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## A comparative study of methods for calculating the oxidative potential (OP) of atmospheric particulate matter†

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The oxidative potential (OP) of particulate matter (PM) is a pivotal metric to evaluate the potential health effects of air pollution. However, the variety of assays and protocols available to measure the OP poses a challenge for comparing one study with another. The present study aims to provide an analysis comparing four calculation methods for determining the OP. These methods include the use of calibration curves (CURVE), absorbance values (ABS), and two concentration-based (CC1 and CC2) methods. Two acellular assays, dithiothreitol (DTT; OP<sup>DTT</sup>) and ascorbic acid (AA; OP<sup>AA</sup>), were chosen to be examined. The application of these assays led to varying OP results depending on the applied calculation method. Regarding results, first of all, there is a notable agreement between the ABS and CC2 methods across both the DTT and AA assays. Second, however, for both assays, the CC1 method consistently leads to higher OP values, with OP<sup>DTT</sup> variations of up to 18% compared to ABS and CC2, and OP<sup>AA</sup> variations of up to 12%. Third, the CURVE method yields OP<sup>DTT</sup> and OP<sup>AA</sup> values that are up to 10% and 19% higher than those calculated by the ABS and the CC2 methods, respectively. Therefore, both the ABS and CC2 methods are recommended for calculating OP values, as they have shown better consistency across different PM samples. These findings underscore the importance of defining standardizing OP protocols which should explicitly include all needed calculation steps in order to further develop the OP metric into a comparable measure linking air quality and human health.

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### Environmental significance

Assessing the oxidative potential (OP) of particulate matter (PM) is crucial for understanding its role in air pollution-related health effects. However, variations in calculation methods can lead to discrepancies in reported OP values, limiting comparability across studies. This work highlights how different computational approaches influence OP estimates and identifies the methods that yield more consistent results. Establishing standardized OP calculation protocols is critical to enhancing the reliability of this metric, ultimately improving its applicability in air quality assessments and public health research. By refining OP quantification, this study contributes to a more robust framework for evaluating the oxidative stress burden associated with PM exposure.

## 1. Introduction

Oxidative potential (OP), the ability of particulate matter (PM) to induce oxidation in the lung environment, is increasingly used as a metric to assess health effects of air pollution.<sup>1,2</sup> Researchers worldwide have extensively explored methodologies for OP assays, recognizing them as more precise measures for predicting the toxicity of aerosol particles.<sup>3–7</sup> So far, the

capacity of aerosol species to catalyze redox reactions and influence the formation of reactive oxygen species (ROS) has been widely investigated in many studies using chemical acellular assays.<sup>8–13</sup> The variability in OP measurements can be attributed to several factors, including the chemical composition of PM,<sup>14–16</sup> emission sources,<sup>17–19</sup> chemical interactions,<sup>20–22</sup> size-segregated PM,<sup>23</sup> reactant concentrations,<sup>24</sup> and operating conditions,<sup>25</sup> all of which have been investigated.

The assessment of aerosol particle toxicity based on oxidative potential assays was initially conducted in the early 2000s.<sup>26,27</sup> Since then, researchers worldwide have developed, optimized and applied a variety of methodologies to determine the OP of PM. Common acellular methods include the dithiothreitol (DTT) assay (OP<sup>DTT</sup>), the ascorbic acid (AA) assay (OP<sup>AA</sup>), the glutathione (GSH) assay (OP<sup>GSH</sup>) and other acellular

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assays.<sup>28,29</sup> Due to the catalytic activity of redox-active PM species in the presence of antioxidants, redox reactions combined with absorbance measurement routines are used to determine the consumption rate of reducing agents, thereby measuring the dynamics of OP values based on air volume and sampled mass normalization.<sup>28,29</sup> However, previous studies have not examined the mathematical approaches to derive OP values from measurements obtained with different assays, and apparently, there is no consensus on the optimal method to determine the OP associated with PM and redox-active species capable of depleting antioxidants. Many of the current protocols for measuring OPs involve the use of known concentrations of reducing agents, such as DTT or AA, incubated with PM extracts. OP values are then deduced from the consumption of the reagents over time, using various mathematical methods. These include calibration curves (CURVE),<sup>30–34</sup> absorbance values linked to consumption rates (ABS)<sup>35–37</sup> or concentration values associated with the decay kinetics of DTT or AA consumption (CC1 and CC2).<sup>16,38–41</sup>

Although previous studies have explored the uncertainties in OP assays by investigating the variability of OP values resulting from factors such as experimental repeatability, regression curves, and operational procedures,<sup>35</sup> no studies have specifically addressed the variability in OP values resulting from different calculation methods. In this study, a critical comparison of different mathematical approaches for estimating the OP of PM is performed. A review of 130 publications resulted in identifying at least four distinct approaches. The methods are applied on specific data sets generated with both the OP<sup>DTT</sup> and OP<sup>AA</sup> assays, as these are the most prominently used assays in the domain. The primary objective of the present study is to assess the variability across different calculation methods for different assays, with the aim of establishing a standardized protocol for OP quantification as a common final step of all OP assays. This will help ensure more consistent and reliable measurements contributing to a better understanding of the toxic potency of aerosols. By promoting the adoption of uniform OP calculation methods, this research should enable more meaningful comparisons across global studies, fostering collaboration and advancing the field of environmental health research.

## 2. Materials and methods

### 2.1. Oxidative potential assays, a kinetic measurement with computational implications

Most oxidative potential measurements are based on the consumption of antioxidants or chemical surrogates (*e.g.*, OP<sup>AA</sup>, OP<sup>DTT</sup>, and OP<sup>GSH</sup>) or the evaluation of ROS production (*e.g.*, OP<sup>OH</sup>) by a PM sample. These analytical protocols involve multiple absorbance measurements over a period of 15 to 45 minutes to evaluate the kinetics of the reaction between the sample and the reactants. The rate of consumption of reducing agents, such as DTT and AA, is determined by applying linear regression to the absorbance data (which is proportional to the concentration loss of DTT or AA) as a function of incubation time. This analysis yields the regression slope and intercept,

which are used to assess the reactivity of the PM samples. Thus, the slope is consistently used in various calculation methods to derive OP values, which are then normalized based on mass and volume. Specifically, the slope corresponds to the rate constant ( $k$ ), which indicates the rate at which the concentrations of reductants (*e.g.*, DTT and AA) decrease over time, in proportion to their respective concentrations, as shown in eqn (1).

$$-\frac{d[\text{reductant}]}{dt} = k'[\text{reductant}]^a[\text{PM}]^b \quad (1)$$

where  $k' = k[\text{O}_2]$ , with  $k'$  representing the rate constant for DTT or AA consumption and “a” and “b” are the reaction orders with respect to DTT or AA and PM, respectively.<sup>42–44</sup> In this context, the concentration of dissolved oxygen is considered negligible, as air is in continuous contact with the surface of the solution throughout the OP assays. However, by maintaining an excess concentration of DTT and AA during incubation with PM components, their influence on the reaction rate becomes negligible and the reaction simplifies to eqn (2).

$$-\frac{d[\text{reductant}]}{dt} = k''[\text{PM}]^b \quad (2)$$

Hence, the rate law simplifies to a dependence solely on the concentration of PM, with the reaction order now being  $b$ . Notably, the presence of reactive PM species increases the overall oxidation of DTT and AA, reflected in a higher  $k$ . Over prolonged incubation periods, the consumption of DTT and AA may become more gradual, leading to a reduction in apparent  $k$ , which is better explained by a pseudo-first-order reaction.<sup>43–47</sup> This has significant implications for routine OP assays, especially when very low concentrations of DTT and AA are used to incubate PM extracts, as it directly affects the reaction order of the OP assay. Another important consideration is the determination of OP values for PM samples with high reactivity, which, in turn, affects the apparent  $k$  and influences the linearity of DTT and AA consumption.

It should be noted that endpoint measurement protocols are also used to determine the oxidative potential based on the initial and final absorbance values.<sup>48,49</sup> However, these protocols do not guarantee linear consumption of reducing agents, leading to significant variations in the measured OP since they typically involve long incubation periods. To address this, initial reaction rates are measured by collecting data at short intervals after the reaction begins to ensure linearity.<sup>13</sup> Calibration curves and appropriate controls are used for accurate quantification of OP<sup>DTT</sup> and OP<sup>AA</sup>, along with critical monitoring of blank absorbance values, especially at time 0. Conditions are optimized to prevent rapid DTT and AA consumption, and automated systems are employed to improve precision and reproducibility.<sup>5</sup>

### 2.2. PM samples

The present study examined six PM<sub>10</sub> samples (#A, #B, #C, #D, #E, and #F) to investigate the variability between different mathematical approaches for estimating OP<sup>DTT</sup> and OP<sup>AA</sup> values. Since this study focuses on method comparability by



analyzing three distinct sources, the small sample size employed here is sufficient to ensure the variability in the OP values across different mathematical approaches. PM samples #A and #B were collected during Saharan dust events, samples #C and #D were collected under European urban conditions, and samples #E and #F were collected during winter in regions affected by biomass burning in Europe. Samples were collected using quartz fiber filters for 24 h of sampling (DA-80 devices, 720 m<sup>3</sup>).

### 2.3. PM extraction and iso-concentration of particles

All PM samples were extracted at 37.4 °C using a combination of dipalmitoyl phosphatidylcholine (DPPC) and Gamble's solution to create a respiratory simulated lung fluid (RSLF).<sup>41</sup> To ensure comparability of the experiments, extractions at iso-concentration of the particles were performed at 25 µg mL<sup>-1</sup> and then incubated with DTT or AA.<sup>50</sup>

### 2.4. Dithiothreitol assay (OP<sup>DTT</sup>)

The OP<sup>DTT</sup> assay was investigated through TNB<sup>2-</sup> formation, which involved multiple absorbance measurements using a TECAN Infinite® M200 Pro spectrophotometer and 96-well CELLSTAR® Multiwell plates from Greiner Bio-One®. The reaction mixture contained 225 µL of phosphate buffer solution (PBS; Carl Roth GmbH + Co KG Karlsruhe, Germany), 50 µL of 250 µM DTT (CAS: 3483-12-3; Carl Roth GmbH + Co KG Karlsruhe, Germany) in PBS, and 20 µL of PM extraction. TNB<sup>2-</sup> formation was monitored at 412 nm with the addition of 50 µL of DTNB (CAS: 69-78-3; Carl Roth GmbH + Co KG Karlsruhe, Germany) at 0, 15, and 30 minutes of incubation. OP<sup>DTT</sup> measurements were carried out in triplicate. Blank measurements ( $n = 6$ ) were carried out using the same protocol as previously described for PM samples. Positive controls consisted of monitoring the OP of 1,4-NQ (24.7 µM) to evaluate measurement quality and reproducibility. The coefficient of variation (COV; %) ranged between 2 and 8% ( $n = 8$ ). Further details can be found in Calas *et al.*<sup>41</sup> and Dominutti *et al.*<sup>18</sup>

### 2.5. Ascorbic acid assay (OP<sup>AA</sup>)

The depletion of AA was investigated using the same instrument as for the DTT assay. A redox reaction was performed by incubating 100 µL of 240 µM AA and 80 µL of the PM extract (25 µg mL<sup>-1</sup>) for 32 minutes. Absorbance measurements were taken every 4 minutes (at 0, 4, 8, 12, 16, 20, 24, 28, and 32 minutes) at 265 nm. The chemical controls involved monitoring and assessing the measurement quality and reproducibility using 1,4-NQ (24.7 µM). The COV ranged between 1 and 5% ( $n = 6$ ). Additional information on the OP<sup>AA</sup> assay can be found in the studies by Marsal *et al.*<sup>3</sup> and Borlaza *et al.*<sup>50</sup> All OP<sup>AA</sup> measurements were carried out in triplicate.

### 2.6. Mathematical approaches for quantifying OP<sup>DTT</sup>

The present study evaluates four mathematical approaches for assessing the OP of PM, identified through a comprehensive literature review (see Section 3.1: Calculation methods for

quantifying oxidative potential). The OP values calculated using these different approaches—including the calibration curve (CURVE), absorbance-based (ABS), and two concentration-based methods (CC1 and CC2)—were initially assessed as the activity rate (nmol min<sup>-1</sup>). These values were then normalized further to account for 1 µg of incubated mass (nmol min<sup>-1</sup> µg<sup>-1</sup>) and 1 m<sup>3</sup> of air volume (nmol min<sup>-1</sup> m<sup>-3</sup>).

For all calculation methods, background absorbance values obtained from blank measurements were subtracted from the absorbance values of the PM samples prior to the calculation step. In addition, we carefully monitored the background absorbance and the autoxidation of DTT and AA in both OP assays, placing particular emphasis on accurately assessing these parameters to ensure data reliability.

**2.6.1. Calibration curve (CURVE) method.** For the OP<sup>DTT</sup>, an analytical curve was constructed using values ranging from 0 to 140 µM ( $N = 6$ ; six concentration levels of DTT) TNB<sup>2-</sup> produced during the DTT and DTNB redox reaction.<sup>13</sup> To examine the impact of different DTT concentrations on the determination of OP<sup>DTT</sup> values, we investigated additional ranges, including 0–100 µM ( $N = 5$ ) and 0–60 µM ( $N = 4$ ). These concentration ranges have been used in previous studies, as described by Yu *et al.*<sup>10</sup> and Dominutti *et al.*<sup>5</sup> For OP<sup>AA</sup>, the AA concentration ranged from 0 to 240 µM ( $N = 6$ ) to facilitate comparison with other calculation methods. Additionally, calibration curves were constructed using different AA concentration ranges, including 0 to 192 µM ( $N = 5$ ) and 0 to 144 µM ( $N = 4$ ), to investigate the impact of varying AA concentration ranges on the determination of OP<sup>AA</sup> values. In this study, we investigated the effect of varying the number of data points selected from the full calibration curves (DTT: 0–140 µM; AA: 0–240 µM) on the slope and intercept of the regression and consequently on the calculated OP values.

Using the CURVE method, the OP is obtained as follows:

(i) An absorbance *vs.* (DTT/AA) concentration regression curve is established, and both the slope ( $m$ ) and intercept ( $c$ ) values are determined.

(ii) During the incubation of the samples with (DTT/AA) in the respective assays, the absorbance values at each incubation time point ( $A_t$ ) are recorded.

(iii) The absorbance ( $A_t$ ) is converted into a (DTT/AA) concentration using the calibration curve parameters obtained in (i) above.

(iv) The OP is determined as the rate of change of (DTT/AA) concentration over time.

(v) The value is multiplied by the solution volume ( $V$ ), to obtain the OP values (nmol min<sup>-1</sup>).

The determination of OP values using the CURVE method can be represented as follows:

$$OP_{\text{CURVE}} = -V \frac{d}{dt} \left( \frac{A_t - c}{m} \right) \quad (3)$$

Further features of the CURVE method are discussed in other studies.<sup>30–34</sup>

**2.6.2. Absorbance-based (ABS) method.** The determination of OP values using the ABS method is described as follows:



(i) Absorbance values related to DTT/AA consumption are recorded every 5, 10, or 15 min for the DTT assay and every 4 or 5 min for the AA assay.<sup>3,16</sup>

(ii) A calibration curve of absorbance values *versus* time is established, and both the slope and intercept are determined.

Accordingly, the OP (nmol min<sup>-1</sup>) using the ABS method is determined as follows (eqn (4)):

$$OP_{\text{ABS}} = -V \frac{[\text{reductant}]_0}{p} \frac{dA_t}{dt} \quad (4)$$

Here,  $p$  refers to the intercept of absorbance *versus* time,  $[\text{reductant}]_0$  to the initial concentration of DTT or AA (in mol L<sup>-1</sup>),  $V$  is the solution volume (L), and  $A_t$  to the measured absorbance values.<sup>35–38</sup>

**2.6.3. Concentration-based method 1 (CC1).** DTT and AA consumption rates can also be quantified using the CC1 method, which include the following procedural steps:

(i) Replicate absorbance measurements of blank field filters are performed shortly after the addition of DTT and AA and represent the absorbance at time 0 ( $A_{B_0}$ ).

(ii) Absorbance values are measured at equal time intervals after adding DTT or AA ( $A_t$ ), during a controlled incubation time.

(iii) The OP values in nmol min<sup>-1</sup> are determined according to eqn (5):

$$OP_{\text{CC1}} = -V \frac{[\text{reductant}]_0}{A_{B_0}} \frac{dA_t}{dt} \quad (5)$$

In this method,  $[\text{reductant}]_0$  is the initial concentration of DTT or AA (in mol L<sup>-1</sup>) and  $V$  is the solution volume (L).<sup>12,39</sup>

**2.6.4. Concentration-based method 2 (CC2).** The OP is obtained using the following steps:

(i) Absorbance values for the PM samples at a given incubation time ( $A_t$ ), as well as the absorbance values for the PM samples at time 0 ( $A_{\text{PM}_0}$ ), are measured.

(ii) The consumption of DTT and AA is determined by measuring absorbance at specific time intervals during the incubation period. For the DTT assay, measurements are typically taken every 5, 10, or 15 minutes, while for the AA assay, intervals of 4 or 5 minutes are commonly used.<sup>3,16</sup> These intervals are chosen to effectively monitor the reaction kinetics and accurately assess the consumption rates of DTT and AA.

For the OP determination (nmol min<sup>-1</sup>), as proposed by eqn (6), the  $[\text{reductant}]_0$  is the initial DTT or AA concentration (in mol L<sup>-1</sup>) and  $V$  is the solution volume (L).<sup>16,40,41,51</sup>

$$OP_{\text{CC2}} = -V \frac{[\text{reductant}]_0}{A_{\text{PM}_0}} \frac{dA_t}{dt} \quad (6)$$

## 2.7. Statistical evaluation

A comprehensive statistical analysis was conducted to assess the variability in OP values derived from the four mathematical approaches (CURVE, ABS, CC1, and CC2). The COV was calculated to quantify the relative variability of OP values, providing a standardized measure of dispersion. Discrepancies between methods were quantified by comparing individual OP values to the overall OP mean. Analysis of variance (ANOVA) was used to determine

statistically significant differences among methods, incorporating replicate measurements. Paired  $t$ -tests and associated  $p$ -values were employed to examine OP value variations across calibration curve ranges and to further investigate the differences between the calculation methods. The mean and median were also used to compare OP values obtained from each method.

## 3. Results and discussion

### 3.1. Calculation methods for quantifying the oxidative potential

The mathematical approaches presented in the previous section are designed to investigate the linear consumption rate of DTT or AA during incubation with redox-active PM species. To fulfill linearity, the methods are applied for protocols with short incubation times, typically less than 45 minutes. However, some studies have used alternative assays that extend the incubation time, typically to four hours, and have used additional mathematical approaches to assess the OP of PM.<sup>42,43,52–55</sup> These extended incubation methodologies are not discussed in the present study, as differences in incubation times potentially affect the dynamics of the redox mechanism and linearity. This issue alone might warrant a separate review-like treatment. Based on OP studies conducted over the past five years (2020–2024), the various mathematical approaches applied for determining OP are grouped to provide a comprehensive overview of the methodologies. Studies lacking sufficient information about their calculation methods are classified as “unknown.”

A review of 130 peer-reviewed papers within this period revealed that the CC2 method (39 publications, 30%) and the ABS method (34 publications, 26.1%) were the most commonly employed approaches for assessing the oxidative potential of particulate matter (Fig. 1). Other OP studies used calibration curves to investigate the rate of DTT or AA consumption; however, this approach was less common, appearing in only

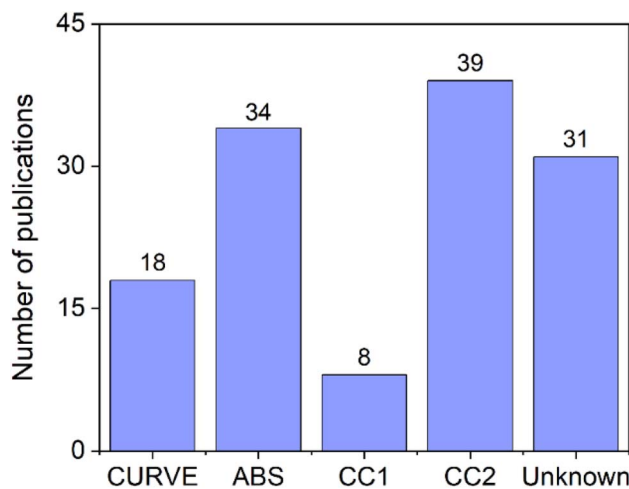


Fig. 1 Number of peer-reviewed publications addressing different calculation methods used to estimate OP values in the scientific literature over the past 5 years ( $n = 130$ ), including previous citations and others.<sup>56–171</sup>



13.8% (18 publications) of the OP studies. Additionally, about 6% (8 publications) of the studies applied the CC1 method in DTT or AA assays. A substantial portion, 23.8% (31 publications), lacked sufficient information to be classified within the categories presented in the previous section.

### 3.2. Variability of $OP^{DTT}$ values across different mathematical methods

**3.2.1. Mass-normalized ( $OP_m^{DTT}$ ) and volume-normalized ( $OP_v^{DTT}$ ) values.** Determination of  $OP^{DTT}$  values normalized by mass and volume was carried out using the four mathematical approaches described above: CURVE, ABS, CC1, and CC2. The values of both  $OP_m^{DTT}$  and  $OP_v^{DTT}$  are shown in Fig. 2(a and b) and also in Table S1 in the ESI.† Similar patterns are observed for  $OP_m^{DTT}$  and  $OP_v^{DTT}$  in terms of variability introduced by the use of different calculation methods. For different PM samples, higher  $OP_m^{DTT}$  is observed for #E and #F (influenced by biomass burning), followed by #C and #D (urban areas with traffic and anthropogenic activities), and finally #A and #B (dominated by mineral dust). In terms of  $OP_v$ , higher values are observed for #B, followed by #E, #F, and #A and finally #C and #D.

When comparing the OP values from different calculation methods, CC1 yields significantly higher results. For instance, in the case of sample #F, the  $OP_m^{DTT}$  values are roughly 25% higher (0.099  $\text{nmol min}^{-1} \mu\text{g}^{-1}$ ) than those obtained using the ABS and CC2 methods. For  $OP_v^{DTT}$ , the ABS and CC2 methods yielded values of 4.24 and 4.19  $\text{nmol min}^{-1} \text{m}^{-3}$ , respectively, while CURVE and CC1 methods result in values of 4.59 and 5.57  $\text{nmol min}^{-1} \text{m}^{-3}$ , respectively. Therefore, both ABS and CC2 methods tend to produce comparable  $OP_m^{DTT}$  and

$OP_v^{DTT}$  values, whereas the CURVE and CC1 methods typically result in higher OPs. A detailed comparison of  $OP_m^{DTT}$  and  $OP_v^{DTT}$  values is presented in Table S1† for all the investigated samples, including information on their statistics, mean, median, and COV.

**3.2.2. Differences in relation to the overall  $OP^{DTT}$  mean.** To assess the variability of  $OP^{DTT}$  values across different calculation methods, we compare the overall OP mean values—calculated as the average of the OP values from all four methods—for each sample with the OP values obtained from each method. The comparisons are given in percentages, as shown in Fig. S1.† The results indicate substantial agreement between the ABS and CC2 methods, with both showing comparable mean and median OP values (Table S1†) and similar differences in relation to the overall OP mean. In contrast, the CURVE method yields OP values that differ by up to 10% from those obtained using the ABS and CC2 methods on average. This difference is statistically significant for  $OP_v^{DTT}$  ( $p < 0.05$ ) but not for  $OP_m^{DTT}$  ( $p > 0.05$ ) (Table S2†).

The ABS method leads to  $OP^{DTT}$  values differing from the overall OP mean by 4.0% to 11%, while the CC2 method shows deviations of 4.5% to 14% ( $p > 0.05$ ; Table S2†). Although both the ABS and CC2 methods exhibit similar profiles, the CC2 method reveals slightly higher variation compared to the ABS method. In contrast, the CURVE method exhibits the least variation, with the deviations in the OP values ranging between 1.1% and 5.7% relative to the overall OP mean.

The CC1 method displays the highest variability observed in this study, with variations of up to 27% (ranging from 14% to 27%). This indicates that CC1 provides OP values that

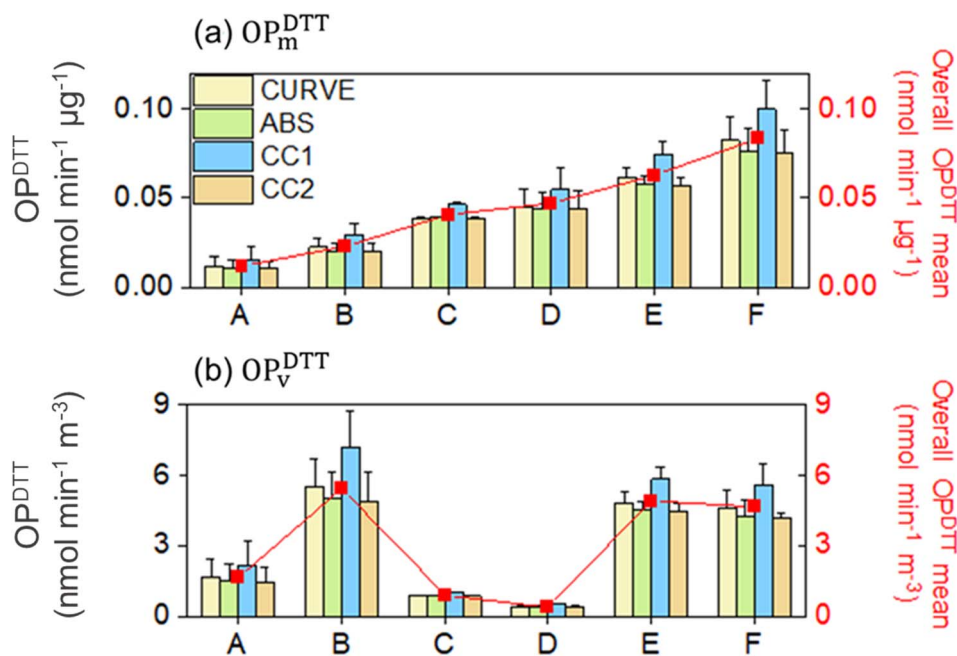


Fig. 2  $OP^{DTT}$  values normalized by both mass (a) and volume (b) for four distinct calculation methods: (first bar: CURVE (0–140  $\mu\text{M}$ ), second bar: ABS, third bar: CC1, and last bar: CC2), along with the overall  $OP^{DTT}$  mean per sample (the average of  $OP^{DTT}$  values calculated by all four methods). Six random PM samples (A, B, C, D, E, and F) were selected to show the variability between the mathematical approaches with a concentration of particles of 25  $\mu\text{g mL}^{-1}$ .  $OP^{DTT}$  measurements were performed in triplicate ( $n = 3$ ), and error bars show the standard deviation.



significantly differ from those obtained through other mathematical approaches ( $p < 0.05$ ; Table S2†). Notably, the parameters utilized for the OP determination in the CC1 method differ from the others, potentially exerting a substantial impact on the distribution of both  $OP_m^{DTT}$  and  $OP_v^{DTT}$  values.

The variability previously observed for  $OP_m^{DTT}$  remains for  $OP_v^{DTT}$  values, as shown in Fig. 2b, since both normalized OPs are primarily derived from the same OP values ( $\text{nmol min}^{-1}$ ). Moreover, the  $t$ -test reveals statistically significant differences for all methods except for the comparison between ABS and CC2, which yield similar  $OP_v^{DTT}$  and  $OP_m^{DTT}$  (Table S2†). To facilitate the interpretation and presentation of our findings, the following sections will focus on discussing the  $OP_m^{DTT}$  values.

In general, the descending order in both  $OP_m^{DTT}$  and  $OP_v^{DTT}$  values for the investigated methods is as follows: CC1 > CURVE > ABS  $\approx$  CC2.

**3.2.3. Intrinsic variability of  $OP_m^{DTT}$  values for each calculation method.** This section evaluates the impact of replicate measurements on the overall determination of OP, highlighting the variability observed in each calculation method due to differences in replicate absorbance values. Higher COVs are observed for the replicates of PM samples #A, #C, and #E when using the CC1 method, compared to the COV values for the CURVE, ABS, and CC2 methods (Table S1 and Fig. S2a†). This implies that the CC1 method shows larger variability relative to the  $OP_m^{DTT}$  mean for these samples, leading to less consistency in  $OP_m^{DTT}$  values among the replicates. On the other hand, higher COVs are observed for PM samples #B, #D, and #F when using the CC2 method (Fig. S2a†).

Although the differences in the mean values for these samples may be low, the relative variability of the results is higher for these PM samples. In other words, the COV for the CC2 method varies significantly between different PM samples, suggesting that its reliability is sample-dependent, as already observed for CC1. This implies that the variability observed in both the CC1 and CC2 methods may depend on the complex interplay of aerosol chemical species across different PM samples, or alternatively, may arise from the inherent effects of the variability caused by the distinct absorbance values at time zero, as incorporated within the framework of these methods.

The  $OP_m^{DTT}$  values of PM samples #A, #B, #D, and #F are not significantly different, regardless of the calculation method used and replicate values (one-way ANOVA,  $p > 0.05$  at the 0.05 level; Table S3†). This was different for the effects of replicate values on the  $OP_m^{DTT}$  of PM samples #C and #E, which showed significant differences between the mathematical approaches. This aligns well with the previous observation, where the high COVs for the CC1 method indicates general variability issues, while the varying COVs suggest that each method's relative performance can be significantly different depending on the PM sample. A complete assessment of the statistical differences between the calculation methods with consideration of their replicated OP values, including ANOVA and  $p$ -values is provided in Table S3(a–f)† for the DTT assay. It should be noted that significant variation already exists in calculating the OP using different methods as previously described. However, the variability increases further due to differences between replicates

for each PM sample, particularly for methods CC1 and CC2. In these cases, the variability, expressed here as the COV, is notably higher compared to those generated by other calculation methods.

**3.2.4. Variability of  $OP_m^{DTT}$  values using the CURVE method.** The calibration curve is generated with a DTT concentration range of 0 to 140  $\mu\text{M}$  ( $n = 6$ ) to allow comparison with the ABS, CC1, and CC2 methods. In this section, the variability of the OP values is evaluated using different ranges of DTT concentrations, including 0 to 100  $\mu\text{M}$  DTT ( $N = 5$ ) and 0 to 60  $\mu\text{M}$  DTT ( $N = 4$ ) (Fig. 3). Further details on the slope and intercept parameters are given in Table S4 in the ESI.†

A consistent pattern in the OP distribution emerges when varying the DTT concentration range between 0 to 100  $\mu\text{M}$  ( $N = 5$ ) and 0 to 140  $\mu\text{M}$  ( $N = 6$ ), as similar  $OP_m^{DTT}$  values are obtained for both ranges, indicating consistency across these calibration intervals. However, across all PM samples, markedly lower  $OP_m^{DTT}$  values are observed when using the calibration curve in the lower range of 0–60  $\mu\text{M}$  DTT ( $N = 4$ ), as shown in Fig. 3. Thus, the  $OP_m^{DTT}$  values calculated with the CURVE method based on the 0–60  $\mu\text{M}$  DTT calibration curve are, on average, up to 43% lower than the values derived either from 0 to 140  $\mu\text{M}$  DTT ( $p < 0.05$ ; Table S5a†) or from 0 to 100  $\mu\text{M}$  DTT ( $p < 0.05$ ; Table S5a†). This difference arises solely from the variation in calibration parameters (slope and intercepts) among the calibration curves used. As such, the slopes and intercepts play a key role in the observed differences in OP values when comparing different calibration curves. As evidenced in Table S4,† the differences in these parameters propagate through the calculation, ultimately resulting in substantial changes in the final  $OP_m^{DTT}$  values. Similar trends are observed for  $OP_v^{DTT}$ , as

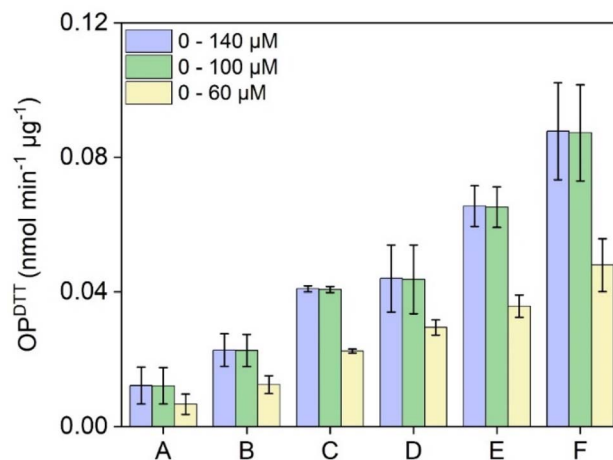


Fig. 3 Mass-normalized DTT activity ( $OP_m^{DTT}$ ) for the CURVE method across different DTT concentration ranges ( $\mu\text{M}$ ) for six PM<sub>10</sub> samples (A–F) at 25  $\mu\text{g mL}^{-1}$ .  $OP_m^{DTT}$  values were calculated using the slope and intercept of calibration curves with concentration ranges of 0–140  $\mu\text{M}$  (first bar;  $n = 6$ ), 0–100  $\mu\text{M}$  (second bar;  $n = 5$ ), and 0–60  $\mu\text{M}$  (third bar;  $n = 4$ ), as described in Section 2.4.1. In this study, we varied the number of data points selected from the full calibration curve to assess their influence on the slope and intercept, and consequently on the calculated OP values.



both values are derived from non-normalized OPs ( $\text{nmol min}^{-1}$ ).

This disparity underscores the potential for significant deviations in  $\text{OP}^{\text{DTT}}$  value determination when employing calibration curves of different concentration ranges. Specifically, variations in both slope and intercept values directly influence the baseline absorbance utilized in the CURVE method's calculation of DTT consumption.

This analysis supports the work of Molina *et al.*,<sup>34</sup> where these authors demonstrated relative uncertainties in  $\text{OP}^{\text{DTT}}$  values for both PM<sub>10</sub> and PM<sub>2.5</sub> samples, highlighting significant variation in linear regression analyses of PM samples. According to the authors, various operational conditions contribute to the variability of OP values in the DTT assay, including reproducibility factors associated with curve fitting analysis.<sup>34</sup>

Although significant variability exists between the mathematical approaches, the chosen concentration range of DTT for generating the calibration curves appears to significantly influence the assessment of OP. This suggests that a significant bias may arise from the concentration ranges used in the calibration curve, potentially due to a loss of linearity and issues related to DTT consumption at low concentrations, such as those observed at 0–60  $\mu\text{M}$ . For routine  $\text{OP}^{\text{DTT}}$  assays, the DTT concentration ranges between 0–140  $\mu\text{M}$  and 0–100  $\mu\text{M}$ , which provide results more consistent with those obtained using the ABS and CC2 methods, are thus preferred.

**3.2.5. Variability of  $\text{OP}^{\text{DTT}}$  values using the CC1 method.** In this subsection, the variability of  $\text{OP}^{\text{DTT}}$  values using the CC1 method (Section 2.3.3) is investigated focusing on variations in the filter blank absorbance at time zero ( $A_{B_0}$ ) and its impacts on the  $\text{OP}^{\text{DTT}}$  values. The CC1 method normalizes the difference in the absorbance by the blank absorbances at time zero, as detailed in eqn (5). Fig. 4 shows the distribution of  $\text{OP}^{\text{DTT}}$  values

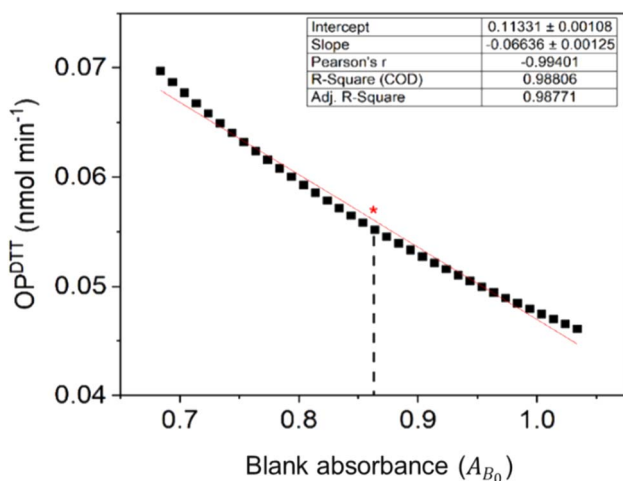


Fig. 4 Variability of  $\text{OP}^{\text{DTT}}$  values ( $\text{nmol min}^{-1}$ ) in relation to variations in filter blank absorbance values at time 0 ( $A_{B_0}$ ). "\*" denotes the average blank absorbance value for this study. The figure illustrates the implications on the use of different initial blank absorbance values on  $\text{OP}^{\text{DTT}}$  values based on the CC1 method.

( $\text{nmol min}^{-1}$ ), accounting for a range of blank absorbance values from 0.683 to 1.033 at time zero.

Fig. 4 shows that the  $\text{OP}^{\text{DTT}}$  values exhibit substantial variability, primarily driven by changes in the  $A_{B_0}$  values in the CC1 method, with  $\text{OP}^{\text{DTT}}$  ranging from 0.046 to 0.070  $\text{nmol min}^{-1}$ . This variability corresponds to an additional variation of up to 34% in the OPs, as demonstrated by the cascading effect observed when varying  $A_{B_0}$ . The results suggest that larger differences between  $A_{B_0}$  and  $A_t$  lead to lower OP values, while smaller variations among these parameters tend to yield higher OPs. Specifically, higher absorbance values at time 0 indicate reduced intrinsic reactivity of the PM samples, resulting in lower OP values that are more consistent with those obtained using the ABS and CC2 methods. Thus, the normalization with  $A_{B_0}$  may explain the differences observed between CURVE, ABS, and CC2 methods. Trace levels of redox-active species and PM catalysts in the blank solution could also lead to increased consumption of DTT and AA, resulting in lower absorbance values at time 0. This, in turn, affects the results of the CC1 method.

Accordingly, minimizing variation in blank absorbances can enhance the reliability of OP values and reduce the variability between replicates. In summary, this section highlights the importance of using consistent absorbance values for blank measurements in routine OP assays, as this has direct implications for the accuracy of OP values. Future OP studies should consider comparing theoretical absorbance values, calculated based on the initial concentrations of DTT and AA, with the actual blank absorbance values obtained through instrumental measurements. Finally, it is crucial to carefully examine the intrinsic reactivity of blank samples in OP analysis, as this can contribute to more consistent OPs.

### 3.3. Contrasting $\text{OP}^{\text{DTT}}$ and $\text{OP}^{\text{AA}}$ outcomes for different methods

The observed variability in  $\text{OP}^{\text{AA}}$  values across different methodologies mirrors previous findings for  $\text{OP}^{\text{DTT}}$  (Fig. S3 and Table S6†). Notably, both CC1 and CURVE methods consistently produced higher  $\text{OP}^{\text{AA}}$  values, while ABS and CC2 methods resulted in comparatively lower values. For  $\text{OP}^{\text{AA}}$ , the CURVE method leads to the highest observed  $\text{OP}^{\text{AA}}$ , followed by CC1, CC2 and ABS. This difference may be partially attributed to the effect of incubation time, as previously reported, or to the normalization approach used in the CC1 method. Specifically, variations in the absorbance values of blank samples at time 0 suggest substantial fluctuations in  $\text{OP}^{\text{AA}}$  values when using the CC1 method, which could influence comparisons across studies.

In terms of replicates, the CURVE and CC1 methods exhibit higher COVs for PM samples #A, #B, and #C. In contrast, the ABS and CC2 methods show higher COVs for samples #D, #E, and #F. Specifically, stronger AA depletion was associated with increased COVs for the ABS and CC2 methods, while weaker AA depletion corresponded to higher COVs for the CURVE and CC1 methods (Fig. S2†). Statistical analysis indicated significant differences among replicate measurements for PM samples #C,



#D, #E, and #F (Table S3;† ANOVA, one way,  $p < 0.05$ ). This finding is consistent with previous results from the DTT assay, highlighting the influence of replicate variability in OP assays and its dependence on the calculation methods employed, where the inherent variability in replicate measurements appears to be sample-dependent. Calibration curves with a narrower AA concentration range (0–144  $\mu\text{M}$ ) yielded lower  $\text{OP}^{\text{AA}}$  values than those with wider ranges (0–192  $\mu\text{M}$  and 0–240  $\mu\text{M}$ ; Fig. S3†). This trend is similar to that previously observed with the DTT assay, which indicates that higher DTT concentration ranges for the calibration curve result in increased  $\text{OP}^{\text{DTT}}$ . This aligns with the findings of Lin *et al.*,<sup>12</sup> who demonstrated that the initial concentration of DTT significantly affects OP values during incubation with PM.<sup>12</sup> Specifically, the authors showed that lower DTT concentrations during incubation generally lead to lower OP values.

As a final comparison, the variability caused by the different calculation methods have a more pronounced effect on  $\text{OP}^{\text{DTT}}$  compared to  $\text{OP}^{\text{AA}}$ , as indicated by the greater variability observed in the  $\text{OP}^{\text{DTT}}$  values (Fig. S1†). However, at higher consumption rates, as previously demonstrated for #D, both the ABS and CC2 methods have a greater influence on the AA compared to the DTT assay. For both assays, increasing the concentration ranges of DTT and AA used in calibration curves resulted in higher OP values compared to other calculation methods. While the CC1 method led to elevated OPs in both assays, its impact was particularly pronounced in the DTT assay.

#### 4. Conclusion and recommended key features of a standardized OP determination

This study presents an in-depth analysis of the mathematical methodologies outlined in the literature for the determination of OP values. These approaches assess the consumption rates of DTT and AA in the presence of redox-active PM species and PM catalysts by utilizing relative absorbance values over time, which are subsequently extended to yield slope and rate constant values. The experimental results revealed distinct OP profiles, with varying calculation methods leading to divergent outcomes. These findings are best summarized as follows:

(i) Both the ABS and CC2 methods exhibited significant similarities in both DTT and AA assays, demonstrating similar OP values and relative variation.

(ii) The CC1 method is prone to yielding higher  $\text{OP}^{\text{DTT}}$  and  $\text{OP}^{\text{AA}}$  values compared to the CURVE, ABS, and CC2 methods. The intrinsic variability of the CC1 method, as observed in measurement replicates, affects the precision and stability of OP values, which in turn influences comparisons across different studies. The blank assessment is the critical step within CC1 to obtain comparable OP values.

(iii) Variation in the concentration ranges of DTT and AA used in the CURVE method significantly influence the variability of  $\text{OP}^{\text{DTT}}$  and  $\text{OP}^{\text{AA}}$  values. Such variations carry important implications for the slope values associated with the rate

constants of the redox and catalytic reactions involved in OP assays.

The findings of the present study highlight the importance of uniform OP protocols with guidelines on the methodological aspects of OP assays, including the adoption of more comparable mathematical approaches for measuring the OP and critical evaluation of blank solutions.

It is important to acknowledge the inherent limitations in the assumptions in the absorbance-to-concentration conversion methods. While the 'CURVE' method employs a more complex approach, other methods appear to utilize a simplified conversion factor ( $[\text{reductant}]_0; p; A_{\text{B}_0}; A_{\text{PM}_0}$ ). This approach implicitly assumes ideal Beer–Lambert behavior and exclusive absorbance due to the reductant. However, as demonstrated in Table S4,† real-world measurements often exhibit a non-zero intercept, indicating deviations from ideality. This intercept suggests that background absorbance or other systematic effects contribute to the measured absorbance, rendering the simplified conversion factor potentially inaccurate. Therefore, for OP standardization, we must consider the limitations of these simplified methods. From an analytical chemistry standpoint, employing linear regression of absorbance *versus* concentration provides a more accurate and scientifically justified approach. Based on the findings of the present study and the observed discrepancies in the OP values obtained through each calculation method in comparison to the overall OP mean, both the ABS and CC2 methods exhibit greater consistency and are recommended for routine  $\text{OP}^{\text{DTT}}$  and  $\text{OP}^{\text{AA}}$  assays. While our analysis identifies statistical similarities among computational approaches, we acknowledge that numerical agreement alone does not imply scientific validity. Rather, our findings highlight the relative differences between methods across diverse PM sources, allowing us to identify approaches that yield more comparable results. These findings are intended as guidance for enhancing comparability across studies, rather than a definitive endorsement of any single method's absolute correctness.

To maintain the accuracy and consistency of measurements across all calculation methods, strict adherence to quality control protocols is essential. Experimental protocols should include an evaluation of the linearity of the regression analysis for both DTT and AA consumption, as well as a critical assessment of their concentration to ensure greater consistency across methods. Failure to account for potential deviations from linearity may lead to misinterpretations of the patterns observed in both the DTT and AA assays. Additionally, future studies could consider extracting OP values from different methods to enable a more detailed comparison across studies, improving the standardization and interpretability of OP assessments.

Overall, the present study contributes to the development of standardized protocols for the OP quantification stage, which will enhance consistency in predicting the toxic potency of aerosol particles and facilitate reliable comparisons across OP studies globally.



## Data availability

The data supporting this article have been included as part of the ESI.†

## Author contributions

Conceptualization: ES and GU; methodology: ES and GU; formal analysis and OP measurements: TM; investigation: ES, GU, KWF and PD; resources: GU, KWF, JLJ and HH; data curation: ES; writing – original draft preparation: ES; writing – review and editing: ES, GU, KWF, PD, JLJ, and HH; supervision: GU, KWF, JLJ and HH; project administration: GU and KWF; funding acquisition: GU, KWF, JLJ, and HH.

## Conflicts of interest

There are no conflicts to declare.

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