Development of an antioxidant assay to study oxidative potential of airborne particulate matter

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Abstract. Oxidative potential is a measure of redox activity of airborne particulate matter (PM) and is often used as a surrogate to estimate one form of PM toxicity. The evaluation of oxidative potential in physiologically relevant environment is always challenging. In this work, we developed a chromatographic method, employing an ultra-high performance liquid chromatograph coupled to a triple-quadruple mass spectrometer, to determine the oxidative potential of PM from different sources. To this purpose, we measured the PM-induced oxidation of glutathione, cysteine, and ascorbic acid, and formation of glutathione disulfide and cystine, following PM addition to simulated epithelial lining fluids, which, in addition to the antioxidants, contained inorganic salts, a phospholipid, and proteins. The new method showed high precision and, when applied to standard reference PM, the oxidative potential increased with the reaction time and PM concentration in the lung fluid. The antioxidant depletion rates were considerably higher than the rates found with the conventional dithiothreitol assay, indicating the higher sensitivity of the new method. The presence of the lung fluid inorganic species increased the oxidative potential determined through glutathione and cysteine, but showed an opposite effect with ascorbic acid, whereas the presence of proteins resulted in a moderate decrease in the oxidative potential. In the presence of $PM_{2.5}$, glutathione and cysteine demonstrated similar depletion patterns, which were noticeably different from that of ascorbic acid, suggesting that cysteine could be used as an alternative to glutathione for probing oxidative potential.

1 Introduction

Air pollution is a major environmental health risk. It has been associated with respiratory and cardiovascular diseases as well as increased morbidity and mortality rates over the past 25 years. Human exposure to fine particulate matter (aerodynamic diameter ≤2.5 μm, i.e. PM$_{2.5}$), is one of the main causes of the adverse effects across the world (WHO, 2016; Cohen et al., 2017; Burnett et al., 2018; Lelieveld et al., 2018; Lelieveld et al., 2019a). Recent studies suggest that exposure to PM$_{2.5}$ significantly reduces the human life expectancy in both low-middle income and developed countries (Lelieveld et al., 2018; Lelieveld et al., 2019a), with fossil-fuel-related emission being responsible for 65% of premature death from anthropogenic air pollutants worldwide (Lelieveld et al., 2019b).

For decades, PM mass concentration has been applied as a metric to relate air pollution with adverse health outcomes. However, a large part of PM is made of chemicals with low toxicity, e.g. sea salt, ammonium sulfates and nitrates, and only a small portion of PM including the organic phase and metals are expected to pose toxic effects (Ayres et al., 2008; Lodovici and Bigagli, 2011). Moreover, the pool of chemicals in PM dynamically changes as it undergoes temporal and spatial evolutions due to reaction with oxidants and mixing in the atmosphere (Pöschl and Shiraiwa, 2015; Paula et al., 2016). Growing evidence links the adverse health effects of air pollution to pulmonary oxidative stress due to increased exposure to atmospheric oxidants or decreased antioxidant defense levels (Kelly, 2003; Li et al., 2003; Künzli et al., 2006; Borm et al., 2007; Weichenthal et al., 2013; Kelly and Fussell, 2017; Bates et al., 2019). Consequently, the oxidative potential of PM$_{2.5}$ has been applied as an additional parameter to PM mass in explaining the air pollution health effects (Weichenthal et al., 2019).

In the context of inhalation toxicity, oxidative potential is defined as the ability of PM-bound chemicals to oxidize the lung antioxidants and catalytically generate reactive oxygen species, e.g. hydrogen peroxide, superoxide radical, and hydroxyl radical (Bates et al., 2019). Through this process, the redox-active constituents of PM, e.g. quinones and transition metals, react with the antioxidants in the epithelial lining fluid (ELF), resulting in the depletion of antioxidants and formation of oxidation products. In the case of thiol-containing antioxidants, such as glutathione (GSH) and cysteine (CSH), their oxidation leads to formation of glutathione disulfide (GSSG) and cystine (CSSC) through formation of a disulfide bridge (Fig. 1). Although quinones and transition metals have been recognized as important redox-active substances, other redox-active chemicals, such as humic-like substances, organic hydroperoxides, highly oxygenated molecules, and environmentally persistent free radicals were also found to have oxidative potential (Charrier and Anastasio, 2012; Verma et al., 2015; Dou et al., 2015; Tong et al., 2016; 2017; 2019; Gonzalez et al., 2017).

To this date, oxidative potential has been primarily determined using colorimetric methods, and the dithiothreitol assay has been used most frequently (Charrier and Anastasio, 2012; Charrier et al., 2014; Verma et al., 2009; Verma et al., 2014; Verma et al., 2015; Calas et al., 2017; Crobeddu et al., 2017; Calas et al., 2018; Tong et al., 2018; Tuet et al., 2019; Bates et al., 2019). Such methods introduce certain challenges; for instance, dithiothreitol is a chemical that is alien to the ELF and the assay was shown to have greater response to PM-bound quinones rather than metals (Ayres et al., 2008; Charrier et al., 2014); metals outweigh quinones in ambient PM. It has been suggested that the dithiothreitol oxidation rates may not correspond entirely to the formation rates of reactive oxygen species (Xiong et al., 2017; Fang et al., 2019). Moreover, dithiothreitol oxidation demonstrates degrees of association to changes in PM

components, sources (e.g. biomass burning, traffic), and temporal variations that are different from those seen with GSH or ascorbic acid (AA) (Fang et al., 2016; Weichenthal et al., 2019). Such observations raise the question of whether the dithiothreitol oxidation rate is a realistic metric for oxidative potential of ambient PM in physiological ELF.

5   Colorimetric methods are not fully compatible with the ELF containing lipids and proteins because the turbidity resulting from their presence in ELF could interfere with the reading of UV probe. Extraction of PM in simulated-ELF (SELF), as opposed to water, as well as SELF formulation could in various ways affect the oxidative potential results (Calas et al., 2017). The presence of lipids and proteins could be particularly important as they influence the ELF characteristics: for instance, dipalmitoylphosphatidylcholine (DPPC), a major ELF surfactant phospholipid, is

10   known to reduce the ELF surface tension (Gregory et al., 1991; Griese, 1999; Johansson et al., 1994; Pison et al., 1986; Boisa et al., 2014). The presence of DPPC has been linked to improved oxidative potential dose-response relationships, supposedly by enhancing suspension stability (Calas et al., 2017). Albumin, a major ELF protein (Bredberg et al., 2012), binds to metals and limits their solubility; hence, influencing ELF antioxidant and PM pro-oxidant capacity (Cross et al., 1994).

15   Chromatographic methods have been applied less frequently to studies of oxidative potential from air samples (Mudway and Kelly, 1998; Zielinski et al., 1999; Künzli et al., 2006; Crobeddu et al., 2017; Calas et al., 2018). These studies determined the consumption of AA in a simplified SELF (0.9% saline, pH 7.4) using high performance liquid chromatograph coupled with electrochemical detection (Iriyama et al., 1984). Crobeddu et al., (2017) advanced this method by measuring GSH and GSSG in addition to AA; however, they did not take into account potential artifacts

20   due to oxidation of GSH during sample processing. This effect was shown to be a major source of uncertainty in measuring oxidative potential using thiol-containing antioxidants in biological samples (Giustarini et al., 2016). ==The artifact can happen during sample processing due to various factors, such as sample acidification or restoration of pH to neutral/alkaline (Rossi et al., 2002), which may lead to variable oxidation of thiols and low reproducibility of measured GSH and GSSG concentrations.==

25   In the present work, a new chromatographic method has been developed to determine the oxidative potential of ambient PM using an ultra-high performance liquid chromatograph (UHPLC) coupled to a triple-quadruple mass spectrometer (MS/MS). This method measures the direct consumption of AA, CSH and GSH and formation of CSSC and GSSG following incubation of PM in SELF which, in addition to antioxidants, contains major ELF inorganic salts, a phospholipid, and proteins. ==The use of UHPLC-MS/MS allows relatively short analytical time down to few==

30   ==minutes, whereas the MS/MS capability allows simultaneous detection of multiple analytes (see Table 2). This is a major advantage compared to conventional HPLC analysis with absorbance detection, and it is important when performing high-throughput laboratory analysis, in particular when analyzing high-molecular mass substances with various organic or water solubility. Pre-analytical sample preparation is key to achieving optimal results in the analysis of antioxidants. In the case of GSH and CSH, masking the –SH group is crucial in preventing their artificial oxidation==

35   ==prior to instrumental analysis (Giustarini et al., 2016; Escobar et al., 2016). In the present study, this has been achieved by derivatizing GSH and CSH with *N*-ethylmaleimide (NEM), which is an effective alkylating reagent for masking –SH group at the pH range of 6.5-7.5.== We investigated the method performance and reproducibility, the oxidative

potential time-dependence and dose-response, and the effect of SELF composition on PM oxidative potential. We compared the current method with the dithiothreitol assay and applied the method to contemporary $PM_{2.5}$ samples.

2 Methods

2.1 Chemicals

5   AA, GSH, GSSG, and isotopically labelled AA-$^{13}C_6$, GSH-$^{13}C_2^{15}N$, CSH-$^{13}C_3$, and GSSG-$^{13}C_4^{15}N_2$ were purchased from Toronto Research Chemicals (Toronto, Canada). CSH, CSSC, *N*-ethylmaleimide (NEM), DPPC, ethylenediaminetetraacetic acid (EDTA), sulfosalicylic acid (SSA), nitric acid, phosphate buffered saline (PBS; Bio-Performance, containing NaCl, KCl, and Na_2HPO_4), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), sodium sulphate (Na_2SO_4), glycine, uric acid (UA), and albumin were purchased from Sigma Aldrich (Oakville, Canada).

10   CSSC-d_6 was purchased from TLC Pharmaceutical Standards (Aurora, Canada). LC-MS grade acetonitrile and methanol were obtained from Caledon Laboratory Chemicals (Georgetown, Canada), and LC-MS grade (Optima) water and formic acid were purchased from Fisher Scientific (Ottawa, Canada). The stock solution of analytical standards AA, AA-$^{13}C_6$, GSH, GSH-$^{13}C_2^{15}N$, CSH, CSH-$^{13}C_3$, GSSG, GSSG-$^{13}C_4^{15}N_2$ were prepared in 50:50 methanol-H_2O mixture, whereas CSSC and CSSC-d_6 were prepared in 10:90 methanol-H_2O; these were used only for

15   preparing calibration standards. In addition, a 50 mM mixture of AA, GSH, and CSH was prepared in 20:80 methanol-H_2O and used specifically to prepare SELF. All antioxidant stock solutions were stored at -80°C. The 20% methanol solution allowed swift thawing (~1 min) of concentrated antioxidant mixture at room temperature and helped prevent oxidation of antioxidants due to long thawing times prior to the experiments. 100 mM stock solution of NEM and a solution containing EDTA (2 mM) and SSA (2%) were prepared in 20:80 methanol-H_2O and stored at 4°C. We

20   avoided glass-ware for sample processing and all plastic-ware used in this study were cleaned with Nalgene L900 soap, 0.1 M nitric acid, deionized water, and LC-MS grade methanol.

2.2 Sample characteristics

2.2.1 Particulate matter

Standard reference material (SRM 1649b) was obtained from the National Institute of Standard and Technology

25   (NIST, Gaithersburg, USA). The sample is characterized for a large number of organic and inorganic species, including some of redox-active substances, i.e. 9,10-anthraquinone (1.8 pg µg^{-1}), 1,2-benzanthraquinone (3.6 pg µg^{-1}), copper (311 pg µg^{-1}), and manganese (337 pg µg^{-1}). In terms of mass size distribution, the volume weighed mean falls in the range of coarse atmospheric particles. In the present study, SRM suspension in LC-MS grade water was freshly made (5 mg mL^{-1}) and used across different experiments. The PM suspension was gently mixed each time

30   prior to spiking into SELF. In addition, $PM_{2.5}$ samples were obtained from the National Air Pollution Surveillance (NAPS) site in Hamilton, Canada and used for validating the antioxidant assay. The samples were collected on quartz fiber filters using Dichotomous Air Sampler (Thermo Scientific, Waltham, USA) each for the period of 24 hr. The sampled air volume was 21.6 m^3 in each case, and a 1-cm^2 filter punch from each sample was used to perform the

2.2.2 SELF formulations

Different SELF formulations representing extracellular fluid of human lung were tested in the present study; a
5   simplified formula was used as the basis for all formulations and contained PBS (pH: 7.4) and a mixture of CSH, GSH, and AA (200 µM each; Table 1). These salts and antioxidants are among constituents of Gamble solution and physiological ELF (Stopford et al., 2003; Boisa et al., 2014). Four other formulations were used in order to explore the effects of SELF composition on oxidative potential results. These formulas, in addition to the above components, contained UA, CaCl$_2$, MgCl$_2$, Na$_2$SO$_4$, DPPC, albumin, and glycine at physiological concentrations (Boisa et al.,
10   2014). The presence of inorganic salts, antioxidants, surfactant lipids, and proteins could affect the dissolution kinetics of redox-active species (Boisa et al., 2014). The pH of SELF formulations was monitored during incubation in an independent experiment.

2.3 Sample preparation

The stock solution of reduced antioxidants (50 mM) was swiftly thawed (~1 min) at room temperature before each
15   experiment and spiked into SELF to achieve antioxidant concentration of 200 µM each using positive displacement pipettes (Microman E, Gilson, Middleton, USA). 2.5 mL of SELF was transferred to pre-cleaned 8-mL low-density polyethylene (LDPE) bottles (Nalgene, Thermo Scientific, Waltham, USA). LDPE bottles containing SELF were spiked with PM stock suspension in order to achieve PM concentrations ranging from 10 to 80 µg mL^{-1}. For experiments involving PM$_{2.5}$ samples from the NAPS site, a 1-cm^2 filter punch was added to SELF. The samples
20   containing PM were gently mixed and incubated along with reference SELF that did not contain PM. The incubation was performed at 37°C and 60 rpm using an incubator-shaker (Benchmark Scientific, Sayreville, USA) at intervals ranging from 30 to 240 min. Following the incubation, 300-µL aliquots ($n = 3$) of each sample were transferred to 1.5-mL centrifuge tubes (Brand, Wertheim, Germany), added with 100 µL of 100 mM NEM, mixed for 5 sec using a vortex mixer, and allowed to react at room temperature for 1 min. This reaction time was found to be sufficient for
25   derivatization of thiols (Escobar et al., 2016). The sample was subsequently added with 200 µL of a mixture containing 2% SSA and 2 mM EDTA (Moore et al., 2013), mixed for 10 sec, and centrifuged (10000 g, 6 min). The SSA in the precipitation mixture was used to precipitate albumin and to reduce the pH to ~2, whereas EDTA was used to chelate transition metals (Moore et al., 2013). Finally, 5 µL of the supernatant from each sample was transferred to a push-filter vial with a polytetrafluoroethylene filter (Whatman, Pittsburgh, USA) containing 485 µL of 20:80 methanol-
30   H$_2$O and spiked with 10 µL of labelled antioxidant standard mixture (10 ng µl^{-1}). We applied this mixture across all SELF formulations in order to ensure consistency in sample processing. Moreover, we compared our method with the dithiothreitol assay in terms of dose-response and reproducibility. The dithiothreitol assay was performed following the procedure described in Tong et al., (2018); more details can be found in Sect. S1 in the Supplement.

2.4 Sample and data analysis

The sample analysis was carried out using an Acquity UHPLC (Waters, Milford, USA) coupled to a Xevo TQ-S MS/MS (Waters). The analysis was performed in electrospray ionization in the negative mode for AA and AA-$^{13}C_6$ and in the positive mode for the rest of the analytes. The auto-sampler temperature was set to 4°C and the injection volume was 2 μl. The target substances were separated using an Acquity HSS T3 UPLC column (3 mm, 50 mm, 1.8 μm; Waters), connected to a pre-column (2.1 mm, 5 mm, 1.8 μm, Waters), and thermostated at 40°C. The mobile phase A was composed of LC-MS grade H_2O containing 0.1% formic acid, and mobile phase B was made of LC-MS grade acetonitrile containing 0.1% formic acid. The mobile phase flowrate was set to 0.4 mL min^{-1}. For the target compounds in the negative mode, the mobile phase gradient started at 20% phase B, ramped to 100% B from 0.3 to 0.5 min and held for 4.5 min, then decreased to 20% B from 5 to 5.1 min and held for 2.9 min. For substances in the positive mode, the gradient started at 1% phase B, ramped to 50% B from 0.1 to 0.3 min and held for 3.2 min, followed by an increase to 100% B over 0.1 min and a hold time of 2.4 min, followed by a decrease to 1% B and re-equilibration time of 1.9 min.

The target compound detection parameters are presented in Table 2. The following MS/MS parameters were used: capillary voltage of 1.5 kV, source temperature of 120°C, nitrogen and desolvation gas flow-rates of 150 and 900 L hr^{-1}, desolvation temperature of 500°C, and collision gas flow rate of 0.15 mL min^{-1}. The analyte quantification was done using the internal calibration method with 6-point linear calibration curves ($r^2 = 0.999$) ranging from 1 to 250 pg μl^{-1}. The chromatographic data analysis was performed using MassLynx software (Waters). Blanks were analysed after the samples and highest-level calibration standard, and analyte carry-over was below the instrument detection limits ($n = 3$).

Antioxidant consumption (μM min^{-1}) was calculated as follows: (CSH$_{REF}$ – CSH$_{PM}$)/(incubation time), where CSH$_{REF}$ and CSH$_{PM}$ are the measured concentrations (μM) of CSH in the reference SELF and SELF containing PM following the incubation (min), respectively. The consumption of AA and GSH were also calculated using the formula above. The oxidation product formation (μM min^{-1}) was calculated as follows: (CSSC$_{PM}$ – CSSC$_{REF}$)/(incubation time), where CSSC$_{PM}$ and CSSC$_{REF}$ denote the measured concentrations of CSSC in SELF containing PM and the reference SELF after the incubation, respectively. The formation of GSSG was calculated using the same formula.

Availability of the concentrations of redox couples CSH/CSSC and GSH/GSSG allows calculating the redox state of SELF before and after addition of PM. In the biochemical literature, the Nernst equation has been frequently used to calculate the reduction potential (Eh, expressed in mV) of antioxidant redox couples such as GSH/GSSG (Schafer and Buettner, 2001). Despite some debate about the interpretation of the reduction potentials (Flohé, 2013), in terms of the presence or absence of equilibrium between the redox couples, such values have been used as the measure of oxidation state within biological systems (Jones et al., 2000; Iyer et al., 2009). The molar ratio of the redox couples, e.g. GSH/GSSG, is another metric that has been used widely to determine the oxidative stress in organisms (Giustarini et al., 2016). Both metrics would provide a meaningful way to interpret oxidative potential data, because they consider redox couples as opposed to a single antioxidant; however, one limitation with using ratios is that they could vary from zero to infinity. In this work, in addition to presenting the results as molar concentrations, we calculated the theoretical redox state of CSH/CSSC and GSH/GSSG redox couples, Eh (mV), using the molar concentrations (mol

$$Eh_{(CSH/CSSC)} = -250 + 30 \log_{10} ([CSSC]/[CSH]^2) \qquad \text{Eq. 1}$$

$$Eh_{(GSH/GSSG)} = -264 + 30 \log_{10} ([GSSG]/[GSH]^2) \qquad \text{Eq. 2}$$

3 Results

3.1 Method performance

The method reproducibility was tested using SELF-a (Table 1). Four samples containing PM (20 µg mL^{-1}) were incubated together with a reference sample for the duration of 180 min. Three replicates were prepared from each SELF sample containing PM and five replicates prepared from the reference SELF. The mean consumption for AA, CSH, and GSH, due to the presence of PM, were 0.29±0.03, 0.43±0.03, and 0.53±0.04 µM min^{-1}, respectively, with relative standard deviation (%RSD) of ≤9% (Fig. 2). These values reflect the *inter-sample* reproducibility, targeting the entire sample preparation procedure. The *intra-sample* variabilities (n = 3) were smaller, with %RSD ≤3%. The formation of CSSC and GSSG were similarly reproducible, i.e. 0.05±0.01 and 0.09±0.01 µM min^{-1}, with inter- and intra-sample RSD of ≤13 and ≤6%, respectively (Fig. 2). The results indicate noticeable enhancement in precision compared to those reported by Crobeddu et al., (2017), where derivatization of the -SH group was not considered during sample processing and RSD values of >50% were observed in several cases. Re-calculating our results following the Crobeddu et al., (2017) approach (i.e. % depletion), the RSD for GSH depletion with our method would be 8% for inter-sample variability and ≤5% for intra-sample variability. High reproducibility is essential for statistically valid comparison of oxidative potential between different samples. Our results further confirm the need for derivatization when analysing thiol-antioxidants (Giustarini et al., 2016).

We investigated if our post-derivatization sample processing influenced the oxidative potential results: in a separate experiment, which was conducted with PM in SELF-a, we replaced the precipitation solution (i.e. 2 mM EDTA/2% SSA) with (a) H$_2$O and (b) 2 mM EDTA. Note that treatment with EDTA/SSA is necessary for effective deposition of proteins prior to instrumental analysis (Moore et al., 2013), when these are present in SELF formulation. The oxidative potential was found to be independent of the processing methods mainly due to the use of internal standards in our method, which accounted for post-incubation changes in concentrations of target compounds in both reference SELF and SELF containing PM. Among the three treatments, RSD of the antioxidant depletion and oxidation product formation rates were ≤3 for CSH and GSH, 6% for AA, and ≤7% for CSSC and GSSG. The addition of EDTA/SSA mixture, however, prevented the *absolute* loss of AA by ≤20% in reference SELF and SELF containing PM, compared to the other two sample treatments. This indicates that the addition of EDTA/SSA helps stabilize AA. There was no considerable difference in the *absolute* abundance of the other target analytes, which is reasonable given that CSH and GSH underwent derivatization immediately following the incubation. In order to ensure the stability of pH in the SELF mixtures, in a separate experiment, we measured the pH in all reference SELF mixtures and SELF mixtures

containing PM (Table 1) over 180 min reaction time. The pH was found to be 7.3±0.1; hence, no noticeable effect of pH on the oxidative potential results could be anticipated.

3.2 Assay kinetics

The assay reaction kinetics were studied using SELF-a at PM concentration of 25 µg mL^{-1} (Fig. 3). The consumptions of antioxidants were similar in the first 60 min (33±2 µM; n = 3); however, beyond this time point, CSH and GSH diverged from AA at increasing rates, and this trend continued with the passing of time (Fig. 3). CSH and GSH showed similar consumptions that were constantly higher than those with AA, with a difference ranging between 3 and 47 µM. The highest consumption rates were found at the last time-step; these were 161±1 for GSH, 154±1 for CSH, and 114±1 µM for AA (Fig. 3).

As was seen with the consumption rates of CSH and GSH, the formation of CSSC and GSSG were similar up to 60 min time-step, i.e. ≤3 µM, after which they grew apart at increasing rates. The highest formations were seen at 240 min interval and were 24±0 and 46±1 µM, respectively (Fig. 3). Considering the molar concentrations of the redox pairs CSSC/CSH and GSSG/GSH, the observed formations of CSSC and GSSG were found to be noticeably lower than the theoretical values anticipated from reaction stoichiometry, i.e. 2 CSH → 1 CSSC and 2 GSH → 1 GSSG. This was more pronounced for CSH/CSSC (i.e. 6 → 1) than GSH/GSSG couple (i.e. 3 → 1). Such observations could be related to the formation of complexes between the organic molecules in PM and deprotonated –SH, prior to formation of CSSC or GSSG, such as glutathionylated quinone species (Song and Buettner, 2010).

3.3 Effect of PM concentration

The response characteristics of the assay to various doses of PM were studied using SELF-a (Table 1), PM concentration ranging from 10 to 80 µg mL^{-1}, and incubation time of 180 min. As shown in Fig. 4, the oxidative potential showed an increasing trend with PM concentration. GSH demonstrated the highest consumption, followed by CSH, and with a relatively larger gap by AA. The highest increase in oxidative potential was between PM concentration of 10 and 40 µg mL^{-1}, and this was relatively more pronounced for CSH and GSH (from 0.32 to 1 µM min^{-1}) than AA (from 0.17 to 0.55 µM min^{-1}). The CSH and GSH depletion rates were linear in the PM concentration of 10-40 µg mL^{-1}, and slowed down beyond this point; the two analytes were found completely consumed at PM concentration of ≥60 µg mL^{-1} (Fig. 4). Similar response patterns of CSH and GSH to PM suggests that CSH could be alternatively used to study the oxidative potential of ambient PM; this has not been considered in the past. The AA depletion covered a larger linear range, i.e. up to PM concentration of 60 µg mL^{-1}, but it slowed down beyond this concentration, with the highest depletion (0.80 µM min^{-1}) found at 80 µg mL^{-1} PM concentration (Fig. 4). The linear response range of antioxidants could depend on variables such as PM composition and mass-size distribution. AA was shown to respond to both Fe and Cu, along with quinones, whereas GSH had a negligible response to Fe (Ayres et al., 2008).

Similar to antioxidants, the formation of CSSC and GSSG increased with PM concentration (Fig. 4) from 10 to 60 µg mL^{-1} and plateaued beyond this range. This plateau is explained by the complete loss of CSH and GSH at PM concentration of ≥60 µg mL^{-1}. The CSSC and GSSG formations were linear up to 40 µg mL^{-1} PM concentration, in agreement with the depletion values seen for CSH and GSH.

5   We estimated the theoretical redox state of CSH/CSSC and GSH/GSSG redox pairs (Eh) using the Nernst equation (Eq. 1 and 2 in Sect. 2.4; Jones et al., 2000; Iyer et al., 2009). The results are presented in Fig. 4 as Eh$_\Delta$ (i.e. Eh$_{PM}$ – Eh$_{REF}$) for consistency with the calculation and presentation of antioxidant depletion rates. As shown in the figure, Eh$_\Delta$ increased with PM concentration and oxidative potential, and the overall patterns followed those of CSH and GSH depletion. This demonstrates that the oxidative potential of ambient PM can be presented as Eh, which may be more

10   meaningful because Eh represents a redox couple as opposed to a single antioxidant.

3.3.1 Method application to PM$_{2.5}$

In order to validate the current method with contemporary ambient samples, we applied the method to PM$_{2.5}$ samples collected from the NAPS station in Hamilton (Fig. 5, x-axis A-C). The samples were incubated in SELF-a for the duration of 180 min. As shown in the figure, the AA, CSH, and GSH mean consumption rates were 0.29-0.48 (RSD:

15   ≤13%), 0.39-0.65 (RSD: ≤1%), 0.40-0.64 (RSD: ≤2%) µM min^{-1}, respectively. The CSSC and GSSG formation rates were 0.05-0.10 (RSD: ≤3%) and 0.12-0.18 (RSD: ≤5%) µM min^{-1} (Fig. 5). The Eh$_\Delta$ calculated for CSH/CSSC and GSH/GSSG redox couples with the PM$_{2.5}$ samples were 46-100 and 37-81 mV; their patterns followed closely those of CSH and GSH depletion (Fig. 5), as we have seen with the results of the standard reference material in Sect. 3.3. Similarly, CSSC and GSSG formations were lower than anticipated given the reaction stoichiometry, as we also found

20   with the kinetic study (Sect. 3.2), with CSH/CSSC and GSH/GSSG molar ratios of 7.2±1.1 and 3.5±0.1, respectively. AA did not follow the CSH and GSH depletion patterns, and consequently the Eh$_\Delta$ (Fig. 5), reaffirming that AA and thiol antioxidants respond to different PM constituents and emission sources (Ayres et al., 2008; Fang et al., 2016; Weichenthal et al., 2019).

3.4 Influence of SELF composition

25   The effect of SELF composition on oxidative potential was examined using SELF formulations that are listed in Table 1. As shown in Fig. 6 and S1a-b, the addition of UA (SELF-b) did not noticeably affect the depletion of antioxidants or formation of CSSC; however, the mean formation rate of GSSG was found to be 0.05 µM min^{-1} higher in SELF-b than SELF-a. Regardless, this did not result in a difference of Eh$_\Delta$ (Fig. S1b). The presence of additional inorganic salts, CaCl$_2$, MgCl$_2$, and Na$_2$SO$_4$ (SELF-c) led to an increase in the depletion of CSH and GSH by ≤0.18 µM min^{-1}

30   (Fig. 6) and formation of CSSC by 0.03 µM min^{-1} (Fig. S1a), and this change resulted in 13 mV increase in the Eh$_\Delta$ (Fig. S1b). This observation may be related to enhanced electron circulation in the redox system due to the presence of additional electrolytes, or due to an increase in the SELF ionic strength, enhancing the solubility of PM-bound transition metals. Interestingly, AA showed an inverse response to this change in SELF formulation, and its depletion was 0.09 µM min^{-1} smaller in SELF-c than in SELF-b. Further changes in AA depletion due to variation in SELF

composition were not observed (Fig. 6). In contrast, the addition of ELF surfactant lipid DPPC (SELF-d) did not result in a noticeable change in OP (Fig. 6 and S1a-b), whereas we observed a small decrease in oxidative potential with the addition of ELF proteins albumin and glycine (SELF-e; $\leq$0.12 µM min^{-1} decrease in CSH and GSH depletion and $\leq$6 mV decrease in Eh$_\Delta$). The addition of DPPC, however, led to slightly better reproducibility of antioxidant depletion rates (Fig. 6), which is consistent with previous reports (Calas et al., 2017). Overall, our results show that, considering the characteristics of PM used in the present study and with the exception of AA, the oxidative potential that is measured through depletion of antioxidants can be $\leq$28% higher in the presence of a SELF which contains additional inorganic salts and lipid, or $\leq$13% lower following the addition of proteins. This suggests that, in order to determine a more realistic oxidative potential, a SELF that better represents the true human ELF should be used, rather than the simplified formulas that have been used in the past.

3.5 Comparison with the dithiothreitol assay

In order to evaluate the performance of the current method, we tested the SRM sample with the dithiothreitol assay and concentrations of 25 to 100 µg mL^{-1}. The method details and results are presented in the Supplement (Sect. S1 and Fig. S2). As can be seen in Fig. S2, the dithiothreitol assay showed a positive near-linear dose-response relationship, similar to our observation with the antioxidant assay. The mean dithiothreitol consumption ranged from 0.02 to 0.06 µM min^{-1}. Evidently, the consumption rates were considerably lower (nearly an order of magnitude) than the rates found for antioxidants AA, CSH, and GSH, which were determined with the chromatographic method. This difference may be related to the relatively low response of dithiothreitol assay to the transition metals present in the PM, as previously reported (Ayres et al., 2008; Charrier et al., 2014). Moreover, the dithiothreitol results showed relatively lower reproducibility (RSD: $\leq$45%) at the studied PM concentration range when compared to antioxidant assay data. This is not surprising considering the previous results from the dithiothreitol assay in the literature (Charrier and Anastasio, 2012; Fang et al., 2016; Crobeddu et al., 2017). Additional work is being conducted to compare the reaction kinetics between the two assays.

4 Conclusions

In summary, we developed a method that uses UHPLC-MS/MS to measure the oxidative potential of atmospheric PM. As new features, we included CSH/CSSC redox pair, involved physiologically relevant ELF components, i.e. inorganic salts, a phospholipid, and proteins, and explored the SELF redox state (Eh) following the addition of PM. The developed method showed high reproducibility of the measured oxidative potential, both inter- and intra-sample, when compared to previous methods, highlighting the importance of derivatization when analyzing thiol-antioxidants. The depletion rates of antioxidants, the formation rates of oxidation products and, consequently, the Eh increased with PM concentration in SELF. The method was further validated using contemporary PM$_{2.5}$ samples, and the results reaffirmed that CSH and GSH have similar depletion rates and patterns, which are different from those found with AA. This similarity suggests that CSH and its redox couple CSSC can be used as alternative target chemicals when determining the oxidative potential of ambient PM. One advantage of CSH/CSSC is that their labelled standards are

more readily available. We found that the dithiothreitol response to PM could be up to an order of magnitude lower than the responses seen with the antioxidant assay. This study showed that the presence of additional electrolytes could influence the measured oxidative potential in various ways, increasing the oxidation of thiol-containing antioxidants and decreasing that for AA, whereas proteins may result in a moderate decrease in antioxidant depletion in the presence of PM. Further work needs to be done to realize the potential of this method by including samples that represent the chemical composition of various types of atmospheric aerosols and their spatial and temporal variations.

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Table 1. Compositions of SELF used in this study

	SELF-a	SELF-b	SELF-c	SELF-d	SELF-e	mg L^{-1}
NaCl[a]	*	*	*	*	*	8065
KCl[a]	*	*	*	*	*	201
Na$_2$HPO$_4$[a]	*	*	*	*	*	1420
CaCl$_2$[b]			*	*	*	256
MgCl$_2$[b]			*	*	*	200
Na$_2$SO$_4$[b]			*	*	*	72
AA[c]	*	*	*	*	*	35
CSH[c]	*	*	*	*	*	24
GSH[c]	*	*	*	*	*	62
UA[b]		*	*	*	*	16
DPPC[b]				*	*	100
Albumin[b]					*	260
Glycine[b]					*	376

[a] composition of standard PBS used (pH 7.4); [b] follows reported physiological concentrations (Boisa et al., 2014); [c] 200 µM of each antioxidant was used; SELF: simulated epithelial lining fluid, AA: ascorbic acid, CSH: cysteine, GSH: glutathione, UA: uric acid, DPPC: dipalmitoylphosphatidylcholine

Table 2. LC-MS/MS detection parameters for target substances

Analytes	Parent ion m/z	Daughter ion m/z	Collision energy	Cone voltage	Retention time (min)
AA	175	115	12	6	0.62
AA-^{13}C$_6$	181	119	12	6	0.62
CSH-NEM	247	158	22	14	1.61
CSH-^{13}C$_3$-NEM	250	158	20	25	1.61
CSSC	241	120	18	2	0.62
CSSC-d$_6$	247	155	14	18	0.61
GSH-NEM	433	201	20	10	1.61
GSH-^{13}C$_2$^{15}N-NEM	436	201	22	26	1.61
GSSG	613	355	22	18	1.60
GSSG-^{13}C$_4$^{15}N$_2$	619	361	22	18	1.60

AA: ascorbic acid, CSH: cysteine, CSSC: cystine, GSH: glutathione, GSSG: glutathione disulfide, NEM: *N*-ethylmaleimide
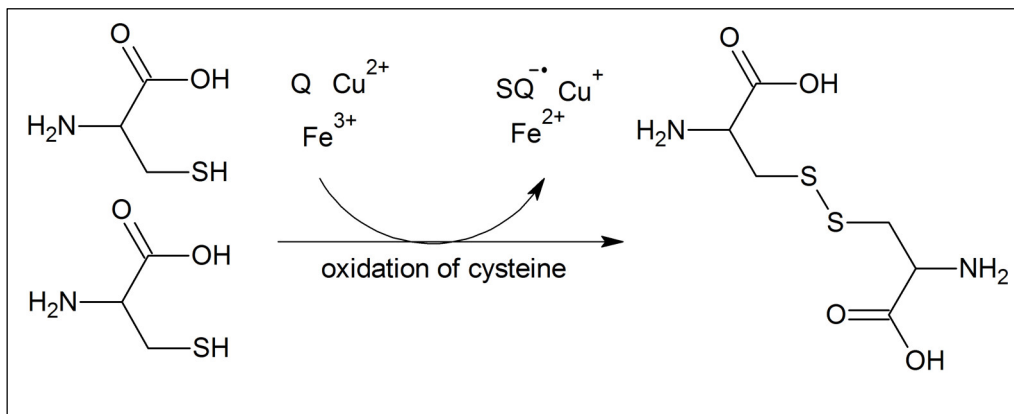
Figure 1. Oxidation of CSH through reaction with quinones (Q) and transition metals (Fe^{3+}, Cu^{2+}) and subsequent formation of CSSC, semi-quinones ($SQ^{-\bullet}$) and reduced transition metals (Fe^{2+}, Cu^+)
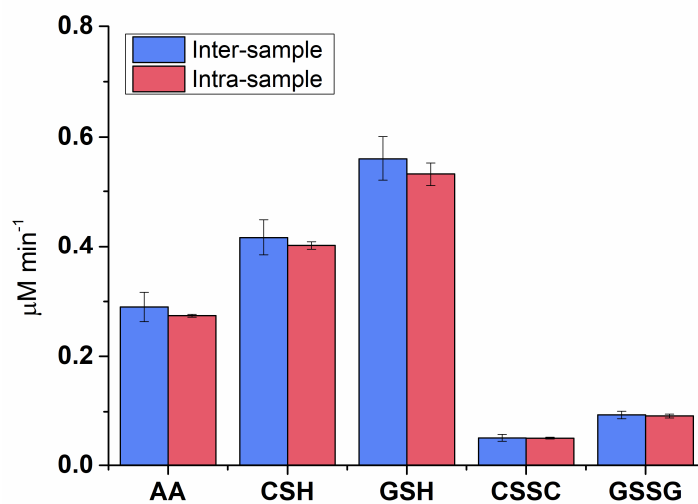
Figure 2. Depletion of antioxidants, AA, CSH, and GSH, and formation of oxidation products CSSC and GSSG ==in SELF-a containing 20 μg mL^{-1} of SRM and following incubation time of 180 min.== *Inter*-sample reflects the reproducibility of entire method ($n = 4$), whereas *intra*-sample shows the post-incubation variability due to sample processing (from replicates of a single sample; $n = 3$)
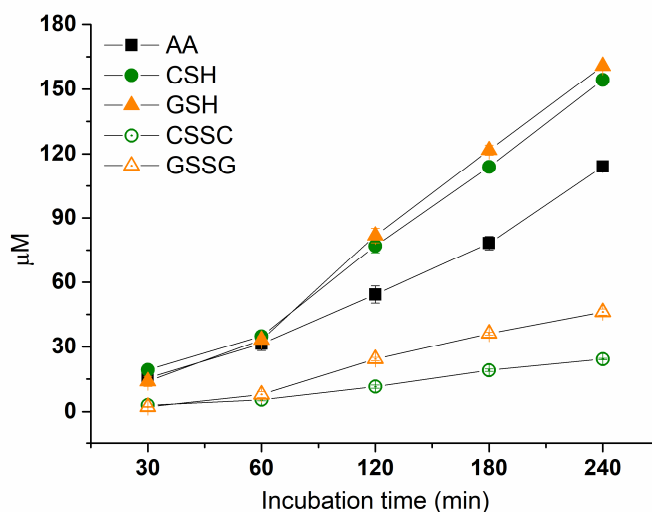
Figure 3. Time-dependent depletion of antioxidants AA, CSH, and GSH, and formation of oxidation products CSSC and GSSG in SELF-a containing 25 µg mL⁻¹ of SRM
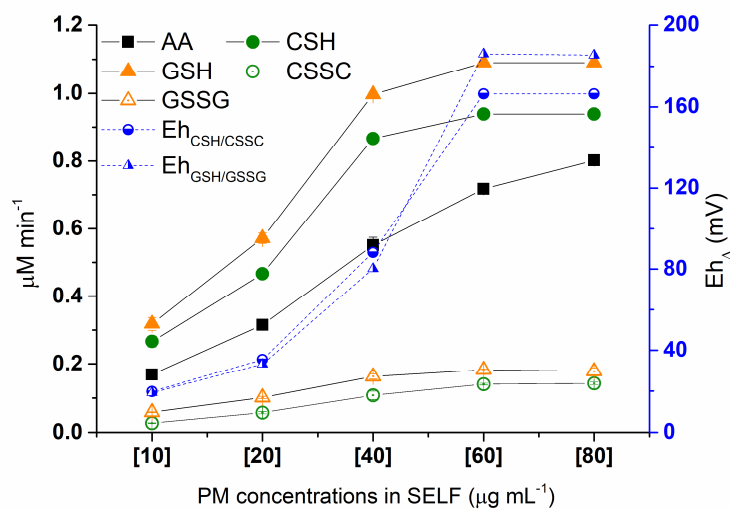
Figure 4. Dose-response of antioxidants AA, CSH, and GSH depletion and oxidation products CSSC and GSSG formation due to increase in SRM concentration in SELF-a (left y-axis) following 180 min incubation; corresponding changes in redox state of SELF calculated using Nernst equation (Eh; right y-axis), where Δ denotes the difference in Eh between SELF containing PM and reference SELF
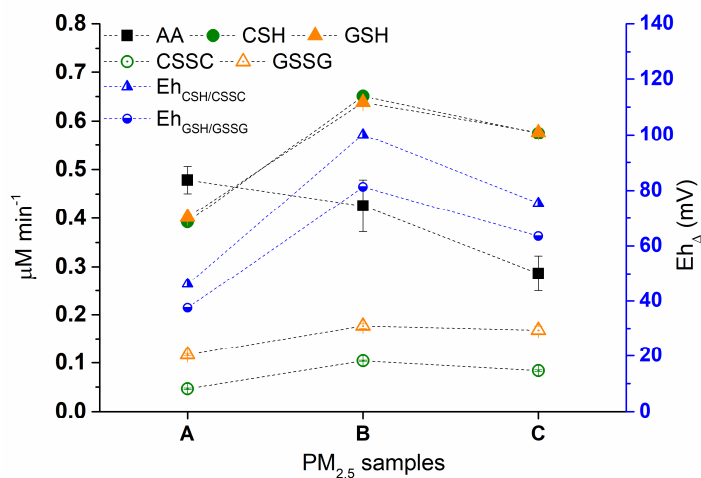
Figure 5. Application of antioxidant assay to PM₂.₅ samples (A-C) from NAPS station in Hamilton. Samples were incubated in SELF-a for 180 min. The lines connecting the markers are used to indicate the trends among the variables
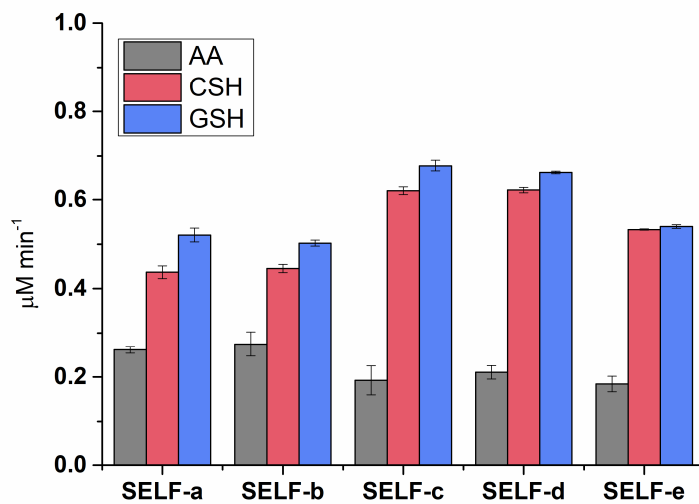
Figure 6. The effects of SELF composition on antioxidant depletion rates in the presence of 20 μg mL⁻¹ SRM following 180 min incubation. The details of SELF formulations are given in Table 1