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Review

Analytical methods to investigate glutathione and related compounds in biological and pathological processes

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Abstract

Reduced glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is a fundamental low-molecular mass antioxidant that serves several biological functions. Upon enzymatic and non-enzymatic oxidation, GSH forms glutathione disulfide (GSSG) and, under particular conditions, may generate other oxidative products. The determination of GSH, its precursors, and metabolites in several bio-matrices is a useful tool in studying oxidative stress. Many separative and non-separative methods have been developed and improved for the assay of GSH and related compounds. At present, high-performance liquid chromatography and capillary electrophoresis are the most used separative techniques to determine GSH and congeners. The review will deal with analytical methods developed over the last few years for the determination of GSH and related compounds, and with the procedures performed in sample pre-treatment in order to minimize analytical errors. Since GSH, GSSG, and related compounds lack of strong chromophores or fluorophores, it is advantageous, in many assays, to derivatize the compounds in order to improve the detection limit with UV–Vis and to allow fluorescence, thus the most commonly used labeling agents are also described.

Keywords: Reviews; Glutathione

Contents

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1. Introduction

Cells are continuously challenged by oxidative stimuli coming either from external sources like the onset of inflammatory processes, exposure to UV radiation, contact with oxidative chemicals, or from internal metabolic pathways producing reactive oxygen species (ROS). Mitochondria, in particular, generate substantial quantities of ROS, mainly due to the uncontrolled leakage of electrons, which occurs during their transfer between mitochondrial complexes [1,2]. Cells oppose several antioxidant defenses in order to counteract the deleterious effects produced by radicals and reactive oxygen intermediates. A battery of enzymes and low-molecular mass reducing substances provides one of the first lines of defense. Sulphydryl-containing molecules are the most reactive reducing species operating at physiological pH found in cells, making them candidates for counteracting free radicals and ROS [3]. Reduced glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is the major non-protein thiol present in elevated amounts in living cells. In fact, GSH found up to a concentration of 12 m*M*, acts as a redox buffer to prevent oxidative damage due to its reducing and nucleophilic properties [4,5]. Lacking the toxicity otherwise associated with free cysteine (Cys), GSH is also responsible for the maintenance of protein thiol status in cells [6]. Glutathione is present mainly in a reduced form, and is the predominant component, while two principal oxidized forms, such as GSSG and protein–GSH mixed disulfide (PrSSG), are detected at lower levels [7]. GSSG is formed by enzymatic oxidation catalyzed by glutathione peroxidase (GPx), generating a disulfide bridge between two molecules of GSH. Similarly, oxidative mechanisms led to the formation of a GSH adduct with sulphydril groups in proteins [8]. GSSG usually accounts, in whole cells, for 1% of reduced glutathione. However, in subcellular compartments, such as mitochondria, the percentage of GSSG may increase to 25% of GSH [9]. Evidence has been gathered about the important role played by GSH in the detoxification and protection from oxidative injuries. As matter of fact, GSH behaves as a free radical scavenger and also helps in regenerating other antioxidants, e.g., vitamin E, and ascorbic acid [10,11]. GSH deficiency induced in animal models by the administration of a transition state inhibitor of γ-glutamyl-cysteine (γ-GluCys) synthetase, i.e. buthionine sulfoxime (BSO), leads to mitochondrial damage that can be prevented by supplementation with GSH esters or ascorbate [12,13]. Furthermore, reduction of GSH content in vivo and in vitro by BSO enhances the toxic effect of insults that are associated with elevated production of ROS [14–16]. On the contrary, elevated availability of Cys through *N*-acetylcysteine (NAC) administration increases

GSH levels and prevents ROS-mediated damage [15,17]. The mitochondrial changes deriving from conditions of altered GSH metabolism have been explored and discussed [1,2]. GSSG is a physiological indicator of intracellular defense system activity against ROS, and it can be used to monitor oxidative stress even in vivo [3,18]. GSSG can be measured in plasma, bile, lymph, and tissues. Increased levels of GSSG in tissues can be used qualitatively to detect enhanced detoxification of ROS in different organs, although concentrations are usually lower than in secretion fluids because of the active extrusion of GSSG from cells. Secretion of biliary GSSG is the most sensitive index of hepatocellular GSSG formation in vivo. Besides GSSG, depending on cellular environmental conditions and availability of nitric oxide (NO) donors, other oxidative metabolites of GSH, such as glutathione sulfonate (GSO_3H), glutathione sulfonamide (GSO_2NH_2), and *S*-nitrosoglutathione (GSNO), can be ultimately formed. In the last few years, interest in these GSH derivatives has been growing due to its likely involvement in cell signaling [19–21].

The following brief discussion, regarding the involvement of altered GSH homeostasis in pathologies, is far from being exhaustive and only supplies reader with a few examples of GSH redox system perturbation. Several pathological conditions are characterized by GSH deficiency or an imbalance in the GSSG/GSH ratio. In animal models, decreased levels of GSH in the brain have been detected after cerebral ischemia, followed by reperfusion [22]. The depleted GSH is converted in a minimal part as GSSG and mainly as PrSSG accompanied by a loss of protein thiols [23]. GSH deficiency in substantia nigra appears to participate in the pathomechanisms of Parkinson's disease and abnormalities of GSH metabolism have been associated with other disorders of the central nervous system, e.g., Alzheimer's and Huntington's diseases [13,24–26].

In experimental diabetes, obtained in animals by using inducers of hyperglycemia, a severe loss of GSH has been observed in the ocular lens associated with increased cellular mortality [27]. Human studies have shown an intracellular GSH deficiency in diabetic patients and, furthermore, the levels of GSH were inversely correlated with the degree of lipid peroxidation, the activation of transcription factors and fasting glycemia [28–30]. Available evidence suggests that the intact immune system depends upon a narrow balance between pro-oxidant and antioxidant conditions maintained by a limited and finely regulated supply of Cys. In acquired immunodeficiency syndrome, GSH and Cys deprivation associated with elevated plasma levels of glutamate have been detected, and a strong intracellular GSH influence on lymphocyte activation and proliferation has been reported [31]. Recently, decreased amounts of GSH in lymphocytes have been detected in patients affected by vitiligo, a skin depigmentary disorder in which oxidative stress has an important pathological role [32]. Physiological decrease in plasma levels of GSH have been observed associated with the age in male and female subjects. On the contrary, GSSG plasma content does not change significantly with age [33]. Currently, the study of GSH metabolism, the assessment of its intracellular and extracellular levels, and the regulation of biological functions take advantage of several analytical procedures. The literature offers different approaches in analyzing GSH, GSSG, related thiols, and the most recently discovered GSH derivatives, such as GSNO, GSO_3H, etc., in several biological substrates [34,35]. The aim of this review is to summarize the most recent analytical methods available to assess levels of GSH and congeners in body fluids, cells, and tissues. Since knowledge on the main features, functions, metabolism, and pathology of the GSH redox system is fundamental in applying informative analytical procedures to study the modification and manipulation of GSH, these aspects will also be covered.

2. Functions and metabolism of glutathione

2.1. Biological functions of glutathione

GSH has evolved to serve diverse functions in biological systems. One well-characterized role of GSH concerns its involvement as a reaction partner in the detoxification of xenobiotics. GSH also acts as a cofactor in isomerization reactions and as a storage and transport form of Cys, the latter being exceedingly cytotoxic [6,36]. Moreover, GSH is essential

for the regulation of cell proliferation [37] and maintains the thiol redox potential in the cells, keeping sulphydril groups of proteins in the reduced form [38,39]. An increasing number of reports have investigated the implication of GSH in apoptosis, a modality of cell death [40–42]. GSH is also involved in protein and DNA synthesis and in amino acid transport. The GSH system is very important for the cellular defence against oxidative and nitrosative stress [10,43,44]. A high intracellular concentration of GSH protects against a variety of different ROS [45]. The GSH redox cycle has a particularly relevant role in the prevention of oxidative damage that can occur in mitochondria, where catalase, the enzyme responsible for the removal of hydrogen peroxide, is lacking. It has been estimated that, in hepatocytes, 10–15% of the cellular GSH resides in mitochondria, where it is concentrated (10–12 vs. 7.0 m*M* in the cytosol) [2].

2.2. Biosynthesis and metabolism of glutathione

Since GSH has poor entry into the cells (except epithelial cells), it is synthesized intracellularly by the consecutive action of two enzymes [46]. As illustrated by reaction (1), L-γ-glutamyl-L-cysteine (γ-GluCys) synthetase uses glutamate (Glu) and Cys as substrates forming the dipeptide γ-GluCys that is then combined with glycine (Gly) in reaction (2), catalyzed by glutathione synthetase finally generating GSH. Adenosine triphosphate (ATP) is a co-substrate for both enzymes. The intracellular levels of GSH are regulated by feedback inhibition of γ-GluCys synthetase by the end product GSH itself [47,48].

$$\text{Glu} + \text{Cys} + \text{ATP} \rightarrow \gamma\text{-GluCys} + \text{ADP} + \text{P}_\text{i} \quad (1)$$

$$\gamma\text{-GluCys} + \text{Gly} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{P}_\text{i} \quad (2)$$

During detoxification of ROS, GSH is involved in two types of reactions: (i) GSH reacts with free radicals such as the superoxide radical anion, nitric oxide or the hydroxyl radical and (ii) GSH is the electron donor in the reduction of peroxides governed by glutathione peroxidase (GPx) [45,49]. In particular, reducing equivalents of GSH are consumed in the demolition of hydroperoxide carried out by GPx, leading to the formation of GSSG (reaction (3)), which is reduced back to GSH with the consumption of NADPH as electron donor by means of glutathione reductase (GR) (reaction (4)).

$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\,\text{H}_2\text{O} \quad (3)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\,\text{GSH} + \text{NADPH}^+ \quad (4)$$

Augmented GSSG levels and GSSG/GSH ratio are often suggestive of a state of oxidative stress [50]. In addition, the increased oxidation of GSH to GSSG can promote protein *S*-thiolation with accompanying loss of protein thiols. Even mixed disulphide between GSH and thiols in small mercaptane molecules like Cys, are formed [51]. Similarly to GR, protein–disulfide reductases including glutaredoxin, protein–disulfide-isomerase, and GSH–insulin transhydrogenase, regenerate GSH and protein thiols [2]. Other oxidation by-products of GSH, such as GSO_3H, GSO_2NH_2, and GSNO (under study to clarify their biological significance) may be generated under particular conditions [19,21,52]. It should be emphasized that these oxidation products differently from homo- and hetero-disulfides, cannot regenerate GSH under the standard reducing conditions [19].

GSH is not consumed during the reactions catalyzed by GPx and GR, but recycled. In contrast, the levels of total intracellular GSH are lowered during the generation of GSH–*S*-conjugates by glutathione-*S*-transferases (GST), or by release of GSH metabolites from cells [36,38]. In fact, when formed, GSSG is promptly and actively transported across the plasma membrane. Extrusion of GSSG and GSH conjugates outwards is a mechanism taking part in the complex of cellular defense system against ROS. [40]. Consequently, measurement of GSSG in plasma or extracellular fluids is useful in assessing the extent of cellular or tissue oxidative insult. GSH consumed for all these processes has to be replaced by re-synthesis from the constituent amino acids. Extracellular GSH and GSH-conjugates are substrates for the ectoenzyme γ-glutamyl transpeptidase (γ-GT) that catalyses the release of the γ-glutamyl moiety from GSH or its conjugate, thereby generating the dipeptide cysteinil–glycine (CysGly) or the CysGly-conjugated, respectively. CysGly can be

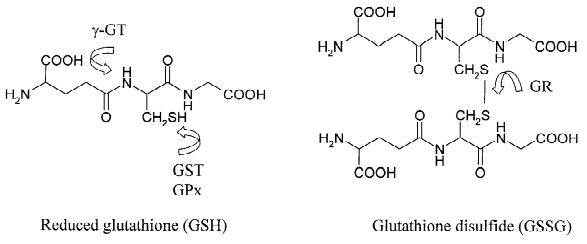

Fig. 1. Chemical structures of GSH and GSSG, and schematization of principal enzymes of glutathione metabolism.

further hydrolyzed by ectopeptidases to Cys and Gly, amino acids that are subsequently taken up by cells and can serve again as substrates for cellular GSH synthesis [15,38]. Fig. 1 depicts chemical structures of GSH and GSSG, reporting also the sites were the enzymes of the glutathione metabolism act.

3. Analytical methods for glutathione, congeners, and biotransformation products

GSH plays a fundamental role in cell homeostasis, and its qualitative and quantitative modifications are widely considered as an index of oxidative damage. In addition, nowadays many tools for manipulating GSH levels in cells and living systems are available and are applied extensively in studying GSH metabolism and oxidative stress. For example, NAC is used as a source of Cys to restore or elevate GSH content, whereas BSO is applied to deplete cells and organs by GSH [15,17,53]. Thus, measurement of actual GSH levels is also extremely useful in assessing the effectiveness of treatments with inhibitors and enhancers of GSH synthesis.

The requirements for an accurate GSH determination are, respectively, specificity to discriminate GSH from other thiols present in cells and biotransformation products, and selectivity to separate GSH and GSSG from possible interfering molecules in complex matrices. Even if sensitivity is one of the goals of many quantitative analytical methods, it is not critical for measurement of physiological levels of intracellular GSH since it is normally present in relatively high concentration. However, high sensitivity is important when GSH is significantly lowered by depleting agents or oxidative chemicals or physical agents or when determined in extracellular matrices. On the contrary, GSSG is present almost constantly at low levels in physiological conditions, accounting for about 1% of the intracellular GSH levels. Moreover, when the amount of GSSG increases consequent to GSH oxidation, it is promptly extruded from the cells, and its intracellular levels remain low. Extracellular compartments also present a low amount of GSSG due to its short half-life. Thus, high sensitivity is an indispensable requirement for GSSG determination in cells and in extracellular compartments. Determination of minor compounds deriving from GSH oxidation also requires an adequate level of sensitivity. Since sample collection, processing, and preparation are crucial steps in the analysis of GSH, GSSG, and congeners, affecting an accurate analyte measurement, the first sections are purposely dedicated to this matter. Further, the analytical methods developed in the last few years to determine GSH and analogues are reviewed in the next sections, and are placed in two principal classes: non-separative and separative techniques. Since sensitivity strongly depends on the detection mode, dedicated sections will report the state of the art by examining the main applications found in the literature according to the detection systems employed.

3.1. Sample pre-treatment

The major weak points in the GSH determination are the ease GSH autoxidation, and the enzymatic proteolysis of GSH by γ-GT. GSH rapidly oxidizes non-enzymatically at pH values greater than 7, and γ-GT, which catalyzes the first step of GSH proteolysis, has an optimum of activity at neutral pH [54,55]. Thus, it is suggested to maintain the pH of media where GSH is processed below pH 7 [9]. Other thiols, such as Cys, are even more susceptible to non-enzymatic oxidation than GSH, producing large losses due to the formation of oxidized forms and mixed disulfides during the sample preparation [54]. The measurement of GSSG, especially in plasma, also requires caution in order to avoid assay artifacts or data misinterpretation. When released in the blood, GSSG is degraded by proteolysis and has a half-life of less than 2 min [56]. Consequently, actual plasma GSSG concentration is the result of cellular release and immediate degradation. Further

precautions that minimize oxidation and proteolysis are refrigeration during sample processing and protein precipitation by acidification. On the contrary, sonication, a means for obtaining cell lyses, may lead to slightly increased GSSG levels. Senft et al. used a redox quenching buffer composed of HCl, diethylenetriaminepentaacetic acid, and ascorbic acid 10 m*M* in order to prevent thiol oxidation [56].

Several approaches have been explored in order to avoid erroneous estimation of GSH, GSSG, and other aminothiols (ATs) in biological substrates. Usually, the procedure for their determination is a function of a number of parameters such as separation technique used, detection selected, sample matrix composition, etc. Accordingly, the following points have to be checked in order to perform an accurate analysis: (i) sample collection, (ii) reduction of disulfides, (iii) deproteinisation, and (iv) derivatisation of reduced thiols.

3.1.1. Sample collection

Several errors can occur during sample collection affecting the accuracy of the results. Especially for blood and plasma, the main sources of errors are: (i) hemolysis of red blood cells, and (ii) storage at room temperature [57]. The former causes over-estimation of GSH in plasma since erythrocytes contain 500-fold higher GSH levels than plasma, whereas the latter leads to under-estimation of GSH since autoxidation and proteolysis are not repressed and GSH is consumed [51]. Measures can be adopted in order to limit these phenomena during sample collection. Jones et al. suggested the use of a butterfly needle for the blood withdrawal into heparinized tubes containing a preserving solution [58]. Agents participating in the preserving solution are inhibitors of γ-GT and chelating agents like 1,10 phenanthroline that, entrapping ferrous ions, prevents some oxidative reactions [59]. Differences between anticoagulants in terms of influence on thiol measurement in plasma have been evaluated [60]. Limitations caused by the long isolation time of cells from blood, separation of subcellular organelles, and preparation of tissues do not allow for a complete elimination of errors in the determination of GSH and GSSG in such specimens [9].

3.1.2. Disulfides reduction

Determination of total GSH and other low-molecular mass ATs requires the reduction of all disulfide species present in biological fluids, cells and tissues [61]. It has been reported that Cys, precursor of GSH, could be detected only after reduction of the extract [54]. The reduction of disulfide can be achieved by (i) enzymatic reaction (adding GR and NADPH to the sample) [62], (ii) electrolysis [63], (iii) chemical reaction. The last method can be performed with a variety of reducing compounds, such as sodium borohydride, several phosphines, dithiothreitol (DTT), 2-mercaptoethanol, etc. [9,33,52–54,56,63–68]. Even if all the reducing compounds effectively reduce GSH disulfides, the selection of the reagent is critical for assay performance. As examples, DTT or 2-mercaptoethanol, being thiol compounds themselves, can negatively affect GSH and GSSG assay because they react competitively with labeling reagents. It has been reported that they react with monobromobimane or *o*-phthaladehyde, leading to the formation of interfering fluorescent compounds [9,33,69]. However, DTT proved to be a superior reducing agent in comparison with commonly used reducing compounds, and the reaction can be performed in a phosphate buffer of approximately neutral pH [33]. Thus, selection of DTT concentration is a compromise between the amount necessary to obtain efficacious disulfide reduction and the amount required to avoid side-reactions and interfering peaks [70].

Sodium or potassium borohydride being rather drastic reducing agents are unstable in aqueous solution and disadvantageous mainly because of foaming, which causes serious problems in solution preparation. To prevent excess foaming, monophosphoric acid can be added to borohydride solutions [51].

Tang et al., reported that tri-*n*-butylphosphine (TBP) acted as an efficacious antioxidant and disulfide-cleaving agent, and enhanced the reaction between thiols and derivatizing agents [65,66]. Moreover, TBP does not produce gas during the reduction reaction [71]. However, TBP is used less frequently due to its explosive properties at relatively high concentration [63]. Triphenylphosphine (TPP) is more convenient due to its satisfactory reactivity and

greater safety. TPP, used in excess, also serves as prohibitory agent for the re-oxidation of ATs before their derivatization. Addition of chlorohydroxic acid to TPP solution sets conditions for catalyzing disulfide reduction [63,64]. Tris-(2-carboxyethyl)-phosphine, is another phosphine derivative solving the problems associated with the above compounds regarding stability and gas production [60]. Dithionite (DT, sodium hydrosulfite) has been also used for GSH disulfide reduction. It is worth noting that stoichiometry of GSSG reduction by DT is different from other reducing reagents, i.e. DT releases 1 thiol equivalent from GSSG instead of 2 obtained with other reducing agents.

3.1.3. Protein removal

Proteins represent the most abundant component in biological samples and, in most cases, must be removed before the chromatographic or electrophoretic analysis of GSH and analogues. In fact, the presence of proteins in injected samples may cause serious problems in the performance of the analytical procedure. For example, proteins in capillary electrophoresis are easily adsorbed on the capillary wall, strongly affecting migration time, peak shape, resolution, as well as detection response of analyzed compounds. Different approaches can be used to eliminate proteins: (i) acidification, (ii) addition of organic solvent, i.e., acetonitrile (ACN), acetone, methanol (MeOH) [60,72,73], (iii) ultrafiltration. Typically, the acidic agents used for this purpose are 5-sulfosalicylic acid (5-SSA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), perchloric acid (PCA), or metaphosphoric acid (MPA) [63,71,74,75], among these 5-SSA acid was indicated as the substance of choice, although it proved to favor GSNO formation from GSH [54,65]. In comparing protein precipitation methods, Caussé et al. established that the best results were obtained with ACN or 5-SSA precipitation in GSH identification upon derivatization with a fluorescent tag [60]. When the thiol group is not protected, precipitation by organic solvents does not prevent GSH autoxidation as in the case of acids. For example, to detect GSH and GSSG in hemolysate and bronchoalveolar lavage fluid (BALF), proteins were precipitated by PCA and MeOH, respectively. While upon storage of blood samples, GSH and GSSG maintained the relative proportions for up 30 min, oxidation of GSH to GSSG occurred rapidly in BALF [76]. Even if protein precipitation by acidification is the most effective, it has the disadvantage that the pH must be increased above 9 prior to derivatization of ATs with a chromophore or fluorophore. Dissolving the fluorogenic reagent in MeOH and treating plasma with the obtained solution saved the neutralization step before derivatization, shortening sample-processing time [33]. Organic solvents are preferable over acids when a mass spectrometer (MS) is used as detector [72,73].

The use of filters, excluding macromolecules on the basis of pore dimensions, is a valid method to remove proteins because it does not require the addition of acids or organic solvents that can affect separation, derivatization, and detection. The separation between most proteins and low-molecular compounds like GSH and GSSG through filters with low molecular cut off is achieved by applying high gravity force [74,77].

3.1.4. Reduced glutathione and glutathione disulfide derivatization

Because of the lack of strong chromophores and fluorophores in the chemical structure of GSH, GSSG and analogues, it is very often advantageous to derivatize the analyzed compounds introducing appropriate tags in the molecule in order to improve the detection limit with UV–Vis, allowing fluorescence-based detection [78]. In same cases, UV–Vis detectors equipped with extend path-length allowed a satisfactory detection limit even for underivatized GSH and GSSG [79]. The ideal labeling reagent should provide high detection sensitivity, specificity and must not be susceptible to major matrix interference reactions. The selected labeling agent should not require solvent extraction steps to remove reagent excess prior to chromatographic or electrophoretic analysis [68]. Even if GSH presents three sites susceptible to derivatization, i.e., carboxylic, amino, and thiol functional groups, labeling at the thiol moiety is preferred for its specificity and for its function as protective group. On the contrary, GSSG can only undergo derivatization on the amino and carboxylic groups. As far as we know, studies on

derivatization of GSH and GSSG on the carboxylic group have not been reported.

3.1.4.1. Introduction of a chromophore. The compounds most commonly used to cover the needs of thiol group protection and improved UV detection are *N*-ethylmaleimmide (NEM) and monoiodoacetic acid (MIAA), yielding thioethers. The reaction with NEM involves the addition of thiols across the double bond of maleimide, whereas MIAA forms an *S*-carboxymethyl (CM) derivative by nucleophilic substitution [72,73,77]. The superior effectiveness of MIAA in protecting GSH from autoxidation in comparison with NEM, was demonstrated by measuring the oxidation product GSSG by HPLC–UV in liver homogenates after addition of exogenous GSH [80]. Studies to optimize conditions for derivatization showed that reaction of thiols with MIAA was complete within a few minutes at pH 9.0 or higher [58,81]. Similarly, NEM is able to react rapidly with thiol bearing substances [72,77].

N-(2,4-Dinitrophenylaminoethyl)-maleimide, eosin-5-maleimide (EMA), and pyrenyl-maleimide are NEM analogues designed to further improve UV–Vis absorbance of GSH and related ATs [82–84].

5′,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) is a widely used reagent that forms a easily detectable adduct in presence of GSH and other thiols in pre-column reaction [67,85,86]. Using the reagent for post-column reaction and subsequent detection by conventional UV–Vis detector, Nozal et al. showed an alternative application of DTNB. Maximum absorbance was obtained when the derivatization was operated at a pH around 8.0. They proved that the addition of cationic micelles of hexadecyl-trimethylammonium bromide (HTAB) in the post-column reaction improved the detection limit of derivatives of three thiol compounds. In particular, the signal doubled for GSH and NAC and the maximum signal was achieved at a DTNB/HTAB ratio of 1:2 [67]. Double labeling may even be performed on ATs. When thiol group is trapped by MIAA, the free amino group of the GSH-CM adduct and also GSSG can be converted to *N*-(2,4-dinitrophenyl) (DNP) derivatives upon reaction with 2,4-dinitrofluorobenzene (DNFB) [59]. Optimal conditions of the labeling reactions and the stability of *S*-carboxymethyl-*N*-DNP derivative of GSH and *N*,*N*′-di-DNP derivative of GSSG were examined [59]. When used alone, DNFB displayed reactivity towards both thiol group and amino group as demonstrated by mass spectral analysis which proved the formation of *N*,*S*-di-DNP-GSH and *N*,*N*′-di-DNP-GSSG in the applied reaction conditions, thereby assisting in GSH protection and detection response [87].

Fluorophore reagents are also used to improve detection limit with UV–Vis detectors, even if it is much lower than that achieved with fluorometer [85,88,89].

3.1.4.2. Introduction of a fluorophore. To further increase the sensitivity of the analytical methods, fluorescence detectors have been used after introduction of fluorophore group into the chemical structure of studied compounds. The most frequently used fluorophores are those reacting with the thiol group being selective and preventing artifacts arising from auto-oxidation of GSH during sample processing and analysis. However, such derivatization does not allow the detection of GSSG. Contrarily, fluorophores reacting on the amino group of the peptides allow simultaneous fluorimetric determination of GSH and GSSG, even if less selective. In fact, the fluorophores can also react with other compounds present in the biological matrix generating interfering peaks.

o-Phthalaldehyde (OPA) is widely employed to form fluorescent adducts of GSH and related ATs. OPA is non-fluorescent until it reacts as a heterobifunctional reagent with a primary amine in the presence of thiol or vice versa, forming a fluorescent isoindole [90,91]. When GHS is derivatised with OPA, a co-reagent, such as 2-aminoethanol or Gly, may participate in the heterobifunctional reaction of OPA. However, other thiols may react in this condition [92]. OPA, in absence of a co-reagent, derivatizes selectively GSH, γ-GluCys and other ATs, supplying both the amino and thiol functions [53,56]. Careser et al. achieved quantification of total GSH at femtomole level by OPA derivatization without co-reagent [52]. Whereas, Parmentier et al. described a procedure where a co-reagent, Gly, added to eluents, directed the post-column OPA reaction selectively towards thiols [53]. Fig. 2 displays HPLC separation of a mixture of thiols deriva-

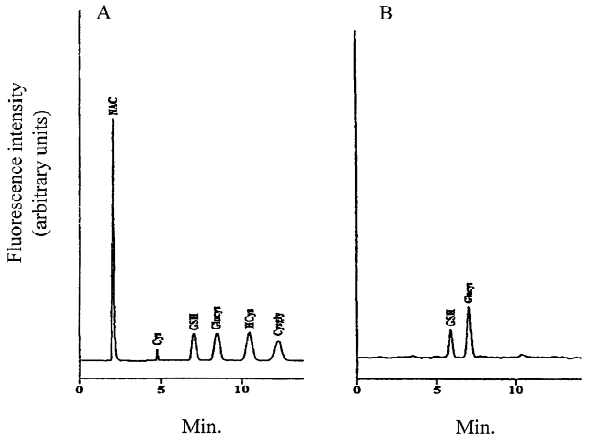

Fig. 2. Modified form Ref. [53]. HPLC-fluorimetric analysis of a mixture of standard thiols (NAC, Cys, GSH, γ-GluCys, HCys, and CysGly) derivatized by (A) thiol-selective OPA reaction and (B) heterobifunctional OPA reaction. HPLC and detection conditions as reported in Table 1. Mobile phase contained 0.5 m*M* Gly when thiol-selective OPA reaction was performed.

tized either with thiol selective OPA reaction or heterobifunctional OPA reaction. Derivatization of GSH with OPA is pH-dependent and maximal fluorescent yield is obtained with a reaction pH between 9.5 and 12 [53]. Although GSSG cannot react with OPA to form a fluorescent derivative, due to its hydrolysis at pH 12.0 producing GSH, a fluorescent derivative is obtained [91]. Using strong alkaline conditions for post-column reaction with OPA, it was possible to analyze fluorescent derivatives of separated GSH and GSSG. The OPA adducts with ATs have proved to be considerably stable under various storage conditions and the method was successfully used for automatic sampling analysis of GSH and analogues [91,92]. OPA also permits GSNO identification after removal of GSH by NEM from biological sample [69]. Naphthalene-2,3-dicarboxaldehyde, a reagent similar to OPA, was used effectively for GSH analysis, producing a fluorescent isoindole adduct with ATs [93].

Monobromobimane (BrB) is a specific reagent for the thiol functional group [54,65,85,94] and forms BrB-thiol adducts with relatively high fluorescence emission that allows the detection of analytes even at low concentration (2×10^{-8} *M*) [95]. Drawbacks of the BrB method include the fact that BrB is self-fluorescent and undergoes photodegradation, yielding fluorescent products, which interfere with thiol determination. Furthermore, BrB is not selective for GSH because other thiols can react, and typically unknown peaks are observed on the chromatogram [63,96]. However, this can be advantageous when the aim of the method is the simultaneous determination of GSH and related thiols Moreover, BrB requires pH adjustment following derivatization and gradient elution liquid chromatography.

Other interesting fluorescent reagents are fluorobenzofurazan derivatives, namely 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F), 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), which exhibit a high specificity for the thiol group [97]. The reaction between fluorobenzofurazans and the organic thiols is favored under basic conditions (pH 8.5–9.5) and at a temperature of 60 °C [88,98,99]. The fluorescent adducts are stable and not associated with reagent interference peaks [66,100]. ABD-F reagent exhibited several advantages in comparison with SBD-F, e.g., fast and quantitative reaction under mild conditions [98,101,102]. Due to these features, it has been shown that ABD-F is suitable for on-column reaction with thiols prior to capillary electrophoretic separation [98].

Recently a reagent called ThioGlo™3 (9-acetoxy-2-(4-(2,5-dihydro-2, 5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran) presenting the reactivity of maleimide has been developed. It has a very high affinity for the thiol group and forms fluorescent products upon reaction with GSH and congeners [62]. Other derivatives of maleimide such as fluorescein-5-maleimide (FM) and *N*-(1-pyrenyl)-maleimide (NPM), and 2-(4-*N*-maleimidophenyl)-6-methoxybenzofuran have been employed for thiol fluorescent labeling [53,62,70,103]. Derivatization with FM is rapid and quantitative at room temperature and pH 7.5. However, the selectivity for thiol group decreases at higher pH because FM begins to react also with amino group [70].

Another approach to derivatizing GSH and congeners is the use of reagents specific for the amino functional group. Among them, *N*-hydroxyuccinimidyl-α-(9-acridine)-acetate (HSAA), and *N*-hydroxysuccimidyl-α-(9-phenanthrene)-acetate (HSM-PA) have been tested as pre-column fluorescent

derivatization reagent for the selective determination of amino acids and oligopeptides. They showed reactivity specifically toward the primary amino group [68,104]. Oligopeptides derivatized with such reagents resulted to be stable and not susceptible to major matrix interference reactions. The reactions of these derivatizing fluorescent compounds take place at an alkaline pH and are completed in 30 min at 60 °C with negligible decomposition of the reagent [68,104].

3.2. Non-separative techniques

GSH and its analogues have been analyzed in different matrices by using non-separative methods such as spectrophotometry, spectrofluorimetry, and amperometry that allowed for the assay of the compounds under studies with a good sensitivity, simplicity and at low cost. However, the above methods are affected by unsatisfactory specificity and selectivity.

According to a classical spectrophotometric assay, total concentration of glutathione was determined in a kinetic reaction based on the oxidation of GSH to GSSG by DTNB, forming the colored product 5-thionitrobenzoate upon reduction. To determine GSSG, GSH was blocked before hand with NEM or vinyl pyridine, the excess of reagent eliminated and the isolated GSSG by solid-phase extraction was reduced to GSH by GR in presence of NADPH [105,106]. This method has found wide application in several biological matrices [107].

A spectrofluorimetric assay of GSH was developed based on the derivatisation of the thiol group with the fluorescent label 5-maleimidyl-2-(*m*-methylphenyl)-benzoxazole (MMBP). The fairly high selectivity of MMPB for GSH in comparison with other thiol containing small molecules such as Cys, offered the benefit of its applicability without previous pre-treatment. The method was applied to directly determine GSH in human whole blood, and in pig liver and heart homogenates; detected GSH levels were 0.75–1.23 m*M*, 1.37–1.94 μmol/g, and 0.37–0.42 μmol/g, respectively [108].

An electrochemical sensor incorporating the redox cofactor pyrroloquinoline quinone as a biocatalyst, into a polypyrrole matrix has been designed for the detection of thiols, including GSH. The amperometric detection performed by measuring the current response at a fixed potential of +0.500 mV as a function of time, allowed the detection of GSH with a limit of detection (LOD) of 11.2 μ*M* at pH 3.45 [109].

Whole thiols can be also quantitatively determined by electro-spin resonance (ESR) with the help of the imidazoline biradical disulfide reagent that, participating in the thiol-disulphide exchange reaction, produces profound changes in the ESR spectra. The label has been demonstrated to be highly sensitive and low concentrations are sufficient for thiol determination at physiological conditions, rendering this technique feasible for non-invasive determination of thiols, even in vivo [110].

3.3. Chromatographic methods

3.3.1. Thin-layer chromatography methods

To detect GSH levels and GST isoenzymes activity in low volume of bovine pulmonary artery endothelial cells extract, a method based on the selective reaction of a fluorescent molecule, the 5-(pentafluorobenzoylamino)-fluorescein (PFB-F), with GSH, has been reported. The specificity of the GSH labeling reaction has been confirmed upon depletion of cells with an inhibitor of γ-GluCys synthetase. The chemically stable adduct of PFB-F with GSH (GS-TFB-F) was separated from the excess of fluorescent reagent by thin-layer chromatography (TLC) and quantitatively determined by fluorimetric detection ($\lambda_{ex}=495$, $\lambda_{em}=515$). The results achieved were compared with those obtained by HPLC. Accordingly, TLC coupled with scanner detection offered advantages over HPLC-based separation for its rapidity and ability to assay several samples simultaneously [111].

3.3.2. High-performance liquid chromatography methods

Several high-performance liquid chromatography (HPLC) methods for the determination of GSH and related compounds using different detection systems, such as UV–Vis, diode array detector (DAD), electrochemical detector (ECD), and mass spectrometry (MS) have been developed. A summary of the HPLC methods reviewed in this article is given in Table 1. The most commonly used stationary phase is octa-

Table 1
HPLC methods for the analysis of GSH, GSSG, and related compounds

Compounds	Label	Matrix	[A]Column	Eluents	Detector	[B]LOD GSH/GSSG	Levels GSH/GSSG	Refs.
GSH, GSSG	MIAA DNFB	Erythrocytes	LiChrospher 100 NH_2 250×4.5	(A) H_2O MeOH 1.4 (B) 2 M CH_3COONa (pH 4.6) MeOH 36.64	UV–Vis 365 nm	–/–	602±36/116±9 μg/ml	[59]
GSH	DTNB	Authentic compounds	Hypersil ODS 1 250×4.6	(A) 100% PBS, (B) PBS MeOH 82.5 17.5	UV–Vis 257 nm	10/-μ*M*	–/–	[113]
GSH, GSSG	DTNB	Pharmaceuticals	Hypersil 5 ODS 250×4.6	H_3PO_4 1 mg/ml: ACN 64:36	UV 214 nm	12/1.2 μ*M*	–/–	[86]
GSH, tGSH, Cys, NAC	DTNB post-column	Rabbit eye tissues	Spherisorb 5 ODS 2 250×4.6; 5	50 m*M* phosphate buffer pH 3.0	UV–Vis 412 nm	19/-μg/l	0.46–6.58/0.03–1.73 μg/g	[67]
GSH, GSSG Cys, cystine	DNFB	HepG2 Cell[a] and mouse liver[b]	Rainin Dynamax C_{18} 250×4.6; 5	(A) H_2O–0.1% TFA; (B) ACN–0.1% TFA	UV–Vis 365 nm	0.01/0.1 μ*M*	2–3/-nmol/10^6 cells[a] and 4–5/0.1–0.4 μmol/g liver[b]	[126]
GSH, Cys, HCys, γ-GluCys, CysGly	*N*-(2,4-Dinitrophenyl aminoethyl)-maleimide	Pre-cancerous rat liver	Cosmosil 5 C_{18} AR-II 150×4.6; 5	(A) 10 m*M* K-phosphate (pH 7); (B) 50% A, 50% ACN	UV–Vis 350 nm	5/-pmol/μl	0.375±0.013/-nmol/0.1 g	[82]
GSH, tGSH Cys	BrB	Human skeletal muscle	Supelcosil LC-18 ODS 150×4.6; 3	(A) ACN–9% H_2O–0.25% $HClO_4$ (pH 3.71); (B) 75% ACN	FD $Ex_{394\ nm}$ $Em_{480\ nm}$	10/-pmol	1320±37/– μmol/kg	[54]
GSH	2-(4-*N*-maleimido phenyl)-6-methoxy benzofurazan	Rat tissue	Ultrasphere ODS 250×4.6; 5	65% 10 m*M* KH_2PO_4–0.1% hexanesulfonic acid, 35% ACN pH 4.5	FD $Ex_{310\ nm}$ $Em_{390\ nm}$	8 fmol/-	0.63–3.78/-μmol/g	[103]
GSH	OPA	Rat liver	Ultrasphere ODS 250×4.6; 5	(A) 0.25% CH_3COOH pH 3.9; (B) MeOH:0.25% CH_3COOH 30:70	FD $Ex_{303-395\ nm}$ $Em_{420-470\ nm}$	12.5/-pmol	90.4±6.5/– nmol/mg prot.	[91]
	BrB					6.25/–pmol	92.5±3.4/– nmol/mg prot.	
GSH, tGSH	BrB	Human plasma of healthy male[a] and female[b]	Econosphere C_{18} 150×4.6; 5	(A) 30% MeOH–30 m*M* TBA; (B) 100% MeOH–30 m*M* TBA, pH 3.1–3.4	FD $Ex_{260\ nm}$ $Em_{474\ nm}$	0.2/-pmol	0.48–0.95[a] and 0.43–1.11[b]/-μ*M*	[33]
GSH, AA	HSMPA	Authentic compounds	Spherisorb-C_{18} 200×4.6; 5	75% MeOH–23% H2O–2% Et_3N	FD $Ex_{310\ nm}$ $Em_{385\ nm}$	68/-fmol	–/–	[104]
GSH, GSSG Cys, mixed disulfides	DC	Human plasma of healthy male[a] and female[b]	3-Aminopropyl 250×4.6; 5	(A) 80% MeOH–20% H_2O; (B) acetate buffer (pH 4.6)–MeOH	FD $Ex_{335\ nm}$ $Em_{515\ nm}$	–/–	2.07±1.29[a] and 2.10±1.02[b]/mean of 0.083±0.090 μ*M*	[58]
GSH, tGSH AA	HSAA	Rat liver	Spherisorb C_{18} 200×4.6; 5	38% MeOH-H_2O-Et_3N [20 m*M* (NH_4)–H_2PO_4 pH 7.0+15 m*M* Et_3N pH 7]–32% MeOH	FD $Ex_{385\ nm}$ $Em_{435\ nm}$	65/-fmol	405±5/79±1 μg/250 mg liver	[68]
GSH, tGSH Cys, NAC γ-GluCys CysGly	NPM post-column	V79cl[a] and V79HGGT[b] Cells	LiChrospher 100 RP18 end-capped 125×4; 5	5%-ACN–10 m*M* phosphate buffer (pH 2.5)–0.1 m*M* EDTA 0.5 m*M* *n*-decylsulfate	FD $Ex_{342\ nm}$ $Em_{389\ nm}$	1.30/-pmol	15.0±1.2[a] and 14.0±1.2[b]/- nmol/mg prot.	[53]
	OPA post-column		Spherisorb ODS-2 or Nucleosil C_{18} 125×4; 5	5%-ACN–10 m*M* phosphate buffer (pH 2.5)–0.1 m*M* EDTA–0.5 m*M* *n*-decylsulfate–0.5 m*M* glycine	FD $Ex_{340\ nm}$ $Em_{440\ nm}$	1.30/-pmol	–/–	
GSH, Cys, NAC, CysGly	SBF-Cl	Enzymic activities in rat liver homogenate	Nova-Pak C_{18} 150×3.9	5% ACN, 95% 0.15 phosphate buffer pH 2.5, 5:95	FD $Ex_{365\ nm}$ $Em_{510\ nm}$	–/–	–/–	[127]

Table 1. Continued

Compounds	Label	Matrix	[A]Column	Eluents	Detector	[B]LOD GSH/GSSG	Levels GSH/GSSG	Refs.
GSH, several thiols	SBD-F	Urine	Cosmosil 5 C_{18} AR	98% 75 m*M* Na-citrate (pH 2.9), 2% MeOH	FD $Ex_{386\ nm}$ $Em_{516\ nm}$	–/–	–/–	[100]
GSH, GSNO	OPA	Human, rat plasma	Nucleosil 100-5C_{18} 250×4.6; 5	7.5% MeOH–92.5%–CH_3COONa buffer (015 *M*, pH 7.0)	FD $Ex_{338\ nm}$ $Em_{458\ nm}$	3/-n*M*	–/–	[69]
GSH, GSSG	OPA post-column	Jurkat T cells[a] and mitochondria[b]	CSC ODS 150×4.6; 5	50 m*M* phosphate buffer (pH 3.0)	FD $Ex_{340\ nm}$ $Em_{425\ nm}$	100/100 fmol	33.2±3.6[a] and 3.8±0.4[b] nmol/mg prot./190±20[a] and 180±40[b] pmol/mg	[9]
GSH, tGSH	BrB	Rat liver	Nova-Pak C_{18} 150×3.9	12% MeOH in 0.25% HCO_2H–NH_4OH–HCl (pH 3.5)	FD $Ex_{370\ nm}$ $Em_{485\ nm}$	–/–	9.3±0.5/0.127±0.01 μmol/g	[56]
GSH, tGSH Cys, HCys	BrB	Human plasma of healthy male[a] and female[b]	Ultrasphere ODS 250×2; 5	(A) 0.1% TFA in H_2O (pH 2.2); (B) ACN	FD $Ex_{300\ nm}$ $Em_{470\ nm}$	2–5/-pmol	6.97±1.82[a] and 6.12±1.88[b]/– μ*M*	[63]
GSH, tGSH Cys, HCys	BrB	Human plasma of healthy male[a] and female[b]	Ultrasphere ODS 75×4.6; 3	(A) 30 m*M* NH_4NO_3 and 40 m*M* NH_4CO_2H (pH 3.6); (B) ACN	FD $Ex_{300\ nm}$ $Em_{470\ nm}$	–/–	7.12±2.61[a] and 6.63±2.49[b]/-μ*M*	[64]
GSH, tGSH Cys, NAC γ-GluCys	SBD-F	Natural water	Waters Symmetry C_{18} 250×4.6; 5	(A) 0.1% TFA in H_2O; (B) ACN	FD $Ex_{385\ nm}$ $Em_{515\ nm}$	23/-n*M*	77±2/– μmol/g chlorophyll *a*	[66]
GSH, Cys	OPA automated	Grape juice[a] and wine[b]	Ultramex 3 C_{18} 100×4.6; 3	(A) 50 m*M* CH_3COONa; (B) MeOH	FD $Ex_{340\ nm}$ $Em_{450\ nm}$	3.3/-n*M*	0–1.28[a] and 2.1–5.1[b]/-mg/l	[92]
GSH, tGSH Cys, CysGly, HCys	BrB	Human plasma of healthy male[a] and female[b]	Milichrom A-02 Nucleosil C_{18} 75×2; 5	(A) 0.1% in TFA in H_2O (pH 2.2); (B) ACN	FD $Ex_{370\ nm}$ $Em_{470\ nm}$	70/-amol	7.67±2.23[a] and 6.88±2.82[b]/-μ*M*	[65]
GSH, tGSH Cys, HCys	ThioGlo™3	Rat tissue[a] and ovarian cells[b]	C_{18} 100×4.6; 3	30% H_2O–70% ACN, 0.1% CH_3COOH, 1% H_3PO_4	FD $Ex_{365\ nm}$ $Em_{445\ nm}$	50/-fmol	0.092–45.00[a] and 27.4±1.7[b]/0.503 –3.44[a] and 0.24±0.12[b] nmol/mg prot.	[62]
GSH, tGSH	OPA	Human red blood cells[a] and fibroblasts[b]	Inertsil ODS 2 250×4.6; 5	(A) ACN; (B) 50 m*M* CH_3COONa buffer (pH 6.2)	FD $Ex_{340\ nm}$ $Em_{420\ nm}$	50/-fmol	2.23±0.15/0.19±0.02 m*M*[a] and 14.41–20.24/ 0.73–0.85 nmol/mg prot[b]	[52]
GSH, GSSG		Rat liver, extracellular microdialysis	Econosphere C_{18} 150×4.6; 5	0.1 *M* $ClCH_2COOH$, 2 m*M* Na-heptansulfonate, 46 m*M* NaOH in 2% ACN	ECD	–/–	4.6–76.5/-μ*M*	[120]
GSH, GSSG		Equine blood[a] and balf[b]	Hypersil ODS 150×4.6; 3	10 m*M* NaH_2PO_4-H_3PO_4 (pH 2.7), 5% MeOH	ECD	5/10 ng/ml	295.41±56.48[a]/ 38.59±16.72[a] and 0.36±0.01[b]/0.04±0.01[b] μg/ml	[76]
GSH, GSSG Cys, HCys, γ-GluCys, CysGly		Blood[a], tissues, coffee	Inertsil ODS 2 250×4.6; 5	0.1 *M* $ClCH_2COOH$, 5% MeOH, 1.75% DMF, 2.25 m*M* Na-heptansulfonate, NaOH (pH 2.8)	ECD	0.4/0.5 pmol	0.834/0.022 μmol/ml[a]	[117]
GSH, GSSG cystamine		Liver[a], watermelon, grapefruit	Sepstik ODS 100×1; 3	95% 100 m*M* phosphate buffer, 5% ACN	ECD	0.2/-pmol	110/46 ppm[a]	[87]
GSH, GSSG		Lung	Phase separation 200×4; 5	50 m*M* NaH_2PO_4, 0.05 m*M* OSA, 2% ACN, H_3PO_4 (pH 2.7)	ECD	0.8/1.63 pmol	2.45–4.52/0.15–0.40 nmol/mg prot.	[115]

Table 1. Continued

Compounds	Label	Matrix	[A]Column	Eluents	Detector	[B]LOD GSH/GSSG	Levels GSH/GSSG	Refs.
GSH, Cys NAC		Rat brain	Hypersil ODS 200×4.6; 5	0.1 m*M* KH_2PO_4 (pH 3.0)	ECD	2.5/-μ*M*	0.35 m*M*/-	[119]
GSH, Cys, other compounds		Urine	Zorbax C_8 250×4.6	0.2 m*M* phosphate buffer (pH 3.0)	ECD	20/-pmol	–/–	[121]
GSH, GSSG other 7 ATs		Plasma	RP C_{18} NBS 150×4.6; 5	50 m*M* phosphate monobasic, 1 m*M* OSA, 2% ACN, H_3PO_4 (pH 2.7)	ECD	5/50 fmol/ml	6.9±0.5/1.5±0.1 μ*M*	[71]
GSH, L-Cys NAC, ASC uric acid		Human blood	Zorbax C_8 250×4.6	0.20 m*M* phosphate buffer (pH 3.0)	ECD	16/-pmol	423.9/-μ*M*	[122]
GSH, GSSG		Pharmaceuticals	Supelcosil LC 18 DB 100×4.6; 5	98% H_2O–0.1% TFA, 2% ACN	ECD	0.60/0.15 ng	–/–	[112]
GSH, GSSG, other ATs and disulfides		Human plasma	Inertsil ODS 2 250×4.6; 5	93.25% 0.1 *M* $ClCH_2COOH$, 1.75% DMF, 2.25 m*M* heptane sulfonic acid, NaOH (pH 2.25)	ECD	–/–	8/10 n*M*	[51]
GSH, Cys, NAC	EMA	Authentic compounds	TSK gel ODS-120T 150×4.6	22.5–54.0% ACN–0.05% TFA	FAB-MS	–/–	–/–	[83]
GSH, GSSG	NEM	Peripheral blood mononuclear cells	Nucleosil-100 OH 250×4.6; 7	ACN:H_2O–1% CH_3COOH (25:75)	ESI–MS	0.01/0.05 μ*M*	74.4±21.2/0.9±0.6 nmol/mg prot.	[72]
GSH, GSSG	MIAA	Rat hepatocytes	Hamilton PRP-X110S anion-exchange 100×2.1; 7	(A) 0.1% aq. HCOOH–ACN (1:1); (B) 2% aq. HCOOH–ACN (1:1)	ESI–MS[1] and ESI–MS/MS[2]	0.651/0.817[1] and 0.163/0.0816[2] μ*M*	65/0.6 μ*M*	[73]
GSH, GSSG		Mice liver	Altima C_{18} 150×4.6; 5	(A) 10% MeOH in 0.1% HCOOH; (B) 10% MeOH	ESI–MS/MS	0.2/2 pmol	2.17/-μmol/g	[125]

[A]Column dimensions, in the order: Length (L)×internal diameter (I.D) mm; particle size (p.s.) μm; [B]S/N ratio = 3.

decylsilica gel (ODS). Methods described so far utilize normal bore columns, except for two separation methods performed with narrow-bore columns. The latter permits to limit solvent consumption, without consistent loss of sensitivity. The standard HPLC separation of the derivatized compounds, due to the relatively hydrophobicity enhanced upon reaction with the fluorescent tag, is performed in most cases by reversed-phase (RP) mode. However, even when GSH and GSSG are analyzed without derivatization, or when post-column derivatization is performed, despite the polar properties, they are also separated in RP mode, using acidic eluents. In fact, eluents buffered at acidic pH are used to maintain GSH, GSSG, and related compound in a non-dissociated form, improving interaction with stationary phase. Moreover, pH values below neutrality minimize thiols auto-oxidation. Moreover, trifluoroacetic acid (TFA) added to mobile phase resolves peak tailing associated specially with GSSG, functioning as ion-pairing reagent [112].

3.3.2.1. HPLC of GSH, GSSG, and congeners with photometric detection. Relatively few HPLC methods with UV–Vis absorbance or DAD have been reported in the recent years, probably due to the low sensitivity and specificity of the UV–Vis detection in comparison with ECD, fluorimetric and MS determinations, and to the diffusion of these latter detectors wider than the past. However, an HPLC–UV system is associated with simplicity and applicability to simultaneous determination of GSH and GSSG. Moreover, a step of derivatization of GSH with UV–Vis absorptive thiol-reactive chemicals may be included in the sample preparation. Doubly derivatized GSH with MIAA and DNFB was separated from GSSG-DNP adduct by HPLC by means of an amino-silica stationary phase [59]. In an analogous assay, DNFB was used alone to determine GSH and GSSG, plus other thiols, in a single RP-HPLC analysis. The method was validated in terms of linearity, reproducibility, LOD, limit of quantification (LOQ), and recovery of exogenous analytes, and included the use of N^{ϵ}-methyl-L-lysine as internal

standard to minimize experimental errors occurring during derivatization reaction, sample processing, injection in the HPLC system, and detector response. Accordingly, quantification of GSH and GSSG was performed in HepG2 cells and mouse liver. The results obtained revealed that differences in the GSH levels in cultured cells might emerge due to dependence on the growth cycle phase and the supply of the culture medium with sulfur amino acids for GSH biosynthesis [87].

An HPLC method with the potential for studying glutathione synthesis, because determining contemporaneously GSH, NAC, and Cys was developed based on the reactivity of thiols with Ellman's reagent. The detection revealed mixed disulfides with DTNB being repressed, by an excess of reagent, the formation of by-products affecting GSH and related thiols quantification [86,113].

3.3.2.2. HPLC of GSH, GSSG, and congeners with fluorimetric detection. HPLC separation of GSH and analogues with fluorimetric detection has been extensively applied to the determination of these compounds in biological matrices where a high sensitivity is usually required. Advantages of HPLC combined with fluorimetry come from the relatively high sample loading, intrinsic to HPLC, and the availability of several fluorescent labels specific for the thiol group and for ATs compounds. Additionally, an HPLC system can be easily connected with a reactor where the underivatized ATs separated in the column are introduced and allowed to react with the fluorogenic reagents. Such a systems can be optimized in order to standardize time and temperature of reaction before the detection. This approach minimizes errors due to pre-analytical processing and saves time [9]. The types of fluorescent labels reported in the literature and tested for their feasibility for GSH, GSSH and congeners derivatizations are reported in the previous section. The BrB method requires pH adjustment following derivatization and usually gradient elution chromatography is performed to effectively separate BrB derivatives with thiols [91]. On the contrary, RP-HPLC separation of OPA-derivatives is achieved in isocratic conditions [56,91]. However, Ivanov et al. described a highly sensitive and reproducible assay for the determination of thiols based on BrB derivatization and isocratic separation of derivatives by narrow-bore RP-HPLC [64,65].

Aqueous–polar organic mixtures were supported with ion pairing reagents, such as tetrabutylammonium hydroxide (TBA), TFA, and sodium *n*-decylsulfate in order to improve chromatographic separation of several ATs derivatives with different fluorescent labels [33,53,63,66]. Whereas, 2% triethylamine was applied to the separation of HSMPA derivative of GSH since it reduced peak broadening, and improved column efficiency for these adducts [68,104].

3.3.2.3. HPLC of GSH, GSSG, and congeners with electrochemical detection. HPLC-ECD represents a significant tool for analyzing redox-reactive compounds, such as thiols and disulfides, since it is specific, rapid and does not require sample derivatization. Nonetheless, a drawback of the analysis by ECD of disulfides, like GSSG, is the applied high oxidation potential that can reduce the performance of ECD [114]. ECD of GSH and GSSG with gold–mercury (Au–Hg) amalgam electrode is based on reduction of GSSG at the upstream electrode surface set to a negative potential followed by oxidation of GSH produced at the downstream electrode surface set to positive potential [9]. When organic thiols are oxidized, electric current is produced. More recent studies concern the use of two porous graphite detectors in series both set at positive potential. The first electrode is used for the detection of GSH, whereas GSSG is detected with the second electrode at a higher positive potential [76,115]. A recently developed coulometric array detector offered several advantages over amperometric ECD, such as minimal electrode oxidation and longer half-life, and baseline stability. While the amperometric detection mode relies on the oxidation of mercury on the electrode's surface, the coulometric detection mode is based on oxidation of the thiol group of the analyte. With amperometric detection only 1–5% oxidation of the compounds is achieved at the Au–Hg electrode, whereas with coulometric detector the analyte is quantitatively oxidized at the porous graphite electrodes. [116]. Validated methods for the quantitative determination of GSH and GSSG in biological samples containing 50–1000 μg proteins and relying on HPLC separation and coulometric

detection was reported [76,115]. Ion-pair HPLC with coulometric ECD was performed to provide a complete profile of plasma ATs in the same plasma sample and within the same chromatographic run. The study included comparisons between pre-analytical reduction steps with either TBP or sodium borohydride for compatibility with coulometric ECD. The influence on ECD of different strong acids used for protein precipitation, optimal concentration of the ion-pairing reagent and pH of the eluent were also investigated [71]. The ion-pairing reagent 1-octanesulfonic acid (OSA) in the mobile phase was critical for the separation of ATs and disulfides and caused increased retention time. Also pointing to an accurate simultaneous determination of a wide variety of thiols and disulfide in blood and tissues, a battery of compounds involved into GSH metabolism have been analyzed by HPLC with dual ECD. The sample preparation was minimal and the applicability to different tissues practicable. Profiles of GSH-related thiols and disulfides were given for a broad panel of rat tissues [117]. Based on the same method, levels, stability, and distribution of GSH and other biologically relevant thiols and disulfides have been assessed in human plasma [51]. Microchromatographic separation associated with pulsed ECD in mildly acidic conditions have been exploited to increase mass sensitivity and chromatographic efficiencies, to lower solvent consumption, and to improve sensitivity for compounds that exhibit poor optical and DC amperometric detection response. In particular, the integrated pulsed amperometric detection (IPAD) waveform was determined to achieve low LODs, baseline stability, elimination of oxide-promoted artifacts and determination of GSH and GSSG in different matrices [87]. When the method was applied to the analysis of ACN extracts of rat brain, the simultaneous presence of GSSG and chloride imposed an optimization that could minimize interference with the analysis of *S*-sulfocysteine (SSC) by means of a dual Au–Hg amalgam electrode detection system. The chromatographic separation of SSC, GSSG, and GSH was accomplished in anion-exchange mode and offered an approach to determine these analytes in mixture. Since SSC lowers GSH content through the inhibition of γ-GluCys synthase, simultaneous monitoring of SSC and GSH in biological systems could give useful information [118]. A combination of microdialysis sampling technique with optimized HPLC separation and ECD has been applied to determine GSH and other thiols in brain of freely moving rats or in extracellular space of anesthetized rats [119,120]. In the former method, a new chemically modified electrode exhibited performance comparable with a glassy carbon electrode towards ATs [119]. Similarly, nafion–indium hexacyanoferrate film modified electrode was prepared in order to efficiently catalyze oxidation of thiocompounds in cyclic voltammetry (CV) and analyze them in natural and artificial urine [121]. Description of the manufacture and application of a multichannel amperometric detection system (MADS) with chemically modified microelectrodes was reported. As in the previous method, the chemical modifier copper tetra-aminophthalocyanine was immobilized on platinum microelectrodes by CV. The use of microarray electrodes set at different potentials, offered the advantage of performing a three-dimensional hydrodynamic voltammogram. The assay based on LC–MADS with either microdialysis or normal sampling, proved to be appropriate for the analysis of ATs plus ascorbic acid and uric acid in biological matrix [122].

3.3.2.4. HPLC of GSH, GSSG, and congeners with mass spectrometry. HPLC and MS hyphenation has significantly enhanced their respective potential for the robust and widely automated analysis of minute amounts of biological samples. Instrumental set-up, as well as chromatographic and mass spectrometric experimental conditions, need to be carefully selected in order to maximize the performance of the hyphenated analytical system [123]. The sensitivity and specificity of the detection system is further improved, coupling an HPLC device with a tandem mass spectrometer (MS–MS) [124]. Among the various chromatographic and mass spectrometric modes available, RP-HPLC and electrospray ionization MS (ESI-MS) have been shown to be most suitable for the direct interfacing of HPLC and MS. Nonetheless, different HPLC systems have been adopted for the analysis of GSH and congeners. Native GSH and GSSG are highly polar and establish improved interaction with ODS stationary phase when uncharged. However, eluent system consisting of water–methanol–formic acid was used for GSH

separation on a C_{18} column and MS–MS analysis. The acidic eluent drove the association of GSH and GSSG with proton forming positively charged pseudo-molecular ions by ESI (308.1 m/z, and 613.3 m/z for GSSG). The transitions from the $[MH]^+$ ion to the 162.0 m/z for GSH and 355.3 m/z for GSSG monitored by multiple reactant monitoring mode (MRM) useful in determining the two analytes are poorly separated in the HPLC system [125]. Acidic eluent, given by ACN and aqueous acetic acid, was used for the isocratic separation of GSH and GSSG on a diol stationary phase followed by detection in an ESI–MS system. The chosen eluent composition showed a high compatibility with MS and drove ionization toward positively charged analytes formation [72]. On the other hand, in the acidic eluent it was possible to identify the compounds as negative ion also, even if with a lower response, when ESI–MS was set in the negative ion mode. The optimized method based on detection in the positive ion mode and selected ion monitoring (SIM) was suitable for quantification of GSH and GSSG in peripheral blood mononuclear cells, since it had good sensitivity, repeatability, and reproducibility [72]. Fig. 3 illustrates the HPLC–MS assay of GSH as NEM derivative, and GSSG in lysate of human lymphocytes. The figure shows also the ESI–MS spectra of GSH in positive and negative ion modes. Loughling et al. performed an HPLC–MS/MS assay to simultaneously quantify GSH and GSSG in hepatocytes. The baseline separation was achieved by ion-exchange chromatography using eluents containing an ion-pairing reagent compatible with ESI, e.g., TFA. The analytes were detected in the positive ion mode using an ion trap MS, or selected reaction monitoring (SRM) using a triple quadrupole MS. The best sensitivity for both compounds was obtained with SRM [73]. Fig. 4 illustrates the HPLC–MS/MS analysis of the GSH-CM derivative and GSSG with a triple quadrupole MS.

3.3.3. Gas chromatography methods

Few gas chromatography (GC) methods for the determination of GSH, GSSG and congeners are

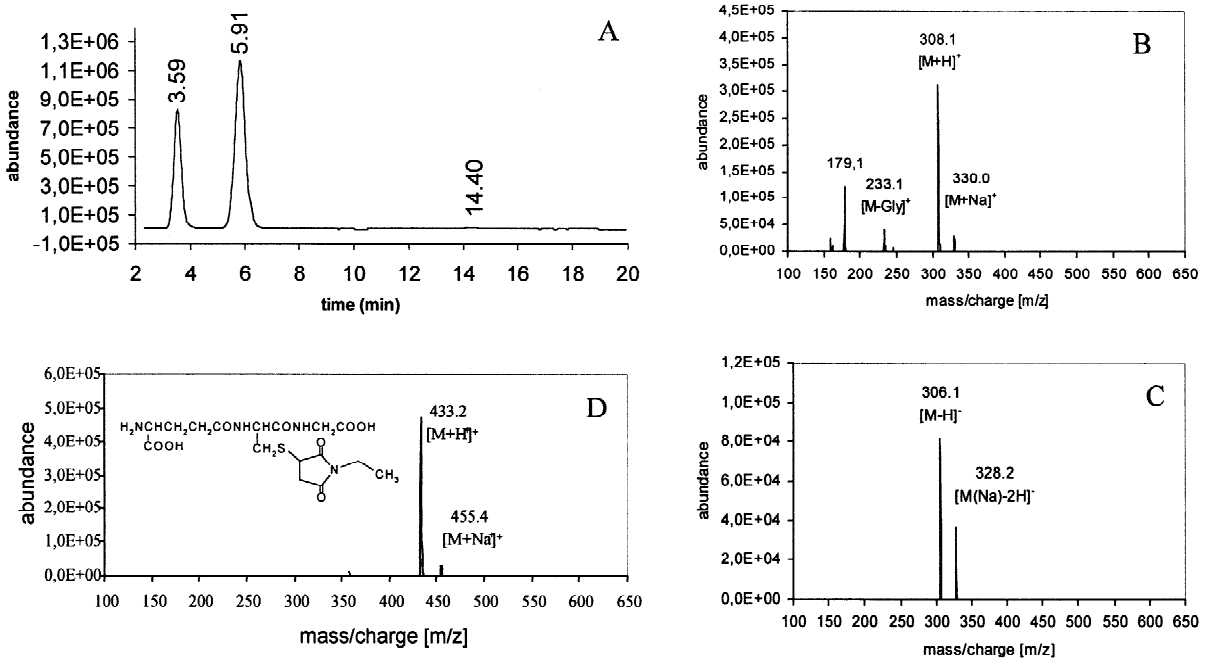

Fig. 3. Modified from Ref. [72]. (A) Reconstructed chromatogram of the analysis of GSH-NEM derivative (3.59) and GSSG (14.40) in human lymphocytes in presence of an internal standard (5.91). (B,C) ESI–MS spectra of underivatized GSH analyzed in positive and negative ion modes, respectively. (D) Positive ion ESI–MS spectrum of GSH–NEM derivative. HPLC conditions as reported in Table 1.

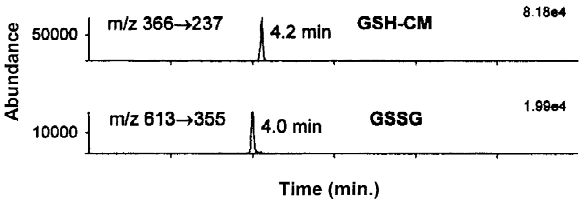

Fig. 4. Modified from Ref. [73]. Reconstructed ion chromatograms of GSH–CM derivative and GSSG analyzed by HPLC–MS–MS with a triple quadrupole MS. The analytes were detected by selected reaction monitoring (SRM) using the transitions m/z 366→237 and m/z 613→355 for GSH–CM and GSSG, respectively. HPLC conditions as reported in Table 1.

reported. Additionally, the elective detection system coupled with a gas chromatographer is the mass spectrometer and GC–MS methods are the only ones mentioned in the literature in the above time period. The analytes detection in a GC system requires volatilization of compounds consequent to a fast evaporation in a heated inlet port. Then, in the gaseous form they reach the MS where they are ionized and detected [128]. Oligopeptides are polar compounds, possess high boiling points and are easily degradable at high temperatures. Thus, in order to analyze them in a GC system, a derivatization step is necessarily required to lower the boiling point to an acceptable level. Examples of derivatizing agents for this purpose are trifuoroacetic anhydride [129] and ethylchloroformate [130]. The former gives a complex bicycloglutarimide with GSH, while the latter converts GSH and other thiol-containing peptides into a *N,S*-ethoxycarbonyl methyl ester derivative in alkaline medium. Both types of GSH derivatives were chromatographed in a crosslinked methyl siloxane capillary column and detected by a mass analyser. The bicycloglutarimide was formed in a real sample, separated by capillary GC, and detected with negative chemical ionization by SIM in the mass range m/z 477.1–480.1. GSH content in whole blood was quantified in presence of synthetic [1,2-$^{13}C_2$-glycyl]GSH as the internal standard. The method had a sensitivity that allowed the detection of the tracer enrichment of the internal standard with a quantification limit in the range of 0.3–0.5 mol% excess [129]. Capitan et al. applied a GC–MS procedure to determine stable isotope-labeled GSH in blood and tissues. They measured GSH synthesis rate in vivo by infusion of [^{15}N]Cys to rats and of [D_2]Cys to human subjects and detected GSH, CysGly, and γ-GluCys modifications over time [130]. Fig. 5 shows the total ion chromatogram obtained analyzing *N,S*-ethoxycarbonyl methyl esters of GSH and related compounds by GC–MS and the mass spectrum of GSH derivative obtained by electron impact (EI).

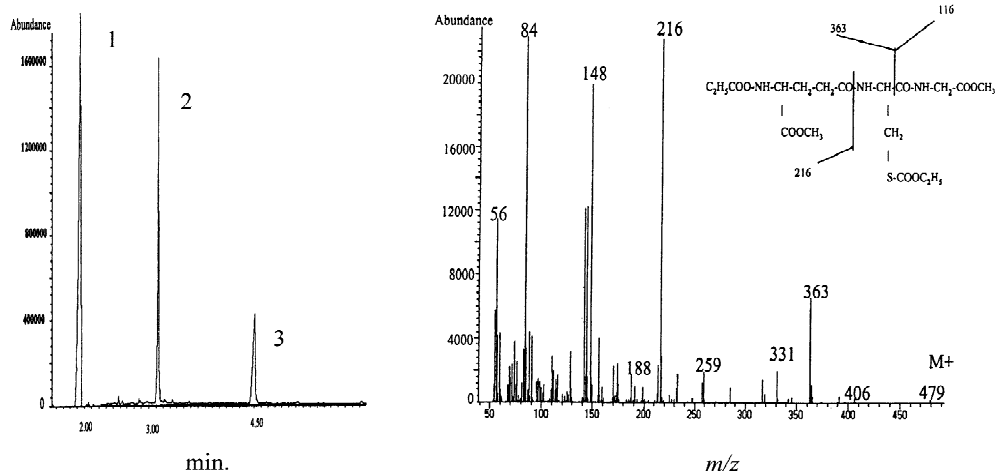

Fig. 5. Modified from Ref. [130]. Left panel: total ion chromatogram obtained by GC–MS analysis of a mixture of CysGly (1), γ-GluCys (2), and GSH (3) as *N,S*-ethoxycarbonyl methyl esters. Right panel: EI mass spectrum of GSH *N,S*-ethoxycarbonyl methyl esters. Chromatographic conditions reported in Section 3.3.3.

3.3.4. Capillary electrophoresis methods

Capillary electrophoresis (CE) is a recently developed powerful separation technique for the analysis of a wide number of analytes belonging to different classes such as organic and inorganic compounds, peptides, proteins, nucleotides, sugars, drugs, herbicides, etc.

The electrophoretic separation is achieved by applying a relatively high electric field to the sample introduced into a capillary (10–100 μm I.D.) containing an appropriate background electrolyte (BGE). Because of the high electric field applied, highly efficient separations can be easily obtained in short time. In CE, several modes of operation are available to improve the selectivity of the separation, namely free zone capillary electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), isotachophoresis (ITP), capillary gel electrophoresis (CGE) and capillary electrochromatography (CEC). Among the other characteristics of CE it is noteworthy to mention that (i) the method is very cheap and with low pollution due to the minute volumes used in the separation process (nl and μl of sample and buffer, respectively), and (ii) aqueous or polar organic solvent can be used [131–133].

Besides the above-mentioned properties, CE techniques present drawbacks such as detection sensitivity due to the limited analytes loading arising from the relatively small volumes injected. Several solutions were proposed to resolve this problem, e.g., use of capillaries with extended path length (bubble- or zeta-shaped cell), more sensitive detectors (multi-wavelength and DAD, conductivity, laser-induced fluorescence (LIF), ECD, MS), two-dimensional CE (ITP coupled to CZE, ITP–CZE), derivatisation reactions, etc. [134,135].

In spite of the wide choice of the CE separation modes experimented to improve the selectivity of the separations, the analysis of GSH and its congeners has been performed by using only CZE and MEKC.

The CE-based methods developed to determine GSH and related compounds are summarized in Table 2, outlining the optimized conditions for capillary electrophoretic separation. The aim of most of the CE-based methodologies reviewed was the simultaneous determination of GSH, GSSG, and related thiols in complex matrices. The determination of such compounds was carried out by CZE selecting the appropriate experimental conditions, e.g., relatively high concentration of phosphate and borate buffer for resolution improvement. The running buffer pH ranged between 2.1 and 10.5, analytes being either positively or negative chargeable, even when derivatized with different tags. Satisfactory results were achieved mostly in untreated fused-silica capillaries; however, in some cases, coated capillaries were chosen to minimize electro-osmotic flow and obtain good resolution in short time window [77,85,88,136]. In some separative methods organic modifiers were used to improve electrophoretic separation [75].

3.3.4.1. CE of GSH, GSSG, and congeners with photometric detection. CE methods with photometric detection have been successfully applied to GSH and GSSG determination even in complex matrices, i.e., in erythrocytes, plasma, animal and vegetable tissues, without any pre-assay derivatization with chromophores [75,79,136,137]. However, most CE methods analyzing GSH and congeners benefited from reactions with several chromophores, such as NEM, NPM, DTNB, etc. Such derivatizations (i) stabilized GSH during pre-analytical and analytical phases, (ii) improved the absorbance of thiol compounds and (iii) enhanced the resolution between GSH and GSSG in the CE system [11,74,77]. For example, the use of DTNB allowed GSH determination even when it was significantly lowered by a strong oxidative challenge in a cell system [85]. A special feature of CE is the possibility of derivatizing analytes on-column before they reach the detector, limiting the time of sample pre-treatment. Moreover, such a method can be standardized and applied to automated CE analysis. On-column reaction with 2,2′-dipyridyldisulfide (DPDS) was performed to detect GSH and other thiols based on the release of a thiopyridone absorbing at 343 nm. In this method MEKC separation mode facilitated the delimitation of part of the capillary length as a reaction chamber where analytes, previously resolved, are mixed with the reagent DPDS [138].

3.3.4.2. CE of GSH, GSSG, and congeners with fluorimetric detection. Fluorescence detection may offer the potential advantage of a detection limit 100–1000 times over UV absorption. Moreover,

Table 2
CE methods for the analysis of GSH, GSSG, and related compounds

Analytes	Label	Matrix	Capillary phase	Capillary dimensions[A]	kV	BGE	CE mode	Detection mode	Time (min)	LOD[B] GSH	GSH content	Refs.
GSH GSSG	NEM	Erythrocytes	Hydrophilic	27×20 cm ∅ 50 μm	30	Phosphate 35 m*M* (pH 2.1)	CZE	UV 200 nm	<4	0.5 μ*M*	5.89±0.36 μmol/g Hb	[77]
GSH GSSG	NEM	Mitochondria	BFS	67 cm ∅ 75 μm	30	Borate 100 m*M* Tris 25 m*M* (pH 8.2)	CZE	UV 200 nm	6.1	1.8 μ*M*	8.1±2.6 nmol/mg prot.	[74]
GSH GSSG NAC ASC		Plant tissue	BFS	70×62.5 cm ∅ 50 μm	30	Borate 200 m*M* ACN 20% (pH 9.0)	CZE	UV 185 nm	17	6.5 μ*M*	/	[75]
GSH GSSG Purines		Heart tissue	BFS	60×52.5 cm ∅ 75 μm	20	Borate 20 m*M* (pH 10.0)	CZE	UV 185 nm	<17	0.4 nmol/mg P	6.17±1.87 nmol/mg prot.	[137]
GSH GSSG ASC	DTNB	Lymphocytes	BFS[a] and polyacrylamide[b]	27×20 cm ∅ 50/75 μm	8	Phosphate 50 m*M* (pH 7.0)	CZE	UV 200 nm	<5[a] and 7.2[b]	0.5 μ*M*	8–12 μ*M* 15×10^6 cells/ml	[85]
GSH GSSG GSNO		Erythrocytes	Polyacrylamide	27×20 cm ∅ 75 μmm	7	Phosphate 40 m*M* (pH 2.2)	CZE	UV 200 nm	22	10 μ*M*	952±148 μ*M*	[136]
GSH HCys Cys	ABD-F SBD-F	Plasma	BFS	57×50 cm ∅ 50 μm	25	Borate 20 m*M* (pH 10.5)	CZE	UV 220 nm	<10	1 μ*M*	/	[88]
			Dimethylpolysiloxane	27×20 cm ∅ 50 μm	15	Phosphate 50 m*M* (pH 2.1)			<6			
GSH Cys ATs	BrB	Plasma	BFS	65×45 cm 50 μm	16	Phosphate 80 m*M* (pH 9.0)	CZE	UV 234 nm	<8	5 μ*M*	7.68±2.43 μ*M*	[89]
GSH GSSG NAC		Plasma	BFS	64.5×56 cm ∅ 50 μm	15	NaH_2PO_4 5 m*M*, Na_2HPO_4, 15 m*M* SDS 50 m*M* (pH 8.0)	MEKC	UV 195 nm	<15	0.8 μ*M*	1.6 μ*M*	[79]
GSH HCys Cys	DPDS on-column reaction	Blood[a] and erythrocytes[b]	BFS	64×58.8 cm ∅ 50 μm	28	Phosphate 50 m*M* SDS 50 m*M* (pH 7.5)	MEKC	UV 200 and 343 nm	<4	5 μ*M*	645±25 μ*M*[a] 2.6±0.08 m*M*[b]	[138]
GSH HCys Cys	ABD-F on-column reaction	Authentic compounds	BFS	55×30 cm ∅ 50 μm	15	Trizma® Phosphate (pH 2.1)	CZE	Argon ion LIF $Ex_{364\ nm}$ $Em_{447-651nm}$	15	0.004 μ*M*	/	[98]
GSH	ABD-F	Rat hepatocytes	BFS	50 cm ∅ 50 μm	30	NaH_2PO_4 20 m*M* Na_2HPO_4 20 m*M* (pH 7.5)	CZE	He-Cd ion LIF $Ex_{325\ nm}$ $Em_{496\ nm}$	3.5	0.25 pmol	30.3±6.94 76.3±9.41 fmol/cell	[97]
GSH Cys	BrB on-line reaction	Cerebral extracellular fluid	BFS	19×15 cm ∅ 25 μm	10	Hepes 100 m*M* (pH 8.0)	CZE	He-Cd ion LIF $Ex_{354\ nm}$ $Em_{450\ nm}$	<1.2	0.02–0,04 μ*M*	2.0±0.1 μ*M*	[95]
GSH γ-GluCys ROS	NDA	V79cl cell line	BFS	37×30 cm ∅ 75 μm	25	Borate 100 m*M* (pH 9.2)	CZE	Argon ion LIF $Ex_{460\ nm}$ $Em_{530\ nm}$	11	0.04 μ*M*	14.7±1.3 nmol/mg prot.	[93]
GSH, HCys Cys CysGly	6-IAF	Plasma and serum	BFS	85×50 cm ∅ 50 μm	30	Boric acid, 50 m*M* CAPS 20 m*M* NaOH (pH 10.0)	CZE	Argon ion LIF $Ex_{488\ nm}$ $Em_{515\ nm}$	<14		/	[60,102]
GSH HCys Cys NAC	FM	Blood and plasma	BFS	57 cm ∅ 75 μm	/	Phosphate 10 m*M* SDS 50 m*M*, ACN (pH 7.0)	CZE	Argon ion LIF $Ex_{488\ nm}$ $Em_{520\ nm}$	<8	0.01 μ*M*	0.64 μ*M*	[70]

Table 2. Continued

Analytes	Label	Matrix	Capillary phase	Capillary dimensions[A]	kV	BGE	CE mode	Detection mode	Time (min)	LOD[B] GSH	GSH content	Refs.
GSH		Erythrocytes	BFS	30 cm ∅ 10 μm	25	Na_2HPO_4 6,1 m*M* NaH_2PO_4 3,9 m*M* (pH 7.0)	CZE	ECD	<4	0.1	1.12±0.25 m*M*	[139]
GSH HCys Cys		Urine (l-Cys)	BFS	85 cm ∅ 75 μm	30	Formate 5 m*M* EDTA 1 m*M*, NaCl 0.1 m*M* (pH 2.7)	CZE	ECD	25	5 μ*M*	/	[131]
GSH Cys		Erythrocytes	BFS	40 cm ∅ 25 μm	25	Na_2HPO_4 NaH_2PO_4 (pH 7.0)	CZE	ECD	<4	0.63 μ*M*	1.08 m*M*	[141]
GSH Cys 6-TP MMI		Tablets plasma (l-Cys)	BFS	70 cm ∅ 25 μm	/	Phosphate 20 m*M* (pH 7.4)	CZE	ECD	<20	2.5 μ*M*	64.0 μ*M* (Cys)	[140]
GSH		Urine	BFS	111 cm ∅ 75 μm	30	Borate 300 m*M* (pH 8.5)	CZE	ESI–MS	9.5	0.08 mg/ml	850 mg/day	[144]

[A]In the order: total length (T.L.)×effective length (E.L.), internal diameter (∅), only T.L. is reported when E.L. is not indicated. [B]*S*/*N* ratio = 2–3.

using lasers to induce fluorescence can improve the flux of the excitation light, thereby further extending the detection limit. An interesting development in improving CE detection sensitivity is the recently evaluated extended light path capillary for use in CE-laser-induced fluorescence (LIF) detection. A fully automated and sensitive CE assay with on-column derivatisation with ABD-F has been developed to detect thiols with LIF detection [98]. A larger spectrum of fluorobenzofurazans, i.e., SBD-F, ABD-F, and DBD-F, has been tested for reactivity with intracellular GSH, since they acquire fluorescence after reaction with thiols, devoid of negative effects on the background signal. Reagents were applied externally to the cells, and diffused inward to different extents according to their hydrophobicity. After permeation, single hepatocytes were introduced into the capillary without previous lysis. In particular, the use of the most permeable ABD-F to detect GSH required relatively few cells for its quantification in hepatocytes. Moreover, the intrahepatocellular GSH concentration was estimated to be between 14 and 103 fmol [97]. GSH and Cys in caudate nucleus were determined in anesthetized rats taking advantage of a fully automated system permitting dialyzation of extracellular fluid, on-line derivatization with BrB, transfer to the CZE separation system, and LIF-based detection [97]. Interestingly, a method for the simultaneous determination of GSH and ROS was applied to monitor oxidative stress-induced in cultured cells with synthetic hydroperoxides. GSH and ROS were detected after reaction with the fluorogenic probes naphthalene-2,3-dicarboxaldehyde (NDA) and dihydrorhodamine-123 (DHR-123), respectively [93].

3.3.4.3. CE of GSH, GSSG, and congeners with electrochemical detection. ECD is a valid detection system that allows overriding the intrinsic low detection limit associated with CE. ECD is based on the reaction of analytes on an electrode surface and only molecules in a thin diffusion layer contribute to the signal intensity. For compounds that cannot be detected favorably by UV-absorption, as in the case of GSH and similar thiols, ECD can be a valuable alternative. A prerequisite for performing ECD in CE is the isolation of the electrophoretic current generated by high voltage used for separation, from the ECD current. To achieve this, there are basically two approaches: the end-column mode and the off-column mode. ECD at a constant potential has been performed using the end-column amperometric approach with a microcurrent voltammeter to detect GSH in single, whole erythrocytes. The lysis of

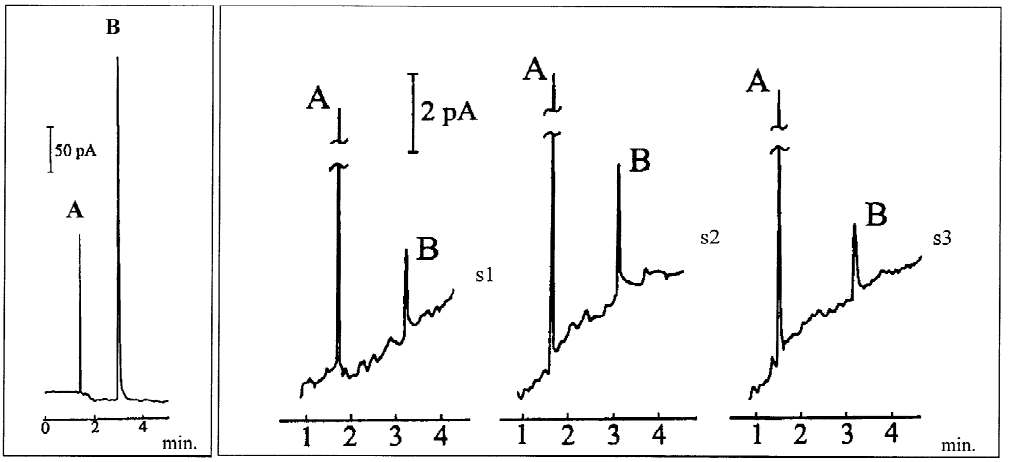

Fig. 6. Modified from Ref. [139]. Left panel: CE-ECD analysis of authentic GSH in PBS. Right panel: electropherograms of GSH in three single human erythrocytes (s1, s2, and s3). Peaks A and B are PBS and GSH, respectively. Details of BGE, capillary and separation voltage are reported in Table 2.

injected erythrocytes was obtained within 60 s when a ''lysis voltage'' of 0.7 kV and an incubation time in the phosphate buffer of 5 s were applied. Fig. 6 illustrates the electropherograms of three individual human erythrocytes lysed on-column in the above conditions. By this method a mean GSH concentration of 97±22 amol per cell was detected in comparison with 68±48 amol obtained with LIF detection [139]. Improved palladium field-decoupler has been employed for off-column detection of reduced thiols using chemically modified electrodes [131]. Determination of thiols following CE separation has been achieved by amperometric detection (AD) at a bare carbon disk electrode in solution at nearly neutral pH. Due to the efficiency of the CE-based separation, the relatively high overpotential associated with thiols can be overlooked, making the method suitable for detecting such compounds in pharmaceuticals and serum samples [140]. End-column AD of GSH in plasma and erythrocytes using an Au–Hg amalgam microelectrode has been presented. The electrode design did not require conductive joint and, additionally, solution deoxygenating was not necessary [141].

3.3.4.4. CE of GSH, GSSG, and congeners with mass spectrometry detection. CE–MS is a hyphenated technique allowing the differentiation of the compounds on the basis of their electrophoretic mobility, molecular mass, and possibly structure-related fragmentation, making this combination one of the most powerful techniques for the identification of unknown substances in complex matrices [142,143]. ESI as an ionization technique for MS analysis has been successfully applied for the MS analysis of the species separated by CE. Nevertheless, to improve ESI and to permit CE–MS interfacing, a sheath liquid flow is used, usually with a coaxial arrangement [144]. GSH and GSSG have a quite good response in both positive and negative ion modes analysis and the sheath liquid, according to its pH, may favor cation or anion formation. Values of BGE and sheath liquid pH coincident with p*I* values of GSH or GSSG must be avoided in order to obtain their CE migration and MS detection.

CE–MS and CE–MS–MS methods have been proved useful in the determination of GSH in urine samples. CE–MS–MS improves the analysis of compounds in complex matrices because, even in case of co-migration with other compounds having the same molecular mass, they generate characteristic fragments following collision-induced dissociation that permits unequivocal identification. In this method, the use of borate buffers as run BGE minimized the problems of capillary obstruction possibly associated with crude biological samples,

and the urine samples were analyzed directly by CE–MS [144].

4. Study of glutathione enzymatic system functionality through GSH and GSSG determination

Measurements of GSH, GSSG, precursors, and metabolites, by chromatographic and electrophoretic methods are a useful tool in investigating defective GSH metabolism systems [144]. The determination of γ-GluCys synthetase activity consists of measuring γ-GluCys as the reaction product in the presence of DTT, Cys, Glu, and ATP, whereas the determination of GSH synthetase activity consists of measuring GSH as the reaction product in the presence of DTT, γ-GluCys and Gly [53,91]. GR activity has been determined in crude tissue samples without previous isolation of the enzyme thank to a sensitive HPLC–ECD method. The sample was incubated in phosphate buffer at a pH of 7.0 in the presence of GSSG and NADPH and the GSH formed was then measured [145]. The method presented by He et al. based on CE–MS assay of GSH in urine has application in the diagnosis of defective γ-GT. In fact, the presence of considerable amounts of GSH in urine is diagnostic of inherited γ-GT deficiency or of efficacious inhibition of the enzyme [100,144].

4. Conclusions

Numerous papers investigating qualitative and quantitative modifications and metabolism of GSH in several biological matrices have been published in the last years, reflecting the special interest around this important bio-molecule. As the analytical technologies have been improved, also refinements of GSH determinations have been achieved, and methods for the analysis of GSH and related compounds cover almost completely the combinations between analytical equipments and detection systems present on the market. At the present time, many methods are available to determine and quantitatively analyze GSH and congeners in several complex bio-matrices. Different HPLC and CE methods present elevated sensitivity and reproducibility thus supplying with useful instrument for investigating even fine variations in GSH redox system. However, despite the availability of validated analytical methods, GSH levels reported in the literature vary over 10-fold, especially for those matrices where are very low. Errors may arise from diverse phases of GSH determination procedures such as sample collection, processing and storage. This is mainly due to the ease GSH autoxidation and to the rapid proteolysis of GSH and GSSG in biological matrices. Moreover, results of GSH and GSSG quantification are expressed using different parameters, such as fluid or lysate volume, tissue weight, cell number, etc., rendering often difficult comparison between methods. Thus, although the availability of several separative and non separative methods allowing for GSH assay with the most common analytical equipments, there is a need of standardized procedures in sample collection and pre-treatment aiming to minimize artifacts and provide comparable results.

Nomenclature

AA	amino acids
ABD-F	4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole
ACN	acetonitrile
AD	amperometric detection
ASC	ascorbic acid
ATP	adenosine triphosphate
ATs	aminothiols
BALF	bronchoalveolar lavage fluid
BFS	bare fused-silica
BGE	background electrolyte
BrB	monobromobimane
BSO	buthionine sulfoxime
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CV	cyclic voltammetry
Cys	cysteine
CysGly	cysteinyl-glycine
CZE	capillary zone electrophoresis
DAD	diode array detector

DBD-F	4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole
DC	dansyl chloride
DCFDA	2′,7′-dichlorofluorescein diacetate
DHR-123	dihydrorhodamine-123
DMF	dimethylformamide
DNFB	2,4-dinitrofluorobenzene, Sanger's reagent
DNP	*N*-(2,4-dinitrophenyl)
DPDS	2,2′-dipyridyldisulfide
DT	dithionite, sodium hydrosulfite
DTNB	5′,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent
DTT	dithiothreitol
ECD	electrochemical detector/detection
EI	electron impact
EMA	eosin-5-maleimide
ESI	electrospray ionization
ESI–MS	electrospray ionization–mass spectrometry
ESR	electro-spin resonance
Et_3N	triethylamine
FAB	fast atom bombardment
FM	fluorescein-5-maleimide
GC	gas chromatography
Glu	glutamate
γ-GluCys	γ-glutamyl-cysteine
Gly	glycine
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione, γ-L-glutamyl-L-cysteinylglycine
GSNO	*S*-nitrosoglutahione
GSO_3H	glutathione-*S*-sulfonate
GSO_2NH_2	glutathione sulfonamide
GSSG	glutathione disulfide
GST	glutathione-*S*-transferase
γ-GT	γ-glutamyl transpeptidase
HCys	homocysteine
Hepes	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethansulfonic acid)
HPLC	high-performance liquid chromatography
HSAA	*N*-hydroxyuccinimidyl-α-(9-acridine)-acetate
HSMPA	*N*-hydroxysuccimidyl-α-(9-phenanthrene)-acetate
HTAB	hexadecyl-trimethylammonium bromide
6-IAF	6-iodoacetamidofluorescein
I.D.	inner diameter
IPAD	integrated pulsed amperometric detection ITP = isotachophoresis
LIF	laser-induced fluorescence
LOD	limit of detection
LOQ	limit of quantification
MADS	multichannel amperometric detection system
MCAA	monochloroacetic acid
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MIAA	monoiodoacetic acid
MMBP	5-maleimidyl-2-(m-methylphenyl) benzoxazole
MMI	methimazole
MPA	monophosphoric acid
MRM	multiple reactant monitoring mode
MS	mass spectrometry
MS–MS	tandem mass spectrometry
NAC	*N*-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NDA	naphthalene-2,3-dicarboxaldehyde
NEM	*N*-ethylmaleimide
NPM	*N*-(1-pyrenil)-maleimide
OCA	1-octanesulfonic acid
ODS	octadecylsilica
OPA	*o*-phthaladehyde
OSA	octane sulfonic acid
PBS	phosphate-buffered saline
PCA	perchloric acid
PFB-F	5-(pentafluorobenzoylamino)-fluorescein
PrSSG	protein-glutathione mixed disulfide
p.s.	particle size
ROS	reactive oxygen species
RP-HPLC	reversed-phase high-performance liquid chromatography

SBD-F	4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate
SBF-Cl	4-chloro-7-sulphobenzofurazan
SDS	sodium *n*-dodecyl sulfate
SIM	selected ion monitoring
SRM	selected reaction monitoring
5-SSA	5-sulfosalicylic acid
TBA	tetrabutylammonium hydroxide
TBP	tri-*n*-butylphosphine
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
ThioGlo™3	9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-phenyl)-3-oxo-3H-naphto[2,1-*b*]pyran
TLC	thin-layer chromatography
6-TP	thiopurines
TPP	triphenylphosphine
Tris	tris(hydroxymethyl)aminomethane
Trizma® Phosphate	mono[tris(hydroxymethyl)aminomethane] phosphate
UV	ultraviolet
UV–Vis	ultraviolet–visible

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