Ascorbate oxidation driven by PM$_{2.5}$-bound metal(loid)s extracted in an acidic simulated lung fluid in relation to their bioaccessibility

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Abstract

The oxidative potential (OP) is defined as the ability of inhaled PM components to catalytically/non-catalytically generate reactive oxygen species (ROS) and deplete lung antioxidants. Although several studies have measured the OP of particulate matter (PM OP) soluble components using different antioxidants under neutral pH conditions, few studies have measured PM OP with acidic lung fluids. This study provides new insights into the use of acidic rather than neutral fluids in OP assays. Thus, the first aim of this study was to clarify the effect of using an acidic lung fluid on ascorbic acid (AA) depletion. This was achieved by measuring the oxidative potential (OP-AA) of individual compounds known to catalyze the AA oxidation (CuSO$_4$, CuCl$_2$, and 1,4-NQ) in artificial lysosomal fluid (ALF, pH 4.5), a commonly used acidic simulated lung fluid, and in a neutral fluid (phosphate-buffered saline (PBS1x), pH 7.4). Our results from these individual compounds showed a significant decrease of OP-AA in the acidic fluid (ALF) with respect to the neutral fluid (PBS). Then, the second aim of this work was to investigate whether the OP-AA assay could be applied to PM$_{2.5}$ samples extracted in acidic conditions. For this purpose, OP-AA and bioaccessible concentrations of metal(loid)s (V, Mn, Fe, Ni, Cu, Zn, As, Mo, Cd, Sb, and Pb) of PM$_{2.5}$ samples collected in an urban-industrial area that were extracted in ALF were analyzed. The mean volume-normalized OP (OP-AAv) value was 0.10 ± 0.07 nmol min^{-1} m^{-3}, clearly lower than the values found in the literature at neutral pH. OP-AAv values were highly correlated with the ALF-bioaccessible concentration of most of the studied metal(loid)s, mainly with Cu and Fe.

Keywords Oxidative potential · Ascorbate · PM$_{2.5}$ · Metal(loid)s · Artificial lysosomal fluid · Bioaccessibility

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Introduction

Exposure to particulate matter (PM) has been significantly linked with cardio-respiratory diseases in numerous epidemiological studies (Kim et al. 2015; Shiraiwa et al. 2017; Pope et al. 2020). PM toxicity has been strongly related to its chemical composition, to its size (Kelly and Fussell 2012), and in particular to its ability to cause oxidative stress in the exposed population (Weichenthal et al. 2013; Ristovski et al. 2012; Bates et al. 2019). Regarding their size, particles smaller than 2.5 μm (PM$_{2.5}$) pose the greatest risk to health because they are mostly transported to the alveolar region (Mukhtar and Limbeck 2013). Furthermore, the most toxic components of PM are transition metals and certain organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), quinones, and phthalates (Seinfeld and Pandis 2016).

Although the mechanisms of action for PM and its compounds on health are still not completely understood,

extensive literature has pointed out that oxidative stress is likely one of the most important mechanisms of PM inhalation toxicity (Ghio et al. 2012; Weichenthal et al. 2013; Møller et al. 2014). Oxidative stress corresponds to an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defenses in cells and tissues (Sauvain et al. 2014; Tian et al. 2017). The ROS can be exogenously introduced to the body via particle inhalation and/or generated *in vivo* inside the cell via cellular redox reactions stimulated by target inhaled PM compounds (Bates et al. 2019; Park et al. 2018). An excess of ROS also increases the inflammatory response, which can cause various diseases, including respiratory and cardiovascular conditions (He et al. 2018; Van Den Heuvel et al. 2016).

In recent years, the oxidative potential (OP)—defined as the ability of inhaled PM components to catalytically/non-catalytically generate ROS and deplete lung antioxidants (Bates et al. 2019; Øvrevik 2019)—has been suggested as a relevant exposure metric to predict PM toxicity. Extensive research has measured PM OP using multiple acellular assays, among which the most widely used are the dithiothreitol (OP-DTT) and the ascorbic acid (OP-AA) tests (Øvrevik 2019; Gao et al. 2020a; Clemente et al. 2023). Nonetheless, there are many protocols for measuring OP within each assay and considerable doubts regarding which assay parameters should be adopted (Hedayat et al. 2015; Bates et al. 2019; Shen et al. 2021; Song et al. 2021).

Recent toxicological research has broadly assumed that the solubility of PM-bound compounds in pulmonary fluids (bioaccessibility) is one of the key factors for toxicological assessments from PM (Wallenborn et al. 2007; Charrier and Anastasio 2012; Mukhtar and Limbeck 2013; Guo et al. 2019). Several studies have measured PM OP when particles are extracted with ultrapure water followed by filtration to remove the insoluble fraction (Fang et al. 2016; Pietrogrande et al. 2018; Khoshnamvand et al. 2023). However, the use of water as a leaching agent is unrepresentative of physiological conditions in the lungs. For this reason, there is a growing number of investigations in which PM is extracted with simulated lung fluids (SLFs) frequently applied in bioaccessibility tests (Calas et al. 2019; Moufarrej et al. 2020; Pietrogrande et al. 2021; Song et al. 2021).

To assess metal(loid) bioaccessibility in the fine fraction of PM, two SLFs are commonly used in the literature: Gamble's solution (pH 7.4) and artificial lysosomal fluid (ALF) (pH 4.5). Gamble's solution is representative of the neutral interstitial fluid of the deep lung, whereas ALF represents the more acidic intracellular conditions found in the lysosomes of alveolar macrophages (Colombo et al. 2008; Mukhtar and Limbeck 2013; Innes et al. 2021).

Several operating factors, such as extraction solvents, extraction techniques, and filter types, have been investigated in OP evaluation (Calas et al. 2017; Frezzini et al. 2022; Khoshkam et al. 2022) and bioaccessibility tests as well (Colombo et al. 2008; Pelfrêne et al. 2017; Expósito et al. 2021a). Nevertheless, OP assay conditions have not been fully studied (Li et al. 2009; Shen et al. 2021); in particular, the composition of the leaching agent and the influence of the pH on the reactions involved in OP measurement still need to be thoroughly investigated (Øvrevik 2019; Shahpoury et al. 2019; Wei et al. 2021).

Some studies have compared PM OP at neutral pH when water, ALF, and Gamble's solution are used in the extraction (Calas et al. 2017; Cigánková et al. 2021; Pietrogrande et al. 2021). However, to our knowledge, only two recent studies have measured OP-AA under acidic conditions after extracting PM with an acidic simulated lung fluid (ALF) (Schiavo et al. 2022; Wu et al. 2022). Only Wu et al. (2022) measured the OP-AA of $PM_{2.5}$ samples; however, this study did not focus on discussing the lower reactivity observed in ALF compared to neutral fluids, nor on evaluating the sensitivity of OP-AA test in ALF to the concentrations of PM compounds and the emission sources.

The OP values were found to depend on the acidity and the composition of the extraction solution and on the soluble metal concentration. On one hand, previous investigations on metal(loid) bioaccessibility have shown that an increase in the solubility of toxic pollutants from PM occurs in acidic media, e.g., bioaccessibility in ALF is higher than in Gamble's solution (Colombo et al. 2008; Hernández-Pellón et al. 2018; Expósito et al. 2021a; Innes et al. 2021). A higher metal(loid) concentration in the OP assay would lead to a higher OP value. However, on the other hand, former research has highlighted that the ascorbate test for catalytic metals is more effective at neutral pH than at acidic pH because the oxidation of ascorbic acid catalyzed by metals is too slow at low pH. As a result, some investigations such as Gosselin and Zagury (2020) decided only to use Gamble's solution in the OP-AA assay because of the higher sensitivity of this assay near neutral pH, although both Gamble's solution and ALF were used to extract PM components in bioaccessibility tests. In fact, ascorbic acid species depend on pH (pka1 4.12, pka2 11.5) (Du et al. 2012), and the OP-AA assay follows the depletion of the ascorbate anion, so its concentration at acidic pH is lower (Weissberger et al. 1943; Khan and Martell 1967; Buettner 1988; Shen et al. 2021). Besides, the composition of SLFs may affect the OP, because species such as chloride, citrate, and glycine, which are found in some SLFs, could also reduce or inhibit the loss of AA (Mapson 1945; Ayres et al. 2008; Pietrogrande et al. 2019; Shahpoury et al. 2019). Therefore, OP assays

should be further investigated in acidic SLFs, since only a few studies have recently reported OP-AA values in these conditions, and poor or null discussion on why increased acidity modifies the PM OP can be found in such references.

It is important to highlight that under a macrophage attack scenario, the leaching of PM-bound metal(loid)s would occur in acidic conditions (Roth 2006; Mukhtar and Limbeck 2013); once solubilized, these metal(loid)s would contribute to the antioxidant depletion.

The purpose of this work was to assess the ascorbic acid PM OP in acidic simulated lung fluid. First, individual compounds known to catalyze the AA oxidation ($CuSO_4$, $CuCl_2$, and 1,4-NQ) in an acidic fluid (ALF, pH 4.5) were considered, and their OP was measured and compared with that obtained with a neutral fluid (phosphate-buffered saline (PBS1x), pH 7.4). Then, the OP-AA assay in ALF media was applied to $PM_{2.5}$ sampled in an urban-industrial area (Maliaño, Northern Spain). ALF-bioaccessible concentrations of metal(loid)s (V, Mn, Fe, Ni, Cu, Zn, As, Mo, Cd, Sb, and Pb) in $PM_{2.5}$ samples were also measured in order to associate the OP-AA values in acidic conditions with the metal(loid) bioaccessible contents.

Methodology

$PM_{2.5}$ sampling campaign

A $PM_{2.5}$ sampling campaign was conducted in the north of Spain, in the region of Cantabria located alongside the southern part of the Santander Bay, specifically in Maliaño. This area is mainly characterized by high air Mn concentrations, identifying the presence of a ferromanganese alloy industry as the main source of Mn (Arruti et al. 2011; Ruiz et al. 2014). The sampling site was located on the rooftop of the "Cultural Center of La Vidriera" (latitude: 43° 24′ 39.6″ N; longitude: 3° 50′ 13.2″ E) some 350m north of a Mn alloy production plant (Fig. 1). The sampling site is located downwind of the Mn alloy plant based on our earlier studies (Otero-Pregigueiro and Fernández-Olmo 2018; Hernández-Pellón and Fernández-Olmo 2019). A more detailed description of the sampling site can be found in Hernández-Pellón and Fernández-Olmo (2019).

$PM_{2.5}$ samples were collected using an impactor (Dekati® PM_{10} Impactor, PM2530) with a flow rate of 30 L/min for 24 h on a 47 mm Teflon filter. Overall, 60 $PM_{2.5}$ daily samples
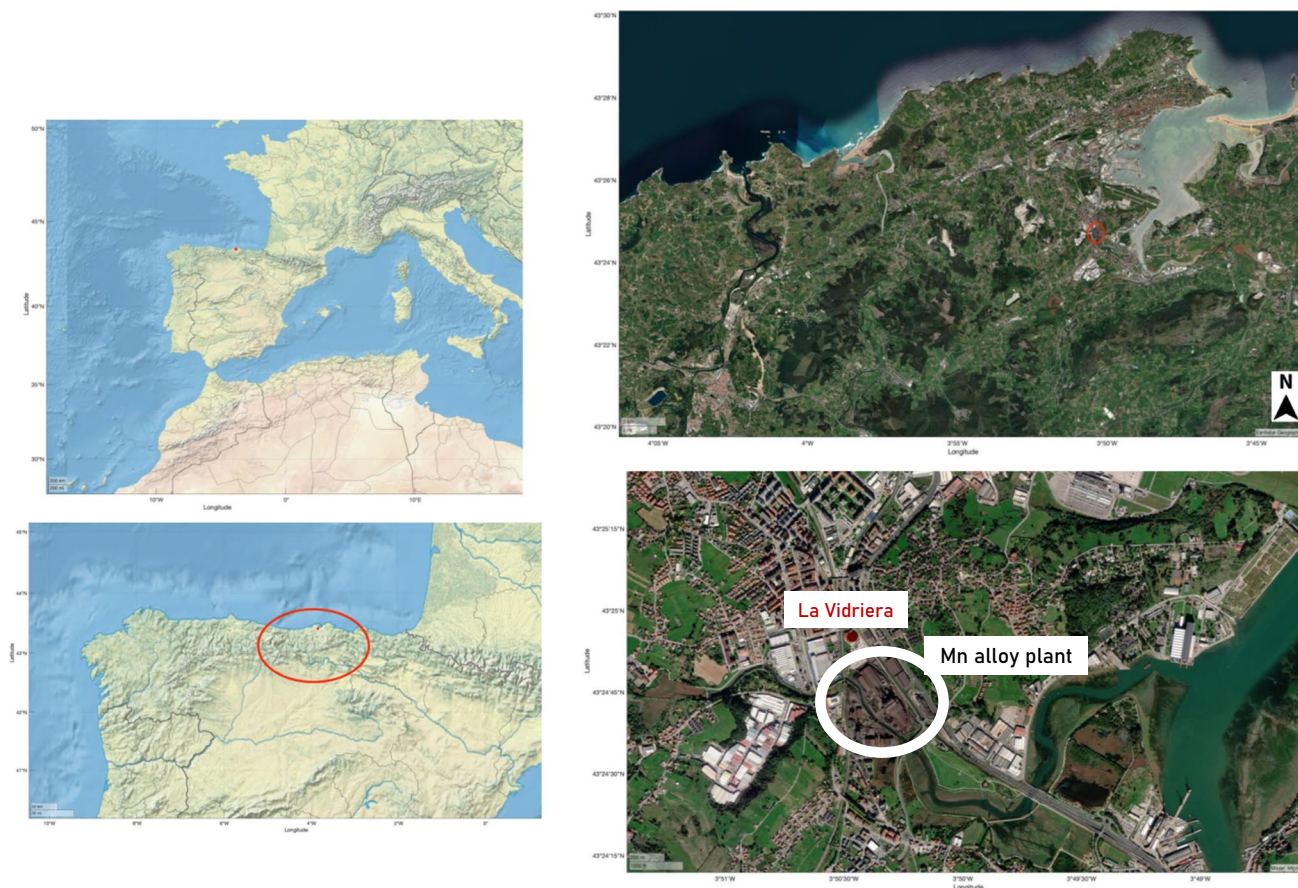

Fig. 1 Location of the sampling site (La Vidriera) and the manganese alloy plant. The figures were generated by MATLAB R2022b

were collected at the Vidriera site, 34 from November 2019 to March 2020, and 26 from September to November 2020 (the sampling campaign was interrupted due to the COVID-19 lockdown).

Once the gravimetric determination was performed, the filters were cut into two pieces using ceramic scissors. Half of the filter was used for the OP-AA analysis, while the other half was used for the determination of the metal(loid) bioaccessible concentrations.

Standard solutions and reagents

All chemicals were of analytical grade from AppliChem GmbH and Sigma-Aldrich and all solutions were prepared in purified water from a Milli-Q system (Millipore, ≥ 18.2 MΩ cm). ALF (pH 4.5 ± 0.1) was daily freshly prepared using ultrapure water and mixing the stock solutions following the order given in Table S1. The pH of the ALF was adjusted to 4.5 by adding dropwise 1 M of nitric acid (HNO_3) or sodium hydroxide (NaOH). Besides, phosphate-buffered saline x1 (PBS1x, 0.01M phosphate buffer, 0.154M sodium chloride) at pH 7.4 ± 0.1 was prepared by diluting phosphate-buffered saline x10 (PBS10x, 0.1M phosphate buffer, 1.5M sodium chloride).

Standard solution of ascorbic acid was prepared at a concentration of 10 mM in ultrapure water and stored at $-20°C$ in an amber glass vial due to its instability at room temperature until the measurement of PM OP values.

Three compounds known to catalyze the AA oxidation were considered: 1,4-naphthoquinone (1,4-NQ), copper (II) sulfate ($CuSO_4$), and copper (II) chloride ($CuCl_2$). Individual standard stock solutions were prepared at a concentration of 0.01 M using ultrapure water for metal salts and methanol for 1,4-NQ as solvents. Then, aliquots of Cu stock solutions were diluted in PBS1x (pH 7.4) and ALF (pH 4.5) at environmentally relevant concentrations (Charrier and Anastasio 2012; Visentin et al. 2016; Pietrogrande et al. 2022a), leading to final Cu concentrations in the microplate wells in the range of 0.34 to 1 μM, while the final concentration of 1,4-NQ was in the range reported by Calas et al. (2018) and Moufarrej et al. (2020), when it was used as a positive control (8 μM).

Ascorbic acid oxidative potential assay

The OP-AA assay was performed following the procedure of Moufarrej et al. (2020), but using ALF as a leaching agent.

PM$_{2.5}$ sample extraction in ALF

Briefly, for each PM$_{2.5}$ sample, half of the filter was incubated with 4.5 mL of ALF in a heated orbital shaker for 24 h at 37°C, at a speed of 250 oscillations/min. Solutions were then filtered

through 0.45-μm nylon filters, and the filtrates were placed in flasks and stored at 4°C until OP analysis. The maximum storage time was 24h.

Determination of ascorbic acid oxidative potential

The OP-AA assay was performed in 96 well plates with UV-transparent flat bottom. 160 μL of PM leachate (or blank or pure compound solution) was placed in each well of the plate, and the latter was incubated at 37°C for 5 min. Then, 40 μL of 1mM AA solution was added into the wells (so that the final AA concentration was 200 μM) and was shaken for 1 min. Finally, the microplate was placed in the spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Finland) at 37°C, and the absorbance was measured at 265 nm every 2 min for 2h with a 30 s shaking step before each measurement. The OP-AA assay only accounts for the oxidation of ascorbate anion (AH^-), since this species shows an absorbance peak at this wavelength. Note that the oxidation rate of the acid form (AH_2) is much slower than that of AH^- (Macartney and Sutin 1983). Prior to the absorbance measurements of chemical species and PM$_{2.5}$ samples, calibration curves were made plotting the absorbance/concentration data of the calibration standards (200, 150, 100, 50, and 0 μM) in ALF or PBS1x.

The AA depletion rates (OP-AA value) of chemical species and PM$_{2.5}$ samples were determined by considering the slope of the linear regression of the remaining AA versus time. In general, a good linearity was found for all samples with a correlation coefficient (R^2) higher than 0.98 between 5 and 60 min. Samples were analyzed in triplicate (RSD<15%). The OP-AA values were expressed per unit of mass of PM (OP-AAm in pmol min^{-1} μg^{-1}) and per unit of air volume (OP-AAv in nmol min^{-1} m^{-3}).

For the detection limit (D.L.) calculation, OP-AA values of 18 half-filters were determined by measuring the depletion rates of AA after extracting laboratory blank filters in ALF. The D.L. was calculated by multiplying the standard deviation of the OP-AA values for the 18 laboratory blank filters with the n-1 sample two-tailed Student's t-test at 95% confidence level (2.11). Furthermore, the OP-AA arithmetic mean of laboratory blank filters was subtracted from the depletion rate of each PM$_{2.5}$ sample. In particular, the mean value to be subtracted was 0.034 μM/min and the D.L. was 0.091 μM/min. The slope of AA depletion curves for most of PM$_{2.5}$ filters could be distinguished from that of blank filters, as shown in Figure S1. For statistical calculations, values below the D.L. were assigned D.L./2.

Bioaccessible metal(loid) concentration analysis in ALF

The metal(loid) bioaccessibility test was performed using ALF as leaching agent. Half of each PM$_{2.5}$ filter was extracted with 10 mL ALF in an end-over-end rotation

incubator system for 24h at 30 rpm and at 37°C using poly-propylene (PP) tubes. The L/S ratios varied between 11,765 and 200,000. After the extraction, the samples were centrifuged at 4200 rpm for 10 minutes, and the supernatants were filtered through a 0.45-μm polypropylene syringe filter. The samples were stored until analysis at 4°C, and the maximum storage time was 48h.

Concentrations of 11 metal(loid)s (V, Mn, Fe, Ni, Cu, Zn, As, Mo, Cd, Sb, and Pb) in ALF were measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500 CE). Blanks were considered by treating 10 laboratory blank filters with the same protocol applied to $PM_{2.5}$ filters. The arithmetic mean of blanks for each metal was subtracted from each measurement. D.L.s of the analyzed metal(loid)s were calculated based on the variability of the results of the 10 filter blanks (two-tailed Student's *t*-test with 95% confidence for n-1 samples (2.26) times the standard deviation of the blanks). Values below the D.L. were replaced with D.L./2 for the statistical analysis. The D.L. values of metal(loid)s are shown in Table S2. Further details of ICP-MS analysis of bioaccessible metal concentrations are described elsewhere (Expósito et al. 2021a).

Statistical analysis

Statistical analysis of the data was performed using IBM SPSS Statistics software (version 22). Relationships between OP-AAv values and bioaccessible concentrations of metal(loid)s were investigated by calculating correlation coefficients and principal component analysis (PCA).

All data distributions were checked for normality using the Kolmogorov-Smirnov test. Since most distributions deviated from the normality, the association between bioaccessible concentrations of metal(loid)s and OP-AA values was evaluated by determining Spearman's correlation coefficients (rho). A probability level of $p < 0.05$ was chosen to establish statistical significance.

Before applying the PCA, the data was first transformed into a dimensionless standardized form to consider the variability of the data set using the following equation:

$$Z = \frac{x - X}{\alpha} \tag{1}$$

where x is the daily bioaccessible concentration of the metal(loid) or OP-AAv, X is the arithmetic mean, and α is the standard deviation. Varimax orthogonal rotation was performed on the resolved factors in order to facilitate the interpretation. Resolved factors with high eigenvalues compared to the unity were considered to have a significant contribution. Additionally, the Kaiser-Meyer-Olkin (KMO) value was above 0.6 and the Bartlett value was found to be significant ($p < 0.01$).

Results and discussion

Behavior of pure compounds in neutral and acidic fluids

Figure 2 compares the AA depletion curves of 1,4-NQ, $CuCl_2$, and $CuSO_4$ over 60 minutes in ALF and PBS1x. The OP-AA values calculated from the depletion curves are shown in Table 1.

Our results show a strong decrease in the AA consumption in ALF with respect to PBS1x. Thus, high OP-AA values were obtained in PBS1x for 1,4-NQ (3.34 μM/min), similar to the value obtained by Moufarrej et al. (2020) (2.74 μM/min), but higher than that obtained by Visentin et al. (2016) at lower concentration of 1,4-NQ (0.64 μM/min at 1 μM). Our OP-AA values with Cu salts in PBS1X (1.29–1.33 μM/min at 1 μM) were lower than those obtained by Visentin et al. (2016) in PB solution (without chloride), 3.52 μM/min, at 0.17 μM Cu concentration. The role of chloride in the reduction or inhibition of copper activity has been studied in the literature. For instance, Pietrogrande et al. (2019) obtained higher OP-AA values when PB was used instead of PBS (2.33 vs 0.57 nmol/min). Although the mechanism of AA oxidation in the presence of chloride is still not well understood, Murekhina et al. (2022) suggested that the deceleration of ascorbic acid oxidation due to a higher concentration of chloride salts could possibly be explained by the formation of intermediate chloride complexes of Cu (I), whose oxidation rate differs from that of the free cation.

However, the AA consumption rate of 1,4-NQ in the acidic solution is so small that the slope cannot be distinguished from that of AA blank (0.08 μM min⁻¹). This compound is usually used as a positive control in neutral media due to its high catalytic capacity in AA oxidation, but not in acidic solutions. Only the OP-AA of $CuCl_2$ and $CuSO_4$ could be calculated in ALF media. Similar values were obtained for the two Cu (II) salts. However, a sharp decrease in the OP-AA value in acidic pH with respect to neutral pH was observed for these salts, approximately a 60 % reduction both at 0.34 and 1 μM. The Cu concentration plays also an important role in the AA depletion rate, resulting in OP-AA values between 2.4 and 2.7 times higher when the Cu concentration increases from 0.34 to 1 μM, both in ALF and PBS1x.

These results agree well with the ascorbate depletion rate equation shown in the literature (Buettner 1988; Murekhina et al. 2022):

$$\frac{d(AH^-)}{dt} = [AH^-] * k_{cat} * [\text{catalyst}] \tag{2}$$

Thus, a higher concentration of the catalyst (Cu) leads to a higher depletion rate (OP-AA value) (Cervellati et al. 2020). In addition, the lower consumption rates of ascorbate
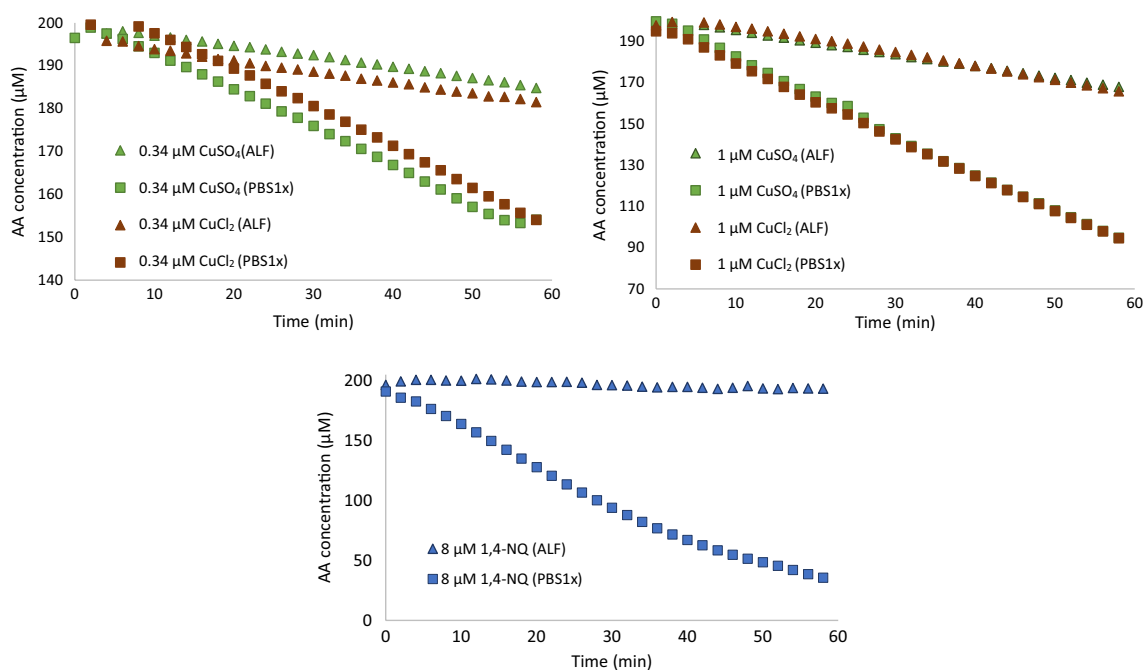
Fig. 2 AA consumption rate of pure compounds (CuSO$_4$, CuCl$_2$, and 1,4-NQ) in acidic (ALF) and neutral (PBS1x) media

Table 1 OP-AA values from catalytic-active compounds in the AA autoxidation at neutral and acidic pH

Species	Concentration tested (μM)*	Depletion rate (μM AA min^{-1})	
		PBS1x (pH 7.4)	ALF (pH 4.5)
CuSO$_4$	0.34	0.47	0.18
CuSO$_4$	1	1.29	0.47
CuCl$_2$	0.34	0.57	0.22
CuCl$_2$	1	1.33	0.54
1,4-NQ	8	3.34	< D.L.

*Concentration in the microplate wells

in ALF compared to PBS1x could be partly explained by the variation of protonated AA species with pH. The AA assay measures the ascorbate species, which is dominant in neutral conditions and its amount decreases at lower pH while the amount of ascorbic acid species (AH$_2$) increases (Khan and Martell 1967; Hayakawa et al. 1973; Buettner 1988). Specifically, the percentage of AH$^-$ is 71% and close to 100% at pH 4.5 and 7.4, respectively. This leads to a decrease in the initial AH$^-$ concentration at an acidic pH (see Fig. S2) leading to a lower reaction rate. However, the reduction in AA consumption in acidic conditions cannot be fully explained by the lower concentration of ascorbate species; the oxidation rate of ascorbate also decreases at acidic pH (Khan and Martell 1967), but this decrease depends on the catalyst (a much higher decrease with 1,4-NQ compared to Cu (II) as shown in Table 1). The lower depletion rate in ALF

can also be explained by the effect of the fluid composition on the kinetic constant (k_{cat}) of each catalyst. In particular, since ALF contains citrate, this may form complexes that affect the activity of the catalyst used. For example, Şahbaz and Somer (1993) reported that the rate of ascorbic acid oxidation depended on the catalytic activity of Cu(II)-citrate formed in the pH range of 2.5–6, following the order Cu(II)>H$_3$CiCu$^+$>HCiCu$^-$>CiCu^{2-}. However, 1,4-NQ was not expected to form strong complexes with the ALF components (Calas et al. 2017).

The results clearly show that the AA assay in PBS1x provides higher sensitive measurements of OP-AA than in ALF media. However, PBS1x is not a realistic representation of the conditions in the lungs and may overestimate the OP. ALF might better simulate the antioxidant depletion driven by PM compounds in biological conditions. In spite of the low sensitivity of OP assay in ALF media, the OP-AA of Cu (II), the most reactive ion reported in the bibliography (Khan and Martell 1967; Visentin et al. 2016), was accurately measured in ALF at a concentration range commonly found in ambient PM (Charrier and Anastasio 2012).

OP-AA of PM$_{2.5}$ filters in an urban-industrial mixed site

The AA assay in ALF was applied to 60 PM$_{2.5}$ filters collected at Vidriera site, obtaining 56 samples above the detection limit. This shows that the OP-AA assay was sensitive to quantify the OP levels even though the PM extraction and OP analysis were done in ALF. The OP-AA values ranged from a

minimum of 0.09 μM min^{-1} to a maximum of 1.14 μM min^{-1}. The mean air volume- and PM mass-normalized OP-AA values in PM$_{2.5}$ were 0.10 ± 0.07 nmol min^{-1} m^{-3} and 13.6 ± 11.94 pmol min^{-1} μg^{-1}, respectively (see Table 2). The PM$_{2.5}$ OP-AA responses, both volume- and mass-normalized values, were far below than those observed in other urban and industrial areas (Fang et al. 2016; Moufarrej et al. 2020; Borlaza et al. 2021; Li et al. 2021; Pietrogrande et al. 2022b). For example, Moufarrej et al. (2020) reported an average AA depletion rate in PM$_{2.5}$ samples solubilized in Gamble's solution of 1.12 ± 1.50 nmol min^{-1} m^{-3} (OP-AAv) and 55 ± 66 pmol min^{-1} μg^{-1} (OP-AAm) in Dunkerque (France) and Pietrogrande et al. (2022b) reported a mean OP of PM$_{2.5}$ samples solubilized in phosphate buffer of 1.08± 0.62 nmol min^{-1} m^{-3} air and 29± 100 pmol min^{-1} μg^{-1} in Milano Pascal (Italy). Similar values were also reported by Perrone et al. (2019) in Salento's peninsula (Italy). However, all of these studies measured the OP-AA in neutral fluids. Only, Wu et al. (2022) obtained a similar OP mean value for PM$_{2.5}$ samples collected in Shanghai (China) when ALF was used in the AA assay (0.33 ± 0.12 nmol min^{-1} m^{-3}); this value was clearly lower than that obtained in PBS in the same study (1.6 ± 0.27 nmol min^{-1} m^{-3}). It is important to highlight that the concentrations of metal(loid)s in ALF are higher than in neutral pH. However, the OP-AA values are lower proving a lower catalytic activity of PM$_{2.5}$ components due to the pH and the composition of ALF.

Overall, the comparison of our results with the other studies supported the idea that lower value measurements of OP-AA are obtained when an acidic simulated lung fluid (ALF) is used in the extraction. In addition, the differences in the OP-AA values compared with other studies could also be caused by different ambient concentrations of PM$_{2.5}$ components and the synergistic and antagonistic interactions among these components (Guo et al. 2020).

Bioaccessibility of PM$_{2.5}$-bound metal(loid)s in ALF

The bioaccessible concentrations of PM$_{2.5}$-bound metal(loid)s in ALF are shown in Table 2. The highest concentrations were found for Mn, Fe, Zn, and Pb. These concentration levels could be explained by the influence of the Mn alloy industry located in the sampling area (Hernández-Pellón et al. 2018; Hernández-Pellón and Fernández-Olmo 2019).

Mn is a metal of concern in the studied area. Although this metal is not regulated by EU air quality Directives, the World Health Organization (WHO) recommends an annual mean value of 150 ng m^{-3} as a guideline (WHO 2000). The mean bioaccessible concentration of Mn in PM$_{2.5}$ at Vidriera site was 358 ng m^{-3} reaching daily values up to 1432.9 ng m^{-3}, which exceeds by far the WHO guideline at this site. The mean Mn value is approximately three times higher than that obtained in a parallel sampling campaign carried out by personal samplers (mean: 109 ng m^{-3} for participants living <1.5 km from the same Mn alloy industry) (Expósito et al. 2021b). This difference can be explained by the different distances between the place of residence of the participants and the Mn alloy industry and the time spent indoors (Otero-Pregigueiro and Fernández-Olmo 2018; Markiv et al. 2022).

Spearman's correlation coefficients between the analyzed metal(loid)s were calculated and are shown in Table 3. In addition, the PCA results are depicted in Table 4. The Varimax rotated solution provided a three-factor model with eigenvalues higher than 1, explaining nearly 65 % of the variance. The time profile of the metal(loid) levels is shown in Fig. 3 grouping them into the three factors obtained from PCA. The first factor explains 27% of the total variance and was assigned to the emissions from the Mn alloy plant located near the sampling site (Mn shows the highest factor loading followed by Zn, Cd, Pb, and Fe). In accordance with these results, strong Spearman's correlation coefficients were

Table 2 Bioaccessible metal(loid) concentrations (ng m^{-3}) in ALF, PM$_{2.5}$ concentration and OP-AA values

	Mean	SD	Median	Max	Min	1st Quart.	3rd Quart.
V	0.34	0.28	0.26	1.30	<D.L.	0.12	0.54
Mn	359	413	190	1433	1.90	38.7	599
Fe	101	122	59.3	685	<D.L.	27.6	124
Ni	0.91	0.99	0.60	4.78	<D.L.	0.18	1.24
Cu	3.99	3.46	3.00	14.9	<D.L.	1.70	4.87
Zn	95.2	97.3	62.5	516	<D.L.	23.1	133
As	0.24	0.35	0.15	2.48	0.02	0.08	0.26
Mo	0.24	0.36	0.16	2.69	<D.L.	0.07	0.32
Cd	0.90	1.15	0.53	5.84	<D.L.	0.16	1.18
Sb	0.44	1.44	0.17	11.2	<D.L.	0.08	0.38
Pb	20.1	29.4	8.73	192	0.70	3.44	24.4
PM$_{2.5}$ (μg m^{-3})	11.1	8.76	7.73	36.9	2.05	4.41	15.7
OP-AAv (nmol min^{-1} m^{-3})	0.10	0.07	0.09	0.31	<D.L.	0.05	0.13
OP-AAm (pmol min^{-1} μg^{-1})	13.6	11.9	8.50	63.8	<D.L.	6.14	17.7

found between these metals, Mn having correlation values of 0.86 with Pb and Cd, 0.74 with Fe, and 0.71 with Zn. These pollutants were also previously associated with this Mn alloy plant (Hernández-Pellón and Fernández-Olmo 2019). The second factor was attributed to non-exhaust road traffic emissions since Cu showed the highest factor loading, followed by Fe and Sb. These three metals are usually considered tracers of non-exhaust road traffic (Pant and Harrison 2013). Moderate correlations were also obtained for these elements (Table 3); the correlation coefficients of Fe-Cu, Fe-Sb, and Sb-Cu were 0.64, 0.55, and 0.65, respectively. The third factor is characterized by a high factor loading of Ni followed by V, Mo, and As. Previous studies in the same area linked Ni and V to ship exhaust emissions from the Santander Bay (Hernández-Pellón and Fernández-Olmo 2019), while the origin of As was not clearly identified (Hernández-Pellón and Fernández-Olmo 2019; Markiv et al. 2022). In addition, Ni showed the lowest correlations with the other elements. The interdependence of these groups of metal(loid)s (those related to the Mn alloy plant, non-exhaust road traffic emissions, and ship emissions) was also observed in Fig. 3, where similar time profiles in each group are shown, confirming the common origin of metal(loid)s in each group.

Association between OP-AA and metal(loid) bioaccessible concentrations in ALF

Spearman's correlation coefficients between OP-AAv and ALF-bioaccessible concentrations of studied metal(loid)s in $PM_{2.5}$ samples are shown in Table 3. Despite the low OP-AAv values because of the acidic conditions of the test, significant positive correlations were obtained between OP-AAv values and most of the studied metal(loid)s. The strongest correlations (rho>0.7) were obtained for Fe, Cu, As, and Sb, followed by Mn (rho=0.64). OP-AAv values showed moderate correlations with Zn, Mo, V, Cd, and Pb (0.5<rho<0.6) and a weak association with Ni. PCA results indicated that OP-AAv is mainly associated with the second

Table 4 VARIMAX rotated factor loadings from PCA applied to metal(loid) concentrations and OP-AAv values

Species	Factor 1	Factor 2	Factor 3
V	0.45		**0.56**
Mn	**0.83**		
Fe	**0.62**	**0.55**	
Ni			**0.90**
Cu		**0.85**	
Zn	**0.79**	0.32	
As	0.39		**0.55**
Mo		0.34	**0.59**
Cd	**0.78**		
Sb		**0.54**	
Pb	**0.66**		0.38
OP-AAv	0.42	**0.74**	0.21
Eigenvalues	3.34	2.18	2.16
Total Variance (%)	27.87	18.19	17.96

Only factor loadings greater than 0.30 are shown; factor loadings greater than 0.50 are presented in bold. Eigenvalues and percentage of variance explained by each factor are also presented

factor (non-exhaust road traffic emissions) with a factor loading of 0.70. The first factor (ferroalloy plant emissions) also had a significant but lower factor loading for OP-AAv (0.45). Numerous studies have identified traffic emissions as a main source contributor to OP-AA (Pietrogrande et al. 2021; Calas et al. 2018; Campbell et al. 2021). The sensitivity of OP-AA to Mn alloy industry emissions is possibly due to Fe (split into factors 1 and 2) and to the high impact of this source in the area. In addition, the potential ability of Mn (III) to contribute to OP-AA cannot be ruled out and should be investigated, since a previous work demonstrated the presence of Mn(III) together with other oxidation states in the studied area (Hernández-Pellón et al. 2019).

Several investigations have studied the correlations between OP and PM species (Gao et al. 2020a) in which OP AA assay was frequently sensitive to metals (Calas et al.

Table 3 Spearman's correlation coefficients between the bioaccessible concentrations of metal(loid)s and between them and the OP-AAv obtained in acidic media (*p* value<0.05)

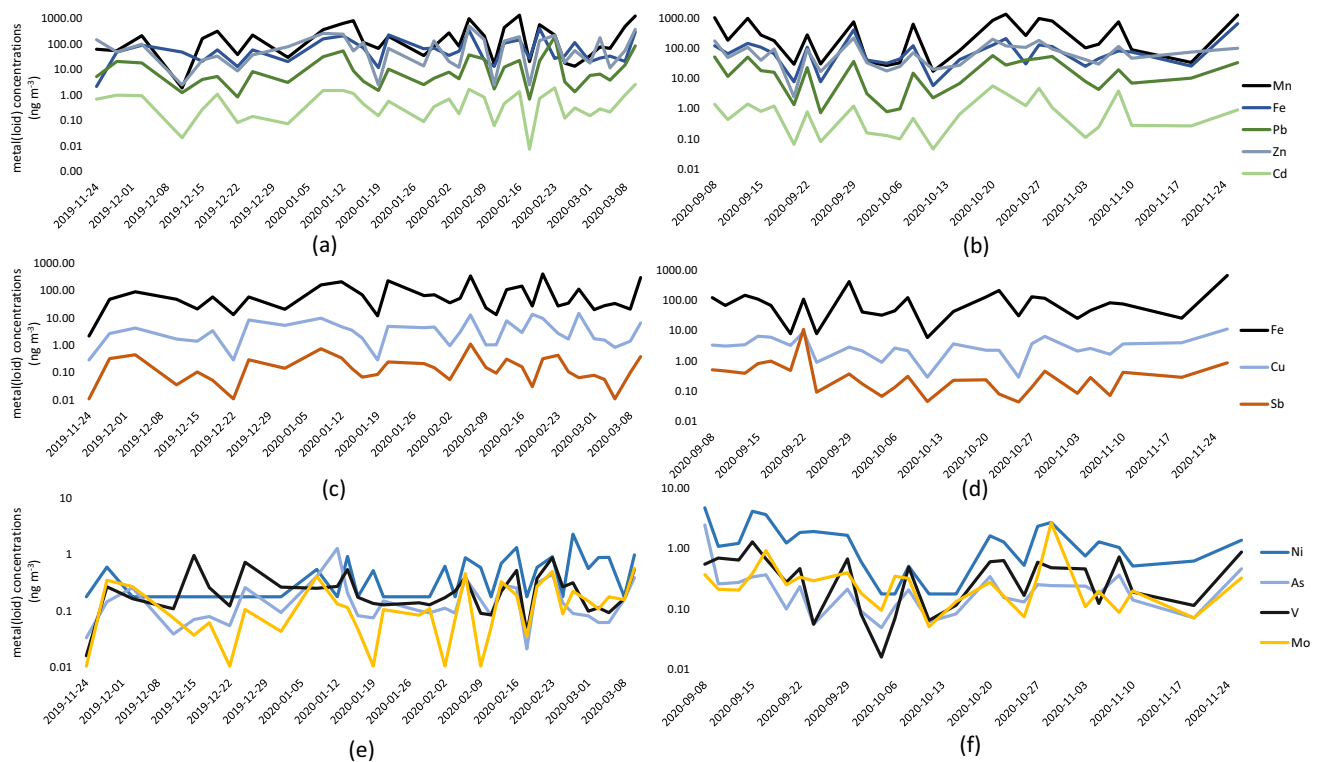|	Mn	Fe	Ni	Cu	Zn	As	Mo	Cd	Sb	Pb	OP-AAv
V	0.68	0.59	0.45	0.42	0.45	0.74	0.49	0.60	0.56	0.64	0.58
Mn		0.74	0.38	0.31	0.71	0.77	0.47	0.86	0.46	0.86	0.64
Fe			0.32	0.64	0.61	0.70	0.58	0.67	0.55	0.65	0.81
Ni				0.21	0.32	0.47	0.66	0.39	0.43	0.43	0.30
Cu					0.35	0.52	0.51	0.26	0.65	0.34	0.75
Zn						0.67	0.45	0.81	0.41	0.81	0.56
As							0.67	0.71	0.75	0.82	0.71
Mo								0.50	0.72	0.56	0.60
Cd									0.41	0.89	0.52
Sb										0.59	0.72
Pb											0.58

Fig. 3 Time series of metal(loid)s associated with the three factors obtained from PCA: **a, b** factor 1 from 11/2019 to 03/2020 and from 09/2020 to 11/2020; **c, d** factor 2 from 11/2019 to 03/2020 and from 09/2020 to 11/2020; **e, f** factor 3 from 11/2019 to 03/2020 and from 09/2020 to 11/2020

2018; Perrone et al. 2019; Bates et al. 2019). In most of the studies, Cu and Fe were found strongly correlated with OP-AAv (Visentin et al. 2016; Fang et al. 2016; Calas et al. 2019; Perrone et al. 2019; Gao et al. 2020b; Gosselin and Zagury 2020). However, there is no consensus about the associations between OP-AA and the rest of the studied metal(loid)s. Moderate or low correlations with OP-AAv responses were obtained for Mn, Zn, Mo, V, Cd, Pb, As, Sb, and Ni (Szigeti et al. 2015; Moreno et al. 2017; Calas et al. 2018; Pietrograndé et al. 2018; Weber et al. 2021).

The different correlations between OP and chemical composition can be affected by many factors such as the solubility of the compounds, the chemical form, the element concentrations, and the OP measurement conditions (Moreno et al. 2017; Bates et al. 2019). Moreover, associations between some species and OP could be due to intercorrelations between chemical compounds rather than a significant causal relationship between OP assay and PM components (Charrier and Anastasio 2012; Borlaza et al. 2021). In particular, in the studied area, almost 60% of metal(loid) concentrations are sourced from the manganese alloy plant (Hernández-Pellón and Fernández-Olmo 2019). In fact, among the studied metal(loid)s, only Cu and Fe are known to be very reactive in the AA assay, at least at neutral pH (Buettner 1988; Ayres

et al. 2008; DiStefano et al. 2009; Guo et al. 2020). Our results with chemical compounds suggest that Cu (II) is able to catalyze the AA oxidation also in acidic conditions at the concentration levels found for $PM_{2.5}$ samples studied in this work. With respect to the other studied metal(loid)s, it is unclear whether they might contribute to the AA consumption. Iron (III) seems also to be active as catalysts for the oxidation of AA while Mn (II), Ni (II), and Zn (II) were found to be inactive (Khan and Martell 1967). However, Visentin et al. (2016) showed that Zn (II) and Ni (II) are reactive ions in OP-AA assay and Mn (II) has a very low AA activity. Martell (1982) suggested that in addition to Cu (II) and Fe (III), many other metal ions are potentially capable of undergoing redox reactions with ascorbate, catalyzing its oxidation. However, Khan and Martell (1967) hypothesized that the electron-transfer process from the ascorbate anion to the metal ion involves the reducibility of the metal cation. Unless the metal cation has a stable lower valence form, such as Cu (II/I) or Fe (III/II), it will not be expected to participate in the electron transfer mechanism and thus will not be active as catalysts in the AA oxidation. Additional research with more chemical compounds (e.g., Mn (III)) is needed to identify the active species in the AA assay, both at neutral and acidic pH.

Conclusions

Our results showed firstly that the AA depletion from three catalytic compounds (Cu salts and 1,4-NQ) in an acidic fluid (ALF) was much lower than in a neutral fluid (PBS1x). The lower AA consumption in ALF is partly explained by considering the lower amount of AH^- in this medium. In addition, the AA depletion rate seems to depend on the sensitivity of each catalytic compound to the pH and composition of the fluid, although further investigations are needed to understand how PM components affect OP consumption in the OP-AA assay at both neutral and acidic pH.

The low OP-AA values found with the studied chemical species in ALF could partly explain the low values obtained in ALF from $PM_{2.5}$ samples collected in an urban-industrial site, compared to the studies found in the literature, carried out at neutral pH conditions. Nevertheless, the sensitivity of the OP-AA assay in ALF is enough to quantify it (i.e., almost all $PM_{2.5}$ OP-AA values were above the detection limit). In addition, significant positive correlations were obtained between OP-AAv and ALF-bioaccessible concentrations of most of the studied metal(loid)s. Copper and Fe showed the highest correlation with OP-AA, which is in agreement with the PCA results, where the highest loadings for the factor attributed to road traffic were obtained for OP-AA, Cu, and Fe; the high correlations of other metal(loid)s with OP-AA (Sb, As, Mn, Mo) could be due to intercorrelations between metal(loid)s. Further investigations are needed to study the role of other metal(loid)s besides Cu in the ascorbate oxidation under acidic conditions.

Declarations

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