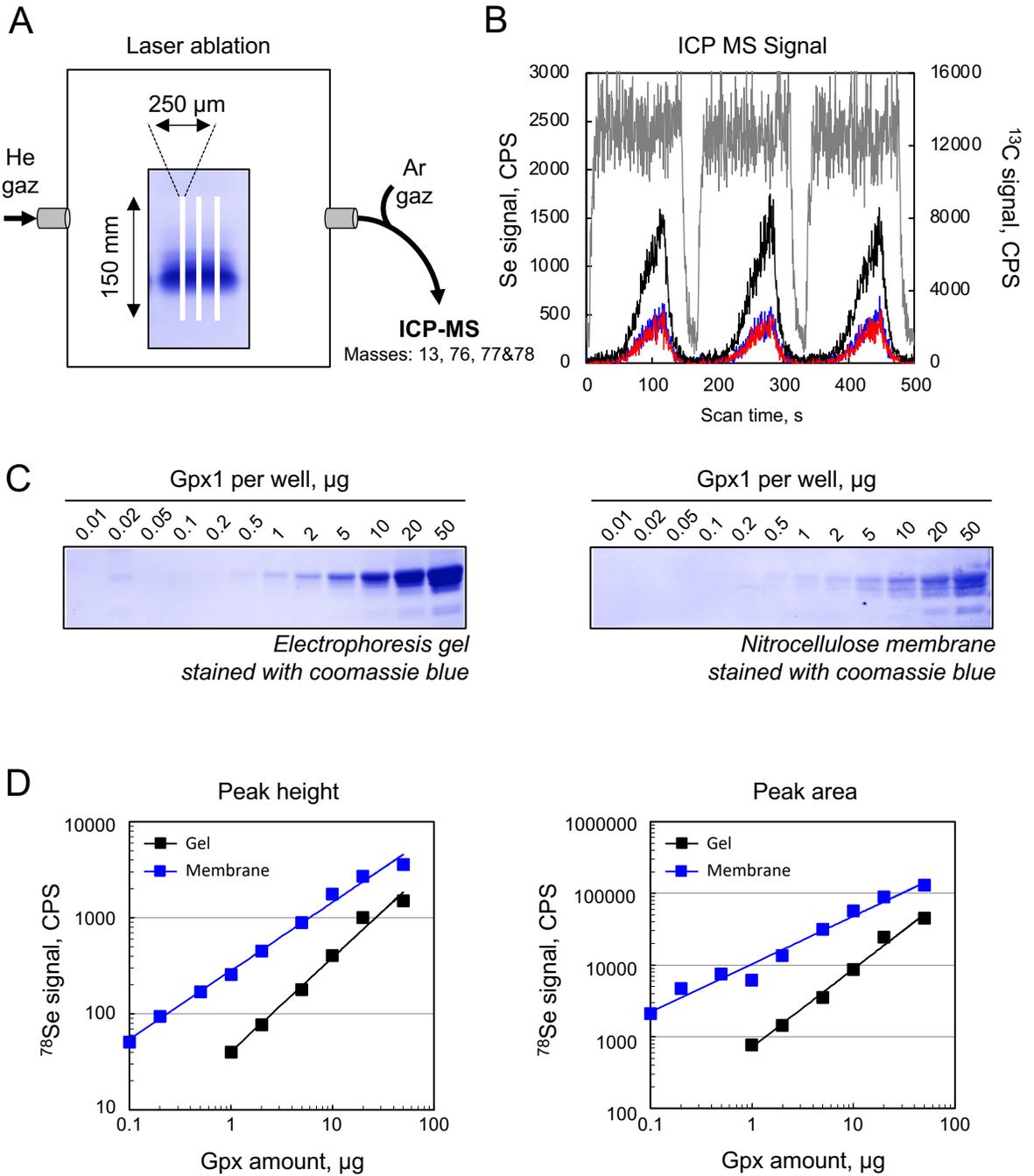
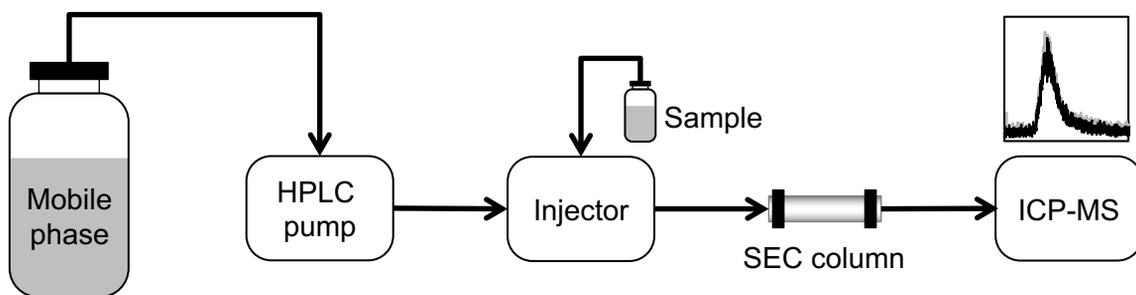
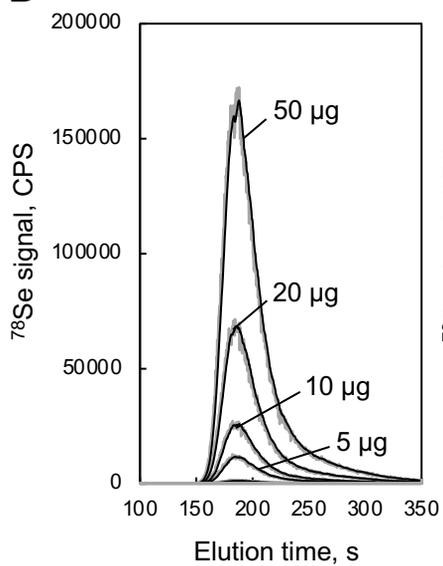


Figure 4

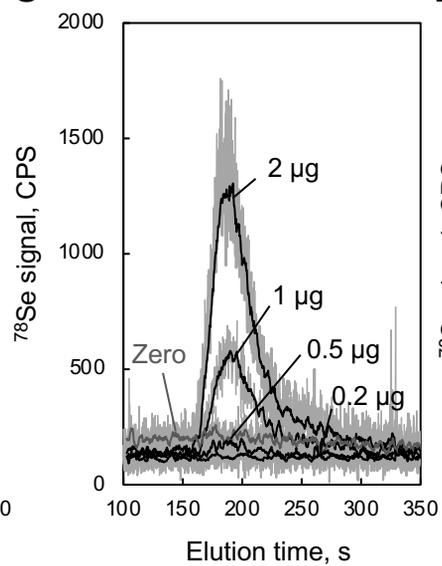
A



B



C



D

