

Review Article

Review: Analytical Procedure for Dithiothreitol-based Oxidative Potential of PM_{2.5}

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ABSTRACT Given the associated health risks, various kinds of methods have been developed to appraise the oxidative potential of particulate matter. However, a standardized experimental approach to assessing the oxidative potential has not yet been established. Researchers need to apply reasonable approaches pertinent to the characteristics of the samples and the purposes of the respective studies and to understand the limitations of each approach. In the present study, the dithiothreitol-based analytical methods of measuring the oxidative potential of particulate matter were appraised for factors that affect the experimental results, such as the sampling substrate, extraction solution, and dithiothreitol reaction. This study reveals the limitations of each experimental approach, which stem from hydrophilic and hydrophobic characteristics, according to simulated lung fluid reactions. Analyses of a Teflon filter and simulated lung fluid were suggested as the most useful procedures based on the results of prior studies.

KEY WORDS DTT, OP, ROS, Human exposure, Health risk

1. INTRODUCTION

From a global perspective, the concentration of particulate matter less than 2.5 μm in diameter (PM_{2.5}) appears higher in densely populated areas due to the rapid urbanization and industrialization of societies around the world (Donkelaar *et al.*, 2010). There have been several historical instances associated with high concentrations of PM_{2.5}, such as the Meuse Valley Fog in 1930, the Smog in Los Angeles in 1943, the Great Smog of London in 1952, etc. Sudden increases in PM_{2.5} concentration are recurring events in developing countries in South-East Asia and China due to the rapid urbanization and industrialization of these areas (Ji *et al.*, 2018; Parrish *et al.*, 2011; Davis, 2002; Nemery *et al.*, 2001). An increase in PM_{2.5} also brings about increased human exposure to PM_{2.5} and has adverse human health effects as it interacts with various underlying conditions such as respiratory diseases, cardiovascular diseases, neuro-degenerative diseases, etc. (Shou *et al.*, 2019; Choi *et al.*, 2018; Maji *et al.*, 2018; Weinmayr *et al.*, 2018).

The term “human exposure” was initially introduced in the early 1980s by Duan (1982) and Ott (1982). The term emphasizes that the human body is the most sig-

nificant receptor of pollutants (Duan, 1982; Ott, 1982). Ott (1982) defined the term “exposure” as “an event in which the human body comes into contact with pollutants,” and expressed exposure in terms of time (t), the human body (i), and the concentration of pollutants (c) (Duan, 1982). Here, two terms, concentration (c) and the human body (i), express the concept of “contact,” which is neither “inhalation” nor “uptake” (Monn, 2001). The term “integrated exposure” was also introduced to extend these terms; however, precisely calculating the time (i) and concentration (c) of contact with the human body (i) is not easy. Due to the inherent difficulty of calculating “integrated exposure,” “average exposure,” which involves calculation of contact with the average concentration of pollutants during a given period of time, is generally used instead (Jung *et al.*, 2012; Monn, 2001). These concepts are ultimately used to appraise the “risk of exposure,” which assesses the time of contact between the human body and pollutants to evaluate the accumulation of pollutants in the human body (Jung *et al.*, 2012).

The concept of “risk of exposure” in the atmospheric environment can be used to determine the time of short-term exposure of the human body to pollutants, rate of inhalation, etc. However, the concept of risk of exposure is limited in its ability to determine the risk to human health of particulate matter (PM) in the air based on the concentration of pollutants and time of exposure. In addition, the particle size distribution and chemical compounds as well as the concentration of PM also affect the human health risk (Dappe *et al.*, 2018; Charrier and Anastasio, 2011; Perrone *et al.*, 2010). PM_{2.5} is known to invade the pulmonary alveoli and terminal bronchioles, while particles in the size range of 2.5–10 µm and over 100 µm invade the primary bronchi and nasopharynx, respectively (Kelly and Fussell, 2012).

In terms of biological interactions with PM, the specific surface area of lung cells increases in accordance with a decrease in the size of PM, resulting in hazardous consequences, which are closely associated with increasing mortality by PM (Perrone *et al.*, 2010; Utell and Frampton, 2000; Schwartz *et al.*, 1999). Thus, the extent of the human health risk of PM increases as the size of the particles decreases even when the concentrations of PM are identical. The principal compounds constituting PM are organic carbon (OC), elemental carbon (EC), heavy metals, and water-soluble ionic compounds (Sandrini *et al.*, 2014; Gentner *et al.*, 2012; Chow *et al.*, 2010). The chemical compounds in PM vary according to the emission

source; however, heavy metals and polycyclic aromatic hydrocarbons (PAHs) account for less than 10% of all compounds in PM (Song *et al.*, 2020; Sandrini *et al.*, 2014; Gentner *et al.*, 2012; Chow *et al.*, 2010). However, the heavy metals As, Cd, Cr (IV), and Ni are classified as group 1 carcinogenic materials to the human body by the International Agency for Research on Cancer (IARC, 2017), while PAHs are also defined as carcinogens (class 1) or potentially presumable carcinogens (class 2A) by the International Agency for Research on Cancer (IARC, 2010). Therefore, the toxicity of PM is broadly dependent on its constituent compounds as well as their concentrations and particle size distributions.

The precise concentration and size of PM as well as the constituent chemical compounds need to be measured to appraise the human health risk of PM. This necessitates complex and various analyses of PM. In particular, there is a risk of underestimation of hazardousness to the human body if the analysis focuses only on specific compounds. The toxicity of PM can be determined if the mechanisms of damage to the human body are identified.

One significant mechanism of air pollution-induced damage to the human body is a continuous state of inflammation (Patel *et al.*, 2013). According to previous studies, exposure to air pollutants for a short period of time can increase inflammation in the respiratory tract and oxidative stress in children (Brown *et al.*, 2012; Berhane *et al.*, 2011). Such inflammatory states can bring about diverse types of human health risks, including inflammation of epithelial cells, hypersensitivity of the respiratory tract, damage to the lungs, etc., through unidentified mechanisms (Auerbach and Hernandez, 2012). Importantly, the reactive oxygen species (ROS) induced by air pollutants are created with triggering such inflammation (Esposito *et al.*, 2014; Auerbach and Hernandez, 2012). Diverse studies have been carried out based on this information, in which suppression of the antioxidant reaction of biological systems and inducement of an unbalanced mechanism of oxidation by the concentration of ROS were reported as key factors triggering toxicity by exposure to PM (Gao *et al.*, 2020; Esposito *et al.*, 2014; Lodovici and Bigagli, 2011). Therefore, the capacity of PM to generate ROS, which is defined as the oxidative potential (OP), is an indicator that is integrated with diverse physicochemical characteristics such as the size of the particles, surface area, chemical composition, etc., and is useful to identify their effects on the human body (Gao *et al.*, 2020; Delfino *et al.*, 2011).

Therefore, various methods have been developed to appraise OP. In the present study, the available methods of utilizing dithiothreitol (DTT) to measure OP were reviewed. The analytical methods that use DTT are non-cellular chemical reactions of the active compound of oxidation-reduction relevant to PM, which is a surrogate measure of biological reduction.

2. DISCUSSION

2.1 DiThioThreitol (DTT) Assay

To conduct the DTT assay, most researchers employ the major methods developed by Cho *et al.* (2005) or Li *et al.* (2009), who simplified the methodology presented by Cho *et al.* (2005) (Lin and Yu, 2019; Fang *et al.*, 2016; Kramer *et al.*, 2016; Verma *et al.*, 2014; Charrier and Anastasio, 2012; Lin and Yu, 2011; Li *et al.*, 2009; Cho *et al.*, 2005). There are several improved methods of DTT assay from Cho *et al.* (2005) or Li *et al.* (2009) (Lin and Yu, 2019; Fang *et al.*, 2016; Kramer *et al.*, 2016; Verma *et al.*, 2014; Charrier and Anastasio, 2012; Lin and Yu, 2011). Thus, the DTT assay by Cho *et al.* (2005) or Li *et al.* (2009) can be considered the representative method.

The experimental DTT assays employed by Cho *et al.* (2005) or Li *et al.* (2009) are divided into three stages, as illustrated in Figs. 1 and 2 (Li *et al.*, 2009; Cho *et al.*, 2005). The stages consist of the collection and extraction

of materials for the analysis, the development of a system for the DTT reaction, and the measurement of 2-nitro-5-thiobenzoic (TNB) through the reaction of DTT solution with 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB). The extractions from collective filters conducted by Cho *et al.* (2005) or Li *et al.* (2009) were carried out using methanol or water; these utilized the ultrasonic extraction process. In addition, the researchers analyzed the extracted samples after measuring the concentrations according to the concentration of OP. In this stage, the solution used for extraction is important. Methanol or water was used in the experiments conducted by Cho *et al.* (2005) or Li *et al.* (2009), wherein DTT assay was organized by a single extraction. Thereafter, dichloromethane and simulated lung fluid (SLF) are added in the modified experiments to measure OP; in these experiments, water in the first stage and methanol in the second stage were used for the extraction (Patel *et al.*, 2018; Pietrogrande *et al.*, 2018; Calas *et al.*, 2017; Gao *et al.*, 2017). The selection of solvent and the processing of several stages for extraction are chosen based on the presence of hydrophilic or hydrophobic sub-fractions in the samples, and SLF can reflect the OP resulting from in vivo reactions (Patel *et al.*, 2018; Pietrogrande *et al.*, 2018; Calas *et al.*, 2017; Gao *et al.*, 2017). Relevant details will be discussed in Section 3.

In the second stage of the DTT assay, the DTT depletion is occurred, while the third stage involves measure-

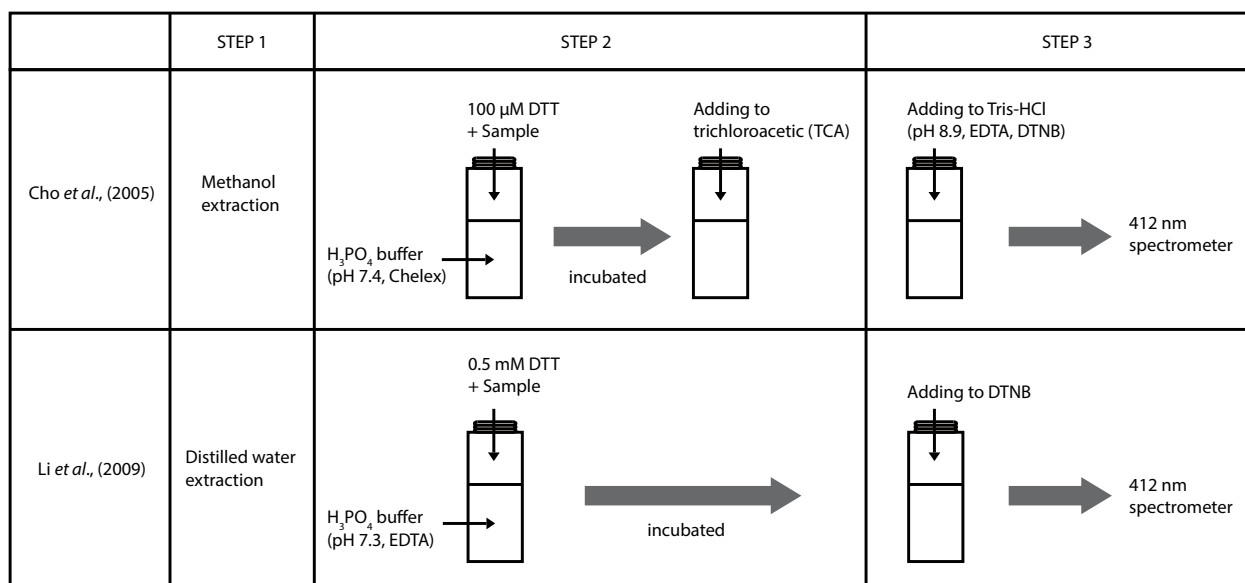


Fig. 1. Schematic illustration of experimental procedures used in the DTT assays by Cho *et al.* (2005) and Li *et al.* (2009).

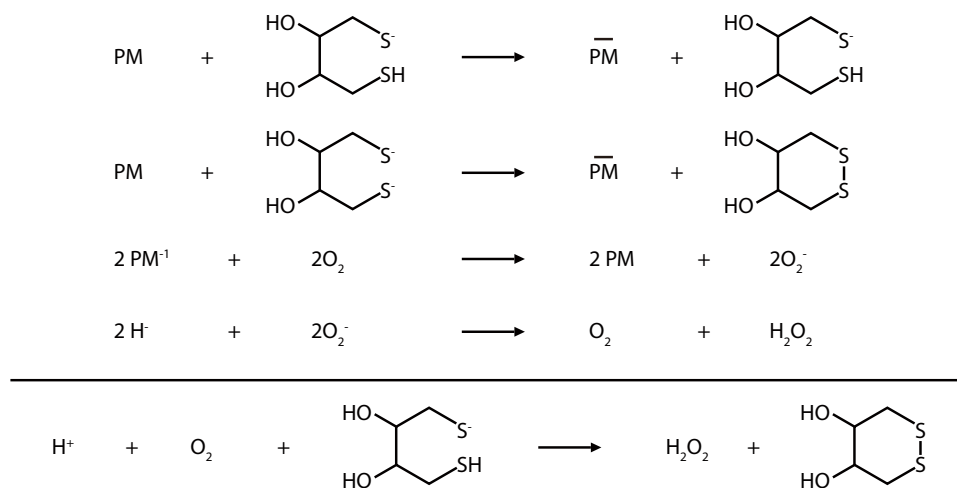


Fig. 2. Chemical reactions in the DTT assay with PM (Cho *et al.*, 2005).

ment of the TNB created by the reaction with DTNB and residual DTT. The oxidation-reduction active quinone, 9,10-phenanthraquinone (9,10-PQ), delivers electrons to oxygen through DTT, thereby inducing the creation of superoxide (O_2^-) (Li *et al.*, 2009). That is, an oxidation-reduction active compound like quinone catalyzes the reduction of O_2 to H_2O_2 by DTT, wherein the DTT is oxidized to DTT-disulfide (Li *et al.*, 2009). Thereafter, in the third stage of DTT analysis, the thiol that remains after DTT reaction reacts with DTNB to create a mixture of bisulfide and TNB, wherein the TNB absorbs UV 412 nm (Cho *et al.*, 2005). The reaction processes in the second and third stages of DTT analysis are similar to the oxidation-reduction of quinone that creates ROS in the human body (Fig. 3). Thus, the results of the DTT assay can be used to measure the biotoxicity of PM, wherein the loss ratio of DTT in the DTT assay is exploited as the measure of OP.

2.2 Sampling Substrate for the DTT Assay

In the first stage of extraction for the OP analysis, the results vary according to the types of filters and solvents that are used (Table 1). As presented in Table 1, the collection of samples for the OP analysis in previous studies employed quartz, Teflon, and Teflon-coated glass-fiber filters; quartz filters were the dominant choice. However, when using a quartz filter, the amount of injected solvent needed for extraction is relatively high due to the higher rate at which moisture is absorbed by a quartz filter, which results in dilution of the extracted solution. This necessitates concentration of the extracted suspension

for the OP analysis (Yang *et al.*, 2014). According to the results of previous studies, quartz fiber filters can be damaged during the extraction process. As a result, OP may be underestimated. In a comparison between Teflon and quartz filters, quartz filters produced lower OP readings than Teflon filters (Yang *et al.*, 2014). This is attributable to the lower efficiency of ultrasonic extraction of OP-related compounds by quartz vs. Teflon filters. The choice of a quartz or Teflon filter affects the absolute value of OP, but does not influence the tendency of the concentration gradient of OP. Thus, some researchers solely presented the trends in OP, or conducted an analysis of OP without removing PM in the suspension to quantify the absolute value of OP collected through the quartz filter (Patel and Rastogi, 2018). Therefore, a Teflon filter is frequently employed as a collection filter for the analysis of OP rather than a quartz filter to attain a higher extraction efficiency and to simplify the experimental procedure.

2.3 Extraction Solution for the DTT Assay

The solvents employed for the extraction of PM collected by filters are distinguished into three types. The first includes water, methanol, dichloromethane, etc. (Patel and Rastogi, 2018; Wang *et al.*, 2018; Janssen *et al.*, 2014; Yang *et al.*, 2014; Eiguren-Fernandez *et al.*, 2010). The efficiency of extraction of each solvent is determined by the polarity of the compounds in the collected PM (Patel and Rastogi, 2018). That is, the samples containing abundant water-soluble particles exhibit higher efficiency of extraction by water, whereas samples containing

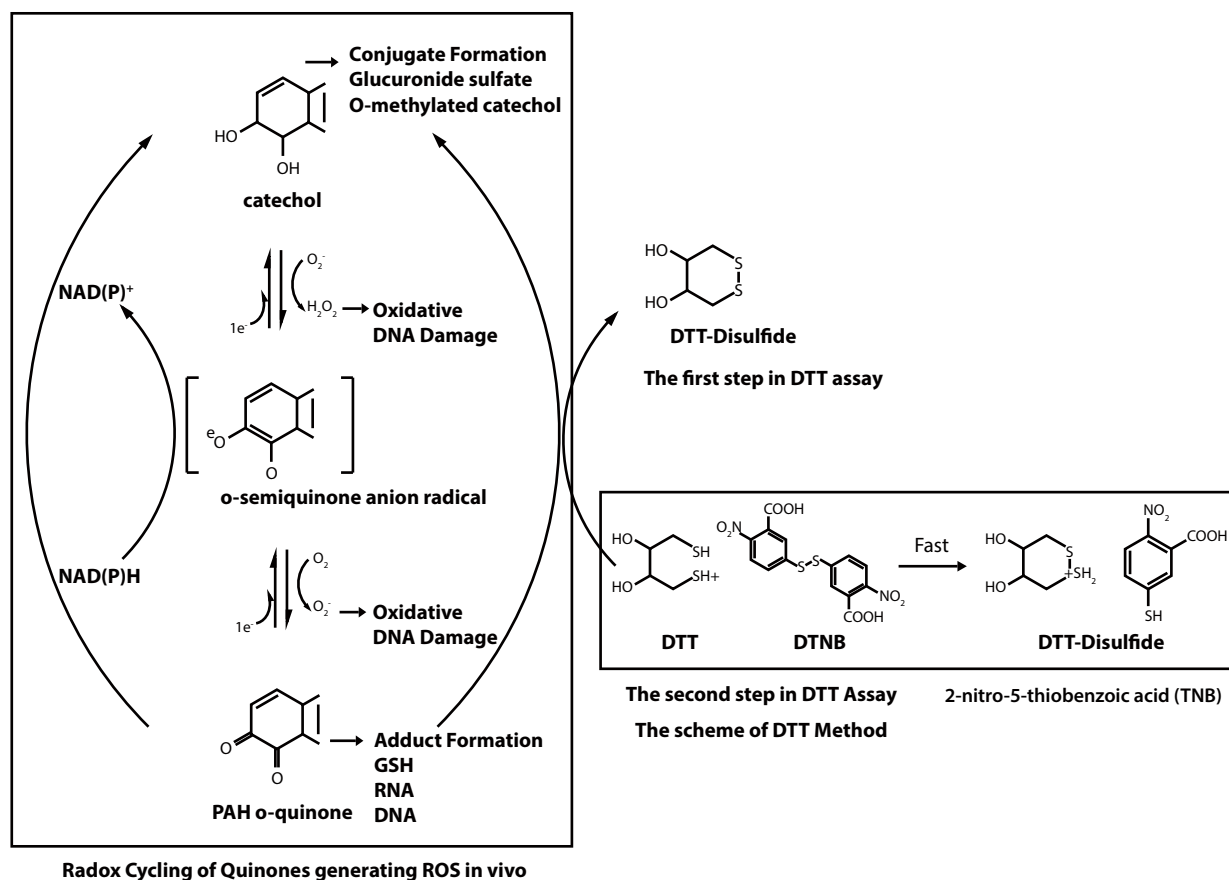


Fig. 3. Redox cycling of quinones generating ROS in vivo and in the DTT assay (Li *et al.*, 2009).

Table 1. Sample collection and extraction method for DTT analysis.

	Sample (Collection filter)	Solvents	Extraction method
Patel and Rastogi (2018)	PM _{2.5} (quartz)	MeOH ¹⁾ : Water = 1 : 1	Ultrasonication for 30 min
Yang <i>et al.</i> (2014)	PM _{2.5} (quartz) PM _{2.5} (Teflon)	MeOH MeOH, Water	Ultrasonication Ultrasonication for 5 min
Janssen <i>et al.</i> (2014)	PM _{2.5} , PM ₁₀ (Teflon)	MeOH	Ultrasonication for 20–30 sec
Wang <i>et al.</i> (2018)	PM _{2.5} (quartz)	Water	Ultrasonication for 1 h
Eiguren-Fernandez <i>et al.</i> (2010)	PM _{2.5} (Teflon-coated glass fiber)	Water DCM ²⁾	Ultrasonication for 30 min Ultrasonication for 30 min
Pietrogrande <i>et al.</i> (2018)	PM ₁₀ (Teflon)	10 mL of 0.1 M buffer at pH 7.4	Ultrasonication for 15 min
Romano <i>et al.</i> (2020)	PM ₁₀ (quartz)	10 mL of 0.1 M buffer at pH 7.4	Ultrasonication for 15 min
Calas <i>et al.</i> (2017)	PM _{2.5} (quartz)	SLF ³⁾	Vortexed at maximum speed during 2 h at 37°C
Calas <i>et al.</i> (2017)	PM _{2.5} (quartz)	SLF	Vortexed at maximum speed during 2 h at 37°C
Weber <i>et al.</i> (2018)	PM ₁₀ (quartz)	SLF	SLF at iso-mass

¹⁾Methanol, ²⁾Dichloromethane, ³⁾simulated lung fluid

abundant non-water-soluble particles exhibit higher efficiency of extraction by dichloromethane. According to the results of previous studies, the OP readings when water was used in the extraction were higher than those when non-polar dichloromethane was used, while measurements taken using methanol were higher than those extracted with water (Patel and Rastogi, 2018; Yang *et al.*, 2014; Eiguren-Fernandez *et al.*, 2010). This indirectly suggests that OP-related compounds are more likely to be water-soluble materials than non-water-soluble materials. In addition, the characteristics of methanol extraction are similar to those of dichloromethane extraction, but the polarity of methanol is greater than that of dichloromethane, so some hydrophilic and hydrophobic compounds can be extracted by the former. Therefore, methanol is frequently employed as an extraction solvent to measure the maximum OP in a single extraction process (Calas *et al.*, 2018).

Meanwhile, extraction with water, methanol, or dichloromethane is selectively used to examine the OP resulting from a specific component. That is, water and dichloromethane are applied to identical samples separately for the extraction so that the relative concentrations of either water-soluble compounds or non-water-soluble compounds can be measured (Eiguren-Fernandez *et al.*, 2010). Furthermore, sequential extinctions using methanol and dichloromethane are exploited in stages to quantify OP after water extraction to calculate the absolute value of OP (Gao *et al.*, 2017; Yang *et al.*, 2014).

The second type of solvent used for filtered extraction is buffer solution (Romano *et al.*, 2020; Pietrogrande *et al.*, 2018; Charrier and Anastasio, 2012). The reason behind the employment of a buffer solution is to reduce blank oxidation. Blank oxidation is mainly triggered by trace metals included in the extraction solution; thus, trace metals should be removed from the extraction solution to avoid blank oxidation (Romano *et al.*, 2020; Charrier and Anastasio, 2012). In previous studies, a buffer solution (pH 7.4) of phosphate and cation-exchange resin was used to remove trace metals. Pretreatment with extraction solution and employment of buffer solution reportedly do not lead to a higher rate of decrease of DTT in the blank solution, and increase the reproducibility of the experiments (Romano *et al.*, 2020; Charrier and Anastasio, 2012).

The third type of solvent used for filtered extraction is SLF. The purpose of using SLF is to attain an accurate assessment of OP in the human body. To accurately mea-

sure the level of hazardousness to the human body, the bioaccessibility of compounds in PM needs to be taken into account. The bioaccessibility in the lungs can be represented by the degree of dissolution of PM compounds in SLF. The physiological conditions that induce reactions of PM in the lungs differ between water, methanol, and DCM, and SLF can be employed to simulate the pertinent physiological conditions (Calas *et al.*, 2018).

When measuring OP using the DTT assay, the measurement is based on the decreasing rate of DTT. The chemical compounds in PM which exhibit strong correlations with a decreasing rate of DTT include elemental carbon, water-soluble organic carbon, and PAHs (Verma *et al.*, 2009; Hu *et al.*, 2008; Ntziachristos *et al.*, 2007; Cho *et al.*, 2005; Li *et al.*, 2003). These materials do not directly interact with DTT in chemical reactions. For example, PAHs include compounds that are not involved in redox reactions, and are therefore regarded as irrelevant to direct loss of DTT. However, PAHs can indirectly decrease the concentration of DTT through chemical reactions with relationships such as quinone (Charrier and Anastasio, 2012). For example, phenanthrenequinone (PQN) and 1,4-naphthoquinone (1,4-NQN) oxidize DTT (Li *et al.*, 2009; Kumagai *et al.*, 2002).

On the other hand, the metals in PM may trigger *in vivo* oxidation damage, though metals are generally not thought to be active in the DTT assay (Chio *et al.*, 2012; Leonard *et al.*, 2004). In early research, an increase in OP was not observed with the addition of Fe and Cu to the DTT analysis (Charrier and Anastasio, 2012). However, the correlation between an increased concentration of metals and a decrease in DTT appeared thereafter, which was attributed to the association between metals and organic compounds (Hu *et al.*, 2008; Ntziachristos *et al.*, 2007). In addition, according to recent studies, the concentrations of heavy metals were found to remain constant even though that of DTT appeared to decrease by approximately 40–100% when the PM was heated. This indicates that semi-volatile organic compounds are responsible for the decrease in DTT (Biswas *et al.*, 2009; Verma *et al.*, 2009). However, Cu(II) and Zn(II) influenced the decrease in DTT, and the presence of metals increased the level of uncertainty in the DTT measurement due to the association between organic compounds (Gao *et al.*, 2017).

Therefore, the most effective way to appraise the level of hazard to the human body is to measure OP by employing SLF, which is similar to compounds in the human

Table 2. Composition (g/L) of extraction fluid (Stopford *et al.*, 2003; Midander *et al.*, 2007).

Chemicals (g/L)	ALF (pH = 4.5)	Gamble's solution (pH = 7.4)
Magnesium chloride, MgCl ₂	0.050	0.095
Sodium chloride, NaCl	3.21	6.019
Potassium chloride, KCl	–	0.298
Disodium hydrogen phosphate, Na ₂ HPO ₄	0.071	0.126
Sodium sulphate, Na ₂ SO ₄	0.039	0.063
Calcium chloride dihydrate, CaCl ₂ · 2H ₂ O	0.128	0.368
Sodium acetate, C ₂ H ₃ O ₂ Na	–	0.574
Sodium hydrogen carbonate, NaHCO ₃	–	2.604
Sodium citrate dihydrate, C ₆ H ₅ Na ₃ O ₇ · 2H ₂ O	0.077	0.097
Sodium hydroxide, NaOH	6.00	–
Citric acid, C ₆ H ₈ O ₇	20.8	–
Glycine, H ₂ NCH ₂ COOH	0.059	–
Sodium tartrate dihydrate, C ₄ H ₄ O ₆ Na ₂ · 2H ₂ O	0.090	–
Sodium lactate, C ₃ H ₅ NaO ₃	0.085	–
Sodium pyruvate, C ₃ H ₃ O ₃ Na	0.086	–

Table 3. Rate of DTT loss vs. molar concentration for water, ALF extraction, Gamble's solution, and Gamble's solution + DPPC extraction (Calas *et al.*, 2017).

Compounds	Milli-Q water			ALF solution			Gamble's solution			Gamble + DPPC solution		
	R ²	N ¹⁾	n ²⁾	R ²	N	n	R ²	N	n	R ²	N	n
Benzo(a)pyrene (BaP)	0.11	6	18	–	6	18	0.56	6	18	0.27	6	18
7H-Benz(de)anthracene-7-one (BA)	0.81	6	18	–	6	18	0.25	6	18	0.22	6	18
Benzo(b)naphtho(2,1-d)thiophene (BNT)	0.19	6	18	0.13	6	18	0.27	6	18	0.29	4	12
3-methylchrysene	0.17	6	18	–	6	18	0.38	6	18	0.18	6	18
1,4-naphthoquinone	0.98	10	25	0.98	8	24	0.9	10	25	0.98	12	36
Levogluconan	0.15	5	15	–	5	15	0.32	5	15	0.15	5	15
NH ₄ ⁺	–	1	2	–	1	2	–	1	2	–	1	2
SO ₄ ²⁻	0.53	5	15	–	5	15	0.45	5	15	0.62	5	15
MnCO ₃	0.71	5	15	0.98	4	12	0.9	5	15	0.94	5	15
Cu ²⁺	0.63	5	15	0.99	5	15	0.79	5	15	0.83	5	15
CuO	–	–	–	0.92	5	15	0.92	4	12	0.95	4	12
Zn ²⁺	0.72	5	15	0.4	5	15	0.84	5	15	0.99	5	15

¹⁾N: number of samples²⁾n: total number of analytes including duplicates or triplicates

body. Three main types of SLF were employed in previous studies. These are Gamble's solution, artificial lysosomal fluid (ALF), and a mixture of Gamble's solution and dipalmitoylphosphatidylcholine (DPPC). The compounds in Gamble's solution and ALF are presented in Table 2 (Midander *et al.*, 2007; Stopford *et al.*, 2003). Gamble's solution represents the interstitial fluid deep within the lungs. Lung macrophages are one of the major producers of ROS in the initial stages of inflammation

(Calas *et al.*, 2017; Marques *et al.*, 2011). Macrophages use the antioxidant defense to control the discharge of ROS (Hamilton *et al.*, 2008; May *et al.*, 2005). ALF simulates the body fluid that is produced after phagocytosis by macrophages residing in the pulmonary alveoli and interstitial tissue; thus, it imitates the in vivo inflammatory state (Lacroze *et al.*, 2009; Colombo *et al.*, 2008). The mixture of Gamble's solution and DPPC is a major phospholipid found in lung surfactant that reduces the surface

tension at the air/water interface in the terminal respiratory tract and simulates the defensive function of the lungs (Lacroze *et al.*, 2009; Colombo *et al.*, 2008).

The correlations between DTT levels in the extractions using the three types of SLF and water, which were employed in previous studies, are shown in Table 3 (Calas *et al.*, 2017). When comparing the extracted solutions resulting from the use of water and three kinds of SLF, the correlations between the concentrations of ionic compounds (NH_4^+ , SO_4^{2-} etc.) and quinone compounds (1,4-NQN etc.) all appear significant and similar to each other. However, with respect to the decreasing concentration of DTT, the extraction solution using SLF showed relatively higher relationships, in contrast to the lower correlations of water extraction for the trace metal compounds frequently represented by Mn, Cu, Zn, etc. DTT content decreased in the extraction solutions in the following order: Gamble's solution, mixture of Gamble's solution & DPPC, water, and ALF. That is, water extraction may result in a higher OP reading than ALF extraction, and water extraction may also result in an underestimation of OP compared to Gamble's solution and Gamble's solution & DPPC. OP measurements taken using each extraction solvent vary according to the particular chemical compounds in the samples. The available data on the effects of the complex of lung lining fluid and ligands on the measurement of air pollutants in PM is insufficient, so it would be unreasonable to conclude that SLF solution extraction is more accurate than water extraction (Calas *et al.*, 2017). However, given the results of previous studies, we can assume that extraction solution containing SLF would produce better results than that containing plain water for the appraisal of hazardousness to the human body with respect to trace metals and PAHs.

2.4 Chemical Reaction for the DTT Assay

The analysis of OP differs depending on the configuration of the DTT reaction system and the measurement tools. In short, the first-stage PM extracts catalyze the reduction of O_2 to H_2O_2 in oxidation-reduction compounds like quinine by DTT in the second stage, thereby oxidizing DTT to DTT-disulfide. In the third stage, the remaining DTT is reacted with DTNB to create TNB, enabling measurement using the light absorption characteristics of TNB. It would be difficult to strictly distinguish the second stage from the third stage because they constitute a continuous integrated process without preser-

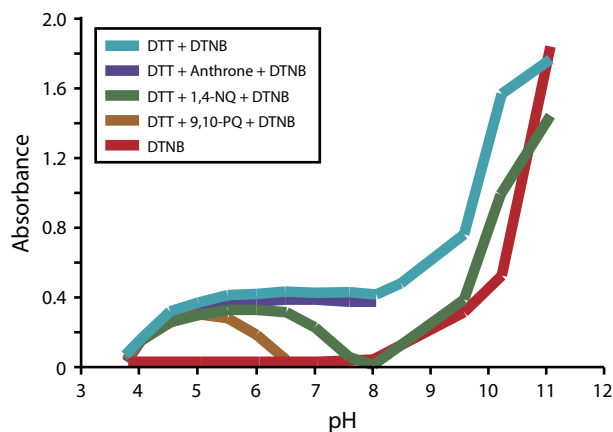


Fig. 4. Effect of pH on DTT reactions and TBN molar extinction coefficient (Li *et al.*, 2009).

vation of separate samples.

The factors involved in the DTT reaction and measurement include the pH of the reaction, solution for the reaction, composition of the reaction stages, and TNB absorbance measurement. The pH affects the speed of the catalyzed oxidation-reduction reaction and the molar absorptivity of TNB upon completion of the reaction. As presented in Fig. 4, the absorbance increases as the pH increases from 3.8 to 5.0 in the reaction between DTT and DTNB, where oxidation-reduction reaction is absent. This phenomenon is attributable to the presence of mercaptide ion (TNB^{2-}), the absorbance of which is higher than that of the conjugate acid reacted with DTT and DTNB (Riddles *et al.*, 1979). In addition, similar absorbance is seen in the pH interval from 3.8 to 5.0 when comparing reactions with DTT + DTNB and DTT + Anthrone + DTNB, DTT + 1,4-NQ + DTNB, and DTT + 9,10-PQ + DTNB. This indicates that absorbance remains unchanged despite the presence of materials that trigger the reaction that decreases DTT, whereas the DTT reaction is almost absent at pH values below 5.0 (Li *et al.*, 2009). In the pH range from 5.5–8.0, TNB appears in the form of TNB^{2-} , and no changes in the absolute values for the DTT + DTNB system are observed. However, the absorbance values of DTT + 1,4-NQ + DTNB and DTT + 9,10-PQ + DTNB decrease significantly. This indicates that the speed of the catalyzed oxidation-reduction reaction increases in accordance with the varying level of pH, while the DTT concentration decreases (Li *et al.*, 2009). At pH values over 8.0, the absorbance in all systems increases rapidly due to alkaline decomposition

of DTNB (Danehy *et al.*, 1971). Thus, the range from pH 5.5 to 8.0 is superior for the DTT reaction. More specifically, 100% of DTT was converted into DTT-disulfide by 9,10-PQ at a pH of 7.3, while approximately 50% of 1,4-NQ was converted into DTT-disulfide. In addition, pH 7.3 is estimated to be optimal for the DTT reaction as it enables the simulation of in vivo reaction in the human body since it is almost identical to the pH of human blood (Li *et al.*, 2009).

The selection of times for the DTT reaction depends on the specific experimental methods that are used; there is no single set of optimal experimental conditions in this regard. Cho *et al.* (2005) measured the rate at which DTT decreased according to various reaction times. The authors induced the DTT reaction for a predetermined period of time and then injected trichloroacetic acid (TCA) to terminate the DTT reaction coercively. Thereafter, each sample was injected with DTNB to complete the DTT reaction at pre-set time intervals to measure the decreasing concentration of DTT corresponding to each reaction time (Eq. 1). OP measurements corresponding to each reaction time were obtained to finally calculate the rate of DTT decrease using the slope of a linear regression equation. On the other hand, Li *et al.* (2009) measured the final rate of DTT decrease of a sample by injecting DTNB after inducing the DTT reaction for a period of over 30 minutes. The method employed by Cho *et al.* (2005) has the advantage of reducing experimental errors by measuring the OP according to the time in culture, and the disadvantage of being more labor-intensive. However, the method employed by Li *et al.* (2009) may show greater experimental uncertainty, but it makes it possible to analyze many samples at the same time.

$$\Delta DTT_t (\mu\text{M}) = \frac{A_0 - A}{A_0} C_{DTT} \quad (\text{Eq. 1})$$

Here, A_0 represents the absorbance (blank) at $t = 0$, while A and C_{DTT} represent the absorbance at a specified time t in culture and the initial concentration of DTT in the reaction solution, respectively.

The DTT assay to reduce errors triggered by a small amount of heavy metals has been used, as discussed previously. The solution that is added to remove a small amount of heavy metals in the reaction solution consists of a cation-exchange resin and buffer solution, as mentioned in the previous section. For the case of organic matter, previous studies reported that the concentration of organic matter and DTT decrease linearly, whereas that of DTT in a reaction with heavy metals varies according to the types of heavy metals (Lin and Yu, 2019; Fujitani *et al.*, 2017; Charrier and Anastasio, 2012).

For example, as illustrated in Fig. 5, the decreasing concentration of DTT exhibits a linear relationship with the concentration of water-soluble compounds in PM, whereas the concentrations of Cu and Mn, which affect the DTT content, appear to have a non-linear relationship (Lin and Yu, 2019). Thus, it is additionally necessary to control the initial concentration of DTT in the DTT reaction in cases containing relatively high heavy metal concentrations that would affect the decrease in DTT in the samples (Lin and Yu, 2019). However, studies that definitively distinguish the decrease in DTT according to heavy metal content to determine the initial concentration of DTT have yet to be reported. Therefore, a small amount of heavy metals needs to be removed to reduce the uncertainty in the OP measurement. As explained in

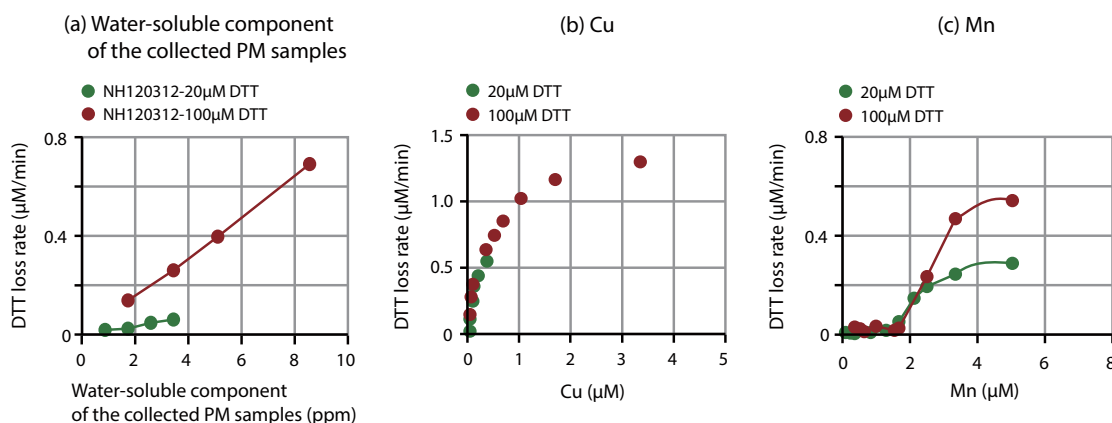


Fig. 5. DTT loss rates in the DTT20 and DTT100 assays by (a) water soluble compounds, (b) Cu, and (c) Mn (Lin and Yu, 2019).

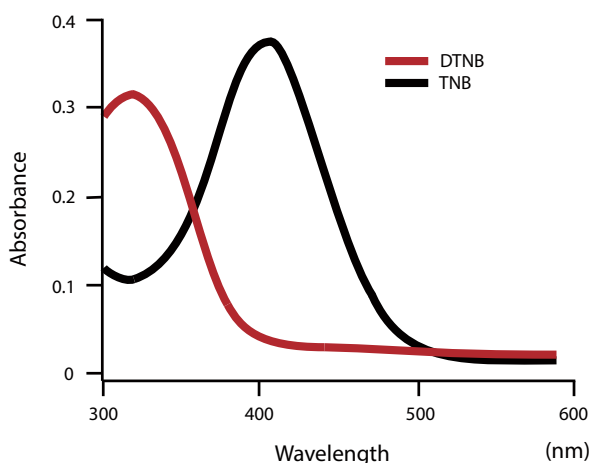


Fig. 6. UV-VIS absorbance by DTNB and TNB (Li *et al.*, 2009).

the previous section, heavy metals cause in vivo inflammation in the human body, and the relationship between a tiny amount of heavy metals and the decreasing rate of DTT appears as explained in the SLF extraction section. Thus, the limitations of each OP measurement method need to be clarified by expressing the processes of the OP assay, whether it be based on the use of an extractive solvent that removes heavy metals.

The decreasing rate of DTT is calculated by measuring the TNB created through the DTT reaction. Absorption at 412 nm is frequently used to measure the TNB concentration. This is because TNB has a higher molar extinction coefficient in the visual range (Li *et al.*, 2009). It should be remembered that, as illustrated in Fig. 6, the DTNB employed for the DTT reaction manifests its peak absorbance at approximately 315 nm, while it also exhibits a bit of absorbance in the neighborhood of 400 nm. Thus, the absorbance of DTNB needs to be removed from the TNB measurement results by taking the conditions of the measuring equipment into account.

3. SUMMARY

In the present study, the procedure, conditions, and factors affecting the DTT assay were investigated. In the DTT assay, OP is measured based on a procedure that can be divided into the following stages: PM extraction, DTT reaction, and measurement. The factors that affect the experimental results include the solvent that is used for extraction, the characteristics of the DTT reaction mechanism, etc. A standardized experimental approach has not

yet been established, and the results of the DTT assay depend on the particular experimental method. Researchers need to apply reasonable approaches considering the characteristics of the samples and the purposes of their studies. In the Korean Peninsula, studying the relation between OP and water-soluble compounds may be more important purpose than that of insoluble compounds due to the presence of aged and transboundary PM. Such considerations can allow useful insight to be gained from an OP analysis. A Teflon filter can be employed for PM collection for OP analysis instead of a quartz filter to attain a higher extraction efficiency and dilution efficiency. Extraction of solution using SLF may provide better results than extraction using water for the appraisal of the hazardousness to the human body with respect to trace metals and PAHs. Additional studies should investigate such topics further, which may lead to an improved, standardized procedure for the measurement of OP.

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