Electronic Supplementary information:

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

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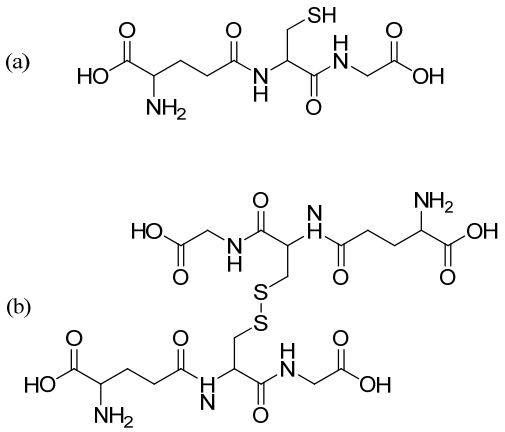

Fig. S1 Structures of (a) glutathione (GSH) and (b) glutathione disulfide (GSSG).

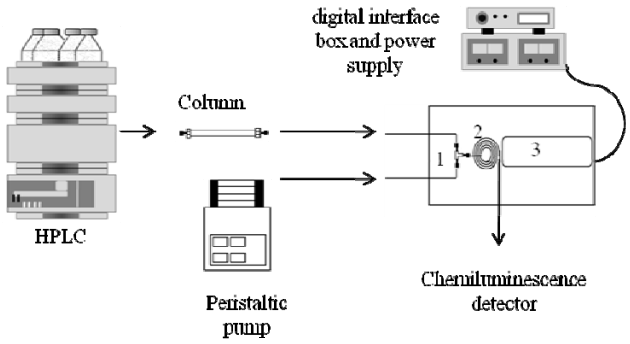

Fig. S2 Instrument setup for HPLC with post-column acidic potassium permanganate chemiluminescence detection. 1, T-piece; 2, transparent PTFE-PFA tubing reaction coil; 3, PMT.

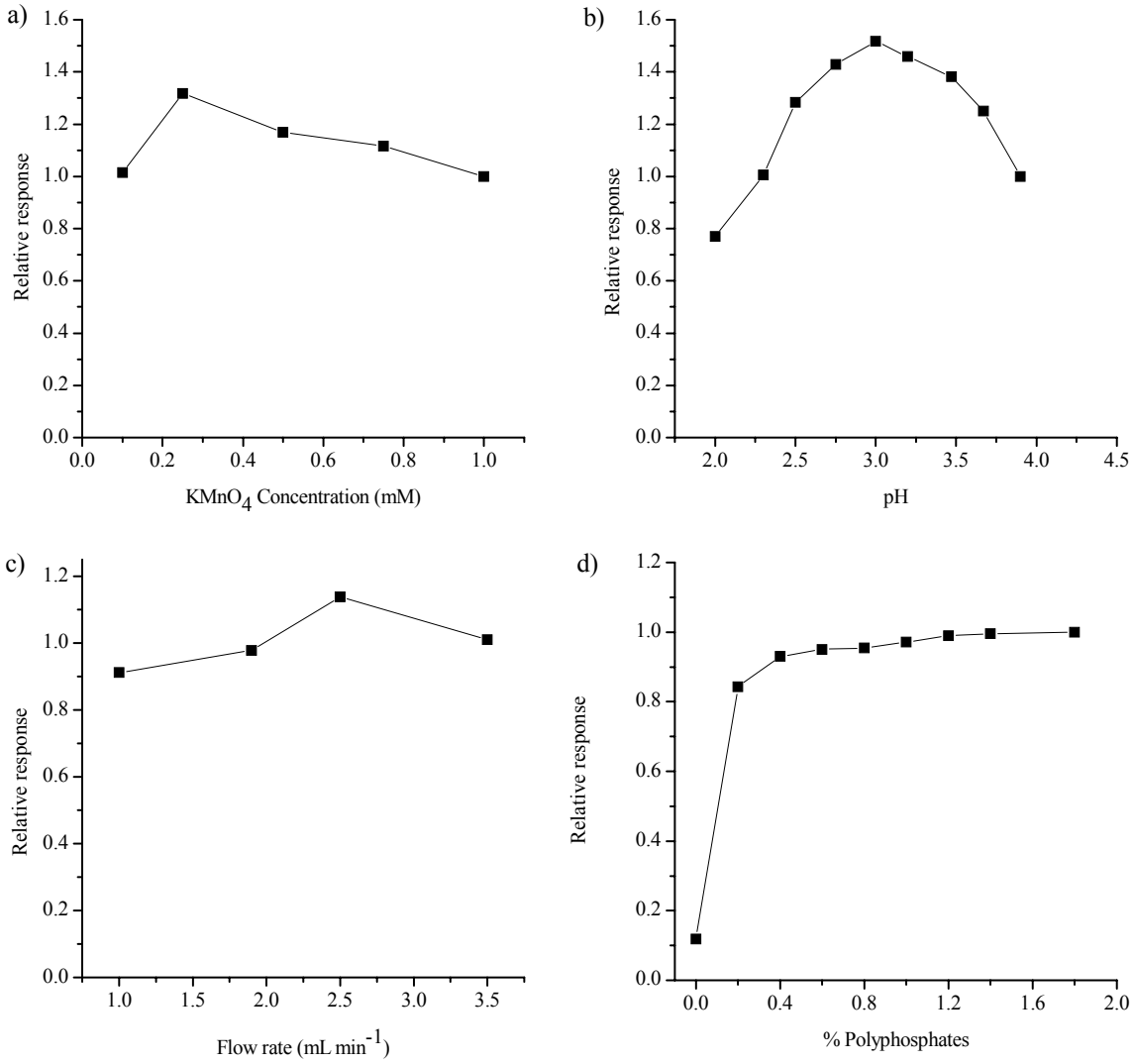

Fig. S3 Effect of (a) potassium permanganate concentration, (b) reagent pH, (c) flow rate through FIA manifold, and (d) percentage sodium polyphosphates (m/v) added to reagent on the chemiluminescence signal of GSH.

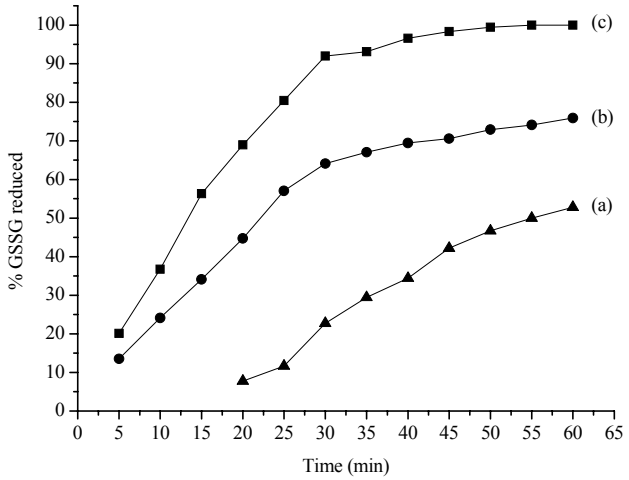

Fig. S4 Optimisation of GSSG reduction using TCEP. The components were mixed together and aliquots taken over time for injection into the HPLC to determine resulting GSH concentration by peak area. The curves represent reactions between GSSG (in formic acid, pH 2.8) and TCEP in: (a) deionised water; (b) Tris-HCl buffer; and (c) Tris-HCl buffer, heated to 50 °C using a hot-plate. Experimental parameters are described in the text above and chromatographic conditions outlined in Table 1.

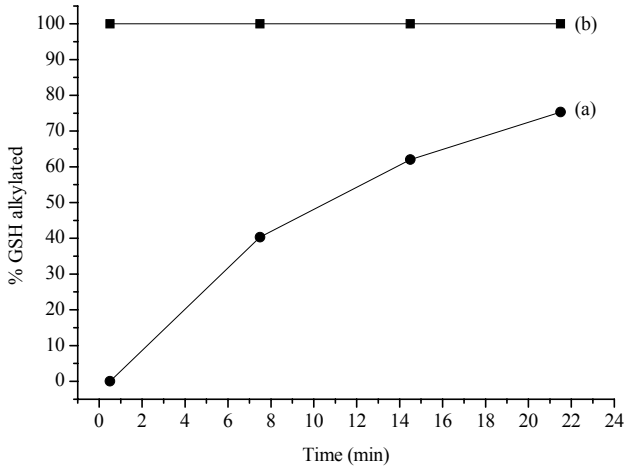

Fig. S5 Optimisation of GSH alkylation using NEM. The components were mixed together and aliquots taken over time for injection onto the HPLC to determine resulting GSH concentration by peak area. The curves represent reactions between GSH (in formic acid, pH 2.8) and NEM in: (a) deionised water; and (b) Tris-HCl buffer. Experimental parameters are described in the text above and chromatographic conditions outlined in Table 1.

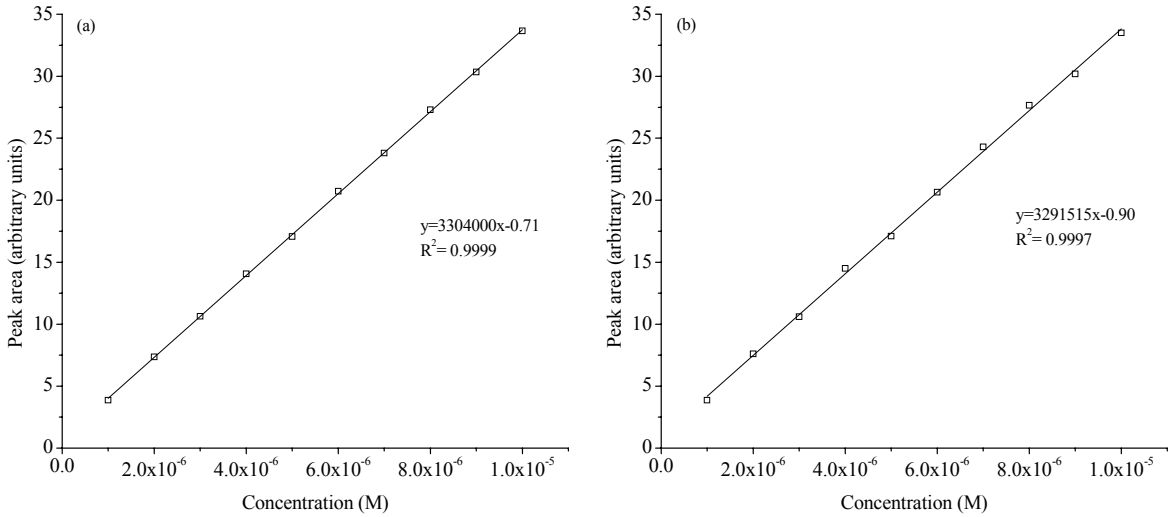

Fig. S6 Calibration plots for (a) GSH and (b) GSSG standards prepared in aqueous formic acid (pH 2.8). Chromatographic conditions described in Table 1.

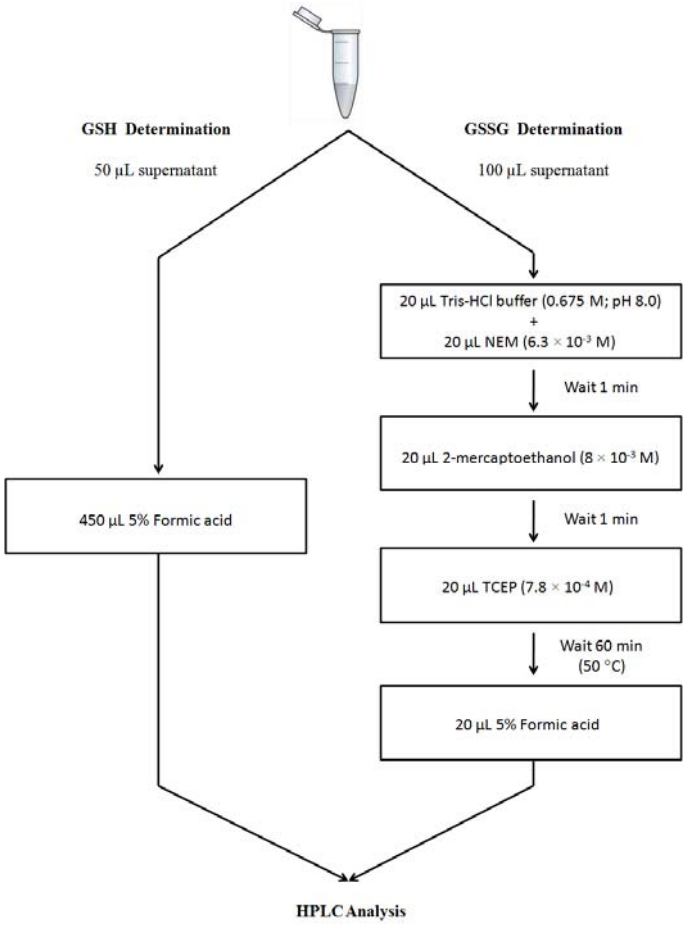

Fig. S7 Summary of the procedure for the determination of GSH and GSSG using HPLC with acidic potassium permanganate chemiluminescence detection.

Table S1. Analytical figures of merit.

	GSH	GSSG
Calibration function	y=3304000x-0.71	y=3291515x-0.90
R^2	0.9999	0.9997
Linear range	7.5×10^{-7} M to 1×10^{-5} M	7.5×10^{-7} M to 1×10^{-5} M
Limit of detection	5×10^{-7} M	5×10^{-7} M
Precision (%RSD)*	0.9	1.3

*Concentration of 2.5×10^{-6} M (n = 6).

ADDITIONAL MATERIALS AND METHODS

Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. *N*-Ethylmaleimide, glucose oxidase, L-glutathione, L-glutathione disulfide, 2-mercaptoethanol, tris(2-carboxyethylphosphine), sodium polyphosphate (+80 mesh) and thiazolyl blue tetrazolium bromide were obtained from Sigma–Aldrich (Castle Hill, New South Wales, Australia). Dulbecco's modified Eagle's medium, fetal bovine serum, Hank's Buffered Salt Solution and horse serum were supplied by Invitrogen (Mulgrave, Victoria, Australia) Potassium permanganate and sodium chloride were obtained by Chem-Supply (Gillman, South Australia, Australia). Analytical grade methanol, sulfuric acid (98% w/v) and tris(hydroxymethyl)methylamine were supplied by Merck (Kilsyth, Victoria, Australia). Hydrochloric acid (32% w/v) was purchased from Ajax Finechem (Taren point, New South Wales, Australia). Formic acid (98% w/v) was obtained from Hopkin and Williams (Chadwell Heath, Essex, England). Stock solutions (1×10^{-3} M) of GSH and GSSG were prepared and diluted in aqueous formic acid (pH 2.8). The acidic potassium permanganate reagent (1×10^{-3} M) was prepared by dissolution of potassium permanganate in a 1% (m/v) sodium polyphosphate solution and adjusted to pH 3 with sulfuric acid.

Determination of cell viability - mitochondrial function (MTT assay) and membrane integrity (ethidium uptake assay)

Mitochondrial function and membrane integrity were used as markers of cell viability, as both are compromised in stressed cells undergoing apoptosis or necrosis. For the MTT assay, C2C12 myotubes were incubated with 0.5 mg/ml thiazolyl blue tetrazolium bromide for 15 min at 37°C and 5% CO_2 to allow for the conversion of thiazolyl blue tetrazolium bromide to dark blue MTT-formation by mitochondrial dehydrogenases. The cells were then solubilised with isopropanol and the intensity of colour development was measured at 570 nm in a micro-plate reader (Bio-Rad, Gladesville, New South Wales, Australia) with 630 nm as the

reference wavelength. The MTT results are presented relative to cells in the control media (0 mU mL^{-1} glucose oxidase), which were considered to be 100% viable.

For the membrane integrity assay, myotubes were incubated with 8 μM ethidium (2 mM stock solution in DMSO; Invitrogen) in Hank's Buffered Salt Solution (HBSS, 5.5 mM glucose) plus or minus glucose oxidase for 10 min at 37°C and 5% CO_2. Ethidium uptake is greater in cells with compromised membrane integrity and this increase in fluorescence intensity was measured with a FlexStation II Scanning Fluorometer (Molecular Devices, Sunnyvale, California, U.S.A; λ_{ex} = 590 nm, λ_{em} = 612 nm). Ethidium uptake is presented as fold-change in absolute fluorescence relative to control media (0 mU/mL glucose oxidase).

Determination of oxidative stress - H_2O_2 assay

H_2O_2 levels were measured with a commercially available Amplex Red H_2O_2 kit (#A22188, Molecular Probes - Invitrogen), according to the manufacturer's instructions. Briefly, C2C12 myotubes were incubated with 50 μM of Amplex Red reagent dissolved in DMSO and 0.1 U/mL horseradish peroxidase in HBSS (5.5 mM glucose) for 10 min at 37°C and 5% CO_2. The increase in fluorescence intensity was measured with a FlexStation II Scanning Fluorometer (Molecular Devices; λ_{ex} = 590 nm, λ_{em} = 612 nm). To confirm the efficacy of the glucose oxidase treatment, H_2O_2 levels were determined with glucose oxidase present in the assay media. All doses of glucose oxidase increased Amplex Red oxidation (data not shown). Results are presented relative to control media (0 mU/mL glucose oxidase). Note: statistically analysed data were not normalised to protein content.