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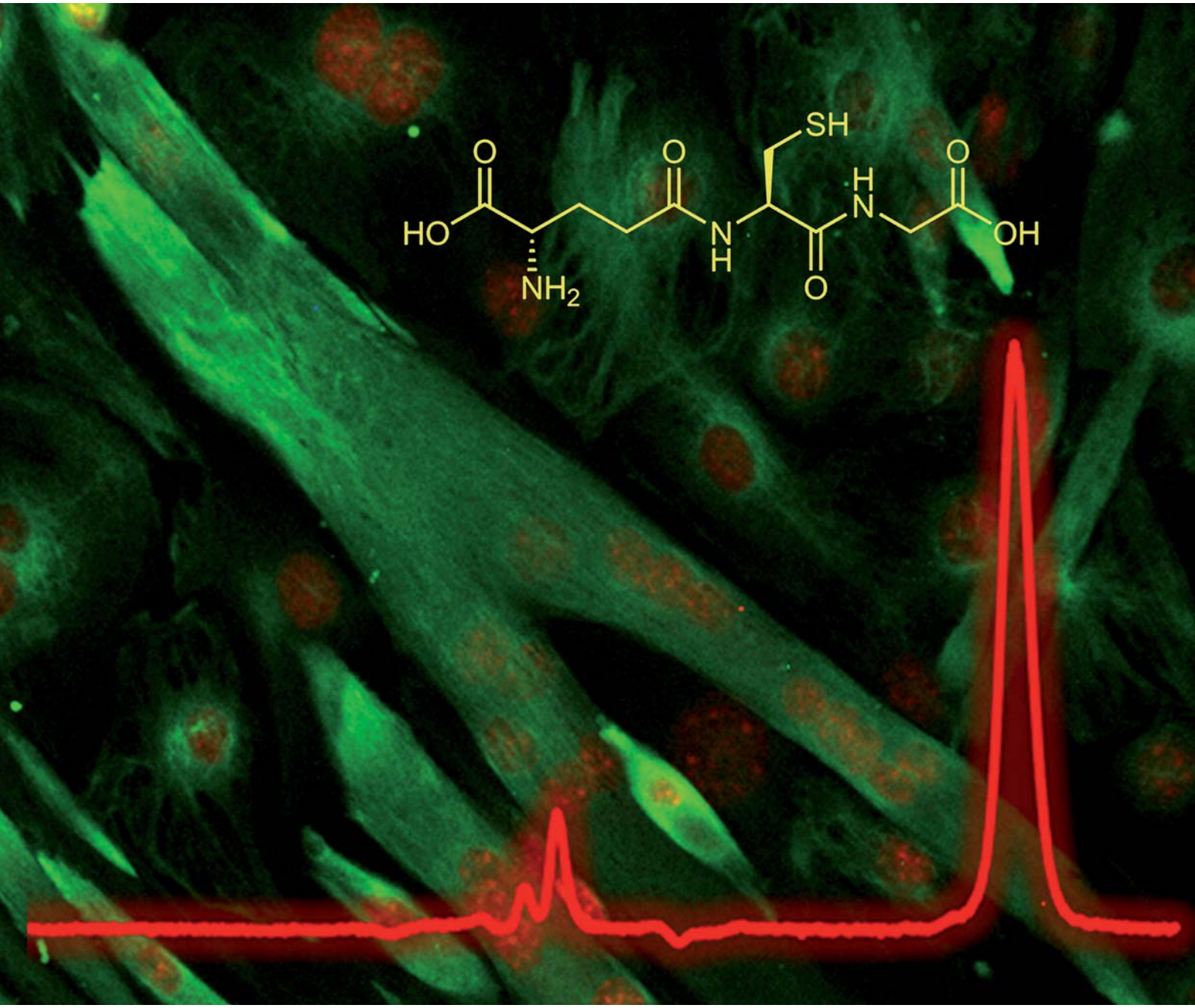

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PAPER

Determination of intracellular glutathione and glutathione disulfide using high performance liquid chromatography with acidic potassium permanganate chemiluminescence detection†

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Measurement of glutathione (GSH) and glutathione disulfide (GSSG) is a crucial tool to assess cellular redox state. Herein we report a direct approach to determine intracellular GSH based on a rapid chromatographic separation coupled with acidic potassium permanganate chemiluminescence detection, which was extended to GSSG by incorporating thiol blocking and disulfide bond reduction. Importantly, this simple procedure avoids derivatisation of GSH (thus minimising auto-oxidation) and overcomes problems encountered when deriving the concentration of GSSG from 'total GSH'. The linear range and limit of detection for both analytes were 7.5×10^{-7} to 1×10^{-5} M, and 5×10^{-7} M, respectively. GSH and GSSG were determined in cultured muscle cells treated for 24 h with glucose oxidase (0, 15, 30, 100, 250 and 500 mU mL^{-1}), which exposed them to a continuous source of reactive oxygen species (ROS). Both analyte concentrations were greater in myotubes treated with 100 or 250 mU mL^{-1} glucose oxidase (compared to untreated controls), but were significantly lower in myotubes treated with 500 mU mL^{-1} ($p < 0.05$), which was rationalised by considering measurements of H_2O_2 and cell viability. However, the GSH/GSSG ratio in myotubes treated with 100, 250 and 500 mU mL^{-1} glucose oxidase exhibited a dose-dependent decrease that reflected the increase in intracellular ROS.

Introduction

Beginning with the early observations of Sir Frederick Gowland Hopkins in the 1920s,[1–3] glutathione (GSH; L-γ-glutamyl-L-cycteinyl-glycine; Fig. S1a, ESI†) has been extensively studied by researchers from a diverse range of disciplines.[4–10] Investigations have primarily focused on the many biochemical properties of GSH as it is the major low molecular weight antioxidant species present in eukaryotic cells, and a regulator of protein and cell functionality.[4,5,8–11] In its capacity as an antioxidant, GSH has the potential to reduce free radicals and reactive oxygen species (ROS), which results in the formation of the corresponding disulfide (GSSG, Fig. S1b, ESI†).[4,5,8–11] To complete this intracellular redox cycle, GSH is regenerated by the enzyme glutathione reductase in a NADPH dependent reaction.[4,5,8–11] The molar ratio of GSH/GSSG in a cell is typically between 10 : 1 and 1000 : 1; however, under oxidative stress, this ratio decreases.[5,8,10,12,13] Since oxidative stress has been implicated in a growing number of pathological and physiological conditions,[5,14–16] assessing redox state is an invaluable tool for many researchers.[4,5,8–10,12,13,17–22]

The importance of GSH and GSSG measurement for the assessment of redox state is reflected by the plethora of analytical methodologies, including liquid chromatography, gas chromatography or capillary electrophoresis separation with fluorescence, electrochemical, mass spectrometry or UV absorbance detection, which are the subject of numerous reviews.[8,12,13,17–22] As identified in these reviews and other papers, there are several major analytical challenges associated with the measurement of GSH and GSSG.[8,12,13,18–38] Firstly, neither contain a strong chromophore or fluorophore. This problem is most often addressed by derivatisation of GSH, but the reactions are time-consuming (up to 60 min) and can add a significant source of error.[8,12,13,18,20–24,26,27,31,34,38] GSSG is then measured by reducing its disulfide bond and subjecting the liberated GSH to the same derivatisation procedure.[8,12,13,20–22] Secondly, GSH is easily oxidised under the alkaline conditions employed for derivatisation and some other sample pre-treatment steps,[8,12,13,19,21,25,28–30,32–37] which can result in artificially high GSSG concentrations.

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† Electronic supplementary information (ESI) available: Optimisation of acidic potassium permanganate chemiluminescence detection, GSSG reduction, and GSH alkylation reaction conditions. GSH and GSSG calibration plots and analytical figures of merit, instrumental setup, summary of GSH and GSSG detection and additional materials and methods. See DOI: 10.1039/c1an0004g

Finally, GSH and GSSG can occur at very low levels (below the limits of detection of some techniques), and GSSG is often present several orders of magnitude lower in concentration than GSH.

Methodologies that employ direct detection and minimal sample handling are therefore more desirable. Electron spin resonance, nuclear magnetic resonance, electrochemical, UV absorbance, fluorescence quenching or mass spectroscopy have been utilised to determine GSH (and in some cases GSSG) without derivatisation, as summarised in the following selected references.[8,12,13,17,20,22,39–41] However, these approaches suffer from limitations in terms of performance, equipment costs, complexity, sensitivity and/or analysis time. An often overlooked alternative for the direct measurement of GSH is chemiluminescence,[42–49] which offers highly sensitive detection using relatively simple instrumentation.[50–54] Beginning in 1984, Hinze *et al.*[45] reported the detection of GSH and several other biologically significant reductants based on the chemiluminescence reaction with lucigenin, but they primarily focused on the effect of micellar systems upon the emission. Several subsequent publications have described the determination of GSH using batch or flow-injection analysis with chemiluminescence detection based on (i) enhancement of the emission from the oxidation of luminol,[42,43,48] (ii) quinine sensitised emission from the oxidation of the analyte,[46,47] or (iii) reaction with permanganate and tris(1,10-phenanthroline)ruthenium(II).[44] Preliminary applications to several blood samples showed a reasonable agreement with a spectrophotometric assay,[43,48] but without chromatographic or electrophoretic separation, these methods lack the selectivity required to accurately measure GSH in complex biological matrices. One attempt to address this issue was recently described by Zhao *et al.*,[49] who determined GSH and other intracellular thiols by microchip electrophoresis with luminol chemiluminescence. The method was applied to single red blood cells from healthy subjects and cancer patients, but the sensitivity was not sufficient to quantify GSH in some samples. Furthermore, none of the chemiluminescence-based procedures reported to date have included strategies for GSSG detection, which is essential to establish cellular redox state.

Herein we describe the determination of intracellular GSH and GSSG based on a rapid chromatographic separation coupled with post-column acidic potassium permanganate chemiluminescence detection. For direct GSH measurement, samples were simply diluted into an acidic solution prior to injection onto the column. In a separate analysis step, GSSG was quantified by masking endogenous thiols, disulfide bond reduction, and detection of the newly formed GSH. Furthermore, this approach was utilised to assess the redox state of cultured muscle cells (C2C12 myotubes) treated with various concentrations of glucose oxidase, which causes oxidative stress through the continuous production of hydrogen peroxide.

Materials and methods

Flow injection analysis

A conventional FIA manifold with a chemiluminescence detector was constructed in our laboratory. A peristaltic pump (Gilson Minipuls 3, John Morris Scientific, Balwyn, Victoria, Australia) with bridged PVC tubing (DKSH, Caboolture, Queensland, Australia) was used to propel solutions through 0.8 mm i.d. PTFE tubing (DKSH). Standards (1×10^{-5} M) were injected (70 μL) with an automated six-port valve (Valco Instruments, Houston, Texas, USA) into a carrier stream (100% methanol unless otherwise stated), which merged with a solution of acidic potassium permanganate at a T-piece, and the light emitted from the reacting mixture was detected with a custom built flow-through luminometer, as described in the following sub-section. The output from the photomultiplier was documented with a chart recorder (YEW type3066, Yokogawa Hokushin Electric, Tokyo, Japan).

High performance liquid chromatography

Chromatographic analysis was carried out on an Agilent Technologies 1200 series liquid chromatography system, equipped with a quaternary pump, a solvent degasser system and an autosampler (Agilent Technologies, Forest Hill, Victoria, Australia). Hewlett-Packard Chemstation software (Agilent Technologies) was used to control the HPLC pump and acquire data from the chemiluminescence detector. Before use in the HPLC system, all sample solutions and solvents were filtered through a 0.45 μm nylon membrane.

Post-column acidic potassium permanganate chemiluminescence was generated using the manifold outlined in Fig. S2, ESI†. The reagent, propelled at a flow rate of 2.5 mL min^{-1} using a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Balwyn, Victoria, Australia) with bridged PVC tubing (DKSH), merged with the HPLC eluant at a T-piece and the light emitted from the reacting mixture was detected with a custom built flow-through luminometer, which consisted of a coiled flow cell comprising of 0.8 mm i.d. PTFE tubing (DKSH), mounted flush against the window of Electron Tubes photomultiplier tube (model 9828SB, ETP) set at a constant voltage of 900 V from a stable power supply (PM20D, ETP) *via* a voltage divider (C611, ETP). The flow cell, photomultiplier tube and voltage divider were encased in a padded light-tight housing, and a Hewlett-Packard analogue to digital interface box (Agilent Technologies) was used to convert the signal from the chemiluminescence detector.

Cell culture and glucose oxidase treatment

Mouse C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM, 5.5 mM glucose) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. To stimulate myotube formation, myoblasts were grown to confluence and then transferred to DMEM supplemented with 2% horse serum (HS). For determination of GSH and GSSG content, cells were grown in 6-well tissue culture plates, and for the oxidative stress and viability assays cells were grown in 96-black well, clear bottom tissue culture plates. To induce oxidative stress, C2C12 myotubes at 4 d post-differentiation were treated with 0, 30, 100, 250 or 500 mU mL^{-1} glucose oxidase in 50 mM sodium acetate buffer (pH 5.1) for 24 h. Glucose oxidase increases ROS levels in the cell culture medium by catalysing the oxidation of glucose to produce D-glucono-δ-lactone and H_2O_2.[55,56] This exposed the myotubes to a continuous, more

biologically relevant source of ROS (that can cross the cell membrane to alter intracellular redox state) than a single concentrated, rapidly metabolised dose.[55–57]

Sample collection and analysis

For determination of GSH and GSSG contents, C2C12 myotubes were harvested by trypsinisation and resuspended in 200 μL of deionised water. A 35 μL aliquot was solubilised in 10% sodium dodecyl sulfate (SDS) to a final concentration of 0.2% SDS (v/v) for protein determination using the bicinchoninic acid (BCA) method (#23225, Pierce) according to manufacturer's instructions. The rest of the sample was treated with formic acid to a final concentration of 0.5%, and stored immediately at −80 °C. Prior to analysis, C2C12 myotubes were thawed on ice and then centrifuged at 12 000 rpm for 5 min at 5 °C using an Avanti 30 Centrifuge (Beckman Coulter, Gladesville, NSW, Australia) to remove precipitated proteins and cellular debris.

For GSH determination, 50 μL of the supernatant was diluted into 450 μL of 5% aqueous formic acid, filtered through a 0.45 μm nylon membrane and analysed. For GSSG determination, the supernatant (100 μL) was combined with Tris–HCl buffer (0.675 M; 20 μL; pH 8.0) and NEM (6.3×10^{-3} M; 20 μL) and left for one minute. Subsequently, 2-mercaptoethanol (8×10^{-3} M; 20 μL) was added and the sample left for another minute. Then TCEP (7.8×10^{-4} M; 20 μL) was added and the solution was left for 60 min at 50 °C to allow complete disulfide reduction. Finally, 5% aqueous formic acid (20 μL) was added, and the sample filtered through a 0.45 μm nylon membrane and analysed. The overall dilutions of the sample aliquots for GSH and GSSG determinations were therefore 10-fold and 2-fold, respectively. GSH and GSSG data are presented normalised to protein content.

The procedures used to assess mitochondrial function (MTT assay), membrane integrity (ethidium uptake assay) and oxidative stress (H_2O_2 assay), and the sources of chemicals used in this study have been included in the ESI†.

Statistical analysis

All data are presented as mean ± SEM. One-way ANOVA with Tukey *post hoc* analysis, when applicable, was used to assess the effect of glucose oxidase treatment on cellular redox state, markers of oxidative stress and cell viability.

Results and discussion

Preliminary chemiluminescence investigations

Li and co-workers reported a weak emission of light from the oxidation of GSH with acidic potassium permanganate, which was significantly enhanced by the addition of quinine.[46] We have previously shown that this chemiluminescence reaction involves two interdependent light-producing pathways;[58,59] the analyte is oxidised to a intermediate capable of transferring energy to the efficient fluorophore ($\lambda_{max} = 458$ nm), and the permanganate is reduced to an electronically excited manganese(II) species ($\lambda_{max} = 735$ nm).[58,59] Considering that the manganese(II) emission from other reactions with acidic potassium permanganate have been significantly enhanced by the addition of sodium polyphosphates to the reagent solution,[50] we sought to use this enhancer to promote the manganese(II) pathway. Removing the need for the quinine would eliminate the background emission from reaction between the sensitiser and oxidant,[46,60] and enable post-column chemiluminescence detection of GSH using a single reagent solution.

Preliminary experiments were conducted using FIA as it provided similar conditions to HPLC, but without the relatively time-consuming separation. The reaction of GSH (1×10^{-5} M) with potassium permanganate (1×10^{-3} M in 1% (w/v) sodium polyphosphate solution, adjusted to pH 2 with sulfuric acid) produced a large chemiluminescence response, but no signal was recorded for the disulfide, GSSG (1×10^{-5} M). To find the parameters that would afford the greatest chemiluminescence response for GSH, a series of univariate searchers were performed (Fig. S3, ESI†). These experiments showed that a 2.5×10^{-4} M potassium permanganate solution containing 1% (w/v) sodium polyphosphates adjusted to pH 3 with sulfuric acid, delivered at a flow rate of 2.5 mL min^{-1} per line, was optimal and therefore utilised in all further experiments.

High performance liquid chromatography

Reverse-phase HPLC has been incorporated into many methods for the determination of GSH, but pre-column derivatisation is often utilised and as a consequence there are very few reports of separations for non-derivatised GSH.[12,13,20–22] In a recent review of chromatographic and mass spectrometric analysis of biological samples for GSH, Iwasaki *et al.*[20] suggested that either highly polar HPLC stationary phases (such as amino, diol) or hydrophobic interaction chromatography is required. However, Zhang *et al.*[38] reported adequate GSH retention using a more conventional non-polar C18 column. Therefore, to resolve GSH from other sample components, an optimisation of HPLC conditions (injection volume, mobile phase composition and pH, temperature and flow rate) using a particle-packed column with either an amino or C18 stationary phase was performed. Conditions found to provide sufficient GSH retention in combination with maximum acidic potassium permanganate chemiluminescence signal are shown in Table 1. These chromatographic parameters allowed for the complete separation of GSH from sample components in less than 6 minutes (Fig. 1).

Sample collection and preparation

Glutathione auto-oxidation in alkaline solutions and enzymatic conversion under neutral conditions are major sources of error in GSH measurement.[8,12,13,19,21,25,28–30,32–37] Therefore, it has been suggested by numerous authors[12,13,19,20,61] that the pH of most matrices to be analysed be kept in an acidic range.‡ Furthermore, acidification is one approach to denature and precipitate proteins; an important requirement for analysis.[8,12,13,17,19,20,22] Although many methods involve acidification of the sample during collection, neutral or alkaline conditions are often

‡ Rossi *et al.* caution against acidification of blood samples as they found it can lead to significant GSH oxidation by unidentified reaction(s) with oxyhemoglobin. Alternatively they suggest blocking the free thiol moiety of GSH using an alkylating agent such as NEM prior to acidification of blood (see ref. 36).

Table 1 Optimised separation conditions for the determination of GSH

Injection volume	20 μL
Mobile phase	97% aqueous formic acid (pH 2.8), 3% methanol
Flow rate	1 mL min^{-1}
Column	Alltech Alltima C18 (250 mm × 4.6 mm i.d., 5 μm)

employed for derivatisation reactions or to improve ionisation when mass spectroscopy is used.[8,12,13,20,22,26] However, since a low pH improved both the chromatographic retention of GSH and the intensity of the chemiluminescence emission with permanganate, the standards and samples (C2C12 myoblasts) were prepared/collected, stored and analysed in an acidic environment (formic acid, pH 2.8). Additionally, using a commercially available BCA protein assay kit, the amount of protein precipitated when using formic acid was found to be similar to the levels removed when acidifying with the more traditional methanesulfonic acid.

Disulfide bond reduction

As previously noted, no measurable quantity of light was recorded from the reaction between GSSG and acidic potassium permanganate, preventing direct detection of this species. The same issue is encountered in methodologies that utilise pre-column derivatisation, as appropriate chromophores or fluorophores are introduced by reaction with the free thiol moiety of GSH, which is absent in GSSG.[8,12,13,18–22] This has been overcome by GSSG disulfide bond reduction, which produces two GSH molecules.[8,12,13,19–22] In this approach, an initial analysis is performed to determine GSH (Fig. 2a). The sample is then subjected to a disulfide bond reduction step before analysis

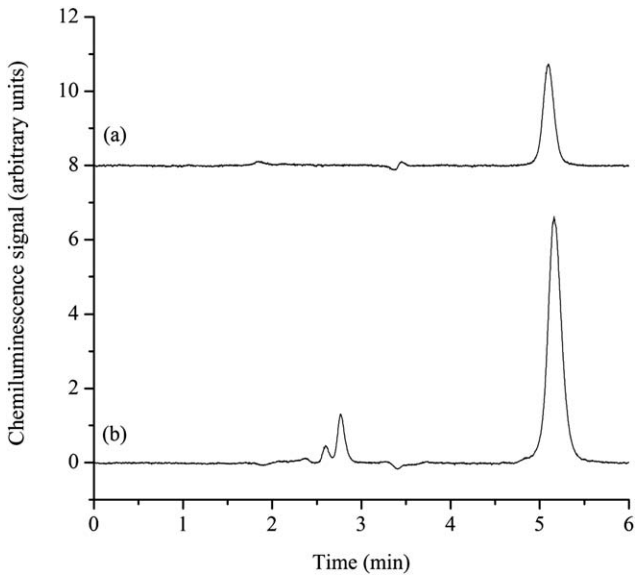

Fig. 1 Typical acidic potassium permanganate chemiluminescence traces from the analysis of: (a) GSH standard (1×10^{-5} M) and (b) mouse C2C12 myotubes (sample diluted 1 : 5 into 5% aqueous formic acid). Chromatographic conditions described in Table 1.

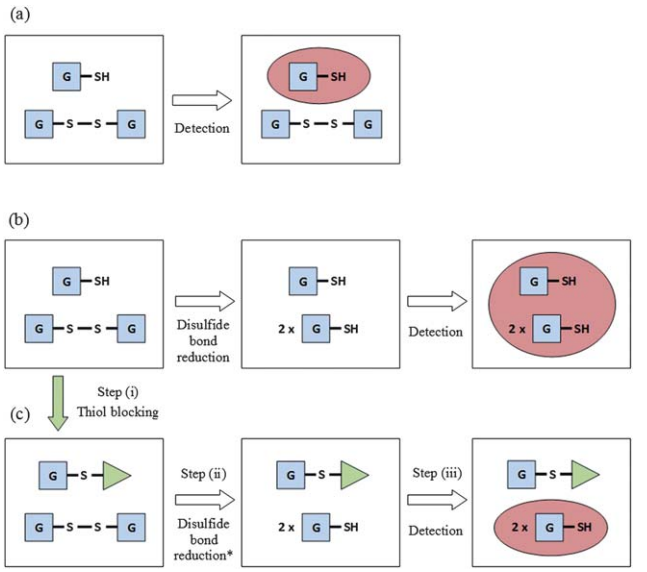

Fig. 2 Strategies employed for determination of (a) GSH, (b) 'total GSH' (GSSG + GSH), (c) GSSG (indirect detection). *This step includes initial removal of excess NEM.

(Fig. 2b), where the signal is now a combination of endogenous and liberated GSH (often referred to as 'total GSH'). The GSSG concentration is therefore the difference between the two aforementioned measurements. Disulfide bond reduction employing tris(2-carboxyethyl)phosphine (TCEP)§ was explored for the indirect determination of GSSG using the methodology outlined in this paper, where the influence of time, temperature and pH on the reaction was considered. It has been reported that a high excess of reagent results in decreased reaction times.[62,63] However, as our preliminary experiments showed that TCEP also elicited chemiluminescence with permanganate, we were limited to a final concentration no higher than 8.75×10^{-5} M to avoid interference in the detection of GSH. Initially, GSSG (1×10^{-5} M in aqueous formic acid, pH 2.8) was combined (1 : 1) with TCEP (1.75×10^{-4} M in deionised water) and the resulting solution was repeatedly injected into the HPLC over time to monitor disulfide bond reduction through the corresponding formation of GSH. Although this reagent reportedly reduces disulfide bonds at low pH,[64] only ~50% of GSSG was reduced in 60 min. Considering the rate-limiting step in this reaction is the attack of the disulfide bond by the phosphine nucleophile (pK_a 7.66[65]), deprotonation of TCEP was postulated to greatly enhance GSSG reduction. Therefore, GSSG (1×10^{-5} M in aqueous formic acid, pH 2.8) was combined with TCEP (3.5×10^{-4} M in deionised water) and a Tris–HCl buffer (0.3 M; pH 8.0) in a 2 : 1 : 1 ratio. Aliquots of the resulting solution were taken,

§ Traditionally, GSSG reduction has been achieved using dithiothreitol (DTT) or 2-mercaptoethanol. However as both of these reagents themselves possess a free thiol moiety, cross-reactivity with derivatising agents is reportedly an issue. This has led researchers to explore alternative reducing agents such as TCEP. Although eliminating the reaction between reducing and derivatising reagents was of little consequence for this study, TCEP was selected for use as unlike DTT and 2-mercaptoethanol it is highly water soluble, odorless, non-volatile, non-flammable, non-corrosive and is less sensitive to air and humidity.

re-acidified, and injected into the HPLC over time. In this case, approximately 80% of GSSG was reduced over 60 min. Finally, increasing the reaction temperature to 50 °C resulted in complete disulfide reduction within 50 min (Fig. S4, ESI†).

Reaction (blocking) of thiol with *N*-ethylmaleimide

Although conditions were found that afforded complete disulfide bond reduction, the ratio of GSH to GSSG under certain physiological conditions may be as high as 1000 : 1, making it analytically challenging to accurately measure small GSSG values by subtracting one large GSH concentration from another. Consequently, we examined an alternative approach to the determination of GSSG, based on the methodology of Østergaard and co-workers.[66] The sample was again split into two, with one part analysed immediately for GSH (Fig. 2a). To the remaining fraction, the thiol alkylating reagent *N*-ethylmaleimide (NEM) was added to form a thioether derivative of GSH that does not elicit any measurable chemiluminescence with permanganate. GSSG is then reduced by the addition of TCEP and the liberated GSH is detected (Fig. 2c). Not only does this approach negate the need to examine the difference between two similar large values, but the addition of NEM also aids in preventing unintended oxidation of GSH. The reaction was initially examined by combining equal volumes of GSH (1×10^{-5} M in aqueous formic acid, pH 2.8) and NEM (1.8×10^{-3} M in deionised water), and injecting the mixture into the HPLC over time to monitor the decrease in GSH. As can be seen in Fig. S5, ESI†, only ~80% of the GSH was blocked by NEM over 25 min. Considering the reaction rate is dependent on thiol deprotonation,[19] the GSH alkylation was re-examined by combining GSH (1×10^{-5} M in aqueous formic acid, pH 2.8) with NEM (3.6×10^{-3} M in deionised water) and a Tris–HCl buffer (0.1 M; pH 8.0) in a 2 : 1 : 1 ratio. Aliquots of the resulting solution were re-acidified and injected into the HPLC over time. Although increasing solution pH can promote auto-oxidation and unwanted side reactions,[8,12,13,19,21,25,28–30,32–37] when employing a Tris–HCl buffer, alkylation of the GSH thiol moiety was practically instantaneous (Fig. S5, ESI†).

Removal of excess *N*-ethylmaleimide

Since NEM is added in excess compared to the level of free thiols, it must be removed prior to disulfide bond reduction, to prevent reaction with the liberated GSH. To achieve this, Østergaard *et al.*[66] added 2-mercaptoethanol because this thiol readily reacts with NEM in an analogous manner to GSH. We evaluated this approach using a mixture of GSH (1×10^{-5} M) and GSSG (1×10^{-5} M) in aqueous formic acid (pH 2.8), which was subjected to the protocols outlined in Fig. 2a and c. Firstly, an aliquot of the mixture was simply filtered and analysed for GSH (Fig. 3a). Secondly, to demonstrate the GSH thiol blocking with NEM (Fig. 2c, step (i) only), the mixture (100 μL) was combined with Tris–HCl buffer (0.675 M; 20 μL; pH 8.0) and NEM (6.3×10^{-3} M; 20 μL), left for 20 s, filtered and analysed (Fig. 3b). The disappearance of the peak at 5.1 min in this chromatogram indicated complete reaction of GSH with NEM. Thirdly, to quantify GSSG, the GSH thiol blocking was followed by removal of excess NEM and disulfide bond reduction prior to analysis (Fig. 2c, steps (i), (ii) and (iii)). In this procedure, 2-mercaptoethanol (8×10^{-3} M; 20 μL) was added to the mixture containing Tris–HCl buffer and NEM and left for 20 s. Next, TCEP (7.8×10^{-4} M; 20 μL) was added and the mixture was left for 60 min at 50 °C to allow complete disulfide reduction (Fig. 2c, step (ii)). Aqueous formic acid (5%, 20 μL) was then introduced to re-acidify the sample before filtration and analysis. The absence of a peak corresponding to GSH in Fig. 3b and its appearance in Fig. 3c (equivalent to 99.7% of the predicted GSH liberated from GSSG) confirm both the successful removal of excess NEM and the complete reduction of GSSG. It should be noted that although the GSSG disulfide bond reduction produces two GSH molecules, the addition of the reagents also diluted the sample 2-fold and therefore the peak in Fig. 3c is similar to the peak in Fig. 3a.

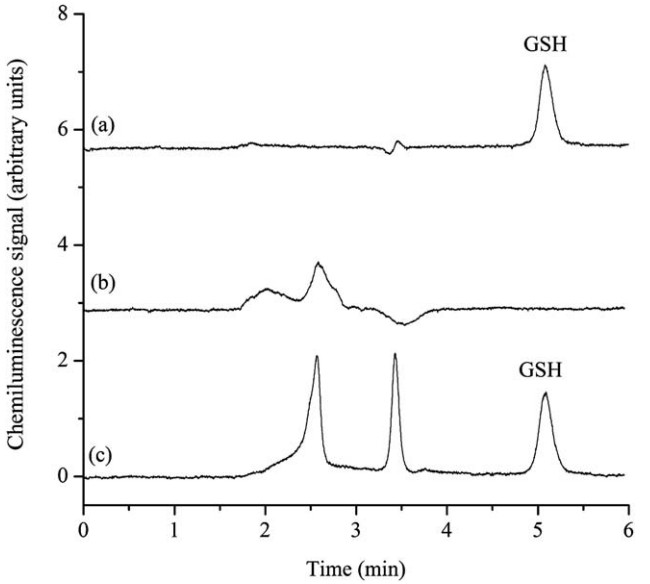

Fig. 3 Typical acidic potassium permanganate chemiluminescence traces from the analysis of a mixture of GSH (1×10^{-5} M) and GSSG (1×10^{-5} M) in aqueous formic acid (pH 2.8). (a) Detection of GSH. (b) Signal after the addition of NEM and Tris–HCl buffer to the sample. (c) Detection of GSSG after addition of 2-mercaptoethanol and TCEP to the sample already containing NEM and Tris–HCl buffer. Experimental parameters are described in the text above and chromatographic conditions outlined in Table 1.

Analytical figures of merit

The procedure was evaluated in terms of linearity, sensitivity and precision (Table S1, ESI†). A calibration curve for GSH prepared using 30 standard solutions over the range of 1×10^{-7} M to 1×10^{-4} M showed an approximate linear relationship from 7.5×10^{-7} M to 1×10^{-4} M (correlation co-efficient, $R^2 = 0.997$). However, within the range of 7.5×10^{-7} M to 1×10^{-5} M the calibration was highly linear with $R^2 = 0.9999$ (Fig. S6, ESI†). The limit of detection, defined as the lowest signal detected with a signal-to-noise ratio of 3, was determined to be 5×10^{-7} M GSH. The precision of repeated injections ($n = 6$) of GSH at low (1×10^{-6} M), medium (2.5×10^{-6} M) and

high (5×10^{-5} M) concentrations was good (R.S.D. of less than 1.5%). Similar figures of merit (Table S1, ESI†) were obtained for the determination of GSSG following the procedure outlined in Fig. S7, ESI†.

Determination of GSH and GSSG in a biological system

The procedure (summarised in Fig. S7, ESI†) was employed to determine GSH and GSSG in C2C12 myotubes that had been treated for 24 h with various quantities of glucose oxidase. This chronically exposed the myotubes to a continuous, biologically relevant source of ROS.[55–57] Hydrogen peroxide produced by the glucose oxidase in the cell culture medium can diffuse into myotubes through the cell membrane, where in order to maintain redox state, it is quenched by various intracellular antioxidants.[55–57] However, if there is an imbalance between H_2O_2 influx and antioxidant capacity, then intracellular ROS levels (including H_2O_2) will increase, cell viability will decrease, and the GSH/GSSG ratio will decrease.

Intracellular H_2O_2 levels were assessed using the commercially available Amplex Red assay, which showed that there was no significant increase in H_2O_2 when the cells were treated with 15 or 30 mU mL^{-1} of glucose oxidase compared to untreated controls (Fig. 4a). However, when myotubes were treated with 100, 250 and 500 mU mL^{-1} glucose oxidase, cellular antioxidant defenses were overwhelmed and a dose-dependent increase in H_2O_2 was observed ($p < 0.05$). C2C12 myotube viability was assessed by measuring mitochondrial function (assessed using the MTT assay, Fig. 4b) and cell membrane integrity (based on ethidium uptake, Fig. 4c). Both mitochondrial function and cell membrane integrity were compromised in cells treated with 100, 250 and 500 mU mL^{-1} glucose oxidase ($p < 0.05$), signifying dead or dying cells undergoing apoptosis or necrosis. Membrane integrity was also reduced in myotubes treated with lower quantities of glucose oxidase (15 and 30 mU mL^{-1}; $p < 0.05$). This attribute is sensitive to oxidative stress because it is exposed to increased H_2O_2, both intracellular and extracellular (where there are essentially no antioxidant molecules). Having established that glucose oxidase treatment causes enough oxidative stress to disrupt myotube homeostasis, the intracellular redox environment was assessed by quantifying GSH, GSSG and their respective ratio using the method described herein.

The concentrations of GSH and GSSG in the supernatant of the cell lysates (corrected for protein content) are shown in Table 2. In myotubes treated with 15 mU mL^{-1} or 30 mU mL^{-1} glucose oxidase, for which there was no significant increase in H_2O_2 or loss of mitochondrial function, the concentrations of GSH and GSSG were similar to the untreated cells. In myotubes treated with 100 mU mL^{-1} or 250 mU mL^{-1} glucose oxidase, much greater concentrations of GSH and GSSG were found ($p < 0.05$), indicating an adaptive response to the oxidative stress stimulus. However, when treated with 500 mU mL^{-1} glucose oxidase, the myotubes had lower GSH and GSSG levels than all other treatments (Table 2; $p < 0.05$), presumably mediated by greater membrane damage (Fig. 4c). The molar ratio of GSH/GSSG in myotubes treated with 15 or 30 mU mL^{-1} glucose oxidase was not significantly different to that in the untreated sample (Fig. 5). Therefore, although membrane integrity was reduced (Fig. 4c), the myotubes maintained their cellular redox state under this level of oxidative stress. However, when the concentration of glucose oxidase was increased, a dose dependent decrease in the GSH/GSSG ratio was observed (Fig. 5; $p < 0.05$), which reflected the increase in ROS (Fig. 4a).

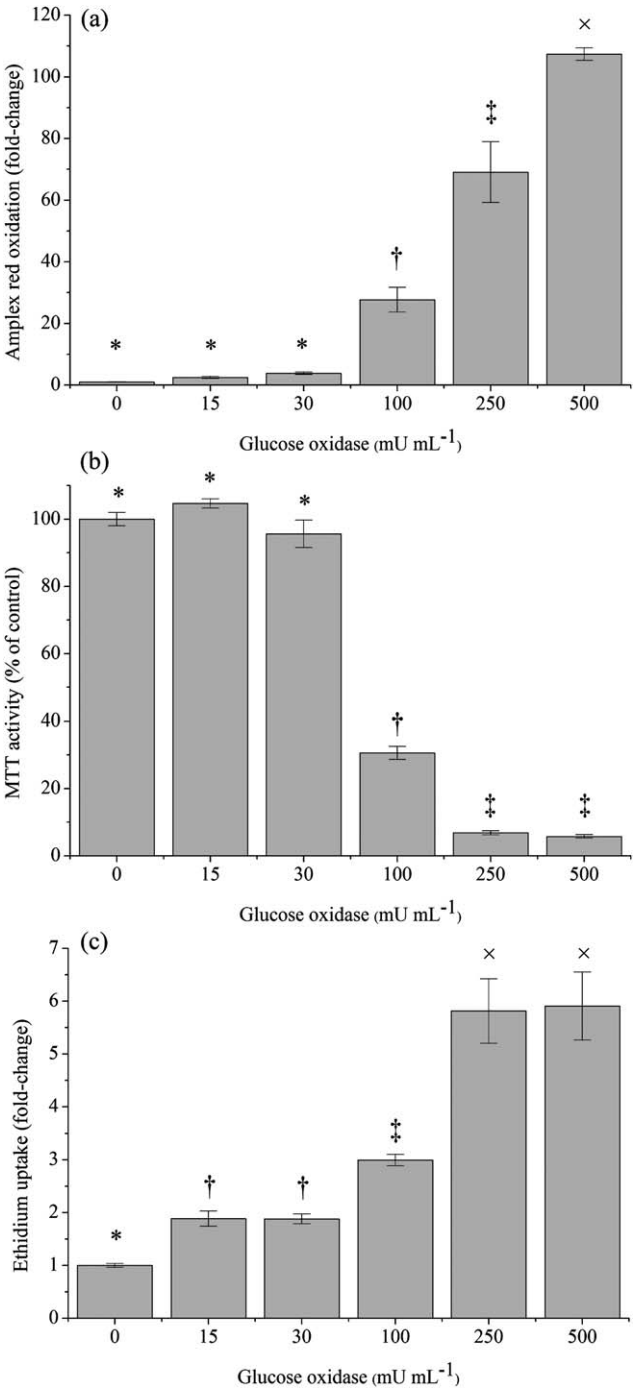

Fig. 4 The effects of a 24 h glucose oxidase treatment on intracellular H_2O_2 levels and cell viability in C2C12 myotubes. (a) Amplex Red assay for intracellular H_2O_2 levels ($N = 1$ experiment with 7 replicates); (b) MTT activity assay to assess mitochondrial function ($N = 1$ experiment with 8 replicates); (c) cellular ethidium uptake to assess membrane integrity ($N = 2$ experiments with 7 replicates). Symbols show significant differences at $p < 0.05$ (one-way ANOVA).

Table 2 GSH and GSSG in mice C2C12 myotubes[a]

GO dose/mU mL^{-1}	GSH/μM per 100 μg protein	GSSG/μM per 100 μg protein
0	8.48 (1.19)*	0.69 (0.11)*
15	5.99 (0.31)*	0.40 (0.028)*
30	6.73 (0.27)*	0.47 (0.046)*
100	14.61 (1.13)†	2.08 (0.17)‡
250	13.33 (0.57)†	2.18 (0.13)†
500	1.43 (0.40)‡	0.36 (0.11)*

[a] These values are reported as means of six determinations (±SEM). For each analyte symbols show significant differences at $p < 0.05$ (one-way ANOVA).

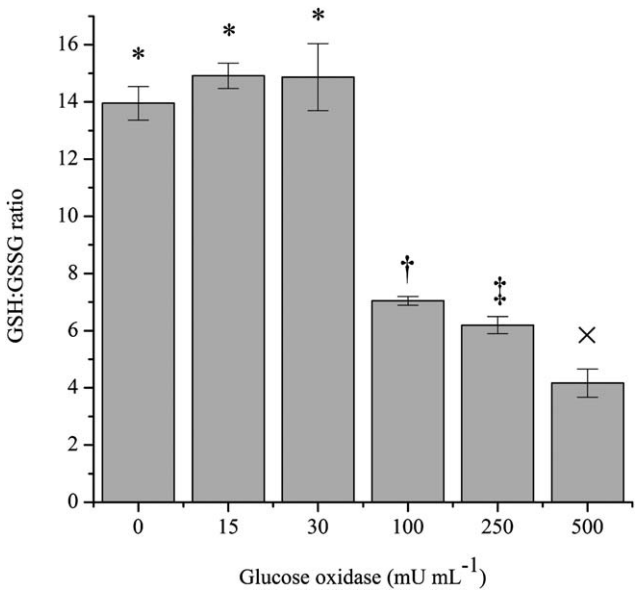

Fig. 5 GSH/GSSG ratio in myotubes treated with different quantities of glucose oxidase ($N = 2$ experiments in triplicate). Symbols show significant differences at $p < 0.05$ (one-way ANOVA).

Conclusions

Rapid HPLC separation coupled with direct acidic potassium permanganate chemiluminescence detection provided a simple and reliable approach to determine GSH, which was extended to GSSG by incorporating thiol blocking and disulfide bond reduction. Unlike conventional approaches with absorbance or fluorescence detection, this procedure does not require derivatisation of GSH (thus minimising error associated with auto-oxidation) and overcomes problems encountered when deriving the concentration of GSSG from 'total GSH'. The procedure was applied to the determination of GSH and GSSG in cultured muscle cells and the trends in the absolute concentrations of these analytes were rationalised by considering intracellular ROS in conjunction with measures of cell viability. The results of this study also highlight the importance of the GSH/GSSG ratio (requiring precise quantification of both GSH and GSSG) to assess the overall intracellular redox environment.

References

1 F. G. Hopkins, *J. Biol. Chem.*, 1927, **72**, 185–187.
2 F. G. Hopkins and M. Dixon, *J. Biol. Chem.*, 1922, **54**, 527–563.
3 F. G. Hopkins and L. J. Harris, *J. Biol. Chem.*, 1929, **84**, 269–320.
4 J. Estrela, A. Ortega and E. Obrador, *Crit. Rev. Clin. Lab. Sci.*, 2006, **43**, 143–181.
5 D. Giustarini, I. Dalle-Donne, D. Tsikas and R. Rossi, *Crit. Rev. Clin. Lab. Sci.*, 2009, **46**, 241–281.
6 D. Grill, M. Tausz and L. De Kok, *Significance of Glutathione to Plant Adaptation to the Environment*, Springer, Dordrecht, 2001.
7 R. Masella and G. Mazza, *Glutathione and Sulfur Amino Acids in Human Health and Disease*, Wiley, New York, 2009.
8 A. Pastore, G. Federici, E. Bertini and F. Piemonte, *Clin. Chim. Acta*, 2003, **333**, 19–39.
9 C. Perricone, C. De Carolis and R. Perricone, *Autoimmun. Rev.*, 2009, **8**, 697–701.
10 D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother.*, 2003, **57**, 145–155.
11 I. A. Cotgreave and R. G. Gerdes, *Biochem. Biophys. Res. Commun.*, 1998, **242**, 1–9.
12 E. Camera and M. Picardo, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2002, **781**, 181–206.
13 P. Monostori, G. Wittmann, E. Karg and S. Túri, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3331–3346.
14 E. Cadenas, *BioFactors*, 1997, **6**, 391–397.
15 M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur and J. Telser, *Int. J. Biochem. Cell Biol.*, 2007, **39**, 44–84.
16 G. Vendemiale, I. Grattagliano and E. Altomare, *Int. J. Clin. Lab. Res.*, 1999, **29**, 49–55.
17 F. Carlucci and A. Tabucchi, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3347–3357.
18 X. Chen, Y. Zhou, X. Peng and J. Yoon, *Chem. Soc. Rev.*, 2010, **39**, 2120–2135.
19 R. E. Hansen and J. R. Winther, *Anal. Biochem.*, 2009, **394**, 147–158.
20 Y. Iwasaki, Y. Saito, Y. Nakano, K. Mochizuki, O. Sakata, R. Ito, K. Saito and H. Nakazawa, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3309–3317.
21 K. Kusmierek, G. Chwatko, R. Glowacki and E. Bald, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3300–3308.
22 T. Toyo'oka, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3318–3330.
23 I. K. Abukhalaf, N. A. Silvestrov, J. M. Menter, D. A. von Deutsch, M. A. Bayorh, R. R. Socci and A. A. Ganafa, *J. Pharm. Biomed. Anal.*, 2002, **28**, 637–643.
24 A. R. T. S. Araujo, M. L. M. F. S. Saraiva and J. L. F. C. Lima, *Talanta*, 2008, **74**, 1511–1519.
25 M. Asensi, J. Sastre, F. V. Pallardó, J. García de la Asunción, J. M. Estrela and J. Viña, *Anal. Biochem.*, 1994, **217**, 323–328.
26 E. Camera, M. Rinaldi, S. Briganti, M. Picardo and S. Fanali, *J. Chromatogr., B: Biomed. Sci. Appl.*, 2001, **757**, 69–78.
27 M. Floreani, M. Petrone, P. Debetto and P. Palatini, *Free Radical Res.*, 1997, **26**, 449–455.
28 D. Giustarini, I. Dalle-Donne, R. Colombo, A. Milzani and R. Rossi, *Free Radical Biol. Med.*, 2003, **35**, 1365–1372.
29 R. Glowacki and E. Bald, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3400–3404.
30 X. Guan, B. Hoffman, C. Dwivedi and D. P. Matthees, *J. Pharm. Biomed. Anal.*, 2003, **31**, 251–261.
31 X.-F. Guo, H. Wang, Y.-H. Guo, Z.-X. Zhang and H.-S. Zhang, *J. Chromatogr., A*, 2009, **1216**, 3874–3880.
32 D. P. Jones, J. L. Carlson, P. S. Samiec, P. Sternberg, Jr., V. C. Mody, Jr., R. L. Reed and L. A. S. Brown, *Clin. Chim. Acta*, 1998, **275**, 175–184.
33 R. Kand'ár, P. Záková, H. Lotková, O. Kucera and Z. Cervinková, *J. Pharm. Biomed. Anal.*, 2007, **43**, 1382–1387.
34 Y. Rao, B. Xiang, E. Bramanti, A. D'Ulivo and Z. Mester, *J. Agric. Food Chem.*, 2010, **58**, 1462–1468.
35 J. M. Rosenfeld, *TrAC, Trends Anal. Chem.*, 2003, **22**, 785–798.
36 R. Rossi, A. Milzani, I. Dalle-Donne, D. Giustarini, L. Lusini, R. Colombo and P. Di Simplicio, *Clin. Chem.*, 2002, **48**, 742–753.
37 A. K. Sakhi, K. M. Russnes, S. Smeland, R. Blomhoff and T. E. Gundersen, *J. Chromatogr., A*, 2006, **1104**, 179–189.
38 F. Zhang, M. J. Bartels, D. R. Geter, Y.-C. Jeong, M. R. Schisler, A. J. Wood, L. Kan and B. B. Gollapudi, *Rapid Commun. Mass Spectrom.*, 2008, **22**, 3608–3614.
39 S. Pelletier and C. A. Lucy, *Analyst*, 2004, **129**, 710–713.

40 O. Yilmaz, S. Keser, M. Tuzcu, M. Guvenc, B. Cetintas, S. Irtegun, H. Tastan and K. Sahin, *J. Anim. Vet. Adv.*, 2009, **8**, 343–347.
41 L. A. Allison and R. E. Shoup, *Anal. Chem.*, 1983, **55**, 8–12.
42 J. Du, Y. Li and J. Lu, *Anal. Chim. Acta*, 2001, **448**, 79–83.
43 A. A. Ensafi, T. Khayamian and F. Hasanpour, *J. Pharm. Biomed. Anal.*, 2008, **48**, 140–144.
44 H.-Y. Han, Z.-K. He and Y.-E. Zeng, *Microchim. Acta*, 2006, **155**, 431–434.
45 W. L. Hinze, T. E. Reihl, H. N. Singh and Y. Baba, *Anal. Chem.*, 1984, **56**, 2180–2191.
46 Y. Li, A. Zhang, J. Du and J. Lu, *Anal. Lett.*, 2003, **36**, 871–879.
47 L. Wang, Y. Li, D. Zhao and C. Zhu, *Microchim. Acta*, 2003, **141**, 41–45.
48 S. Wang, H. Ma, J. Li, X. Chen, Z. Bao and S. Sun, *Talanta*, 2006, **70**, 518–521.
49 S. Zhao, Y. Huang, F. Ye, M. Shi and Y.-M. Liu, *J. Chromatogr., A*, 2010, **1217**, 5732–5736.
50 J. L. Adcock, P. S. Francis and N. W. Barnett, *Anal. Chim. Acta*, 2007, **601**, 36–67.
51 P. S. Francis, N. W. Barnett, S. W. Lewis and K. F. Lim, *Luminescence*, 2004, **19**, 94–115.
52 B. A. Gorman, P. S. Francis and N. W. Barnett, *Analyst*, 2006, **131**, 616–639.
53 C. A. Marquette and L. J. Blum, *Anal. Bioanal. Chem.*, 2006, **385**, 546–554.
54 M. Tsunoda and K. Imai, *Anal. Chim. Acta*, 2005, **541**, 13–23.
55 S. B. Bankar, M. V. Bule, R. S. Singhal and L. Ananthanarayan, *Biotechnol. Adv.*, 2009, **27**, 489–501.
56 J.-C. Lee, Y.-O. Son, K.-C. Choi and Y.-S. Jang, *Mol. Cells*, 2006, **22**, 21–29.
57 A. Barbouti, P.-T. Doulias, L. Nousis, M. Tenopoulou and D. Galaris, *Free Radical Biol. Med.*, 2002, **33**, 691–702.
58 J. L. Adcock, P. S. Francis and N. W. Barnett, *J. Fluoresc.*, 2009, **19**, 867–874.
59 J. L. Adcock, P. S. Francis and N. W. Barnett, *Anal. Chim. Acta*, 2009, **652**, 303–307.
60 B. Gómez-Taylor Corominas, M. Catalá Icardo, L. Lahuerta Zamora, J. V. García Mateo and J. Martínez Calatayud, *Talanta*, 2004, **64**, 618–625.
61 J.-L. Luo, F. Hammarqvist, I. A. Cotgreave, C. Lind, K. Andersson and J. Wernerman, *J. Chromatogr., B: Biomed. Appl.*, 1995, **670**, 29–36.
62 P. Husek, P. Matucha, A. Vránková and P. Simek, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2003, **789**, 311–322.
63 P. Sevcíková, Z. Glatz and J. Tomandl, *J. Chromatogr., A*, 2003, **990**, 197–204.
64 J. A. Burns, J. C. Butler, J. Moran and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 2648–2650.
65 J. Podlaha and J. Podlahová, *Collect. Czech. Chem. Commun.*, 1973, **38**, 1730–1736.
66 H. Østergaard, C. Tachibana and J. R. Winther, *J. Cell Biol.*, 2004, **166**, 337–345.