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Review

Determination of glutathione and glutathione disulfide in biological samples: An in-depth review☆

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ABSTRACT

Glutathione (GSH) is a thiol-containing tripeptide, which plays central roles in the defence against oxidative damage and in signaling pathways. Upon oxidation, GSH is transformed to glutathione disulfide (GSSG). The concentrations of GSH and GSSG and their molar ratio are indicators of cell functionality and oxidative stress. Assessment of redox homeostasis in various clinical states and medical applications for restoration of the glutathione status are of growing importance. This review is intended to provide a state-of-the-art overview of issues relating to sample pretreatment and choices for the separation and detection of GSH and GSSG. High-performance liquid chromatography, capillary electrophoresis and gas chromatography (as techniques with a separation step) with photometric, fluorimetric, electrochemical and mass spectrometric detection are discussed, stress being laid on novel approaches.

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Abbreviations: AA, amino acid; ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; AFS, atomic fluorescence spectrometry; APAD, activated pulsed amperometric detection; ASC, ascorbic acid; AT, aminothiol; BODIPY, difluoroboradiaza-*s*-indacene; CCE, carbon ceramic electrode; CNT, carbon nanotube; CySS, cystine; CVGAFS, cold vapor generation atomic fluorescence spectrometry; CZE, capillary zone electrophoresis; DABODIPY, 8-(3′,4′-diaminophenyl)difluoroboradiaza-*s*-indacene; DAMBO, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)difluoroboradiaza-*s*-indacene; DBD-F, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DHR-123, dihydrorhodamine-123; 4-DPS, 4,4′-dithiodipyridine; DQC, double quantum coherence; DT, dithionite, sodium hydrosulfite; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid), Ellman's reagent; DTT, dithiothreitol; ECD, electrochemical detector/detection; EMLC, electrochemically modulated liquid chromatography; FD, fluorescence detection; FDNB, 1-fluoro-2,4-dinitrobenzene; FEM, *N*-(2-ferroceneethyl)maleimide; FMEA, ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide; FPLC, fast protein liquid chromatography; GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; GCE, glassy carbon electrode; GCL, glutamate–cysteine ligase; GEMBE, gradient elution moving boundary electrophoresis; GEITP, gradient elution isotachophoresis; GGT, γ-glutamyl transpeptidase; GR, glutathione reductase; GS, glutathione synthase; GSSC, glutathione–cysteine mixed disulfide; GST, glutathione-*S*-transferase; HCys, homocysteine; HQS, 8-hydroxyquinoline-5-sulfonic acid; IA, iodoacetamide; IAA, iodoacetic acid; 5-IAF, 5-iodoacetamidofluorescein; 6-IAF, 6-iodoacetamidofluorescein; ITP, isotachophoresis; LIF, laser-induced fluorescence; M2VP, 1-methyl-2-vinylpyridinium trifluoromethane sulfonate; M4VP, 1-methyl-4-vinylpyridinium trifluoromethane sulfonate; MBB, monobromobimane; MIAC, *N*-(2-acridonyl)maleimide; MIPBO, 5-methyl-2-(*m*-iodoacetylaminophenyl)benzoxazole; MPA, metaphosphoric acid; MPPTAB, 1-[3-(4-maleimidylphenoxy)propyl]trimethylammonium bromide; NAC, *N*-acetyl-L-cysteine; NDA, naphthalene-2,3-dicarboxaldehyde; NEM, *N*-ethylmaleimide; NIRS, near infrared reflectance spectroscopy; NMDG, *N*-methyl-D-glucamine; NPM, *N*-(1-pyrenyl)maleimide; OPA, *o*-phthalaldehyde; PCA, perchloric acid; PHMB, *p*-hydroxymercurybenzoate, 4-(hydroxymercuric)benzoic acid, sodium salt; PQQ, pyrroloquinoline quinone; PRESS, point-resolved spectroscopy; Rh-123, rhodamine-123; RMME, reverse micellar microextraction; $Ru(phen)_3^{2+}$, tris(1,10-phenanthroline)ruthenium(II); SBD-F, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate; SOX, sulfhydryl oxidase; 5-SSA, 5-sulfosalicylic acid; TBP, tri-*n*-butylphosphine; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; 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; tp-ITP, transient pseudo-isotachophoresis; TPP, triphenylphosphine; UA, uric acid.

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1. Biological functions and metabolism of glutathione

Structurally, glutathione (GSH) is γ-L-glutamyl-L-cysteinylglycine, containing an unusual peptide linkage between the γ-carboxyl group of the Glu side-chain and the amino group of the Cys [1,2]. The GSH–glutathione disulfide (GSSG) system is the most abundant redox system in eukaryotic cells [2], plays a fundamental role in cell homeostasis [1,3] and is involved in signaling processes associated with programmed cell death, termed apoptosis [4–6]. Qualitative and quantitative alterations of the GSH–GSSG redox system are considered indices of oxidative damage [1,2,7]. The potential tissue injury induced by reactive oxygen and nitrogen species (ROS and RNS), termed oxidative and nitrosative stress, has been implicated in the pathogenesis of certain chronic illnesses, comprehensively reviewed by Dalle-Donne et al. [8].

The biologically active site of GSH is represented by the thiol group of the Cys residue. The high nucleophilicity of the thiol functionality facilitates the role of GSH as a free radical scavenger both under physiological conditions and in xenobiotic toxicity [2,9–11]. GSH also helps in the regeneration of other antioxidants, e.g. vitamin E and ascorbic acid (ASC) [1,12,13]. GSH is a cofactor for glutathione peroxidase in the decomposition of hydrogen peroxide or organic peroxides; for glyoxalase 1 in the detoxification of methylglyoxal and other α-oxo-aldehydes; and for maleylacetoacetate isomerase in the conversion of maleylacetoacetate and maleylpyruvate to the corresponding fumaryl derivatives [14].

GSH can also react with a number of endogenous moieties, resulting in the generation of bioactive endogenous GSH adducts [15,16]. Although some adducts can be formed directly, glutathione-*S*-transferase (GST)-mediated reactions generally predominate.

S-Nitrosothiols contain a nitroso moiety covalently bound to the sulfhydryl group of proteins or low-molecular-mass thiols, such as GSH and Cys, yielding *S*-nitrosoglutathione and *S*-nitrosocysteine. Despite the great interest in these thiol derivatives, the mechanisms responsible for *S*-nitrosothiol formation and the possible biological roles and even levels of *S*-nitrosothiols, at present, are controversial [17–20].

A notable amount of GSH can be bound to the –SH of protein cysteinyl residues by a mechanism called *S*-glutathionylation. *S*-Glutathionylation can be reversed, and therefore regulated, by means of deglutathionylation, with reactions catalyzed by the thiol-disulfide glutaredoxines (also known as thioltransferases) [21,22]. *S*-Glutathionylation may also play a role in the modulation of cell signaling [23]. *S*-Glutathionylation protects sensitive protein thiols from irreversible oxidation during oxidative stress and might also serve as a storage form of GSH to prevent loss of GSH under oxidative conditions, because GSSG would otherwise be exported from the cell [21]. Modifications in the *S*-glutathionylation state of specific proteins may be implicated in the pathogenesis of diabetes, cardiovascular and lung diseases, cancer and neurodegenerative diseases; this has been comprehensively reviewed in [24].

The *de novo* biosynthesis of GSH requires two ATP-dependent enzymes. The rate-limiting step is catalyzed by glutamate–cysteine ligase (GCL), which converts Glu and Cys to γ-GluCys, which in turn is converted into GSH by glutathione synthase (GS) [15,25]. When transported to the extracellular milieu, GSH is rapidly metabolized by γ-glutamyl transpeptidases (GGTs), which catalyze cleavage of the γ-glutamyl amide bond to Cys and transfer the Glu residue to another amino acid (AA) (usually cystine (CySS)). This reaction results in the formation of CysGly and γ-Glu-CySS. CysGly is then cleaved by extracellular dipeptidases to Cys and Gly, which are next transported back into the cell. γ-Glu-CySS can be transported back into the cytosol and is reduced to γ-GluCys, ready for conversion to GSH by GS. This salvage pathway helps maintain GSH levels in the short term, such as during rapid GSH utilization [15]. On the other hand, export of GSH from the erythrocytes may contribute significantly to the extracellular GSH pool, thereby cooperating with the liver and other tissues to the dynamics of the interorgan GSH metabolism [26].

Both the direct and the glutathione peroxidase-mediated enzymatic oxidation of GSH lead to the formation of GSSG, from which GSH is regenerated intracellularly by glutathione reductase (GR) in an NADPH-dependent reaction [27,28]. Physiologically, the activity of GR and the availability of NADPH are sufficient to maintain the molar ratio of GSSG:GSH at about 1:100 to 1:1000 [21,29]. However, if the level of stress increases or the GR activity is limited (e.g. due to attenuated NADPH synthesis caused by a glucose-6-phosphate dehydrogenase deficiency), GSSG accumulates and the molar ratio of GSSG/GSH increases. A high amount of GSSG is exported from the cell and is metabolized extracellularly [30]. Thus, the loss of GSSG from the cells increases the cellular requirements for *de novo* GSH synthesis [15]. Other metabolites, such as glutathione-*S*-sulfinate (GSO_2H), glutathione-*S*-sulfonate (GSO_3H), glutathione sulfonamide (GSO_2NH_2) and other sulfonamides, can ultimately be formed besides GSSG. The sulfinate and sulfonate may be reduced back to GSH, whereas the sulfonamides cannot [31,32].

Study of the levels of GSH and GSSG and their manipulation in cells and living systems are valid tools for an assessment of the functionality of the GSH–GSSG redox system. *N*-Acetyl-L-cysteine (NAC) has been used pharmacologically as a source of Cys to restore or elevate the GSH content [25], while buthionine sulfoxime has been applied to deplete GSH in cells [33]. The chronic administration of EUK-189, a superoxide dismutase/catalase mimetic, prevents heat stress-induced liver injury by decreasing the oxidative damage. This

is mainly related to an improvement in the intracellular redox status (including the molar ratio of GSSG/GSH) and a marked reduction in hepatocellular lipid peroxidation [34]. Moreover, the use and pharmacology of a novel GSSG mimetic, NOV-002, was recently reported by Townsend et al. [35–37]. NOV-002 is the disodium salt of GSSG complexed with cisplatin at a molar ratio of approximately 1000:1. The cisplatin may serve to stabilize the GSSG. When administered in combination with standard chemotherapeutic regimens, NOV-002 increased the efficacy (survival and tumor response) and improved the tolerance to chemotherapy (e.g. hematologic recovery) in advanced non-small-cell lung cancer patients. Among other mechanisms, modulation of the intracellular redox state and stimulation of the GSH metabolism could explain these findings [35,36]. Thus, the measurement of GSH levels is crucial in assessment of the influence of treatments with modulators of the GSH–GSSG redox system.

2. Analytical methods for the determination of glutathione and glutathione disulfide

2.1. Sample pretreatment

The major weak points in GSH determination are the ease of non-enzymatic GSH autoxidation at pH >7, and enzymatic conversion of GSH, the first step of which is mediated by GGTs, which exhibit optimal activity at neutral pH. Thus, it is recommended to maintain the pH of the media in the acidic range. Other thiols, such as Cys, are even more susceptible to non-enzymatic oxidation [38]. As demonstrated by Rossi et al. [39,40] and Caussé et al. [41], the measurement of GSSG in whole blood and erythrocytes [39,40] as well as in plasma and serum [41] requires caution in order to prevent assay artifacts or data misinterpretation. Accordingly, the following points must be checked in order to achieve accurate analyses of GSH and GSSG: sample collection, reduction of disulfides, deproteinization, and derivatization of thiols.

2.1.1. Sample collection

The most important sources of artifacts associated with sample collection, with potential influence on the results of GSH and GSSG assays in whole blood and plasma, are the hemolysis of the red blood cells and the storage at room temperature. Hemolysis can either cause overestimation of GSH in the plasma because the GSH level in erythrocytes is 500-fold higher than that in plasma, or, without refrigeration, lead to underestimation of GSH and overestimation of GSSG in whole blood and plasma when autoxidation and proteolysis of GSH in the plasma are not repressed [38,39,42]. Caution during blood withdrawal, refrigeration during sample processing and, for plasma determinations, immediate centrifugation of the samples are recommended [41,42]. Tubes for blood sampling and preserving solutions are also suggested to contain EDTA. This serves as an anticoagulant and chelating agent entrapping many transition metals (including Fe^{2+}), and therefore avoiding some oxidative reactions [38,41,42]. As a very efficient Fe^{2+} chelator, the addition of diethylenetriaminopentaacetic acid (DETAPAC) has also been suggested [43].

2.1.2. Reduction of disulfides

For the determination of free plus bound GSH and other low-molecular-mass aminothiols (ATs), the reduction of the disulfide bonds formed between them and other thiols or proteins needs to be accomplished. However, GSH formed during reduction can be reoxidized before derivatization, and a variable degree of such reoxidation is a source of erratic results [44]. This phenomenon can be avoided with thiol-masking agents, such as *N*-ethylmaleimide (NEM) or iodoacetic acid (IAA).

The selection of the reducing reagent is critical for assay performance. Sulfhydryl-containing reducing agents, such as dithioerythritol, dithiothreitol (DTT) or 2-mercaptoethanol, can negatively affect assays measuring free plus bound GSH because they react competitively with certain labeling reagents such as monobromobimane (MBB) or *o*-phthalaldehyde (OPA), leading to the formation of interfering fluorescent compounds [38].

Sodium and potassium borohydride are potent reductants that are unstable in aqueous solution [45]. The reduction takes a few minutes at high concentrations of the reducing agent, but requires up to 30 min or the application of heating at lower concentrations (40–100 mM). The formation of gas and foaming during the reaction may be overcome by adding monophosphoric acid (MPA) or a surface-active agent, e.g. octanol [38,44].

Trialkylphosphines, e.g. tributylphosphine (TBP) or triphenylphosphine (TPP), are powerful reductants that need to be present in only slight molar excess for the complete reduction of disulfides; moreover, most trialkylphosphines do not react with thiol-specific reagents and do not give rise to gas formation during the reaction [44]. Another member of this family is tris-(2-carboxyethyl)phosphine (TCEP), which is highly soluble in water, nonvolatile and not greatly sensitive to humidity and air, and its use may provide more reproducible assays than with TBP and TPP [46]. However, TCEP can interfere with certain disulfide-containing derivatizing agents, including DTNB [47]. Mono-, di-, and trimethyl ester analogs of TCEP offer extended reactivity at lower pH. At pH 5.0, trimethyl-TCEP is 35-fold more reactive than TCEP. Esterification of TCEP also increases the lipophilicity, allowing trimethyl-TCEP to penetrate rapidly through phospholipid bilayers. Although more reactive toward small-molecule disulfides at pH 7.5, all phosphines were markedly less reactive than DTT toward protein disulfides at this pH [47].

Dithionite (DT, sodium hydrosulfite) has also been used for disulfide reduction. The stoichiometry of the disulfide reduction by DT is different from that with other reducing reagents: DT releases 1 thiol equivalent from disulfides instead of the 2 obtained with other reducing agents [38].

2.1.3. Protein removal

Proteins are the most abundant components in biological samples and their presence in injected samples may attenuate the performance of the analytical procedure and shorten the lifetime of the instrumentation. In most cases, therefore, proteins must be removed prior to the analysis of GSH and GSSG. Only a few methods (e.g. NMR) are suitable for measurements in intact cells or non-deproteinized samples. Acidification, addition of an organic solvent, such as ACN, acetone or methanol, and ultrafiltration are employed as means of protein elimination.

Typically, the acidic agents used for this purpose are 5-sulfosalicylic acid (5-SSA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), perchloric acid (PCA), or MPA [48,49]. In an earlier study in which these reagents were compared, 5-SSA did not provide acceptable sample stability at any concentration tested, MPA was found to leave substantial amounts of protein in the samples, and TCA interfered at times with the peaks of interest. These results led to a final concentration of 15% PCA being recommended for the analysis of GSH in whole blood [49].

From a comparison of protein precipitation methods, Caussé et al. established that the best results were obtained with ACN or 5-SSA precipitation in the identification of GSH with the fluorescent derivatizing agent 6-iodoacetamidofluorescein (6-IAF) [41]. However, when the thiol group is not protected, precipitation by organic solvents does not prevent GSH autoxidation as in the case of acids. Moreover, Rossi et al. revealed that the restoration of neutral-alkaline pH in acidified samples also leads to a rapid decrease of thiol concentration if no previous treatment

with thiol-masking agents has been performed [39,50]. The latter finding is important in view of the fact that the pH must be increased to neutral or >9 prior to the derivatization of ATs with certain chromophores and fluorophores. These include IAA, OPA, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [38]. On the other hand, organic solvents are preferable to acids when a mass spectrometer is used as the detector [38].

The use of filters, excluding macromolecules on the basis of their pore dimensions, is a useful method to remove proteins [51–53] because it does not require the addition of acids or organic solvents that can affect the separation, derivatization and detection. The separation of most proteins and low-molecular-mass compounds such as GSH and GSSG can also be achieved by applying membrane filtration in combination with centrifugal microconcentration [54].

2.1.4. Derivatization of free and bound thiol functionalities in glutathione and other compounds

The derivatization of GSH, GSSG and their analogs with an appropriate derivatizing agent improves the LOD with spectrophotometry or spectrofluorimetry. This is because there are no strong chromophores or fluorophores in their chemical structures. It also suggests the possibility of their determination by means of indirect detection. Assays with indirect detection are based on the modification (decrease) in a uniform background signal (absorbance, fluorescence or current). The principles of capillary electrophoresis (CE) coupled with indirect detection have been reviewed previously [55]. Extended path-length UV absorbance detectors may also provide satisfactory detection limits for underivatized GSH and GSSG.

Although GSH has three sites susceptible to derivatization, i.e. the carboxylic, amino and thiol functionalities, labeling of the thiol moiety is preferred for its specificity and for its function as protective group. In contrast, GSSG can undergo derivatization only on the amino and carboxylic groups. Glutathione assays based on electrochemical detection (ECD) are not dependent on derivatization.

2.1.4.1. Introduction of a chromophore. Analytical methods using colorimetric reagents and UV absorbance detection are inferior in terms of sensitivity, but simpler as compared with fluorescent detection (FD) or ECD [44]. The compounds most commonly used are NEM [56–60], IAA [61] and iodoacetamide (IA) [62], yielding thioethers. NEM has also been used in combination with FD and MS for the measurement of GSSG, making use of the thiol-masking properties of NEM [63–66]. The reaction with NEM involves the addition of thiols across the double bond of maleimide, whereas IAA and IA form *S*-carboxymethyl and *S*-carboxamidomethyl derivatives, respectively, by nucleophilic substitution. With IA, however, undesired products are also formed [62]. The application of NEM with the GR-coupled enzymatic recycling method presents difficulties due to the inhibition of GR by NEM. The reagent excess must therefore be eliminated either by solvent extraction [59] or by solid-phase extraction [58,60]. It should be noted that NEM also binds amino groups at pH >7.5, even if more slowly as compared with thiol functionalities [39]. Thus, if titration procedures require long incubations at alkaline pH, or if a derivatization agent of the amino group, such as 1-fluoro-2,4-dinitrobenzene (FDNB), is to be used, the excess of NEM must be removed before the alkalization of the medium [39]. Other analogs of NEM designed to improve the UV absorbance are *N*-(1-pyrenyl)maleimide (NPM) [67], *N*-(2,4-dinitrophenylaminoethyl)maleimide and eosin-5-maleimide.

5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) is utilized for the derivatization of GSH and GSSG in the classical spectrophotometric GR-coupled enzymatic recycling or GSH-recycling assay developed by Tietze [59]. It is based on the progressive reduction of GSSG to GSH with GR in the presence of NADPH, while the rate of formation of the colored product 5-thionitrobenzoate is measured. DTNB has been employed in several studies for the determination of both GSH and GSSG [58–60,68–70], in the first three ones coupled with NEM.

4,4′-Dithiodipyridine (4-DPS) has been used for the quantification of protein thiols and dithiols in picomolar amounts. In this application, 4-DPS has a great advantage over DTNB due to its hydrophobic nature, its somewhat smaller size and the possibility of its use at low pH, where thiol-disulfide exchange reactions normally do not occur [45].

FDNB, a derivatizing agent of the amino group, has the advantage of allowing the simultaneous evaluation of GSH and GSSG in a single run. FDNB was introduced in the HPLC assay of GSH and GSSG by Reed et al. [71]. Asensi et al. utilized NEM for GSH masking to prevent the autoxidation of GSH [72], often observed with the IAA used in the original assay [71]. Their method allowed the measurement of GSSG alone [72]. More recently, Giustarini et al. reported an improved HPLC-UV assay that possesses the advantages of both methods: the simultaneous and sensitive determination of GSH and GSSG and the prevention of artifactual autoxidation of GSH. However, the excess of NEM must be removed prior to derivatization with FDNB, due to the slow binding of NEM to amino groups [50].

Recently, 1-methyl-2-vinylpyridinium trifluoromethane sulfonate (M2VP) and 1-methyl-4-vinylpyridinium trifluoromethane sulfonate (M4VP) have attracted attention as thiol-masking agents. Both have the advantages over NEM that they do not inhibit GR and do not react with amino groups. Avoidance of the extraction step significantly shortens the sample preparation time, and allows the development of a microtiter plate assay [69] and even an automated system [70], based on enzymatic recycling. The applicability of M2VP and M4VP for the determination of GSSG has so far been explored only in spectrophotometric assays. It is proposed that future studies evaluate whether M2VP and M4VP are also suitable for use in combination with HPLC or CE.

2.1.4.2. Introduction of a fluorophore. FD allows much lower detection limits than UV absorbance, and the use of lasers (mainly argon, or in some studies mercury or krypton ion lasers) to induce fluorescence is associated with further improvements. Useful reagents must form GSH adducts with sufficient fluorescence yield to permit the measurement of GSH at picomolar amounts or less. Moreover, the ideal reagent should be nonfluorescent when in an unreacted form, and react rapidly and specifically with GSH and other thiols to form stable products. Fluorophores that react with the thiol group are the most selective and also prevent artifacts arising from the autoxidation of GSH during sample preparation and analysis, but do not allow the detection of GSSG. Reagents targeting the amino group allow the simultaneous fluorimetric determination of GSH and GSSG, even if less selectively [38,44].

OPA is nonfluorescent until it reacts as a heterobifunctional reagent with a primary amine in the presence of a thiol or *vice versa*, when it forms a highly fluorescent isoindole derivative. Attractive features of OPA derivatization include mild reaction conditions, widespread experience with its use, and the possible determination of other thiols and AAs. GSH reacts with OPA to form a tricyclic isoindole derivative without the need for a thiol co-reagent [73]. However, a co-reagent such as 2-aminoethanol, Gly, 2-mercaptoethanol, NAC, 3-mercaptopropionic acid or ethanethiol may participate in the heterobifunctional reaction, thereby modulating it [38,74]. For example, in the absence of a co-reagent, OPA derivatizes GSH, γ-GluCys and other ATs, supplying both the amino and thiol functionalities, whereas Gly, added to eluents, directs the post-column OPA reaction selectively towards thiols [75]. Although the primary amino groups of GSSG can react with OPA, derivatization of the thiol functionality of GSSG can also be achieved by the previous reduction of GSSG [76]. Whereas 2-mercaptoethanol is preferred as a co-reagent, the use of ethanethiol in certain appli-

cations may provide advantages over 2-mercaptoethanol, such as in the determination of various AAs (especially Gly, histidine, β-alanine, ornithine and lysine), due to the increased stability of the derivatives [77].

Naphthalene-2,3-dicarboxaldehyde (NDA), a reagent similar to OPA, has been successfully used for the simultaneous assessment of ROS (such as superoxide, hydroxyl radical or hydrogen peroxide) and the levels of GSH [78–80].

Bimanes such as MBB couple rapidly with various thiols at room temperature at pH 8.0 to produce thioethers with relatively high fluorescence emission; this allows the detection of analytes at low (nanomolar) concentrations. The drawback is that the reagent is self-fluorescent and undergoes photodegradation, yielding fluorescent hydrolysis products [38,44].

Fluorobenzofurazans have also been used for the determination of thiols. The unreacted reagents are not fluorescent, the thiol adducts are stable, and no fluorescent hydrolysis products are formed. Thus, their use results in chromatograms that show only known peaks. SBD-F is a low-reactive reagent and requires drastic conditions to react quantitatively with thiols (pH 9.5 and 60 °C for 1 h). In contrast, ABD-F offers fast and quantitative reaction under mild conditions, and is therefore suitable for on-column derivatization with thiols prior to CE [38,44].

IA-type fluorophores are widely used, taking advantage of the affinity of the IA moiety to thiols, which also protects GSH from autoxidation. 5-IAF and 6-IAF contain fluorescein as fluorescent groups, whereas 5-methyl-2-(*m*-iodoacetylaminophenyl)benzoxazole (MIPBO) is a benzoxazole derivative [81]. Another benzoxazole compound, 5-maleimidyl-2-(*m*-methylphenyl)benzoxazole, has the advantage over some fluorophores of higher selectivity for GSH than for Cys under optimized conditions, and is therefore appropriate for the direct spectrofluorimetric determination of GSH in samples containing both thiols [82].

Derivatives of maleimide such as fluorescein-5-maleimide, 2-(4-*N*-maleimidophenyl)-6-methoxybenzofuran, 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) [83] and the newly synthetized *N*-(2-acridonyl)maleimide (MIAC) [84] have also been employed. Both ThioGlo™3 and MIAC have very high affinity for the thiol group, offer low baseline fluorescence, demonstrate high specificity for and fast reactivity with thiols and yield highly fluorescent products [83,84]. When MIAC is used, the specific configurations of the reaction products related to the large planar acridone ring make them very stable [84].

The difluoroboradiaza-*s*-indacene (BODIPY) family of fluorophores have the advantages of high extinction coefficients, high fluorescence quantum efficiency, stability to light and availability in a wide range of pH [85]. Two new members of the BODIPY family were recently introduced for the determination of *S*-nitrosothiols in whole blood: 8-(3′,4′-diaminophenyl) difluoroboradiaza-*s*-indacene (DABODIPY) [86] and 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)difluoroboradiaza-*s*-indacene (DAMBO) [85]. They display very low self-fluorescence and highly fluorescent derivatization products; both the reagent and the reaction products are stable.

A number of fluorophores have been synthetized with a view to covering the needs of the *in vivo* assay of thiols. A dithiolic fluorophore has been derived from fluorescein and rhodamine, which is fluorescent over a wide pH range, including neutral pH. Separation of the two moieties in the cell led to a large increase in fluorescence for fluorescein, allowing real-time measurements [87]. Other fluorophores include cell-permeable latent (“turn-on”) probes. Aniline-based compounds have been designed with a sulfonamide linkage [88], and rhodamine-based ones with either a Se–N bond [89], two disulfide bonds [90] or a sulfonamide linkage [91]. Cleavage of the respective functionally important bonds by thiols *in vivo* or *in cyto* results in the liberation of the fluorescent aniline [88], rhodamine-6G [89] or rhodamine-110 [90,91], with a subsequent increase in the fluorescence.

2.1.4.3. Derivatization for mass spectrometry. The derivatization technique of the analytes is dependent on the chemical properties of the compound and the instrument used for separation (HPLC, GC or CE). The detection of analytes in a GC system requires the volatilization of compounds. GSH is a polar compound and is readily altered at high temperatures. Thus, for its analysis in a GC/MS system, a derivatization step is required to decrease polarity and to enhance thermal stability [38]. Common derivatizing agents are trifluoroacetic anhydride, pentafluoropropionic anhydride and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

Isotope tagging coupled with derivatization of the compounds by means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) allows monitoring of GSH metabolism by the measurement of the incorporation of stable isotopically labeled precursor AAs or ^{2}H from deuterated water ($^{2}H_2O$). This technique, however, raises additional requirements for the derivatizing reagents, such as the limited addition of carbon atoms to the analyte. Among others, ethyl chloroformate/methanol derivatization may be an appropriate choice [92,93].

In contrast, most analytes elute from the column in LC–MS/MS without prior derivatization [94–96]. Novel techniques to enhance the separation and measurement of thiols with MS include the adsorption of positively charged peptides on the surface of negatively charged bare (uncapped) silica nanoparticles [97], and a one-step reverse micellar microextraction (RMME) procedure for the determination of thiol-containing peptides and AAs [98]. Reverse micelles were formed by the cationic surfactant methyltrioctylammonium chloride [98]. Omitting the derivatization step greatly simplifies the sample preparation process and increases the sample throughput [99], but can result in excessive autoxidation and consequently erroneous results (detailed in Section 2.1). Several studies have benefited from the inclusion of derivatization [61,100–102].

1-[3-(4-Maleimidylphenoxy)propyl]trimethylammonium bromide (MPPTAB) makes use of the maleimidyl moiety as a selective thiol labeling group, and a positively charged quaternary ammonium moiety for the improvement of ionization efficiency; these two moieties are combined by the bifunctional linker *p*-aminophenol. A comparative study by MS indicated that, after derivatization with MPPTAB, the signal intensities of the thiol peptides with more strongly polar residues were increased 3–5-fold, while those with less polar residues could be enhanced more than 100-fold [100].

For the selective detection of the various thiols, *N*-(2-ferroceneethyl)maleimide (FEM), originally developed for thiol determination by HPLC with ECD, has been used as a mass tag for the derivatization of free thiol functionalities. After its quantitative reaction and a subsequent reduction of the disulfide-bound thiols by TCEP, the newly formed thiol functionalities were reacted with the newly developed derivatizing agent ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) [101,102]. IAA was also employed for the simultaneous detection of free thiols and disulfides by means of LC–MS/MS [61].

2.2. Techniques without separation

2.2.1. Spectrophotometric methods

Tietze published his classical spectrophotometric method in 1969, often referred to as the GR-coupled enzymatic recycling assay or the GSH-recycling assay [59]. In the presence of DTNB and GR, the addition of NADPH to the reaction mixture initiates the pro-

gressive reduction of GSSG to GSH which, in turn, reacts with DTNB, the colored product 5-thionitrobenzoate being formed upon reduction. The rate of color change at 412 nm, typically followed over 3 or 4 min, is proportional to the total glutathione (i.e. GSH plus GSSG) concentration. GSSG is determined via the blocking of GSH with NEM in a separate sample [59]. The GR-coupled recycling assay is still one of the most widely applied techniques to detect GSH and GSSG, due to its simplicity, satisfactory sensitivity and low cost. Importantly, Rossi et al. reported similar levels of whole blood GSH and GSSG with the method of Tietze (1161 ± 151 and 3.51 ± 2.09 μM, respectively) and with a validated, artifact-free HPLC-UV absorbance assay, using FDNB derivatization and the thiol-masking agent NEM (1199 ± 201 and 3.99 ± 2.04 μM for GSH and GSSG, respectively) [39]. The main drawbacks of the original assay were the unsatisfactory specificity and selectivity and, in the assay of GSSG, the need for the elimination of the excess of NEM due to its GR-inhibiting properties [59], which made the technique time-consuming. Slight modifications have previously been made to the original method, such as solid-phase extraction instead of solvent extraction to remove the excess of NEM and isolate GSSG [58,60]. More recently, assays based on the same principles make use of 96-well microtiter plates [68,69] or an automated sequential injection analysis system [70]. This switch was facilitated by the replacement of NEM by M4VP [69] or M2VP [70] as new thiol-masking reagents for the determination of GSSG in human whole blood [69] and rat liver tissue and bile samples [70], as their excess does not need to be removed. The application of M2VP and M4VP makes the recycling method less time-consuming and less laborious, so that the technique is expected to become even more attractive. The GR-coupled recycling assay is also appropriate to be combined with CL techniques and biosensors (described in detail in the respective sections).

Other spectrophotometric techniques frequently used are the GST 1-chloro-2,4-dinitrobenzene (CDNB) endpoint method, measuring solely GSH, first reported by Brigelius et al. in the assessment of hepatic GSH [103], and a GSSG-endpoint determination, developed by Güntherberg and Rost in 1966 for the determination of erythrocyte GSSG [104]. The latter assay is based on the measurement of NADPH consumption by GR, a process that reduces GSSG present in the sample [104]. Even if quite specific, these methods offer much lower sensitivity than that of the GSH-recycling assay.

2.2.2. Spectrofluorimetric methods

Several spectrofluorimetric methods have been developed for the analysis of GSH, GSSG and related compounds in different matrices. Wang et al. measured GSH in human blood serum with a fluorescent Zn(II)–8-hydroxyquinoline-5-sulfonic acid (HQS) system [105]. The use of OPA together with 1-pyrenemethylamine nanoparticles is associated with stronger fluorescence and an improved LOD than those with OPA alone [106]. Novel methods utilizing a dithiolic fluorophore [87], other rhodamine-based fluorescent probes [89–91], most of them reacting with all thiol functionalities in the cell, and newly synthetized members of the BODIPY family, DAMBO [85] and DABODIPY [86] (both used for *S*-nitrosothiol measurements in combination with Hg^{2+}), have also been published. A number of fluorophores [87,89,90] allow real-time assaying of thiols *in vivo* (for details, see Section 3.2). Some of the most recent methods have not yet been applied to the determination of GSH.

An interesting novel approach for the detection and identification of multiple species is the use of multiparameter fluorescence detection (MFD) [107] or multiparameter fluorescence imaging (MFDi) [108]. In MFDi, the three spatial coordinates are stored in addition to the simultaneous monitoring of the fluorescence information. MFDi allows a real-time assessment of the dynamics of molecules in solution and in cells [108]. The quantitative value of the techniques and their potential application in peptide and glutathione analysis are yet to be determined.

2.2.3. Chemiluminescence methods

CL is an attractive means of detection for biological substance analysis in consequence of its low LOD values (nanomolar amounts) and wide linear working ranges, with relatively simple instrumentation. The first method in which CL was used in the measurement of GSH was developed by Hinze in 1984 [109], but its LOD was higher than that of the spectrophotometric methods in which Ellman's reagent was applied. In 1998, Romero and Mueller-Klieser detected total GSH via coupling of the DTNB-GR recycling assay (with which to amplify the NADPH decrease) with the highly sensitive luminescent FMN oxidoreductase/bacterial luciferase reaction (to detect the consumption of NADPH) [110]. The first CL assay for GSSG, published in 2000 by Mourad et al., was based on the same principles, but used 4-chloro-7-trifluoromethyl-1-methylquinolinium to mask GSH [111]. Since then, other bioluminescent systems for the determination of total GSH and Cys have been developed, including luminol–$NaIO_4$ [112,113], luminol–H_2O_2 [114], quinine–Ce(IV) [115,116], $Ru(phen)_3{}^{2+}$–Ce(IV) [117] and $Ru(phen)_3{}^{2+}$–$KMnO_4$ [118]. Most assays are based on the oxidation reaction between a strong oxidant and GSH or Cys, and the subsequent oxidation of the luminophore to produce CL. In the latter two assays, the weak basal CL of the $Ru(phen)_3{}^{2+}$–Ce(IV) [117] or $Ru(phen)_3{}^{2+}$–$KMnO_4$ [118] system is amplified by the addition of GSH or Cys.

2.2.4. Nuclear magnetic resonance spectroscopic methods

NMR is a technique that allows the determination of GSH and GSSG in the brain *in vivo* and in intact cells *in cyto*. Trabesinger et al. pioneered a method applicable to the human brain in 1999. They used ^1H NMR with double quantum coherence (DQC) filtering in combination with point-resolved spectroscopy (PRESS) volume selection with a relatively low strength field of 1.5 T, and estimated the GSH concentration in the frontal lobe of 12 healthy volunteers as 2–5 mM [119]. The same research group improved the DQC filtering in a subsequent study, so as to minimize the contamination of the DQC-filtered GSH signal with contributions from GABA [120]. Later, further improvements in the DQC filtering were achieved, which eliminated the need for calibration scans to optimize the signal yield and removed the influence of water saturation pulses on the GSH yield. The authors suggested that the new filter was expected to be less sensitive to patient motion by virtue of its single shot nature [121]. In 2006, the contribution of both GSH and GSSG to MEGA-PRESS (a frequency-selective refocusing technique) signals assessed by ^1H NMR were evaluated *in vitro* and *in vivo*. To mimic the *in vivo* conditions, PBS solutions containing GSH and GSSG in concentrations similar to those existing *in vivo* were prepared. Solutions containing creatine, GSH, aspartate and γ-aminobutyric acid were also prepared. In such solutions, GSH gave a single positive signal, whereas GSSG yielded a multiplet of reversed signals. The concentration of GSH in the parietal lobe of healthy volunteers was 1.9 ± 0.37 mM; no signal for GSSG was detected [122]. The simultaneous determination of GSH, GSSG and ASC was achieved with ^1H NMR by using double editing with (DEW) MEGA-PRESS at 4.0 T. The GSH and ASC levels in the occipital lobe of 4 healthy subjects were 1.0 ± 0.1 and 0.8 ± 0.1 μmol/g, respectively. GSSG was suggested to give detectable signals *in vivo* only at exceptionally high concentrations [123].

GSH concentration can also be assayed in blood through ^{19}F NMR by measuring the products of the exchange reactions of thiols with a newly synthetized fluorinated disulfide 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid [124].

Another interesting field for NMR measurements is the investigation of intact cells. To resolve the issue of whether erythrocytes can utilize extracellular GSH to enhance the intracellular free GSH

pool, the intracellular and extracellular GSH redox status was measured by means of ^{1}H spin-echo NMR and ^{13}C[^{1}H-decoupled] NMR. It was concluded that the intracellular GSH in glucose-starved erythrocytes cannot be increased from extracellular sources by the release of bound GSH from mixed disulfides with membrane proteins [125], in contrast with what an earlier report suggested [126].

2.2.5. Electrochemical techniques

A linear sweep cyclic voltammetry method at a hanging mercury drop electrode (HMDE) as a working electrode, polarized between potentials of −0.2 and −0.8 V, was developed by Kizek et al. for the measurement of GSH and GSSG. The surface area of the working electrode was 0.4 mm^2; Ag/AgCl/3 M KCl served as a reference electrode, and Pt wire as an auxiliary electrode [127].

A glassy carbon electrode (GCE) is a good material for modification, and the attachment of carbon nanotube (CNT) layers results in an electrode with widespread detection possibilities [128]. It should be noted that CNT-modified GCEs (and also carbon ceramic electrodes (CCEs)) do not primarily serve as stand-alone detectors (except with authentic compound mixtures), but rather as ECDs after chromatographic separations and transducers of the biorecognition event in biosensors for the analysis of biological samples [128,129].

Salimi and Hallaj reported the preparation of a preheated GCE modified with multiwall CNTs and its use in the detection of GSH, Cys and thiocytosine [129]. Han and Tachikawa tested the electrocatalytic oxidation of 14 thiols, including GSH, homocysteine (HCys), Cys and NAC, at a GCE coated with a single-wall CNT film. The enhanced catalytic properties of this electrode, as compared with bare GC and diamond electrodes, was further improved by its modification with pyrroloquinoline quinone (PQQ) [130].

A renewable three-dimensional sol–gel CCE chemically modified with Ru[(tpy)(bpy)Cl]PF_6 was constructed by Salimi and Pourbeyram, and used for the amperometric detection of GSH and Cys. This modified electrode had the advantages of high sensitivity, a short response time and long-term stability [131].

A biosensor is a device constituted by a biorecognition layer, the purpose of which is the biomolecular recognition of the analyte, and a transducer responsible for the conversion of the biorecognition event into a useful electrical signal. Enzymes, antigens, antibodies, nucleic acids, receptors and tissues have been used as biorecognition elements. Depending on the nature of this element, it is possible to divide biosensors into enzymatic (involving a biocatalytic event) and affinity (involving an affinity event) biosensors [128]. The widespread use of biosensors and electronic tongue systems has been reviewed recently [132].

Timur et al. integrated a membrane modified with GR and sulfhydryl oxidase (SOX) onto the surface of spectrographic graphite rods. The working principle of the biosensor was based on the monitoring of O_2 consumption, which is correlated with the concentration of GSSG or GSH during the enzymatic reaction. The combination of GR and SOX allows the detection of GSSG and GSH in the same matrices. This biosensor has also been applied as an ECD after HPLC separation [133].

A biosensing approach including the indirect detection of current has also been reported. Horseradish peroxidase, immobilized on a rotating disk, catalyzes the oxidation of catechols in the presence of H_2O_2, back electrochemical reduction of which is detected on a GCE surface. GSH or Cys added to the solution participates in Michael addition reactions with catechols to form the corresponding thioquinone derivatives, which decreases the peak current obtained proportionally to the increase in their concentration. The detection of GSH and Cys in nanomolar concentrations is possible, in the processing of as many as 25 samples per hour [134].

Cha and Meyerhoff reported the development of an amperometric NO gas sensor modified with a thin hydrogel layer for the detection of *S*-nitrosothiols. Immobilized 3,3′-dipropionicdiselenide (an organoselenium catalyst) is covalently coupled to the primary amine groups in polyethylenimine, which is further cross-linked to form a hydrogel layer, reported to be capable of decomposing *S*-nitrosothiols to generate NO [135]. However, it should be noted that biosensors are rather of qualitative value in *S*-nitrosothiol analysis at present. Moreover, it is questionable whether low-molecular-mass *S*-nitrosothiols are present in measurable amounts in the circulation [18].

2.3. Methods with a separation step

2.3.1. High-performance liquid chromatographic methods

A number of HPLC and two ultra-performance liquid chromatography (UPLC) methods [95,136] for the determination of GSH, GSSG and related compounds have recently been developed or improved. Detection systems used in combination with them include UV absorbance, FD, ECD, MS, MS/MS and atomic fluorescence spectrometry (AFS). Most assays are reversed-phase (RP)-based, although anion-exchange HPLC has also found application [137]. Common characteristics in almost all these methods are that eluents are buffered at acidic pH (to maintain GSH and congeners in a nondissociated form, so as to improve interaction with the stationary phase, and to attenuate thiol autoxidation), and that TFA is added to the eluent (TFA is an ion-pairing reagent which helps the resolution of peak tailing associated with GSSG). To enhance the separation selectivity of HPLC, electrochemically modulated liquid chromatography (EMLC) has recently been introduced by Saitoh et al. [138,139]. EMLC works by altering retention through changes in the potential applied to the conductive stationary phase [139]. Even if EMLC is potentially suitable for the determination of GSH and GSSG, it has not yet been adopted. A summary of the HPLC methods reviewed in this article is given in Table 1.

Only three recent HPLC methods have employed UV absorbance as a means of detection. Hansen et al. quantified protein thiols and dithiols in picomolar amounts by using 4-DPS as a derivatizing agent [45]. Vignaud et al. assayed disulfides produced from the oxidation of GSH and Cys using by using RP-HPLC coupled with either direct UV absorbance or ECD (coulometric) detection. In order to avoid derivatization, size exclusion was previously performed on a fast protein liquid chromatography (FPLC) system. ECD was found to provide a sensitivity 100 times higher for the disulfides than UV absorbance at 220 nm for underivatized samples. This method allowed the determination not only of GSH, Cys and their symmetric disulfides, but also of their mixed disulfide, GSSC (Fig. 1) [140].

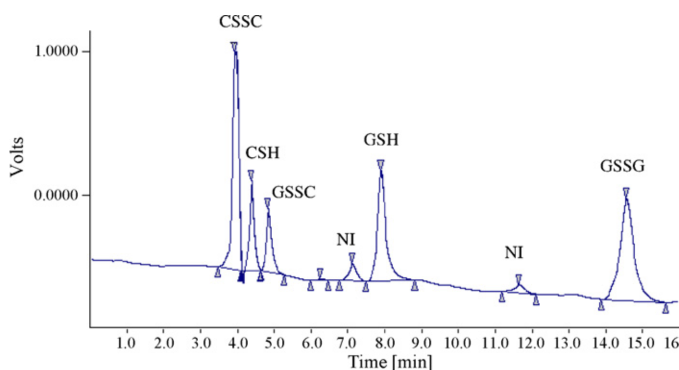

Fig. 1. Coulometric HPLC chromatogram of a standard mixture of cystine (CySS, 50 μM), cysteine (Cys, 50 μM), glutathione–cysteine mixed disulfide (GSSC, 50 μM), glutathione (GSH, 40 μM) and glutathione disulfide (GSSG, 30 μM). Size exclusion was previously performed on a fast protein liquid chromatography system. NI: non-identified peak. Reproduced with permission from [140].

Table 1
Summary of recent HPLC methods for the determination of glutathione (GSH) and related compounds.

Detection	Matrix type	Analytes	Label	Limit of detection		Concentration (human blood)		Reference
				GSH	GSSG	GSH	GSSG	
UV 355 nm	Human whole blood	GSH, GSSG	FDNB	0.5 μM	0.5 μM	1401 ± 113 μM	3.12 ± 0.59 μM	[50]
UV 254 nm (after FPLC) and UV 220 nm and ECD	Standards	GSH, GSSG, GSSC, Cys, CySS	None	5.0 nmol	2.5 nmol	N.A.	N.A.	[140]
UV 338 nm and FD	Human and rat plasma	GSH, *S*-nitrosoglutathione	OPA	70.0 nm	N.R.	N.R.	N.R.	[141]
Ex/Em 338/458 nm				3.0 nm				
FD	Human plasma	GSH, HCys, Cys, CysGly	SBD-F	N.R.	N.R.	N.R.	N.R.	[46]
Ex/Em 385/515 nm								
FD	Human whole blood, plasma, rat hepatocytes	GSH, GSSG	OPA, NEM	14.0 fmol	5.6 fmol	4.69 ± 0.93	0.28 ± 0.12 μmol/g Hb (whole blood), 0.154 ± 0.044 μM (plasma)	[63]
Ex/Em 350/420 nm						μmol/g Hb (whole blood), 1.82 ± 0.55 μM (plasma)		
FD	Rat liver, lung, brain	GSSG	NPM	N.R.	2.5 nm	N.A.	N.A.	[67]
Ex/Em 330/376 nm								
FD (dual)	Human plasma	GSH, GSSG	MBB (for GSH), OPA (for GSSG)	0.20 μM	0.05 μM	N.R.	N.R.	[76]
Ex/Em 390/478 (GSH) and 340/425 nm (GSSG)				0.5 pmol	0.04 pmol			
FD	Standards, human urine	GSH, Cys, NAC	MIPBO	3.5 fmol	N.R.	N.A.	N.A.	[81]
Ex/Em 310/375 nm								
FD	Rat brain, kidney, lung, liver	GSH, GSSG, HCys, Cys,	ThioGlo™3	50.0 fmol	N.R.	N.A.	N.A.	[83]
Ex/Em 365/445 nm								
FD	Human plasma	GSH, HCys, Cys	MIAC	2.0 pmol	N.R.	2.11 μM	N.R.	[84]
Ex/Em 260/416 nm								
FD (for GSH) Ex/Em 390/478 and ECD (for GSSG)	Human plasma	GSH, GSSG	MBB (only for GSH)	1.4 nm	8.0 nm	N.R.	N.R.	[142]
FD Ex/Em 385/515 nm	Human plasma	GSH, HCys, Cys, CysGly	SBD-F	N.R.	N.R.	N.R.	N.R.	[143]
FD Ex/Em 365/510 nm	Standards, synthetic urine samples	GSH, HCys, Cys,	None (post-column: $Cd(HQS)_2^{2-}$)	0.2 μM	N.R.	N.A.	N.A.	[144]
FD Ex/Em 365/510 nm	Standards	GSH, GSSG, plus HCys, Cys and their disulfides	None (post-column: $Cd(HQS)_2^{2-}$)	0.3 μM	4.3 μM	N.A.	N.A.	[145]
ECD	Standards	GSH, GSSG, HCys, Cys and their disulfides, NAC, other thiols	None	nmol, μmol amount	nmol, μmol amount	N.A.	N.A.	[137]
APAD								
ECD	Standards, rat brain microdialysates.	GSH, Cys	None	2.0 ± 0.06 μM	N.R.	N.A.	N.A.	[146]
ECD	Standards, rat striatum microdialysates	GSH, Cys	None	0.12 μM. 5 μM	N.R.	N.A.	N.A.	[147]
ECD	Human serum, maize tissue	GSH, GSSG, HCys, Cys, CySS, NAC, other thiols	None	19.0 fmol	80.0 fmol	N.R.	N.R.	[148]

Table 1 (*Continued*)

Detection	Matrix type	Analytes	Label	Limit of detection		Concentration (human blood)		Reference
				GSH	GSSG	GSH	GSSG	
ECD	Standards, human promyelocytic leukemia HL-60 cell line	GSH, Cys, NAC, γ-GluCys, CysGly	None	2.0 fmol	N.R.	N.A.	N.A.	[150]
ECD	Standards, pig urinary bladder wall	GSH, GSSG, ASC, UA	None	50 nm	N.R.	N.A.	N.A.	[151]
ESI-MS	Rabbit erythrocytes, plasma	GSH	NEM, ^{2}H labeling	N.R.	N.R.	N.A.	N.A.	[64]
ESI-MS	Human whole blood, erythrocytes	GSH, GSSG	NEM	N.R.	N.R.	1.31 ± 0.12 mM	0.64 ± 0.22 μM	[65]
ESI-MS	Human peripheral blood mononuclear cells	GSH, GSSG	NEM	0.01 μM	0.05 μM	74.4 ± 21.2 nmol/mg protein	0.9 ± 0.06 nmol/mg protein	[66]
ESI-TOF-MS	TK6 cells from a lymphoblastic cell line	GSH, and other metabolites	None	N.R.	N.R.	N.A.	N.A.	[95]
ESI-MS	Rat brain, lung, liver, heart, kidneys, erythrocytes, plasma	GSH, GSSG, plus HCys, Cys and their disulfides	DTNB	0.16 μM	0.16 μM	N.A.	N.A.	[152]
ESI-MS	Monocyte/macrophage RAW 267.4 cell lines	GSH, GSSG	ABD-F ^{13}C, ^{15}N labeling	0.01 μM	N.R.	13.0–16.0 nmol/mg protein	0.12 nmol/mg protein	[153]
ESI-MS/MS	Mouse liver	GSH, GSSG, plus HCys, Cys, and their disulfides, γ-GluCys, CysGly	IAA	N.R.	N.R.	N.A.	N.A.	[61]
ESI-MS/MS	Standards, human plasma	GSH, HCys, Cys, GysGly, other aminothiols	None	1.0 ng/ml	N.R.	2.94 ± 0.31 μM	N.R.	[94]
ESI-MS/MS and ECD + MS	Standards	Thiol functionalities in proteins (free or disulfide-bound)	FEM, FMEA	N.R.	N.R.	N.A.	N.A.	[101]
ESI-MS/MS	Human urine	GSH, GSSG, HCys, Cys, CysGly, NAC and their disulfides	FEM, FMEA	N.R.	N.R.	N.A.	N.A.	[102]
MS/MS	Mouse liver	GSH, GSSG	None	0.2 pmol	2.0 pmol	N.A.	N.A.	[148]
ESI-MS/MS	*S. cerevisiae* strain S288c	GSH, GSSG, HCys, Cys, γ-GluCys, other thiols	None ^{15}N labeling	25.0 ng/ml	25.0 ng/ml	N.A.	N.A.	[154]
ESI-MS/MS	Human plasma, urine	HCys, Cys	None ^{2}H labeling	N.R.	N.R.	N.R.	N.R.	[155]
ESI-MS/MS	Human plasma, urine	HCys, Cys, methionine	None ^{2}H labeling	N.R.	N.R.	N.R.	N.R.	[156]
DAD-CVGAFS	Human whole blood	GSH, HCys, Cys, CysGly (plus GSSG indirectly from total GSH)	PHMB	0.8 nm	N.R.	539.4 ± 70.9 μM	90.4 ± 52.7 μM	[157]

N.A.: not applicable, N.R.: not reported.

Giustarini et al. utilized FDNB for the derivatization of GSH and GSSG; NEM was used to prevent artifactual autoxidation of GSH in GSSG measurements [50]. They reported that whole blood GSH and GSSG values are 1401 ± 113 and 3.12 ± 0.59 μM, respectively. This improved HPLC-UV absorbance method was based on the previous assays of Reed et al. [71] and Asensi et al. [72]. OPA derivatives of GSH are also suitable for UV absorbance detection, as reported by Tsikas et al. [141].

In contrast with UV absorbance detection, several fluorophores have found application for the measurement of GSH with HPLC, including NPM [67], OPA [63,76], MBB [76,142], MIPBO [81], SBD-F [46,143], ThioGlo^{TM}3 [83] and MIAC [84]. For the determination of GSSG in one study, free thiol functionalities were blocked with NEM and GSSG was derivatized with OPA without previous reduction of the disulfide bond [63]. In this paper, the levels of GSH and GSSG were 4.69 and 0.28 μmol/g Hb, respectively, in whole blood, and 1.82 and 0.154 μM in the plasma [63], consistent with the plasma GSH levels 2.11 μM, reported by Benkova et al. [84], and 2.94 μM, measured by Jiang et al. by means of HPLC-ESI-MS/MS [94]. In another study, 2-vinylpyridine was used for thiol masking and, following reduction of GSSG, the newly generated GSH was derivatized with ThioGlo^{TM}3 [83]. The measurement of GSH and GSSG from a single injection was achieved by Sakhi et al. through the use of column-switching HPLC. The MBB derivative of GSH was detected by means of FD, whereas underivatized GSSG was assayed by ECD (voltammetry). The limitations of the method include a frequent exchange of analytical column 1 due to the injection of a rather large volume with low pH, a limited specificity for GSSG and relatively large sample sizes. Details of the technique and the configuration of the column-switching system are provided in [142]. In order to improve the method so as to make it feasible in clinical studies involving premature children or animal experiments with small sample sizes, the amino functionalities of GSSG have instead been derivatized with OPA in a post-column reaction and detected by means of a second fluorimeter [76].

As concerns methods involving indirect FD, Pelletier and Lucy [144] reported an HPLC method with a post-column reaction with the highly fluorescent Cd(II)–HQS system, which permits the separation of the analytes in their native forms. Thiols (GSH, HCys and Cys) in the HPLC effluent compete for the complexation of Cd(II), resulting in a decrease in the fluorescence response. Through modification of the method, determination of thiols and disulfides is also possible. After HPLC separation of the thiols and disulfides in their native forms, a post-column solution of TCEP is added to reduce the disulfides on-line to the corresponding thiol. The effluent is then merged with a second post-column solution of the fluorescent complex Cd(II)–HQS [145].

In addition to the previously mentioned reports of Vignaud et al. [140] and Sakhi et al. [142], GSH, GSSG and Cys were also assayed with ECD in other studies [146–148]. The simultaneous determination of various thiols such as GSH, GSSG, HCys, Cys and NAC was described in three reports. The LOD values were in either the nano- and micromolar range, as in a study in which activated pulsed amperometric detection (APAD) was used [137], or the femtomolar range [149,150]. Simultaneous determination of GSSG and the low-molecular-mass antioxidants GSH, ASC and uric acid (UA) has also been carried out with satisfactory sensitivity (Fig. 2) [151].

The advantages of MS detection combined with HPLC include wide automation possibilities, small sample amounts and excellent sensitivity and specificity, which may be further improved through the use of MS/MS [38]. Most methods coupled RP-HPLC and electrospray ionization (ESI)-MS or ESI-MS/MS, and only two recent studies used UPLC. New and Chan compared the chromatographic performances of three UPLC columns for the assay of GSH, GSSG and ophthalmic acid [136]. Patterson et al. reported a complex approach comprising quantitative UPLC-ESI-TOFMS-

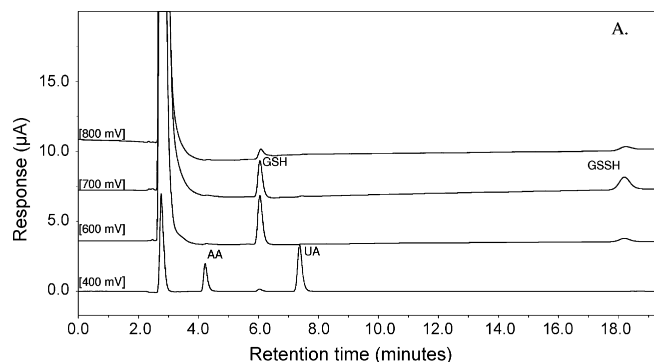

Fig. 2. HPLC chromatograms of oxidized glutathione (GSSG) and antioxidants glutathione (GSH), ascorbic acid (ASC) and uric acid (UA) in a standard mixture at selected cell potentials. The concentrations of GSH, ASC, UA and GSSG were each 10 μM. Reproduced with permission from [151].

based metabolomics and an algorithm for the analysis of the dynamics of gene expression as tools for a better understanding of the cellular response to ionizing radiation [95].

To prevent the autoxidation of the thiol groups, DTNB [152], NEM [64–66], ABD-F [153] or IAA [61] was utilized. It should be noted that DTNB can serve as a thiol-masking agent only when used in a great molar excess [152]. Otherwise, other thiols react with the DTNB-derivatized GSH molecule to form disulfides, reactions utilized in the GR-coupled enzymatic recycling method. In other studies, sequential labeling of free and disulfide-bound thiol functionalities was achieved with the ferrocene-based maleimide reagents FEM and FMEA [101,102]. Some MS and MS/MS methods have made use of stable isotope dilution [64,153–156]. In the three papers that report GSSG levels for whole blood, 0.64 μM or 0.012 μmol/g Hb was reported in one study [65], which was lower than that (0.28 μmol/g Hb) measured in [63]. Although both studies utilized NEM for thiol masking, the reagent was added to the samples at different stages of the preparation, which may cause differences. The level of whole blood GSSG was reported by Bramanti et al. [157] to be much higher (90.4 μM), probably because of the completely different sample preparation and measurement technique, detailed in the next section.

A method based on coupling RP-HPLC on-line with cold vapor generation atomic fluorescence spectrometry (CVGAFS) was recently published by Bramanti et al. [157]. Thiols (GSH, HCys, Cys, CysGly) in the sample were derivatized with *p*-hydroxymercurybenzoate (PHMB). Following HPLC separation, an on-line post-column digestion of organic mercury compounds was effected with bromine (generated *in situ* from KBr/$KBrO_3$ in an HCl medium). The subsequent reduction of Hg^{2+} to Hg^0 was performed with sodium borohydride solution containing hydrazine. The mercury vapor was delivered into the atomizer (a miniature Ar/H_2 diffusion flame) and detected by means of AFS [157].

2.3.2. Gas chromatographic methods

Except for the assay reported in [158], utilizing a flame photometric detector, all GC methods introduced so far for the measurement of GSH, Cys or their *S*-nitroso derivatives have utilized MS for detection [64,92,93,159–162]. GC/C/IRMS was used to assay GSH and Cys in erythrocytes from very-low-birth-weight infants, with ethyl chloroformate as a derivatizing agent. This method permitted the determination of low ^{13}C enrichments in GSH following incubation with ^{13}C-Gly, thereby providing a good tool for study of the turnover of GSH in infants, via determination of the fractional synthesis rate. The authors suggested that this facilitated research into the assessment of *de novo* GSH synthesis [92]. In another study by the same research group, GSH and

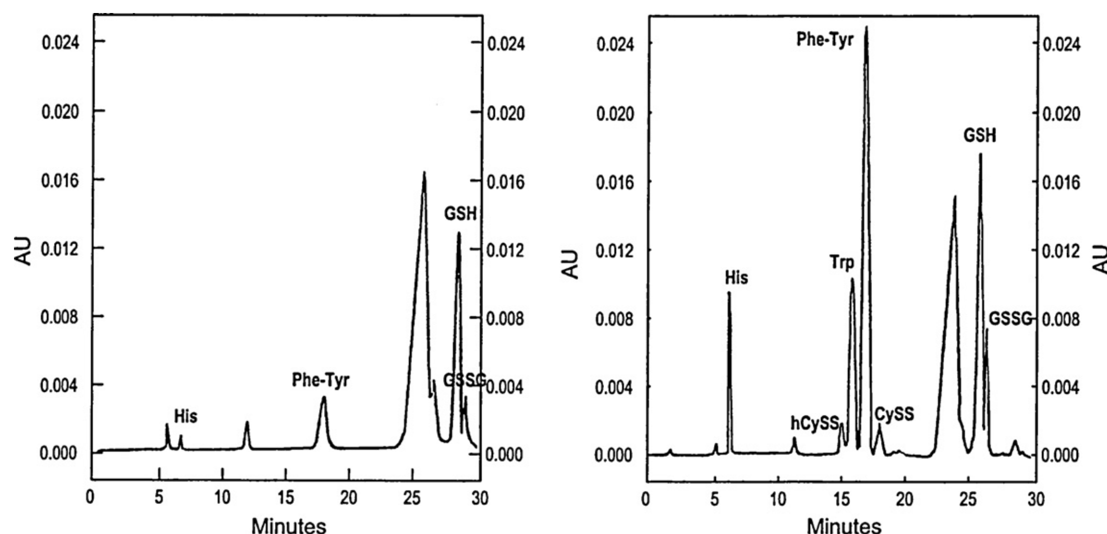

Fig. 3. Capillary electropherograms of underivatized human capillary blood samples (diluted 12-fold) without (left) or spiked (right) with a standard mixture of examined compounds, detected by UV absorbance at 200 nm. Reproduced with permission from [173].

Cys concentrations were determined in erythrocytes from micro-volumes of neonate blood through the use of GC/MS. Additionally, ^{2}H-labeling was used to measure ^{2}H-enrichment in GSH and Cys, which provided a means of kinetic assessment of the AT metabolism in neonates [93].

Cabral et al. reported the introduction of $^{2}H_2O$ for the estimation of GSH synthesis. The use of the less invasive $^{2}H_2O$ as tracer offers advantages over radiolabeled AA infusions: the concentrations of precursors in the biosynthetic pathway are not increased, the cost is lower and there is a possibility to follow the synthesis over long time periods. The authors were able to maintain constant $^{2}H_2O$ enrichment for 2 weeks and to follow the synthesis until a plateau was reached, corresponding to the turnover of the entire GSH pool [64].

2.3.3. Capillary electrophoresis methods

CE is a technique in which the analytes are separated in a narrow (internal diameter: 10–100 μm) capillary containing a background electrolyte. Because of the large resistance of the capillary, a high electric field must be applied. The heat produced is readily dissipated due to the large surface/volume ratio of the capillary.

The advantages of CE include a short duration of analysis, small sample amounts, efficient separation, easy automation and wide applicability. Because of the low amount of analytes arising from the relatively small volumes injected, the LOD is an important issue. Techniques with which the separation efficiency can be enhanced and consequently the sensitivity can be increased include sample stacking [163,164], microchip and microchannel CE [78–80,165], and the novel techniques gradient elution moving boundary electrophoresis (GEMBE) [166,167] and gradient elution isotachophoresis (GEITP) [168]. During sample stacking, analytes present at low concentrations in a long injected sample zone are concentrated into a short zone (stack). The stacked analytes are then separated and individual zones are detected [163,164].

For the separation and assay of GSH and congeners, different CE approaches with different detectors (UV absorbance, FD, laser-induced fluorescence (LIF), ECD and MS) have been used, some reviewed earlier in [169]. All methods employed bare fused-silica capillaries, except for two studies involving the application of polyimide-coated [170] or polybrene-coated capillaries [48]. A summary of the most recent CE methods reviewed in this article is given in Table 2.

2.3.3.1. Conventional capillary electrophoresis. UV absorbance detection is more widely used with CE than with HPLC. Interestingly, in all recent CE-UV absorbance methods [51,52,170–174], thiols (GSH, GSSG, and in [172,173], also HCys and Cys) were assayed in their underivatized forms. These methods used sample stacking or, in one paper [172], a factorial design to optimize the separation parameters, so as to lower the LOD value. However, omitting the derivatization step can increase autoxidation of thiols, which can greatly affect the results.

To prevent the use of acidic reagents, often associated with artifactual oxidation of GSH, one study employed ACN for protein removal [171], whereas Carru et al. used filtration through a Microcon-10 membrane before assaying GSH and GSSG by means of capillary zone electrophoresis (CZE) [51,52]. Their method took only 2 min to complete [51,52]. However, none of these studies utilized thiol-masking agents so as to minimize autoxidation of GSH during sample preparation. Carru et al. reported similar erythrocyte GSH levels, detected with UV absorbance (approximately 7 μmol/g Hb) [51,52] and with LIF (6.78 μmol/g Hb) [175].

Thirty AAs and peptides were determined in not more than 30 min by means of CZE with indirect absorbance detection at 254 nm [172]. The same authors employed a full factorial design to optimize the measurement parameters of a CZE method for the simultaneous direct determination of GSH, GSSG, HCys, Cys and other AAs in human capillary blood (Fig. 3) [173]. Another technique, transient pseudo-isotachophoresis (tp-ITP), has the advantage of permitting the injection of large volumes of samples; in the method for GSH and GSSG developed by Kong et al., the sample length could reach 25% of the efficient length of the capillary [174].

FD offers better sensitivity than that of UV absorbance and the introduction of LIF leads to further improvements. This is especially important with CE in view of the small amounts of samples analyzed. All recent papers have employed exclusively 5-IAF [175–181] or 6-IAF (Fig. 4) [42] as fluorophores for the determination of GSH, HCys, Cys or CysGly. One investigator group added *N*-methyl-D-glucamine (NMDG) to the running buffer in order to obtain a better resolution of the thiol peaks [175,177–181]. By masking the silanol functionalities on the surface of the uncoated capillary wall, NMDG decreased the electroosmotic flow and permitted complete peak resolution [178].

ECD with multiwall CNT microelectrodes [182], coenzyme PQQ-modified electrodes [183] and gold/mercury amalgam micro-disk

Table 2
Summary of recent CE methods for the determination of glutathione (GSH) and related compounds.

Detection	Matrix type	Analytes	Label	Limit of detection		Concentration (human blood)		Reference
				GSH	GSSG	GSH	GSSG	
UV 200 nm	HaCaT cell extracts	GSH, GSSG	NEM	4.0 µM	4.0 µM	N.A.	N.A.	[48]
UV 200 m	Human erythrocytes	GSH, GSSG	None	15.0 µM	7.5 µM	6.77 µmol/g Hb	0.48 µmol/g Hb	[51]
UV 200 nm	Human erythrocytes	GSH, GSSG	None	75 fmol	37 fmol	7.14 µmol/g Hb	N.R.	[52]
UV 214 nm	Rat liver microdialysates	GSH, GSSG	None	0.75 µM	0.25 µM	N.A.	N.A.	[170]
UV 214 nm	Human erythrocytes, dog myocardial tissue and erythrocytes	GSH, GSSG	None	N.R.	N.R.	0.87 mM	0.06 mM	[171]
UV 254 nm	Standards, human plasma, rat peritoneal macrophage cultures	GSH, GSSG, HCys, Cys and other AAs and peptides	None	2.35 µM	1.93 µM	N.R.	N.R.	[172]
UV 200 nm	Standards, human whole blood	GSH, GSSG, HCys, Cys, AAs	None	5.0 µM	0.9 µM	1078.1 ± 17.0 µM	110.6 ± 7.3 µM	[173]
UV 200 nm	Human plasma	GSH, GSSG	None	0.5 µM	0.3 µM	N.R.	N.R.	[174]
Argon ion LIF Ex/Em 488/515 nm	Human plasma, serum	GSH, HCys, CysGly, Cys, NAC	6-IAF	N.R.	N.R.	7.01 ± 0.51 plasma 6.51 ± 0.50 serum	N.R.	[42]
Hg ion LIF Ex/Em 510/550 nm	Unlysed MGC 803 gastric cancer cell line cells	GSH, reactive oxygen species	NDA, DHR-123	N.R.	N.R.	N.A.	N.A.	[78]
LIF Ex/Em 473/520 nm	Acute promyelocytic leukemia-derived NB4 cell lines	GSH, reactive oxygen species	NDA, DHR-123	0.5 µmol	N.R.	N.A.	N.A.	[79]
Argon ion LIF Ex/Em 488/520 nm	Human erythrocytes	GSH, reactive oxygen species	NDA, DHR-123	6.9 amol	N.R.	N.R.	N.R.	[80]
LIF Ex/Em N.R.	Human erythrocytes	GSH, Cys, γ-GluCys, CysGly	5-IAF	N.R.	N.R.	6.78 ± 0.92 µmol/g Hb	N.R.	[175]
Argon ion LIF Ex/Em 488/520 nm	HepG2 hepatoma cells	GSH (free and protein-bound)	5-IAF	N.R.	N.R.	N.A.	N.A.	[176]
LIF Ex/Em N.R.	HUVEC, ECV304, R1 stem cells	GSH, HCys, Cys, γ-GluCys, CysGly	5-IAF	0.1 amol	N.R.	N.A.	N.A.	[176]
Argon ion LIF Ex/Em N.R.	Human plasma	GSH, HCys, Cys, CysGly	5-IAF	N.R.	N.R.	N.R.	N.R.	[178]
LIF Ex/Em N.R.	Human plasma	GSH, HCys, Cys, CysGly	5-IAF	N.R.	N.R.	3.51 ± 0.38 µM	N.R.	[179]
LIF Ex/Em 488/520 nm	HUVEC, ECV304 cell cultures	GSH (free and protein-bound)	5-IAF	N.R.	N.R.	N.A.	N.A.	[180]
LIF Ex/Em N.R.	Human plasma	GSH, HCys, Cys, γ-GluCys, CysGly, NAC	5-IAF	N.R.	N.R.	N.R.	N.R.	[181]
ECD	Standards	GSH, HCys, Cys, NAC	None	2.9 µM	N.R.	N.A.	N.A.	[182]
ECD	Human urine	GSH, HCys, Cys, NAC	None	0.134 µM,	N.R.	N.A.	N.A.	[183]
ECD	Human hepatocarcinoma cells	GSH	None	1.7 µM,	N.R.	N.A.	N.A.	[184]
ECD	Human whole blood, rat brain	GSH, HCys, Cys, ASC, UA	None	2.58 µM	N.R.	N.R.	N.A.	[185]
ESI-MS	Human urine	GSH	None	0.08 mg/ml	N.R.	N.A.	N.A.	[186]
ESI-MS	Cell and plant extracts	GSH, phytochelatins	None	N.R.	N.R.	N.A.	N.A.	[187]
CL	Human erythrocytes	GSH	Diazo-luminol	0.05 amol, 3.6 nm	N.R.	64.9 amol/cell	N.A.	[190]

N.A.: not applicable, N.R.: not reported.

electrodes [184] have also been employed for the assay of GSH and related compounds (HCys, Cys and NAC). Low-molecular-mass antioxidants GSH, ASC and UA were determined simultaneously with CE separation [185].

Only a few methods have coupled MS with CE for thiol analysis [186,187]. A coaxial sheath liquid flow is typically employed to maintain electrical continuity and to bridge the difference in flow rates between the electroosmotic flow of CZE and that required for optimal ion formation in the ESI source [188].

2.3.3.2. Microchip and microchannel capillary electrophoresis. Similarly as for conventional CE, optical, fluorescent, electrochemical and MS detection modes have become routine for microchip CE. Microchip CE has received much attention because of its high degree of integration, portability, minimal solvent/reagent consumption, high performance and speed. ECD offers great promise for microsystems, with features that include noteworthy sensitivity, inherent miniaturization of both the detector and control instrumentation, independence of sample turbidity or optical path length, low cost, minimal power demands and high compatibility [189]. Until very recently, only FD and LIF have been employed in combination with microchip or microchannel CE for the detection of GSH and its congeners [78–80,165]. Most methods have been introduced for the simultaneous determination of the levels of GSH and ROS using the fluorophores NDA and dihydrorhodamine-123 (DHR-123) [78–80]. ITP with indirect FD, performed on an NS-95 borosilicate microchip, was used to focus and separate initially mixed markers and analytes into their respective zones in the sequence of reducing mobility. The fluorescent markers Oregon Green carboxylic acid, fluorescein, and BODIPY segregated nonfluorescent analyte zones and yielded quantitative mobility and concentration information on the analytes [165]. Recently, Zhao et al. combined microchip CE with CL detection for the assessment of GSH in single red blood cells, providing 100-fold lower LOD (0.05 amol or 3.6 nM) as compared with FD [190]. They reported that the average GSH content of single human erythrocytes was 64.9 amol [190].

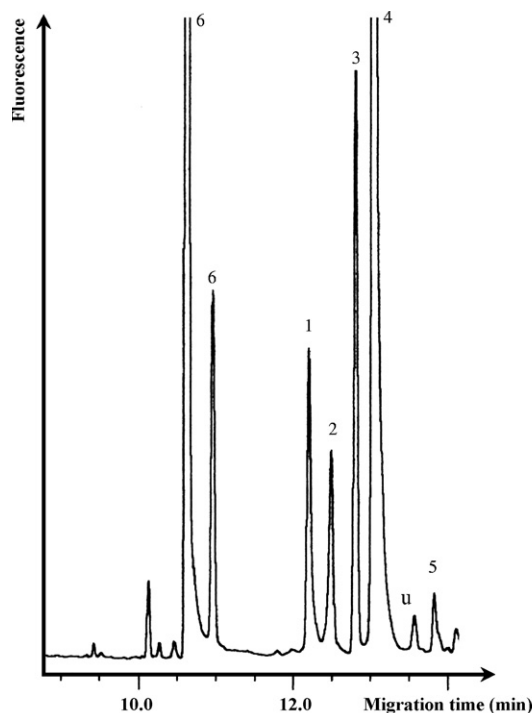

Fig. 4. CE separation of 6-iodoacetamidofluorescein (6-IAF)-labeled thiols in a pathological (hyperhomocysteinemic) human plasma sample, detected by argon-ion laser-induced fluorescence (LIF). Peaks: (1) homocysteine (HCys); (2) cysteinylglycine (CysGly); (3) *N*-acetyl-L-cysteine (NAC); (4) cysteine (Cys); (5) glutathione (GSH); (6) 6-IAF; (u) unknown. Reproduced with permission from [42].

3. Measurement of glutathione as part of an integrative approach

The measurement of GSH and related compounds in various body fluids is an important means of gaining information about redox reactions and signaling pathways. However, such an approach gives information only on the redox status in general, without providing data relating to the extent, duration and compartmentalization of the redox reactions or regarding the compounds participating in these processes, consequently leading to possible misinterpretation of the results. For example, an increased molar ratio GSSG/GSH in whole blood may be caused by either an increased inactivation of ROS, or a decreased rate of GSH synthesis or regeneration in blood. Thus, in addition to the improvement of accuracy of the methods, research is required to establish profoundly different novel approaches, too.

3.1. Multiple analyte measurements

One possible approach is the simultaneous determination of multiple interrelated compounds. Patsoukis and Georgiou reported a methodology consisting of a set of assays for the measurement of free and protein-bound thiols, and also their symmetric and mixed disulfides, and an Excel sheet for easier calculation of the individual components [191]. Multi-analyte sensing in single samples have been reported in three recent papers, based on FD [107,108], or ECD [192]. In the latter study, a single electrode modified with multiple peptides was compared with a multisensor-array containing electrodes modified with one or other of different peptides.

A rapidly advancing field that aims to characterize changes in the concentrations of all small molecules existing in tissues or cells is termed metabolomics. The application of metabolomic technologies for an understanding of physiology, toxicology and disease progression has led to advances through the determination of novel drug and carcinogen metabolites, and also biomarkers of disease. The metabolomic technologies have additionally contributed to a general understanding of how metabolites and their concentrations change under defined conditions [95]. As an example, a TK6 lymphoblastic cell line was exposed to ionizing radiation. To quantitate dose-dependent changes over time in various metabolite concentrations, UPLC-ESI-TOFMS analyses were performed, while metabolite identity was confirmed by means of MS/MS [95].

3.2. Determination of glutathione in vivo or in cyto

The chemical analysis of single cells requires methods for the rapid and quantitative detection of a diverse array of analytes from extremely small volumes (femtoliters to nanoliters) with very high sensitivity and selectivity. Microelectrophoretic separations involving the use of both traditional CE and emerging microfluidic methods are well suited for handling the unique size of single cells and limited numbers of intracellular molecules. Another promising direction in single-cell cytometry is the development of microfluidics for integrated cellular manipulation, chemical processing and the separation of cellular contents [193].

For the *in vivo* assay of thiols, spectrofluorimetry provides the most promising potentialities. Monochlorobimane and 7-amino-4-chloromethylcoumarin have been used for the *in vivo* staining of cellular GSH in human neurons and neuroblastoma cells [194] and in rabbit erythrocytes [195]. These dyes label cellular GSH with satisfactory selectivity, as demonstrated in the comparison with the GSH-recycling assay [194]. Other probes include a dithiolic fluorophore [87] and cell-permeable latent ("turn-on") fluorophores,

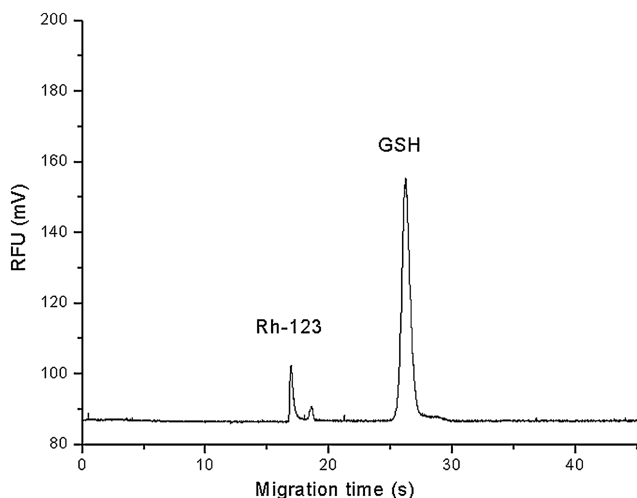

Fig. 5. Typical microchip capillary electropherogram of naphthalene-2,3-dicarboxaldehyde (NDA)-derivatized glutathione (GSH) and rhodamine-123 (Rh-123), derived from reactions with reactive oxygen species (ROS) in NB4 acute promyelocytic leukemia-derived cell line extracts (1.0×10^5 cells/ml), detected by argon-ion laser-induced fluorescence (LIF). The sample was diluted 3-fold prior to injection. Reproduced with permission from [79].

with which the *in vivo* or *in cyto* cleavage of the respective functionally important bonds by thiols results in the liberation of a fluorescent compound and a subsequent increase in the fluorescence [88–91]. However, the latter dyes, except for the probe reported in [90], react with all thiol functionalities in the cell, and are not selective for GSH.

The *in vivo* determination of GSH in the brain or intact cells is also possible by means of NMR methods (see Section 2.2.4). The simultaneous *in vivo* and *in cyto* determination of ROS and the levels of GSH [78,79] furnishes valuable data regarding both the oxidative and antioxidative parts of the redox equilibrium (a detailed description is to be found in the following section).

Monitoring the protein thiol-disulfide state *in vivo* is likewise an important issue. The unique chemical properties and high reactivity of the free thiol functionality make Cys a versatile component of catalytic centers and metal-binding sites in many cytosolic proteins, and its oxidized form, CySS, a stabilizing component in many secreted proteins. Moreover, Cys moieties readily react with ROS and RNS. As a result, these reversible thiol modifications are emerging as regulatory nano-switches in an increasing number of redox-sensitive proteins. These redox-regulated proteins are able to adjust their activity quickly in response to changes in their redox environment. Most assays for the *in vivo* determination of protein thiols and disulfides have been reviewed previously [196]. Since then, near-infrared reflectance spectroscopy (NIRS) as a detector for TLC has also been demonstrated to offer an efficient alternative to LC/MS and CE analysis for peptide reaction monitoring [197].

3.3. Simultaneous assessment of reactive oxygen species and the levels of glutathione

A microchip electrophoresis method coupled with LIF detection has been established for the simultaneous determination of ROS (such as superoxide, hydroxyl radical or hydrogen peroxide) and GSH, which are intracellular signaling molecules related to apoptosis and oxidative stress, in acute promyelocytic leukemia-derived cell lines. As the probe DHR-123 can be converted intracellularly in reactions with ROS to the fluorescent rhodamine-123 (Rh-123), and the probe NDA can react quickly with GSH to produce a fluorescent adduct, rapid determination of Rh-123 and GSH has been achieved on a glass microchip, with LOD values of 1.9 pmol and 0.5 μmol, respectively. The proposed method allows the ready evaluation of the generation of ROS in response to H_2O_2 treatment, which occurs together with the consumption of the GSH (Fig. 5) [79]. The same technique has been applied for the assay of Rh-123 and GSH in single erythrocytes. On-chip electrical lysis, characterized by extremely fast disruption of the cellular membrane, was exploited to minimize enzymatic effects on analyte concentrations during the determination. The average cell throughput was 25 cells per hour. The LOD values for Rh-123 and GSH were 0.5 and 6.9 amol, respectively [80]. More recently, the simultaneous analysis of ROS and the levels of GSH in individual intact cells from gastric cancer cell lines has been achieved. The method was based on fluorescence images of cells in a microchannel with an average analysis rate of 20 cells/min. Moreover, when fluorescent derivatized GSH and the ROS generation were assessed separately, an average detection rate of 80–120 cells/min was achieved [78].

4. Conclusions

The GSH–GSSG system is still being investigated extensively, despite the availability of a broad range of knowledge related to redox homeostasis. The number of published papers has recently increased appreciably, and shortly after the development of novel techniques, they have found application in methods for the measurement of GSH and congeners too. To date, there is no consensus or standardization of the assays. Thus, standard values of GSH, GSSG and related compounds in biological samples are still lacking. Several reports on GSSG concentrations may be affected by artifacts, especially as consequences of improper sample handling and acid deproteinization during sample preparation. These artifacts can be avoided by the application of procedures described in validated methods, such as those by Rossi et al. [39,50]. To overcome the difficulties associated with NEM elimination in this assay and in GR-coupled enzymatic recycling assays, the applicability of novel thiol-masking agents such as M2VP or M4VP should be evaluated. These issues point to a need for an integrative approach; the simultaneous assay of diverse elements of a given process, the investigation of biological molecules in their natural milieu, or at least the exclusion of artifacts related to sample preparation and measurement. This approach should also be extended to the assessment of other thiol-containing biomolecules.

Acknowledgments

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