Effects of Operating Conditions on PM Oxidative Potential Assays

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Abstract

Oxidative potential (OP) has been suggested as a biologically relevant exposure metric for estimating particulate matter (PM) capacity to induce oxidative stress in living organisms. However, standardized experimental procedures are not yet available. This study explores how a variety of operating conditions influences responses of several different assays for measuring OP: the 2',7'-dichlorofluorescein (OP^{DCFH}), the ascorbic acid (OP^{AA}) and the dithiothreitol (OP^{DTT}) assays. A recently optimized method for the evaluation of PM reducing properties, based on the 2,2-diphenyl-1-picrylhydrazyl assay (RP^{DPPH}), was also included in the study. Two monitoring campaigns were carried out in Central Italy by using co-located PM_{10} samplers working in parallel, for comparing results obtained from different operating procedures simultaneously applied on equivalent samples.

Extraction efficiency and repeatability of three different water-extraction methods (rotating agitator, ultrasonic bath, and vortex), and the influence of storage duration and conditions on OP results were examined. OP^{DCFH} values were found to be significantly higher when ultrasonic bath (US) was used for extraction, probably due to the formation of free radicals induced by US; for all the OP assays, the highest repeatability was obtained by extracting samples with rotating agitator (RA). Sample storage was confirmed to be a very critical issue as all the assays, except OP^{DTT}, showed a marked dependence on storage time and conditions. The influence of membrane filters used to collect PM was also assessed. No significant differences were observed between samples collected on quartz and polytetrafluoroethylene (PTFE) membrane filters, except for OP^{AA}, that gave significantly higher results for samples collected on PTFE membranes. Lastly, the contribution of water-insoluble PM components to OP was examined and warrants further investigations.

Keywords: ascorbic acid (OP^{AA}) assay, dithiothreitol (OP^{DTT}) assay, 2',7'-dichlorofluorescein (OP^{DCFH}) assay, extraction method, PM filters' conservation.

1. Introduction

Airborne particulate matter (PM) pollution is one of the most significant threats for human health (Shiraiwa et al., 2017; Dong et al., 2019). It is now broadly confirmed that PM critically impacts human well-being through exposure to particles that can lead to a wide range of adverse health implications including respiratory and

cardiovascular disease, cancer, diabetes, as well as neurodegenerative disease (Uttara et al., 2009; Andersen et al., 2010; Øvrevik et al., 2019).

An actual common thesis claims that one of the biological key mechanisms involved in developing damaging health effects is the PM ability to induce cellular generation of reactive oxygen species (ROS), at the expense of antioxidant defenses, resulting in oxidative stress responses and in several chronic and acute systemic inflammations (Yang et al., 2014; Øvrevik et al., 2019; Cervellati et al., 2020). Oxidative potential (OP) of PM is considered one of the most relevant predictive factors for the assessment of PM toxicity (Delfino et al., 2011; Gupta et al., 2019), since it is intrinsically influenced by different physiochemical properties governing PM ability to cause oxidation of target molecules, including size, surface area as well as chemical composition (Andrade et al., 2020). Therefore, OP is frequently proposed as a more biologically appropriate metric for addressing human exposure than bulk PM mass concentration (Yang et al., 2016; Bates et al., 2019; Nishita-Hara et al., 2019).

Multiple acellular tests, based on the offline analyses of PM field filters, are available for quantifying particles oxidative potential (Bates et al., 2019), among which the most widely used are the ascorbic acid (OP^{AA}) and dithiothreitol (OP^{DTT}) assays, that evaluate the potential of PM components to deplete a physiological antioxidant and a cellular reductant surrogate, respectively (Cho et al., 2005; Stoeger et al., 2009; Campbell et al., 2019). In fact, ascorbic acid (AA) is the prevalent natural occurring antioxidant in the lung (Godri et al., 2010, 2011; Campbell et al., 2019), while dithiothreitol (DTT) acts as a chemical surrogate of cellular reducing agents, such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Kumagai et al., 1997, 2002). The consumption of these antioxidants occurs when PM components catalytically transfer one electron from AA or DTT molecules to molecular oxygen, generating superoxide anion mimicking the crucial initial step of *in vivo* producing ROS (Kumagai et al. 2002; Gupta et al., 2019). Therefore, OP^{AA} and OP^{DTT} have considerable physiological relevance in the assessment of particle toxicity.

2',7'-dichlorofluorescein (OP^{DCFH}) assay is commonly used in biological field as an indicator of oxidative stress for quantifying cellular ROS generation, through a fluorescent-based probe (Venkatachari et al., 2005, 2007; Fuller et al., 2014). However, the test has been adapted to be performed on PM samples for determining total particle-bound ROS and, in the literature, it is conventionally included in OP assays (Hung and Wang, 2001; Simonetti et al., 2017; Zhou et al., 2018; Zhang et al., 2021).

Since there are still doubts and uncertainties regarding the most representative assay to quantify the OP of PM, the synergic application of different acellular methods on the same PM sample is often considered advantageous in providing insightful assessment of particles OP (Ayres et al., 2008; Frezzini et al., 2019; Lin and Yu, 2020; Manigrasso et al., 2020).

All the acellular assays are easy to reproduce in laboratories, they require fewer resources than cellular ones, and give quicker readouts of OP measurements (Yu et al., 2020; Bates et al., 2019; Gupta et al., 2019). In recent years, OP appeared to be the central paradigm in the assessment of PM toxicity, however, there are still several criticisms regarding the effectiveness of this metric to quantify the effects of ambient particles on human health. In fact, OP measurements are strongly influenced by synergic actions of multiple operating

conditions altogether. Among these, the PM filters extraction methods seem to alter OP quantification. In fact, although sonication is the most common approach, some studies underlined the impact of ultrasounds on OP measurements as a result of both the increase of free radicals in the systems, and the degradation of some compounds (Mutzel et al. 2013; Khurshid et al., 2014; Miljevic et al., 2014). This evidence has provided motivation for conducting further investigations to identify an extraction method not affecting OP results. Alternative extraction methods to sonication, such as rotating agitator and vortex, were used in some studies (Perrone et al., 2016; Frezzini et al., 2019), but, to the best of authors knowledge, a systematic comparison among the different extraction techniques has never been undertaken so far.

Another potential factor altering OP measurements is the time delay between filter sampling and OP analyses, that seems to play a crucial role in underestimating particles OP, due to the possible decomposition and/or chemical transformation of the highly reactive components, prior to analysis (Fuller et al., 2014; Campbell et al., 2019). Indeed, it has already been suggested that the aging of the particles on membrane filter surfaces can cause an underestimation of collected reactive species (Hedayat et al., 2015). This is further supported by the previously estimated presence of reducing species in PM (Frezzini et al., 2019) that could reasonably react with oxidizing ones over time, leading to an underestimated OP. Therefore, the assessment of the influence of sample storage conditions and duration on the quantification of PM oxidative potential is essential. In addition, in the literature, most of the OP assays have been applied to polytetrafluoroethylene (PTFE) filters (Yang et al., 2014). However, other types of filters have also been used, , such as quartz filters. Indeed, there is still a lack of information regarding the effect of the type of filter used for the OP measurement.

Lastly, another additional challenge in optimizing OP acellular measurements is assessing the contribution of insoluble species to PM redox activity. OP assays are usually performed on water-soluble fraction of PM samples, that is considered more bioaccessible than the water-insoluble fraction (Shao et al., 2017; Gao et al., 2020). However, a growing scientific evidence underlined the considerable toxicological potential of insoluble particles, showing that it also plays an important role in generating oxidative damage, such as the disruption of the cell membrane (Knaapen et al., 2002; Daher et al., 2011; Zou et al., 2016; Conte et al., 2017; Gao et al., 2017; Piacentini et al., 2019). For example, Akhtar et al. (2010) found that redox-active substances could be strongly bound to solid particles, not completely extracted by water. Furthermore, Yi et al. (2014) demonstrated the capacity of PM insoluble fraction to induce oxidative stress and damage in human lung epithelial cells. Overall, an increasing number of recent findings suggest that the integration of the contribution of water-insoluble species in the OP assessment would be closer to a realistic PM exposure, elucidating actual PM induced health risks.

In general, the use of so many different analytical procedures, coupled with the lack of standardized operating conditions, adds variation in experimental design among laboratories that may contribute to differences between OP results, making a challenge to representatively compare inter-laboratory data (Guo et al., 2019; Lin and Yu, 2019). Therefore, this study is aimed to investigate variability in OP (OP^{DCFH}, OP^{AA}, OP^{DTT}) responses depending on the operating conditions under which the tests are performed. In particular, the influence of extraction method, filter-storage and time delay between PM sampling and analysis, on OP

measurements, were assessed. Furthermore, the influence of the type of filter used on OP results was evaluated. Redox equilibria among PM native species were deepened, due to the use of an assay for estimating the amount of reducing species in PM, defined reducing potential method (RP^{DPPH}; 2,2-diphenyl-1-picrylhydrazyl assay, DPPH) (Frezzini et al., 2019). Then, the contribution of water-insoluble PM components to aerosol OP was examined, in order to assess their role in particle oxidative activity. To the best of authors knowledge, no study has been published so far on the effects of so many operating conditions altogether on OP measurements.
The goal of this study is to gain more information about the driving forces of OP measurements, thus giving a contribution to the standardization of experimental procedures.

2. Materials and Methods

2.1. Sampling sites and methods

A summer and a winter monitoring campaign were carried out to collect PM_{10} field filters to be used for the experimental procedures as reported in Figure 1 that shows the block diagram of the conducted experimental tests. For the summer monitoring period, six PM_{10} sequential samplers working in parallel at the flow rate of 2.3 m^3/h (SWAM5a Dual Channel Monitor, FAI Instruments, Fonte Nuova, Rome, Italy) were employed for 15 days, from July 18th to August 01st , 2019 at the C.N.R. Institute of Atmospheric Pollution Research, located in Montelibretti (geographical coordinates: 42°06′20.55″N; 12°38′24.53″E), a peri-urban area near Rome (Central Italy)
For the winter period, two single-line and one double-line samplers, working in parallel at 2.3 m^3/h, were used (Giano and Gemini, respectively; Dadolab Srl, Cinisello B., MI, Italy) for 42 days, from February 2nd to March 19th, 2020, at the Experimental Botanic Garden of the Sapienza University of Rome, an urban area of Rome (Central Italy; geographical coordinates: 41°54’8.63’’N; 12°31’3.45’’E). All the samplers were equipped with a sampling head for PM_{10} certified UNI EN 12341 (2014). Polytetrafluoroethylene membranes (PTFE, 47 mm diameter, pore size 2µm, Cobetter Filtration Equipment Co., Ltd, Hangzhou, China) were used for the samplings and 24-h PM_{10} field filters were daily collected for both the monitoring campaigns. Quartz PM_{10} filters were parallelly sampled (QM-A quartz filters, 37 mm, Whatman) for 10 days during the winter monitoring campaign. In total, over 250 PM_{10} field filters were collected and analyzed.

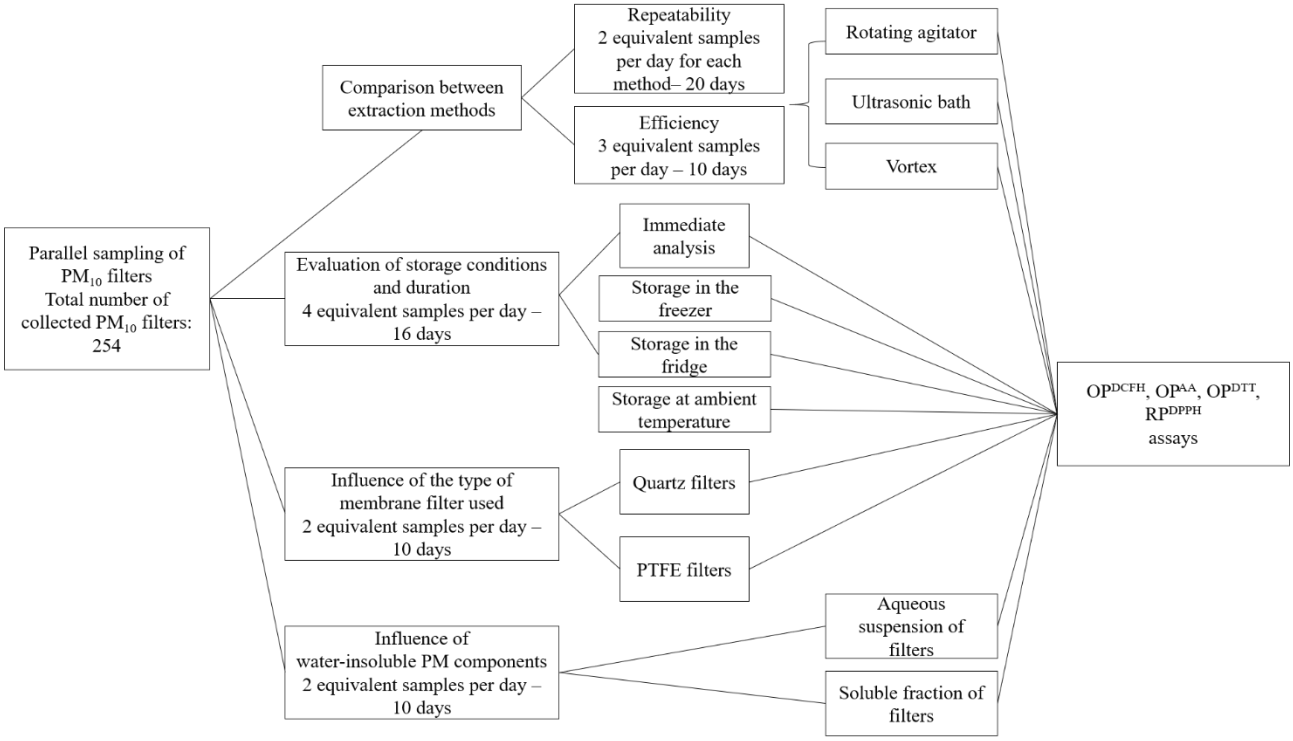

Figure 1. Block diagram summarizing the conducted experimental test of the study.

2.2. Experimental design

PM_{10} collected on PTFE membrane filters was treated by following a previously optimized and detailed procedure (Massimi et al., 2017; 2020a). Briefly, after the removal of the supporting polymethylpentene ring from each sampled filter, apart from some exceptions specified in the text, PTFE membranes were immersed in 10 mL of deionized water (produced by Arioso UP 900 Integrate Water Purification System, USA) and then treated by subsequent different experimental approaches as described below and filtered through a nitrocellulose filter (NC filter; pore size 0.45 µm, Merck Millipore Ltd., Billerica, MA, USA) before analysis.

2.3. Oxidative and Reducing potential measurements

The DCFH, the AA and the DTT assays were used to assess the OP of PM_{10} samples, while the DPPH assay was used for the measurement of the reducing potential.

2.3.1. DCFH assay

DCFH is a non-fluorescent reagent becoming fluorescent dichlorofluorescin (DCF) upon reaction with ROS (Venkatachari et al., 2005) and it is used in combination with horseradish peroxidase (HRP; Sigma–Aldrich, USA), a redox enzyme mainly reacting with hydrogen peroxide and organic hydroperoxides, to catalyze the reactions (Bates et al., 2019; Fuller et al., 2014). DCFH solution was prepared from 2', 7'-dichlorofluorescin diacetate (DCFH-DA; Sigma–Aldrich, USA) according to the procedure provided by Simonetti et al. (2018). Briefly, 125 µL of DCFH reagent (5 µM) and 5 mL of HRP (0.5 units mL^{-1}) dissolved in a sodium phosphate buffer (pH 7.4; 25 mM) were added to 1.5 mL of the extracted solution of PM samples. The reaction mixture was placed in the thermostatically controlled water bath at 37 ºC. After 5 minutes, the concentration of DCF was measured by using fluorescent spectroscopy (Jasco FP-920; excitation at 427 nm, emission at 530 nm).

Standard H_2O_2 solutions (5×10^{-8}, 1×10^{-7}, 2×10^{-7}, 5×10^{-7} and 1×10^{-6} M) were daily used to plot a calibration curve to convert the obtained fluorescence intensity into H_2O_2 equivalents, which are used as indicators of the reactive species reactivity, thus obtaining OP^{DCFH} values (nmol H_2O_2 m^{-3}).

2.3.2. AA assay

For the measurement of AA depletion, the method reported by Fang et al. (2016) was followed with slight modifications. 300 µL of phosphate buffer (0.5 mM) and 100 µL of AA reagent (2 mM; Sigma–Aldrich, USA) were added to 2.5 mL of sample solution. Then, absorbance of the reaction mixture was recorded at 265 nm wavelength, at different reaction times (0, 10 and 20 minutes) by using UV-Vis absorption spectrometry (Varian Cary 50 Bio UV-Vis; Varian Inc., Palo Alto, CA, USA). Blanks were always measured in parallel. OP^{AA} was calculated as the AA consumption rate per sampled volume (nmol AA min^{-1} m^{-3}) according to the equation reported in supplementary material S1.

2.3.3. DTT assay

For the OP^{DTT} protocol, three aliquots of sample solution (0.7 mL) were incubated at 37 °C with 0.1 mL of DTT (1 mM; Sigma–Aldrich, USA) and 0.2 mL of potassium phosphate buffer (1 M) for different reaction times (0, 10 and 20 minutes). Then, 1 mL of trichloroacetic acid (10%TCA; Sigma–Aldrich, USA) was added to the mixture to quench DTT reactions. An aliquot (1 mL) was taken from the solution and mixed with 2 mL of tris-buffer (0.08 M, containing EDTA 4 mM) and with 50 µL of 5,5-dithiobis-2-nitrobenzoic acid (DTNB; Sigma–Aldrich, USA) to form 2-nitro-5-mercaptobenzoic acid (TNB) by reacting with the residual DTT, then measured at 412 nm by using UV-Vis spectrometer. Furthermore, blanks were measured in parallel to samples. OP^{DTT} was expressed as DTT consumption rate per sampled PM volume (nmol DTT min^{-1} m^{-3}), according to the equation reported in supplementary material S1.

2.3.4. DPPH assay

The assay is based on the quantitative measurement of the scavenging capacity of antioxidants towards DPPH free radical by the decrease in absorbance. DPPH assay was previously applied to PM samples with the aim to evaluate the presence of reducing species, thus integrating information about PM redox properties (Frezzini et al., 2019).

Operating in the dark, 2 mL of EtOH 96% and 0.5 mL of DPPH 0.1 mM ethanolic stock solution (Sigma–Aldrich, USA) were added to one aliquot (1.5 mL) of the water-extracted sample solution and the mixture was shaken for 30 min by rotating agitation. The absorbance of the solutions was recorded by UV-Vis spectrophotometry set at 517 nm, by measuring the sample absorbance decrease against the control (blank solution). The DPPH radical scavenging effect resulted in solution decolorization and was calculated in terms of percentage consumption of DPPH per sampled PM volume (RP^{DPPH}; %DPPH Cons m^{-3}), according to the equation reported in supplementary material S1.

2.4. Comparison between extraction methods

The repeatability of OP and RP measurements obtained using the selected extraction techniques was evaluated carrying out binary comparison between twin filters collected in both the monitoring campaigns. Therefore,

for each extraction method, 20 pairs of equivalent samples were considered. The PM_{10} duplicate filters (filter A and filter B), immersed in 10 mL of deionized water were subjected to different extraction methods for 30 minutes: the rotating agitation (RA; Rotator, 60 rpm; Rotator, Glas-Col, USA), the sonication (US; Ultrasonic bath; Proclean 10.0 ultrasonic cleaner, Ulsonix, Germany) or the vortexing (V; Vortex Genie 2, 2000 rpm; Scientific Industries, Bohemia, New York). Then, the obtained solutions were filtered through a nitrocellulose filter (NC filter). The water-extracted solutions were thus split in their respective aliquots for the subsequent analyses. Then, OP assays (OP^{DCFH}, OP^{AA} and OP^{DTT}) were performed on the soluble fraction of samples. To evaluate the repeatability of analytical results of OPs performed on PM samples extracted by the different procedures, for each OP assay, the mean relative percentage differences (Δ%) are calculated as averages of the relative errors of each pair of PM_{10} twin filters (δ_i%), as follows:

$$\delta_i\% = \left[\frac{\left|\left(OP^X_{A,i} - OP^X_{B,i}\right)\right|}{\left[\left(OP^X_{A,i} + OP^X_{B,i}\right) \div 2\right]}\right] \cdot 100$$

where OP_A and OP_B are the OP values obtained for the filter A and B, respectively, X can indicate DCFH, AA, DTT or DPPH and i is the considered pair of PM_{10} filters.

The repeatability of each extraction method, and for each assay, was also assessed by calculating the linear regression between the duplicate filters (A vs B):

$$OP_A = mOP_B + q$$

where $m = 1$ and $q = 0$ indicate a perfect equivalence between A and B.

RA, US, and V were also compared in terms of extraction efficiency, that was quantitatively assessed by comparing OP values of daily equivalent samples subjected to different extraction methods. In this case, 3 equivalent samples were collected during 10 days of the winter monitoring campaign. The paired sample *t*-test was used to observe the significance of the differences of results obtained by each extraction method. A *p*-value less than 0.05 was considered statistically significant.

2.5. Sample storage conditions and duration

The effects of both sample storage conditions and time delay between filters sampling and analyses were observed by using four lines (A, B, C and D) of PM_{10} filters sampled in parallel for 15 days during the winter monitoring campaign.

PM_{10} equivalent filters were treated by following a different experimental design for each line:

- line A: each sampled filter was taken from the unloader and immediately analyzed;
- line B: each PM_{10} field filter was taken immediately after its collection, put in petri dish sealed with parafilm, and stored in a freezer at -20°C for 15 days under controlled temperature and humidity before being analyzed;
- line C: each sampled filter remained for 15 days inside the sampler's unloader under constant and controlled temperature due to a Peltier conditioning system, before being subjected to the subsequent analytical procedures;

- line D: each PM_{10} field filter was left into the sampler's unloader for 15 days under constant and controlled temperature due to a Peltier conditioning system and then, put in petri dish sealed with parafilm and stored in a fridge at 4°C for 15 additional days under controlled temperature and humidity, prior to the OP procedures.

In this experimental stage, all the collected PM_{10} filters were extracted by rotating agitator, filtered (NC filters), and then analyzed. The paired sample *t*-test was used to observe significant differences between PM filters from the four sampling lines for each OP assay.

2.6. Influence of membrane filter used

The comparison between OP and RP results obtained from quartz and PTFE filter (Q and P, respectively) was performed by sampling a pair of equivalent PM_{10} filters during 10 days of the winter monitoring campaign. Two sampling lines were thus equipped with quartz and PTFE filters. Then, OP and RP assays were applied to the water-soluble fraction of both Q and P filters as described in 2.3. section. The paired sample *t*-test was used to observe whether the filter type used significantly influenced the OP and RP results.

2.7. Water-insoluble PM components redox activity

The contribution of the water-insoluble components to PM redox potential was evaluated by sampling a pair of equivalent PM_{10} filters for 10 days during the winter monitoring campaign, on the same days as PM_{10} filters were collected for Q and P comparison. The two equivalent samples were used for performing OP and RP directly on the aqueous suspension of the field filters to determine total redox properties (i.e. soluble plus insoluble; OP^T; RP^T). The obtained OP^T and RP^T were compared to OP and RP of water-soluble fraction (OP^{WS}; RP^{WS}) measured on P membranes (section 2.6.). In order to measure OP^T and RP^T, each PM_{10} filters was cut into four equal parts (8 equivalent pieces in total) and used as described above:

- a quarter of each filter was extracted in 1.5 mL of deionized water. Then the DCFH assay was directly applied on the water-extracted solution by following the procedure detailed in 2.3.1. section;
- three quarters of each filter were extracted in 2.5 mL of deionized water, and then used for the three reaction times of AA procedure described in 2.3.2. section;
- three quarters of each filter were extracted in 0.7 mL of deionized water. The obtained suspensions were used for the three reaction times of DTT procedure described in 2.3.3. section;
- a quarter of each filter was extracted in 1.5 mL of deionized water. The DPPH procedure was applied on the obtained suspension as described in 2.3.4. section.

The paired sample *t*-test was used to observe significant differences between OP^T and OP^{WS} for each OP method.

3. Results and Discussion

3.1. Influence of extraction methods on PM redox measurements

The influence of the considered extraction methods (RA, US, and V) on OP and RP results was investigated by assessing the repeatability on 20 twin pairs of PM filters (A and B), extracted by the three selected techniques. The regression parameters obtained between the duplicate filters were reported in Table 1, along

with the mean relative percentage difference (Δ%) of each pair of filters for each extraction method and the range of OP and RP values.

Data showed that RA extraction allows obtaining the best repeatability of results. In the case of OP assays, the Δ% were always lower than 20%, the R^2 always greater than 0.90, the *m* very close to 1 (range 0.95 – 0.97) and the *q* were well below the minimum of OP values (less than 10% of those values). The lowest repeatability was obtained by using V that leads to high Δ% (> 30%) and to unsatisfactory linear regression parameters ($0.30 < R^2 < 0.70$). RP^{DPPH} showed regression parameters less acceptable than the OP assays, with all the studied extraction methods, with R^2 ranging from 0.62 to 0.75, probably due to the lower variability range. However, Δ% values were still acceptable in the case of RA extraction (Δ% = 16%).

The regression parameters showed the good linearity of RA for all the three OP assays, as opposed to US and V that do not guarantee a good analytical repeatability of OP measurements. The OP^{DCFH}, OP^{AA}, OP^{DTT} and RP^{DPPH} values obtained for each considered twin pair of PM_{10} filters are reported in supplementary material S2 (Table S2).

Figure 2 reports the OP and RP values related to the evaluation of the extraction efficiency of the three considered methods and the *p*-values of the sample paired *t*-test. Differences between results obtained from samples extracted by different methods were not significant for all the OP and RP assays, except for OP^{DCFH} (panel a), that showed significantly higher values when PM filters were extracted with US ($p < 0.05$ for US vs RA, and US vs V). This is in accordance with previous studies that underlined the role of sonication in producing free radicals, due to thermal reactions and degradation (Hung and Wang, 2001; Kurshid et al., 2014). In fact, as already known, ultrasonic waves can generate cavitation bubbles in the extraction solution, and their collapse leads to high temperature and pressure conditions. Consequently, the molecules inside the cavitation bubbles can undergo pyrolysis, that results in free radicals' generation (Mutzel et al., 2013; Miljevic et al., 2014; Massimi et al., 2020b). The formation of these species may originate positive artifacts altering the obtained OP values, thus overestimating radicals' content in PM samples (Mutzel et al., 2013; Miljevic et al., 2014). Therefore, the highest OP^{DCFH} values found in samples extracted by US are not indicative of higher extraction efficiency of US, but of the generation of radical species.

Although RA and V gave similar OP values, thus appearing to be efficient extraction methods for OP analysis, RA emerged as the most suitable method for the extraction of PM filters and subsequent OP analysis, since it showed high repeatability and avoided the generation of free radicals upon ultrasonic irradiation.

Table 1. Linear regression values, variability range (minimum–maximum) and mean relative percentage differences (Δ%) of the 20 pairs of A and B twin filters.

	OP^{DCFH}				
	R^2	*m*	*q* (nmol H_2O_2 m^{-3})	Range (nmol H_2O_2 m^{-3})	Δ%
RA	0.98	0.96	$-4.1 \cdot 10^{-10}$	$2.8 \cdot 10^{-9}$ - $6.5 \cdot 10^{-8}$	19
US	0.67	0.95	$1.1 \cdot 10^{-9}$	$1.8 \cdot 10^{-9}$ - $1.5 \cdot 10^{-8}$	26
V	0.54	0.56	$3.2 \cdot 10^{-9}$	$2.1 \cdot 10^{-9}$ - $2.8 \cdot 10^{-8}$	34

OP^{AA}

	R^2	*m*	*q* (nmol AA min^{-1} m^{-3})	Range (nmol AA min^{-1} m^{-3})	Δ%
RA	0.95	0.95	0.0097	0.91 - 15	20
US	0.77	0.88	0.51	0.18 - 7.5	98
V	0.71	0.81	0.42	0.39 - 3.4	44

OP^{DTT}

	R^2	*m*	*q* (nmol DTT min^{-1} m^{-3})	Range (nmol DTT min^{-1} m^{-3})	Δ%
RA	0.91	0.97	-0.019	0.46 - 2.7	16
US	0.62	0.86	0.22	0.16 - 1.8	23
V	0.31	0.81	0.26	0.38 - 3.1	97

RP^{DPPH}

	R^2	*m*	*q* (%Cons $DPPHm^{-3}$)	Range (%Cons $DPPHm^{-3}$)	Δ%
RA	0.75	0.81	0.23	0.14 - 1.7	16
US	0.69	0.61	0.13	0.031 - 1.2	50
V	0.62	0.69	0.075	0.11 - 0.67	31

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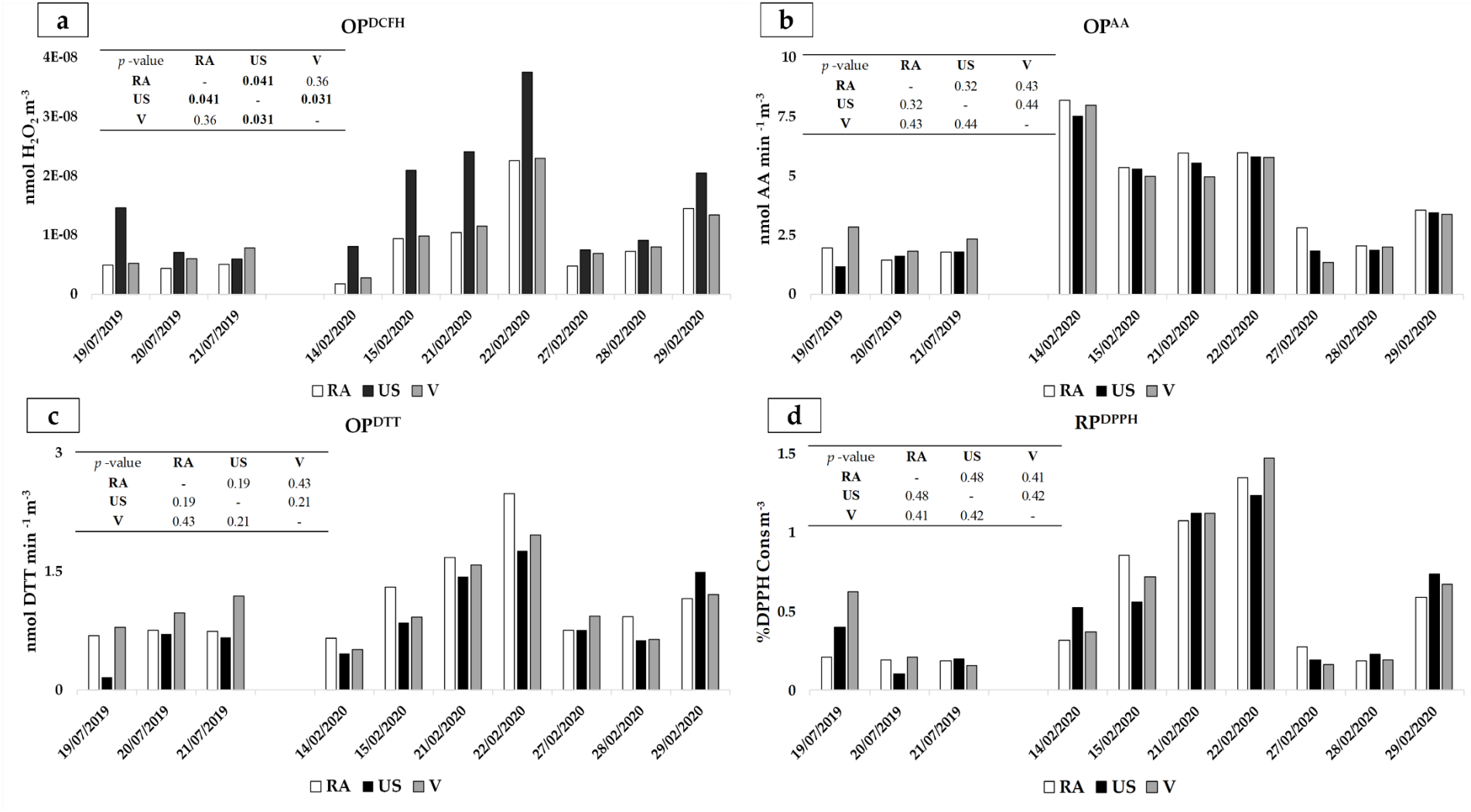
a
OP^DCFH
nmol H2O2 m-3
4E-08
3E-08
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1E-08
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p -value RA US V
RA - 0.041 0.36
US 0.041 - 0.031
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RA US V
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OP^AA
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V 0.43 0.44 -
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nmol DTT min -1 m-3
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p -value RA US V
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V 0.41 0.42 -
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RA US V

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Figure 2. Comparison of OP^{DCFH} (panel a), OP^{AA} (panel b), OP^{DTT} (panel c) and RP^{DPPH} (panel d) between equivalent filters extracted by using the three different techniques: rotating agitator (RA), ultrasonic bath (US) and vortex (V) and the *p* values of the sample paired *t*-test between the three extraction methods. The *p*-value in bold indicate significant differences ($p < 0.05$).

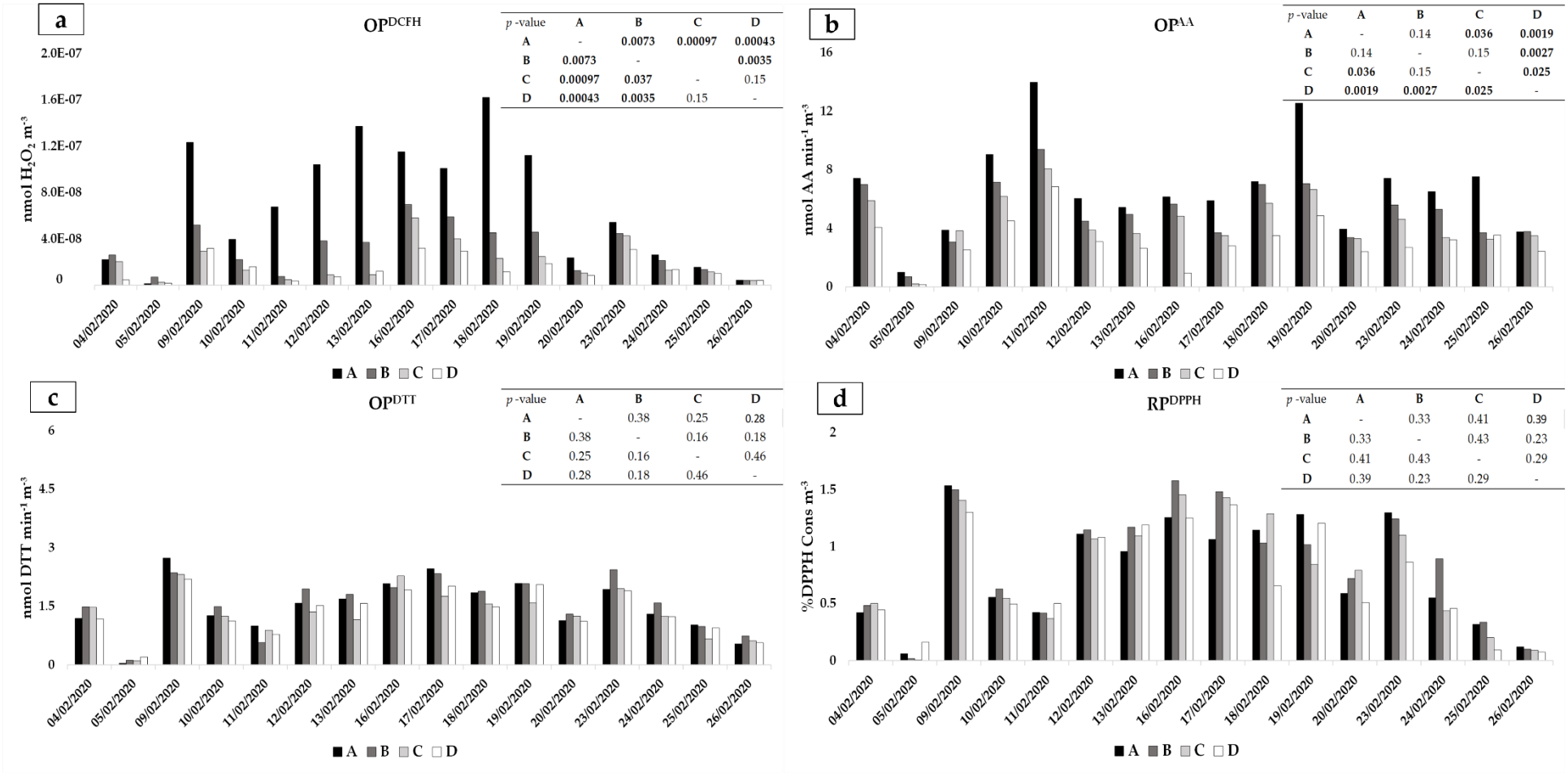

Figure 3. Comparison of OP^{DCFH} (panel a), OP^{AA} (panel b), OP^{DTT} (panel c) and RP^{DPPH} (panel d) between filters subjected to different sample storage condition and duration: A, filters were immediately analyzed after sampling; B, filters were stored in a freezer at -20°C for 15 days before being analyzed; C, filters remained in the samplers' unloader for 15 days before being analyzed; D, filters remained in the samplers' unloader for 15 days and then, stored in a fridge at 4°C for 15 additional days before being analyzed. The *p*-values of the sample paired *t*-test between the four conditions are reported. The *p*-value in bold indicate significant differences ($p < 0.05$).

310

311 *3.2. Influence of sample storage conditions and duration on PM redox measurements*

312 According to the results from 3.1 section, all the PM_{10} filters used for the evaluation of storage conditions and
313 duration were water-extracted by RA. Results of OP^{DCFH}, OP^{AA}, OP^{DTT} and RP^{DPPH} and the *p*-values of the
314 sample paired *t*-test are reported in Figure 3.

315 OP^{DCFH} (panel a) values highlighted that DCFH measurements were simultaneously influenced by both sample
316 storage conditions and duration. In fact, OP^{DCFH} was generally higher in samples from line A, whose filters
317 were immediately analyzed after their collection. Line B, whose filters were rapidly placed in the freezer after
318 their collection and analyzed after 15 days, showed OP^{DCFH} values mostly lower than those from line A.
319 Samples from line C were left inside the sampler's unloader for 15 days prior to their analyses and exhibited
320 DCFH values lower than those from lines A and B. Differences between line B and C showed that, within the
321 same sample storage duration, the conditions of filters storage can greatly influence DCFH measurements.
322 Lastly, the lowest DCFH values were obtained from line D filters, that were left into the sampler's unloader
323 for 15 days and then stored in the fridge for 15 additional days, prior to measurements. The paired sample *t*-
324 test showed a significant effect of different storage conditions and duration (A vs C, A vs D, A vs B, B vs D;
325 $p < 0.01$) for DCFH assay. These results agree with previous studies that underlined the short lifetime of
326 sampled particle-bound ROS, generally ranging from only a few minutes to one or more days (Venkatachari
327 et al., 2005; Bates et al., 2019). Although online techniques to quantify the short-lived and labile fraction of
328 particle-bound ROS with DCFH are strongly suggested (Wragg et al., 2016), ROS are still mainly quantified
329 by offline assays in extracts of aerosol particles collected on filters (Fuller et al., 2014; Daellenbach et al.,
330 2020). Therefore, OP^{DCFH} results from this study confirm that the application of this assay to sampled PM
331 filters may be particularly critical, as only the long-lived fraction of ROS remains over time. Therefore, OP^{DCFH}
332 measurements should be performed as rapidly as possible after sampling and, anyway, after an appropriate
333 standardization of storage conditions and durations.

334 The OP^{AA} showed a clear trend (panel b) with values in the order A > B > C > D with significant differences
335 between B vs D and A vs D ($p < 0.01$) and between A vs C and C vs D ($p < 0.05$). It is well known that the
336 OP^{AA} assay is particularly sensitive toward transition metals (i.e. Cu, Fe, Mn, Sb) generated by non-exhaust
337 traffic emissions, such as brake abrasion and re-suspended dust (Simonetti et al., 2018; Perrone et al., 2019;
338 Lin and Yu, 2020). Consequently, these results may be then interpreted by considering that PM collected on
339 filters might undergo chemical alteration during sampling or storage, such as change in the oxidation state of
340 metals, or alteration of the solubility of redox-active metals (Majestic et al., 2007; Fang et al., 2017; Puthussery
341 et al., 2018). Therefore, collected species responsible for OP^{AA} seem to be subjected to changes over time
342 determining a decrease in their oxidizing capability.

343 Results from DTT assay (panel c) revealed no significant differences between sample storage conditions and
344 duration, remaining almost constant over time, probably because of the high stability of species responsible
345 for OP^{DTT} (Bates et al., 2019). To give an example, OP^{DTT} has large organic species dependence, among which

quinones, that can be directly emitted from traffic or formed from secondary oxidation, are considered remarkably persistent for a considerable time (Valavanidis et al., 2005; Wang et al., 2018).

The DPPH radical scavenging activity of sampled PM (panel d) did not seem to change in relation to filters storage time and conditions. Although not much is yet known about the chemical species responsible for the PM reducing activity, the results from this experimental stage highlighted the stability of this assay for the prediction of the chemical species involved in PM reducing properties.

The results obtained from this experimental stage are closely related to the key research topic focused on testing OP as an air quality metric and to study its biological relevance, which is still under research and investigation. Some studies already reported important findings that, for example, identified short lifetime oxidant species in a fraction of organic aerosols (Brown et al., 2020; Zhang et al., 2021). In this experimental work, the speciation of redox species to identify stable and unstable compounds is not available and is out of the scope of the study.

Overall, at least for the OP^{DCFH} and OP^{AA} assays, these results showed a very significant role of samples storage conditions and durations, which have been poorly investigated in the literature. It is worth noting that the decrease of the values seems not to be constant for each sample and that, for this reason, the variability among samples may be dependent on storage conditions and duration. Consequently, although the OP measured on filters stored for a long time still represents the most adopted procedure (Bates et al., 2019) it could not be truly representative of the real potential of ambient particles to generate reactive species. In support of this evidence and based on current literature, the OP^{DTT} is the assay most relevant to health, due to its greatest associations to health endpoints (Bates et al., 2015, 2019; Fang et al., 2016; Abrams et al., 2017). Otherwise, OP^{AA} still presents a limited utility in epidemiological studies due to the lack of direct associations with adverse health endpoints (Atkinson et al., 2016; Fang et al., 2016).

Given the ROS decay behavior, recent studies presented online measurement technologies to measure OP of PM and could constitute a valid alternative to avoid the loss of reactive species before analyses reducing the time delay before sampling and analysis and possible degradation of compounds on filters. These studies confirmed that OP online measurements, through direct PM sampling into the liquid phase and measurement within a few minutes, are useful and necessary for reliable ROS quantification (King and Weber, 2013; Fuller et al., 2014; Wragg et al., 2016; Eiguren-Fernandez et al., 2017; Puthussery et al., 2018; Zhou et al., 2018; Brown et al., 2019; Campbell et al., 2019). Some of these systems have also given excellent results in terms of collection efficiency of particles (Orsini et al., 2003; Brown et al., 2019). However, although OP online measurements represents an exciting challenge in atmospheric pollution research, offline assays are still mostly performed, as mentioned above (Bates et al., 2019).

3.3. Influence of the type of filter used on PM redox measurements

The comparison between results obtained measuring OP on Q or P filters that were sampled in parallel is reported in supplementary material S3 (Figure 1.S3). OP^{DCFH}, OP^{DTT} and RP^{DPPH} did not appear to be significantly influenced by the choice of the membrane filter. Conversely, a significant effect of the type of filter used was observed for the AA assay ($p < 0.01$). In fact, OP^{AA} on PTFE was found to be generally higher

than OP^{AA} on quartz. These results partially reflect those reported by Yang et al. (2014): the attenuation of the OP^{AA} of Q filters might suggest lower extraction efficiency of the OP reactive species from the quartz filters compared to PTFE filters. On the other hand, OP^{DCFH}, OP^{DTT} and RP^{DPPH} values were less affected by the type of filter used, indicating that reactive components for these assays were efficiently extracted from the quartz as well as from the PTFE filters.

3.4. Influence of water-insoluble PM components redox activity on PM redox measurements

The active role of PM insoluble fraction in determining OP and RP values was assessed by performing OP and RP assays directly on the aqueous suspension of filters, after the extraction with RA and without any preliminary filtration step. Consequently, the effect of both water-soluble and water-insoluble components of PM on its redox potential were simultaneously observed, by adding reagents of each assay directly in contact with the sampled filter (Khurshid et al., 2019). The comparison of OP and RP results between the total and the water-soluble fraction of PM filters (T and WS, respectively) is reported in supplementary material S3 (Figure 2.S3).

Regarding the OP^{DCFH} and OP^{DTT} assays, the p value between the total (soluble plus insoluble) and the water-soluble fraction is 0.41 and 0.06, respectively, indicating that the contribution of the water-insoluble PM components redox activity on OP^{DCFH} and OP^{DTT} measurements is not quite significant, at least as far as this set of samples, also considering that the size of the dataset is rather limited. Results showed that OP^{T} is comparable (or even lower than) to OP^{WS}, contrary to what emerged from studies that demonstrated a very relevant contribution of the PM insoluble fraction to OP (Verma et al., 2012; Conte et al., 2017; Piacentini et al., 2019). A possible explanation to these results is that most of the particles of the sampled PM were fixed too deeply in the filter to fully interact with the reagents of the assays. This also represents a possible contribution to the uncertainty of the results. Nevertheless, different studies have claimed that the PM water-soluble fraction shows a higher correlation with OP metrics compared to the insoluble one (Szigeti et al., 2015). Conversely, OP^{AA} results highlighted significant differences between values obtained for T and WS fraction ($p < 0.01$). It is well known that OP^{AA} is particularly sensitive to elements tracing non-exhaust traffic emissions, that mainly constitute the insoluble fraction of PM (Conte et al., 2017; Massimi et al., 2020b). It is therefore possible that a part of these elements significantly influence OP^{T}. RP^{DPPH} shows a similar pattern to that of OP^{AA}, with OP^{T} values significantly higher than OP^{WS} ($p < 0.01$). These findings confirm previously obtained results that have shown higher RP^{DPPH} values for the insoluble fraction of PM, attributable to the contribution of non-exhaust vehicular traffic to RP (Frezzini et al., 2019).

The results of this experimental step does not allow to clarify the real contribution of PM water-insoluble fraction to PM redox potential. Undoubtedly, also the choice of extraction solvent definitely influences the extraction of PM species (Yang et al., 2014). Previous works investigated the contribution of PM insoluble species extracted through different extraction media, such as organic solvents (Calas et al., 2017; Gao et al., 2017; Pietrogrande et al., 2021). In this work the selected extraction media was the water in order to attempt

the evaluation of OP due to the water-insoluble fraction of particles. Therefore, results from WS vs T comparison were due to the limitation of the detection method and not to the concrete lack of contribution of the insoluble fraction to the OP. Furthermore, the operatively difficult of performing OP assays directly on particles that are embedded in the membrane support is not negligible.

To conclude, knowledge of the insoluble chemical species responsible for the possible contribution to redox potential of particles, as well as the mechanisms involved in PM redox activities, would be useful to elucidate the relative health risks for specific health endpoints and, thus, need to be deepened.

4. Conclusions

In this work, the effects of different operating conditions on redox measurements of PM_{10} field filters was assessed. This study highlights the influence of multiple operating conditions on measured oxidative and reducing potential from which necessarily derives the need of standard protocols for obtaining reliable and comparable data, in order to improve the ability of OP to predict health outcomes.

The extraction methods (rotating agitator, ultrasonic bath, and vortex) were compared in terms of repeatability and efficiency and were shown to influence the repeatability of OP^{DCFH}, OP^{AA} and OP^{DTT} and RP^{DPPH} whose best values were obtained by rotating agitator that has proved to be the most suitable PM_{10} extraction method for obtaining repeatable OP data. Results of the extraction efficiency confirmed that sonication of PM_{10} filters leads to the generation of ultrasound-induced radical species that particularly affect OP^{DCFH} values. These positive artifacts should be considered in interpreting results of redox measurements.

The most relevant findings are related to the effects of storage conditions and duration of PM_{10} filters. Although the filters conservation had not influence on OP^{DTT} and RP^{DPPH} values, storage conditions and duration were shown to be extremely significant for OP^{DCFH} and OP^{AA}, leading to outstanding concerns of the reliability of these offline assays for the prediction of the ROS generation pathways in real-word conditions. This aspect should therefore be carefully deepened in order to fully understand which oxidative species are short living, and which ones are long living, thus ensuring more robust evaluation systems in determining OP of PM.

The type of filter used was found to be relevant for the measurement of OP^{AA} values that appeared to be significantly higher when PTFE filters were used.

Ultimately, the role of the PM water-insoluble particles in determining PM redox potential was assessed. Although the redox activity of the insoluble fraction of PM provided a significant contribution to the OP^{AA} and RP^{DPPH} values, its study still merits further investigations.

This study highlights the influence of multiple operative conditions on redox measurements from which necessarily derives the need of standard protocols for obtaining reliable and comparable data, in order to improve the assurance of OP to predict health outcomes.

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Author statement

M.A. Frezzini: Conceptualization, Investigation, Methodology, Data curation, Writing-Original draft preparation **N. De Francesco:** Investigation, Methodology **L. Massimi:** Reviewing and Editing **S. Canepari:** Conceptualization, Supervision, Reviewing and Editing.

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