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Application of DPPH Assay for Assessment of Particulate Matter Reducing Properties

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Abstract: Different acellular assays were developed to measure particulate matter's (PM) oxidative potential (OP), a metric used to predict the ability of PM in generating oxidative stress in living organisms. However, there are still fundamental open issues regarding the complex redox equilibria among the involved species which could include reducing compounds. The aim of this study was the pilot application of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to PM in order to evaluate the presence of reducing species. The assay, commonly applied to biological matrices, was adapted to PM and showed good analytical performances. It allowed the analysis of conventional 24 h airborne PM samples with suitable sensitivity and good repeatability of the measurements. The assay was applied to seven samples representing possible PM contributors (certified urban dust NIST1648a; brake dust; Saharan dust; coke dust; calcitic soil dust; incinerator dust; and diesel particulate matter certified material NIST1650b) and to PM_{2.5} field filters. The same samples were also analyzed for elements. Preliminary results indicated that the assay gave a linear response and that detectable amounts of reducing species were present in PM samples. The combined application of DPPH and conventional OP assays could then permit, in the future, to gain more knowledge about the reaction and/or competition between oxidative and reducing processes.

Keywords: oxidative potential; PM redox properties; 2,2-diphenyl-1-picrylhydrazyl antioxidant assay; air quality monitoring

1. Introduction

Particulate matter (PM) pollution is a serious global problem, especially in urban and industrialized areas [1], threatening both human and environmental health [2]. A growing number of scientific studies have demonstrated links between exposure to ambient PM and adverse health outcomes in human, especially related to cardio-pulmonary diseases [3] and neurological disorders [4]. Over the last few decades, oxidative stress has been identified as one of the key mechanisms by which PM exerts its negative impacts on cellular systems and, thus, on living organisms [5,6]. Oxidative stress is due to the imbalance between the generation of reactive oxygen species (ROS) alongside reactive nitrogen species (RNS) and the antioxidant defenses [3]. Particulate matter's capacity to elicit damaging oxidative reactions and inflammations is defined as oxidative potential (OP) [7]. Measurements of particles' OP are considered a promising and integrative method for assessing health impacts induced by PM [8]. In fact, it is increasingly recognized that this PM property is more closely associated with adverse

health impacts than ordinarily used PM mass concentrations [9]. In this context, many acellular assays were developed to measure oxidative potential in order to gain a proxy of this PM capability [10]. There is still no agreement regarding the most representative assay to determine oxidative potential [11], but most commonly used acellular tests include the ascorbic acid (AA) [12] and dithiothreitol (DTT) [13] assays which consist of mimicking the consumption of a physiological and a surrogate antioxidant, respectively, and 2',7'-dichlorofluorescein (DCFH) by which particle-bound ROS are determined [14]. Oxidative potential acellular assays present several advantages such as ease of application, a clear and wide description in the literature that make them replicable, and applicability to a high number of samples during monitoring campaigns by generating large data sets with the aim of studying different redox components for detailed investigation [11,15,16]. However, several studies showed that each assay is sensitive to different pathways of ROS/RNS formation [17], and results are also responsive to different ROS/RNS generated by PM components and sources, meaning that each OP assay is seemingly linked to different health endpoints [15]. Therefore, the combined application of different acellular methods on the same samples is strongly suggested. Although some recent studies were focused on the *in vivo* evaluation of PM's negative impacts on living organisms [18–20], knowledge about the relationships between OP and PM's toxicological effects still presents some important gaps [6,20,21].

Other still unresolved issues are related to the assay's design and the influence of the operative conditions by which OP tests are performed. In fact, over recent years, many researchers underlined how test conditions could influence collected data. For example, recent studies analyzed the effect derived by the choices of extraction solvent (methanol and/or water) and sampling filter type (quartz or Teflon filter) on different OP assays [17,22]. Other researchers studied the impact of sonication-derived free radicals on OP results and suggested alternative extraction methods such as vortexing, magnetic stirrer or orbital shaking to avoid ultrasound-induced radicals [23,24]. The influence of filter-storage conditions also merits further investigation. Moreover, OP assays are commonly performed on PM water-soluble fractions, and another still debated issue concerns the lack of an appropriate standard protocol for measuring the water-insoluble oxidative potential [15]. An additional important point is related to the stability of the species: short-life oxidant species can react and redox equilibria, and among PM native species, this could occur during the sample storage and extraction phase. In this regard, it is possible that, within the conditions that influence OP results, there is also the reaction and/or competition between oxidant and reducing species naturally occurring in PM. In fact, PM is a heterogeneous and complex mixture of particles [25] that varies in composition and it is very difficult to identify the exact chemical constituents [26]. Some studies proved the presence of species with likely antioxidant and reducing characteristics such as phenols from wood burning [27], phenolic compounds from different sources [28], pollen [29], airborne bioaerosols that are biological in origin [30], and biological and vegetable components [31].

This research was aimed at clarifying the latter aspect by considering the application of an acellular procedure to evaluate the presence of reducing species in PM samples. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a rapid, simple, and widely used method to evaluate the antioxidant potential of a compound or an extract [32], and it is commonly used for vegetable juice [33], olive oils, and wines [34]. The assay is known to be sensitive towards some classes of reducing species such as phenolic and polyphenolic compounds [35]. 2,2-Diphenyl-1-picrylhydrazyl is a stable free radical by virtue of the delocalization of the spare electron over the molecule [36], and it accepts electrons or hydrogen radicals from donor compounds [37].

The assay is based on the quantitative measurement of the scavenging capacity of antioxidants towards DPPH free radicals [32] by the decrease in absorbance [38]. This test provides information on the antioxidants' capacity to donate hydrogen atoms or electrons [37,39]. To date, no DPPH standard experimental procedures exist; various researchers have used widely different protocols [40,41] based on their treated samples which differ in DPPH concentration, reaction time, and reaction solvent. To our knowledge, this assay has never been applied to PM before.

The aim of this work was to evaluate the applicability of the DPPH assay to PM for measuring reducing potential (RP) due to the presence of antioxidant species which could, in the future, integrate the information about redox properties of PM obtained by OP assays. In this work, the DPPH assay was applied to seven types of widespread components of PM produced by specific emission sources and characterized by very different chemical compositions [19,20] (i.e., urban dust certified for its elemental content, UD; brake dust, BD; Saharan dust, SD; coke dust C; calcitic soil dust, CSD; incinerator dust, ID; and certified diesel particulate matter, D). Moreover, the DPPH assay was applied to PM_{2.5} field samples collected during a short monitoring campaign.

2. Materials and Methods

2.1. Reagents and DPPH Assay

The DPPH solution was prepared according to the procedure described by Chen et al. [42] with slight modifications. The stock solution, prepared daily, was used at a 0.1 mM final concentration: 2 mg of DPPH reagent (Sigma–Aldrich, USA) were weighed (Analytical Balance Gibertini Elettronica E505, sensitivity 0.01 mg) and suspended in 50 mL of ethanol (EtOH) 96%. The mixture was vigorously shaken for 30 min under magnetic stirrer agitation (ARE Heating Magnetic Stirrer; Velp Scientifica, Usmate, Italy) and kept at room temperature in the dark. Experimental data were acquired on a spectrophotometer (Varian Cary 50 Bio UV-Vis; Varian Inc., Palo Alto, CA, USA), set at 517 nm under dim light, by measuring the sample absorbance decrease against the control (blank solution). The DPPH radical scavenging effect results in decolorization and is calculated in terms of percentage reduction of DPPH according to the following equation [43]:

$$\% \text{ DPPH Reduction} = \frac{(A_0 - A_s)}{A_0} \times 100$$

where A_0 represents the absorbance of the control and A_s is the absorbance of the samples.

The % DPPH reduction values were normalized per sampled air volume (m³; %DPPHv) or per PM mass amount (mg; %DPPHm).

2.2. Collection and Chemical Characterization of Samples

Certified urban dust (urban particulate matter, NIST168a, Sigma–Aldrich, USA) was used as urban dust. Certified diesel particulate matter (diesel particulate matter, NIST1650b, Sigma–Aldrich, USA) was used as diesel dust. Besides the certified materials UD and D, some of the other major PM sources were considered. Brake dust, collected from the brake linings of three different cars, represents the part of road dust containing the highest concentration of heavy metals and other pollutants [44]. In the Mediterranean area, Saharan dust represents the major contribution to PM after events of long-range transport from North Africa [45]. Saharan dust was collected in Algeria, in the north of the Sahara Desert. Coke dust contains very high concentrations of organic toxic species [46] and was taken from the ground near a coal park. Calcitic soil dust, one of the major natural compounds of PM [20], was collected in rural areas around the city of Rome. Incinerator dust was sampled by a fine-mesh filter placed in a waste-to-energy plant chimney in Northern Italy. Incinerator dust is known to contain various atmospheric compounds, including organic ones [47], such as polycyclic aromatic hydrocarbons (PAHs) [48] and steroids from plant or animal materials not entirely combusted [49]. All types of PM were sieved at 50 μm and stored at −18 °C until use. More specific details about sample collection are reported by Marcocchia et al. [19] and Simonetti et al. [50]. Although the use of these types of PM is not completely representative of real atmospheric dust, it allows to compare different experimental procedures on a homogeneous sample by performing different replicates.

The dimensional distribution of UD, BD, and C was estimated by Simonetti et al. [50] using optical microscopy (Zeiss Imager M1m; Carl Zeiss Inc., Thornwood, NJ, USA). Images were then converted into black/white and automatically enumerated by NIH ImageJ software v.1.46r (National Institute of

Health, Bethesda, MD, USA). Although the system did not allow to measure particles smaller than approximately 1 μm , 50% of particles had diameters lower than 10 μm in all the considered samples. This estimation was not possible for the other considered types of PM because of the low contrast of the images.

The 24 h $\text{PM}_{2.5}$ field samples were collected from 29 March to 17 April 2019 by a sequential sampler working at 2.3 m^3/h (SWAM5a Dual Channel Monitor, FAI Instruments, Fonte Nuova, Rome, Italy) on PTFE membrane filters (46.2 mm diameter, 2 μm pore size; Whatman, Maidstone, UK) near Ferrara, Italy (Cassana, coordinates: 44°51'4'' N; 11°32'56'' E). Although it was not a long monitoring sampling, it was enough for applying the experimental procedure for demonstration purposes. The $\text{PM}_{2.5}$ concentration was automatically measured by beta attenuation. At the same site, the mixing properties of the lower atmosphere were determined by an automated monitor of the natural radioactivity due to the radon progeny (PBL Mixing Monitor, FAI Instruments, Fonte Nuova, Rome, Italy).

The $\text{PM}_{2.5}$ filters were extracted by following the procedure detailed by Massimi et al. [51]. Briefly, after removal of the supporting polymethylpentene rings from each sampled filter, PTFE membrane filters were extracted in 10 mL of deionized water (produced by Arioso UP 900 Integrate Water Purification System, USA) by 30 min of rotating agitation (60 rpm; Rotator; Glas-Col, USA). After the extraction, the solutions were filtered through a nitrocellulose filter (NC; pore size 0.45 μm ; Merck Millipore Ltd., Billerica, MA, USA). The extracted solutions were split in their respective aliquots for the analyses. Then, both the NC filter and sampled PTFE filter were acid-digested in a microwave oven (Ethos Touch Control with Q20 rotor, Milestone, Italy) using 2 mL of HNO_3 (67%, Promochem, Wesel, Germany) and 1 mL of H_2O_2 (30% Suprapur, Merck Millipore Ltd., Billerica, MA, USA). Details about the digestion program are reported by Massimi et al. [51]. The PM filters were chemically analyzed by adapting an analytical procedure for the determination of micro and trace element on PM samples, optimized and validated by Canepari et al. [52,53] using inductively coupled plasma mass spectrometry (ICP-MS, Bruker 820-MS, Billerica, MA, USA). The concentrations (ng/m^3) of elements (Al, As, Bi, Cd, Ce, Co, Cr, Cs, Cu, Fe, La, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, Sb, Sn, Sr, Ti, Tl, V, Zn, Zr) were determined in both water-soluble and mineralized residue fractions.

2.3. Oxidative and Reducing Potential Assays on Selected Types of PM

The DPPH assay was applied to the seven selected samples by following 4 different protocols to evaluate which experimental procedure better suited which samples. Four DPPH assay experimental procedures with different extraction solutions were compared in terms of limits of detection (LODs), repeatability, and efficiency. First, ethanol and water were compared as extraction solvents ($\text{DPPH}_{\text{EtOH}}$ and $\text{DPPH}_{\text{H}_2\text{O}}$, respectively). Although in the literature, ethanol is commonly used as an extraction solvent to perform the DPPH assay [42,43], water extraction was included in this study as it could permit the integration of this acellular method to the whole chemical characterization procedure on a single PM sample. Regarding water extraction, the effect of the addition of ethanol to the reaction mixture was evaluated ($\text{DPPH}_{\text{H}_2\text{O}/\text{EtOH}}$), as it could increase the solubility of species involved in the DPPH reaction. Finally, the DPPH assay was directly applied to the suspension of samples without any extraction step in order to verify the possible contribution of insoluble species (DPPH_{TOT}).

2.3.1. DPPH Assay on EtOH Extracted Samples

First, the extraction method involved EtOH, according to Mishra et al. [54]. Five milligrams of samples, exactly weighed on analytical balance, were extracted in 5 mL of EtOH 96% by 30 min of rotating agitation (60 rpm). Extracted solutions were filtered through a PTFE syringe filter (PTFE; pore size 0.45 μm ; Fulltech Instruments, Rome, Italy), and then 3 mL were mixed with 1.5 mL of DPPH 0.1 mM solution. The mixture was shaken for 30 min by rotating agitation, and then analyzed by UV-Vis spectrophotometry set at 517 nm ($\text{DPPH}_{\text{EtOH}}$).

2.3.2. DPPH Assay on H₂O Extracted Samples

Five milligrams of each sample were exactly weighed and extracted in 5 mL of deionized water by rotating agitation for 30 min. The extraction solutions were filtered through a nitrocellulose filter (NC filters), and then 2 mL were mixed with 1 mL of DPPH 0.1 mM solution. The mixture was stirred for 30 min by rotating agitation and then analyzed by UV-Vis (DPPH_{H₂O}).

2.3.3. DPPH Assay on H₂O Extracted Samples with EtOH Addition

Five milligrams of selected types of PM were exactly weighed, suspended in 5 mL of deionized water, and extracted by rotating agitation for 30 min. After the extraction, the solutions were filtered through a nitrocellulose filter (NC filters). According to Scalzo [55], 1.5 mL of extracted samples were mixed with 2 mL of EtOH 96% and 0.5 mL of DPPH 0.1 mM stock solution for 30 min by rotating agitation and then analyzed by UV-Vis as already described (DPPH_{H₂O/EtOH}). This procedure was also applied to different amount of the two considered certified materials, UD and D (range 2–10 mg), in order to verify the linearity of response.

2.3.4. DPPH Assay on whole Dusts Samples

The RP^{DPPH} on whole samples was directly performed on 4 mg of each type of PM, without any previous extraction step using 1.5 mL of deionized water, 2 mL of EtOH 96%, and 0.5 DPPH 0.1 mM stock solution. The mixture was shaken for 30 min by rotating agitation and filtered through PTFE syringe filter (PTFE filters) prior to UV-Vis analyses (DPPH_{TOT}).

Ten blank measurements were performed for each experimental procedure. Limits of detection (LODs) were estimated as three times the standard deviation. Furthermore, for each experimental procedure; three replicates of each type of PM were performed.

2.4. Oxidative and Reducing Potential Assays on PM_{2.5} Filters

Since OPs are commonly evaluated in literature on water-soluble fraction [56,57], in this study, OPs and RP were performed only on water-soluble fraction of PM_{2.5} filters. Ascorbic acid (AA), dithiothreitol (DTT) and 2',7'-dichlorofluorescein (DCFH) assays were applied to proper aliquots of the extracted solution (Section 2.2.). The followed OPs procedures are detailed by Simonetti et al. [50,58]. Briefly, DTT and AA assays measure the depletion rate of chemical proxies for cellular reductants and antioxidants, respectively [16], by recording the decrease in absorbance by UV-Vis spectroscopy at 412 nm and 265 nm, respectively.

The chemical OP^{AA} protocol is very similar to the OP^{DTT} experimental procedure [15]. During the OP^{DTT} protocol, three aliquots of water-extracted samples (0.7 mL) were incubated with 0.1 mL of DTT 1 mM and 0.2 mL of potassium phosphate buffer (1 M) at 37 °C for times varying from 0 to 20 min. Then, 1 mL of trichloroacetic acid (TCA) was added to the mixture to quench DTT reactions. An aliquot (1 mL) was removed from the solution and mixed with 2 mL of tris-buffer (0.08 M, containing EDTA 4 mM) and with 5,5-dithiobis (2-nitrobenzoic acid; DTNB; 50 µL) to form 2-nitro-5-mercaptobenzoic acid (TNB) by reacting with the residual DTT which was then measured using a spectrometer.

For the AA assay, three aliquots of water-soluble sample fraction (2.5 mL) were mixed with 0.3 mL of potassium phosphate buffer (0.5 mM at pH 7.4) and 0.2 mL of ascorbic acid 2 mM, and the mixture was kept at 37 °C. The absorbance decrease was measured for 20 min by UV-Vis spectroscopy. Absorbance of the blank solution was measured and subtracted from the sample absorbance readings.

The OP^{DTT} and OP^{AA} were expressed as DTT or AA consumption rate per sampled PM volume or per mg of PM (nmol min⁻¹ m⁻³; nmol min⁻¹ mg⁻¹) [58].

The DCFH, the most common probe used to quantify particle-bound ROS, is a nonfluorescent reagent that becomes fluorescent (DCF) when oxidized in presence of ROS. 125 µL of DCFH solution (5 µM) and 5 mL of Horseradish Peroxidase (HRP) dissolved in a sodium phosphate buffer (25 mM at pH 7.4) were added to 1.5 mL of the sample soluble fraction prior to analysis to catalyze reactions [16].

The DCFH-HRP reagent was added to each sample and then analyzed through the emitted radiation at 530 nm by a fluorescence detector (Jasco FP-920; excitation wavelength: 427 nm). The OP^{DCFH} was expressed in nmol H_2O_2 per sampled volume or mass (nmol H_2O_2 m^3 ; nmol H_2O_2 mg^{-1}) by converting fluorescent intensity to H_2O_2 concentration using least squares regression with a H_2O_2 calibration curve to obtain the final particle-bound ROS measurement [16]. The calibration curve was obtained daily by using a standard H_2O_2 solutions at different concentrations (5×10^{-6} , 1×10^{-7} , 2×10^{-7} , 5×10^{-7} , and 1×10^{-6} M) [49].

One aliquot (1.5 mL) of the extracted solution was also used for the DPPH assay: 2 mL of EtOH 96% and 0.5 mL of DPPH 0.1 mM stock solution were added, and the mixture was shaken for 30 min by rotating agitation. Then, the absorbance of the solutions was measured at 517 nm by UV-Vis spectrophotometry and compared to blank measurements. The DPPH reduction percentage was calculated as already described.

3. Results and Discussion

3.1. DPPH Assay Application on Selected Types of PM

First, four DPPH assay experimental procedures with different extraction solutions were compared in terms of limits of detection (LODs), repeatability, and efficiency.

The LODs were in the range 2–7% of consumed DPPH; the lowest value (2%) was obtained for $DPPH_{EtOH}$ and the highest for $DPPH_{H_2O}$ (7%). Our findings about ethanol extraction performances ($DPPH_{EtOH}$) agreed with previous studies reporting that DPPH assay method seems to work well on ethanol-extracted samples, without any interference with the reaction [43]. The $DPPH_{H_2O}$ showed the highest LOD, and the worst repeatability can be explained considering that the dissolution of DPPH should be done in organic solvents (e.g., methanol or ethanol) [59] limiting the application of this reagent to pure aqueous systems. The simultaneous use of H_2O and EtOH as reaction media allowed better control of the reaction conditions by increasing DPPH solubility: LOD values for $DPPH_{H_2O/EtOH}$ and $DPPH_{TOT}$ were 4%. These results agree with previous studies reporting that antioxidants commonly require polar solvents to be extracted, while DPPH reaction occurs quickly when organic solvents are used as extraction solvents [60]. Ethanol is an organic and polar solvent and resulted to be appropriate for performing DPPH assay [60].

With the aim to evaluate DPPH assay sensitivity and its ability to differently react towards various PM components, seven types of PM, substantially differing for their chemical composition, were used for this experimental stage. Figure 1 reports DPPH reduction (%DPPHm) for each experimental procedure and for the selected samples. All the experimental procedures gave different results among selected types of PM, underlining the expected role of chemical composition on the obtained values, which is reported in Table S1 (Supplementary Materials).

The DPPH assay values were higher when performed on whole dust samples ($DPPH_{TOT}$), compared to the other experimental procedures, especially for UD, BD, and D, confirming the significant contribution of water insoluble species to PM reducing properties [50,61]. Overall, the $DPPH_{EtOH}$ and $DPPH_{H_2O}$ performances were comparable, although $DPPH_{H_2O}$ gave higher values than $DPPH_{EtOH}$ for UD; otherwise, $DPPH_{EtOH}$ exhibited higher values than $DPPH_{H_2O}$ for D. This is probably due to the different solubility of organic species in water and ethanol. In fact, according to Simonetti et al. [50], UD contains relevant water-soluble organic carbon species (WSOC) that could contribute to the $DPPH_{H_2O}$ results. Diesel exhaust particles (D) represent one of the major components of PM in urban areas [26] and are composed of a solid carbon spheres with a high surface area on which a variety of water insoluble organic compounds are adsorbed [62,63].

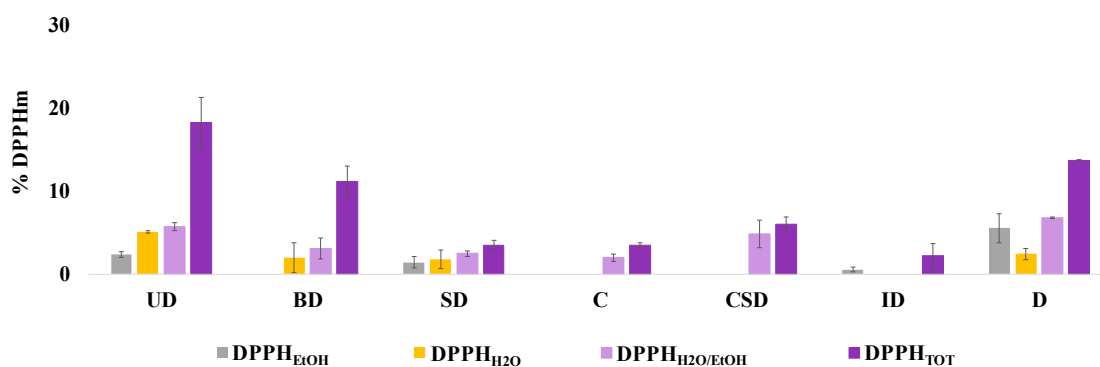


Figure 1. Reducing potential (%DPPHm) of the seven selected types of PM with the four experimental procedures obtained through a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay performed on EtOH-extracted samples (DPPH_{EtOH}), H₂O-extracted samples (DPPH_{H₂O}), H₂O-extracted samples with added EtOH (DPPH_{H₂O/EtOH}), and whole dust samples (DPPH_{TOT}). The mean \pm SD of three replicates are reported. Values below limits of detection (LODs) are not reported. UD: certified urban dust (NIST168a); BD: brake dust; SD: Saharan dust; C: coke; CSD: calcitic soil dust; ID: incinerator dust; D: certified Diesel particulate matter (NIST1650b).

In general, apart from ID and C, the considered types of PM contain detectable amount of species that can react with DPPH. It is well known that DPPH reacts with species that can donate a hydrogen atom or an electron to free radicals [32]. Although this method had never been applied on PM, these results permit to hypothesize that DPPH acts like free radicals commonly found or formed in PM (especially ROS or other radicals) by simulating their reaction with reducing species. This kind of reaction can also occur during the OP assay's application thus consuming ROS or other radicals and leading to an underestimation of PM's oxidative potential. In fact, the reaction between oxidizing and reducing species could reasonably occur during the filter storage and/or the extraction phase; in these cases, the native particle-bound ROS could be consumed before DCFH assay application. Furthermore, it is worth noting that ascorbic acid is often used as target reducing species for the DPPH calibration curve [43]. Considering that ascorbic acid reacts with ROS/RNS (both during the application of OP^{AA} and in biological fluids [64]), it is reasonable to hypothesize that other reducing species could similarly react with ROS/RNS during OP assays application or in biological systems. The reactive species generated in presence of AA or DTT could be consumed by the mentioned competitive reaction thus lowering their measured formation rate. A more extensive application of DPPH assay could help to gain information about this important issue.

In most literature studies, OP assays are applied to a water-soluble fraction of PM samples collected on membrane filters [16,50,61,65]. Therefore, with a view to add the RP^{DPPH} assay to the detailed PM chemical characterization during monitoring campaigns, water extraction with added EtOH after extraction (DPPH_{H₂O/EtOH}) could better operate alongside OPs and other chemical analyses.

The existence of a linear relationship between the dust amount and DPPH reduction (%) was tested for two samples, UD and D, by applying a regression model to data that showed a good fit ($R^2 > 0.97$ for both the samples weighted in the range 2–10 mg; Figure S3, Supplementary Materials).

Taking into account these considerations, the DPPH_{H₂O/EtOH} experimental procedure was used to perform a DPPH assay on PM_{2.5} field filters. However, it is worth noting that the contribution to RP results, due to the water insoluble species, merits further investigation.

3.2. Oxidative and Reducing Potential Assays on PM_{2.5} Field Filters

A short monitoring campaign was carried out in order to prove the applicability of combined RP^{DPPH} and OP (AA, DTT and DCFH) assays on a single PM sample.

The studied period was characterized by a low wind intensity, nighttime temperature included in the range 7–18 °C and daytime temperature in the range 10–23 °C (data from ARPAE Emilia-Romagna,

regional agency for prevention, environment and energy). Meteorological data registered during the monitoring period are reported in the Supplementary Materials (Figure S1). The natural radioactivity, which can be considered a reliable proxy of the lower atmosphere mixing properties [66], indicated, in general, a good dilution of the atmosphere (Figure 2a). During night, an increase in the stability was observed in the first five days of the campaign (29 March to 2 April); then, nighttime stability was also observed until 9–10 April. In the following days, a good mixing was again registered from 13 April, followed by a progressive increase of nighttime stability. In agreement with the great relevance of the low atmosphere dilution properties on air pollutants' concentrations and in the absence of relevant occasional contributors, PM_{2.5} concentrations followed the stability trend (Figure 2b). The PM_{2.5} mass concentration varied in the range 3.8–17 µg/m³, values that can be considered typical in the studied area during the spring season [67].

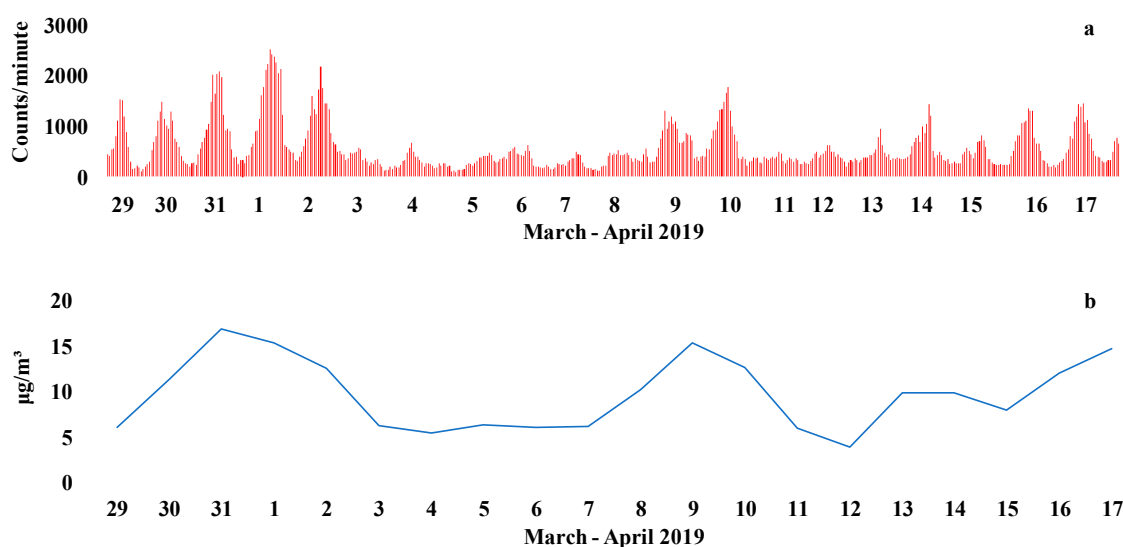


Figure 2. (a) Time patterns of natural radioactivity, expressed in counts per min, and (b) PM_{2.5} mass concentration (µg/m³) from 29th to 17th March-April at Cassana (FE), Italy.

Elemental concentrations (mean and min–max range) in the soluble and residual fractions are reported in Table 1. On the whole, the concentrations during the studied period were similar or lower with respect to that previously obtained in the same area during the warm season, and much lower than the typical winter concentrations which are strongly affected by prolonged periods of intense atmospheric stability [67,68].

The RP^{DPPH} and OP assays were performed on water-soluble fractions of collected PM_{2.5} field samples; the results are reported in Figure 3 and Figure S2 (Supplementary Materials), respectively.

Although sampled PM masses were low (about 0.5 mg), the obtained values were above the LOD (0.07% of consumed DPPH per m³) in every sample, except for one. This indicates that the assay was sufficiently sensitive to be applied to most PM samples. At this early stage of knowledge, the variability of the results among samples can be evaluated by comparison with PM_{2.5} mass concentration and with OP results. In Table 2, the correlation matrix of these parameters is reported. The DPPH assay showed moderate correlation with PM_{2.5} mass concentration ($r > 0.84$).

As can be observed from the correlation matrices, among all the considered parameters (elements in soluble and insoluble fractions of PM filters, RP, OPs; Tables S2 and S3, Supplementary Materials), PM_{2.5} concentration was, in turn, moderately correlated with most of the elements. This evidence indicates a low variability in the sampled PM chemical composition that was mainly modulated by the mixing properties of the low atmosphere, rather than by the strength of local emission sources. In this context, PM chemical composition remained almost constant, while sampled PM mass concentration

varied. This consideration was further confirmed by the moderate positive correlation between OP^{DTT} and PM_{2.5} ($r > 0.7$).

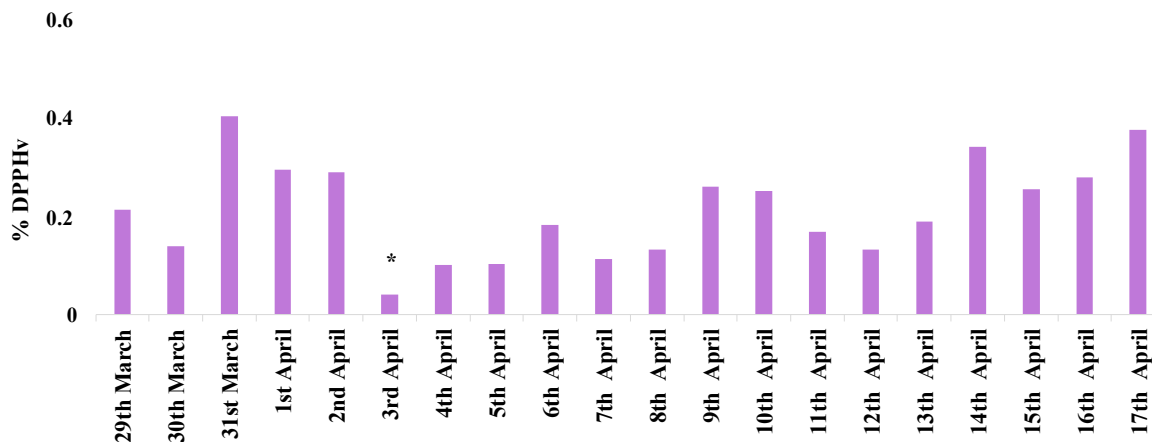


Figure 3. Reducing potential (%DPPHv) obtained through DPPH assay on 24 h PM_{2.5} filters collected in Cassana (FE), Italy, from 29th to 17th March-April 2019. * The value was set at LOD/2.

Table 1. Elemental composition of water-soluble and residual fractions of PM_{2.5} filters collected in Cassana (FE), Italy, from 27 March to 15 April 2019. * The mean was calculated by replacing values below LOD with LOD/2.

Soluble Fraction	LOD (ng/m ³)	Mean (ng/m ³)	Min–Max (ng/m ³)	Residual Fraction	LOD (ng/m ³)	Mean (ng/m ³)	Min–Max (ng/m ³)
Al	2.5	2.2*	2.5–7.1	Al	11	13*	11–91
As	0.29	0.24*	0.29–0.48	-	-	-	-
Bi	0.011	0.017*	0.011–0.049	Bi	0.004	0.036*	0.004–0.078
Cd	0.022	0.068*	0.022–0.19	Cd	0.051	0.046*	0.051–0.22
Ce	0.004	0.005*	0.004–0.012	Ce	0.047	0.025*	0.047–0.051
Co	0.005	0.011*	0.005–0.024	-	-	-	-
Cr	0.054	0.063*	0.054–0.13	Cr	4.1	2.4	1.6–3.5
Cs	0.002	0.006	0.002–0.016	Cs	0.006	0.003*	0.006–0.013
Cu	0.057	0.69	0.22–1.5	Cu	0.87	0.69*	0.87–1.8
Fe	9.2	8.1*	9.2–36	Fe	21	18*	21–44
La	0.005	0.004*	0.005–0.018	La	0.029	0.015*	0.029–0.034
Li	0.015	0.013*	0.015–0.026	Li	0.011	0.013*	0.011–0.019
Mg	6.1	6.2*	6.1–26	Mg	13	7.1*	13–16
Mn	0.12	0.66	0.19–1.4	Mn	0.35	0.66*	0.35–1.1
Mo	0.004	0.11	0.031–0.26	Mo	0.029	0.096	0.029–0.24
Na	7.9	28	11–75	-	-	-	-
Ni	0.58	0.33*	0.58–0.73	Ni	0.82	0.51*	0.82–1.8
Pb	0.21	0.92	0.21–2.4	Pb	0.17	1.1	0.44–3.1
Rb	0.11	0.17*	0.11–0.36	-	-	-	-
Sb	0.004	0.25	0.038–0.63	Sb	0.17	0.24*	0.17–0.39
Sn	0.006	0.071	0.026–0.19	Sn	0.11	0.39	0.17–0.71
Sr	0.22	0.22*	0.22–0.78	-	-	-	-
Ti	0.046	0.075*	0.075–0.18	Ti	0.79	0.42*	0.79–0.81
Tl	0.0005	0.002	0.0004–0.011	Tl	0.002	0.003*	0.002–0.006
V	0.015	0.33	0.031–1.8	V	0.079	0.097*	0.079–0.38
Zn	2.7	9.6	4.1–40	Zn	15	5.3	1.5–12
Zr	0.006	0.012	0.006–0.032	Zr	0.028	0.071	0.027–0.32

Table 2. Correlation matrix among PM mass concentration (PM), 2,2-diphenyl-1-picrylhydrazyl assay results (RP), and oxidative potential assays values (ascorbic acid, AA; dithiothreitol, DTT; 2',7'-dichlorofluorescein, DCFH).

Parameters	PM	RP	AA	DTT	DCFH
PM	-				
RP	0.77	-			
AA	0.19	0.056	-		
DTT	0.63	0.47	0.14	-	
DCFH	0.33	0.52	-0.23	0.34	-

It was also true that the short PM sampling period (thus, the DPPH preliminary application to a small sample set and the very low variability in terms of PM chemical composition), did not permit to identify species or sources responsible for DPPH results. However, obtained results confirmed the linearity of the DPPH assay; %DPPHv values correlated with sampled PM mass concentration by assuming a nearly constant PM chemical composition. At this stage of knowledge, the relevance of information that could be obtained by applying a DPPH assay to PM for evaluating its reducing properties still requires an evaluation. In fact, further studies should be addressed both to the identification of species and sources that drive DPPH results and to deepening the environmental significance of the proposed assay. However, with this preliminary study, the applicability of the DPPH assay as a new experimental approach for estimating the presence of PM reducing species was proved.

4. Conclusions

In this study, the DPPH radical scavenging assay was adapted and preliminarily applied to PM with the aim of verifying its possible use as an acellular method for estimating the presence of reducing species. The results showed that the assay was sufficiently sensitive to be applied to 24 h PM samples collected by samplers working at 2.3 m³/h with a good repeatability and linearity.

The described preliminary application of the DPPH assay revealed the presence of reducing species in several components of atmospheric PM derived from various emission sources and, thus, with very different chemical composition; the highest values were measured in urban dust, brake dust, and diesel dust. These outcomes seem to be mainly due to the reactions involving DPPH and organic fraction, but further studies are required to identify the species responsible for DPPH scavenging.

The RP^{DPPH} test is a cost-effective, rapid, and simple acellular method thus offering the same advantages of the OP assays. In the future, the combined application of all these tests to wider sample sets will permit to better understand redox equilibria among PM native species that might occur during sample storage, extraction phase, and/or application of oxidative potential assays. Furthermore, it is worth considering that reducing species could reasonably react with oxidizing ones, also when PM gets in contact with biological systems, thus exerting a possible opposition to oxidative stress.

The availability of a suitable assay for routinely estimate the amount of reducing species in PM samples could constitute a new and potentially useful tool for acquiring new insight in the field of its redox behavior and health effect.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4433/10/12/816/s1>, Figure S1: Meteorological data collected from 29th to 17th March-April 2019 at Cassana (FE), Italy: wind speed (a), rainfall (b), temperature (c), wind direction (d) and pressure (e), Figure S2: Oxidative potential obtained through 2',7'-dichlorofluorescein (OP^{DCFH}), ascorbic acid (OP^{AA}) and dithiothreitol (OP^{DTT}) assays on PM_{2.5} filters collected at Cassana (FE), Italy, from 29th March to 17th MarchApril 2019. Values below limits of detection (LODs) are not reported, Figure S3: Reducing potential (%DPPHm) linearity of response of (a) UD (urban particulate matter certified material, NIST168a) and (b) D (diesel particulate matter certified material, NIST1650b), Table S1: Chemical composition of the total fraction (water-soluble and insoluble) of brake dust (BD), coke (C), Saharan dust (SD) and calcitic soil dust (CSD). Mean ± standard deviation of three replicates is reported, Table S2: Correlation matrix among elemental concentrations, PM mass concentration (PM), 2,2-diphenyl-1-picrylhydrazyl assay (RP) and oxidative potential assays (ascorbic acid, AA; dithiothreitol, DTT; 2',7'-dichlorofluorescein, DCFH) of extracted fraction of PM_{2.5} filters collected in Cassana (FE), Italy, Table S3: Correlation matrix among elemental

concentrations, PM mass concentration (PM), 2,2-diphenyl-1-picrylhydrazyl assay (RP) and oxidative potential assays (ascorbic acid, AA; dithiothreitol, DTT; 2',7'-dichlorofluorescein, DCFH) of residual fraction of PM_{2.5} filters collected in Cassana (FE), Italy.

Author Contributions: M.A.F. and S.C. designed the research; M.A.F. elaborated the data and wrote the manuscript; M.A.F. and N.D.F. optimized the procedure and performed the DPPH assay, oxidative potential, and chemical analyses; F.C. and M.R. contributed to the analytical phase; S.C. coordinated the group and supervised the manuscript.

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