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73 **Abstract**

74 This paper presents the findings from a collaborative interlaboratory comparison exercise designed to assess
75 oxidative potential (OP) measurements conducted by 20 laboratories worldwide. This study represents an
76 innovative effort as the first exercise specifically aimed at harmonising this type of OP assay, setting a new
77 benchmark in the field.

78 Over the last decade, there has been a noticeable increase in OP studies, with numerous research groups
79 investigating the effects of exposure to air pollution particles through the evaluation of OP levels. However, the
80 absence of standardised methods for OP measurements has resulted in variability in results across different
81 groups, rendering meaningful comparisons challenging. To address this issue, this study engages in an
82 international effort to compare OP measurements using a simplified method (with a dithiothreitol (DTT)
83 assay).

84 Here, we quantify the OP in liquid samples to focus on the protocol measurement itself, while future ILCs should
85 aim to assess the full-chain process, including the sample extraction. We analyse the similarities and
86 discrepancies observed in the results, identifying the critical parameters (such as the instrument used, the use
87 of a simplified protocol, the delivery and analysis time) that could influence OP measurements, and provide
88 recommendations for future studies and interlaboratory comparisons. Even if other crucial aspects, such as
89 sampling PM methods, sample storage, extraction methods and conditions, and the evaluation of other OP
90 assays, still need to be standardised. This collaborative approach enhances the robustness of the OP-DTT assay
91 and paves the way for future studies to build on a unified framework. This pioneering work concludes that
92 interlaboratory comparisons provide essential insights into the OP metric and are crucial to move toward the
93 harmonisation of OP measurements.

94

95 **1. Introduction**

96 Over the last decade, many studies demonstrated associations between exposure to ambient air pollution and
97 adverse human health outcomes (Hart et al., 2015; Laden et al., 2006; Lepeule et al., 2012; WHO, 2021b, a).
98 Adverse health effects attributable to particle matter (PM) are complex and diverse. Among environmental
99 factors, PM is considered to be the largest contributor to morbidity and mortality globally (WHO, 2017). The
100 casual mechanisms underpinning these adverse associations are diverse, with oxidative stress and
101 inflammation, genomic alterations, damage to the nervous system function, and epigenetic alterations, among
102 others, all cited as potential contributing pathways (Huang et al., 2022; Nicholson et al., 2022; Wilker et al.,
103 2023; Zare Sakhvidi et al., 2022). Across these broad domains, the capacity of particles and particle-derived
104 chemicals to cause damaging biological oxidations appears to be a unifying mechanism, both through the
105 introduction of pro-oxidants and stable free radicals into the body, but also through secondary radical/oxidant
106 generation through altered metabolism and induction of inflammation (Li et al., 2008). By definition, oxidative



107 stress is a condition where excess production of reactive oxygen species (ROS) and nitrogen species (RNS)
108 overwhelm endogenous antioxidant defences (Shankar and Mehendale, 2014). Generally, ROS/RNS production
109 in the cells is regulated within physiological limits, through the actions of antioxidant enzymes (e.g. superoxide
110 dismutase, catalase, etc), low molecular weight water-soluble (e.g. glutathione, ascorbate) and fat-soluble
111 (vitamin E) antioxidants (Alkoussa et al., 2020). This antioxidant system plays a valuable key role by limiting
112 ROS/RNS damage, which is associated with cytotoxicity and the induction of inflammation due to changes in
113 the cellular redox balance (Casseo et al., 2013; Gao et al., 2020; Kelly and Fussell, 2017; Sies, 2018). The capacity
114 of PM to invoke biological oxidations has therefore been proposed as a proxy measure of their toxicity, and has
115 been referred to as their oxidative potential (OP); either that intrinsic to their possession of pro-oxidants, or
116 encompassing their capacity more wholistically to simulate ROS/RNS through interaction with cells (Ayres et
117 al., 2008; Bates et al., 2015; Cho et al., 2005; Sauvain et al., 2008; Uzu et al., 2011).

118 Consequently, the OP of PM is increasingly being studied as a potentially health-relevant metric to evaluate
119 effects due to exposure to PM, in addition to PM mass concentration, in multiple regions across the globe (Bates
120 et al., 2019; Bhattu et al., 2024; Daellenbach et al., 2017; Weichenthal et al., 2019). OP is a relatively simple
121 estimation of PM redox activity that reflects a complex interplay of all physico-chemical properties (chemical
122 composition, surface-area, solubility and particle size) contributing to the ROS/RNS generation and the
123 oxidation of target biomolecules, or probes. Implicit within this approach is the contention that not all
124 constituents of ambient PM are equally as harmful, and that those that drive damaging redox reactions, either
125 directly, or indirectly present a greater hazard. Thus, PM composition should be considered a factor more
126 directly linked to adverse health effects than PM mass concentration, highlighting the need to study additional
127 health-relevant metrics such as the OP (Park et al., 2018).

128 In last decades, there has been an increased interest in measuring and developing OP studies, applying different
129 in vivo or in vitro assays and aerosol characterisation techniques to estimate the main sources of OP related to
130 PM (Guascito et al., 2023), and attempting to integrate this metric into health studies. Several acellular chemical
131 methods have been applied for the estimation of the OP of atmospheric particles since these assays allow faster
132 measurement and are less labour-intensive than cell culture or in vivo methods (Bates et al., 2019). In addition,
133 these assays aim to mimic the interaction between PM and different lung antioxidants (e.g. glutathione,
134 ascorbate..., etc) during inhalation. The acellular assays which are most commonly applied include several
135 probe approaches based on antioxidants or surrogates, such as the dithiothreitol assay (DTT), ascorbic acid
136 assay (AA), glutathione assay (GSH), Ferric-Xylenol Orange assay (FOX), 9,10-bis (phenylethynyl) anthracene-
137 nitroxide (BPEAnit) ROS assay and 2,7-dichlorofluorescein assay (DCFH) for bound-particles. Molecular probes
138 display variable sensitivities to PM components due to their unique redox potentials and chemical reaction
139 routes, contributing to aerosol OP values. Therefore, it may be necessary to use several assays simultaneously
140 for a broader assessment of the chemical species in PM potentially triggering oxidative stress and to evaluate
141 which of these probes might be most indicative and closely linked to health effects. Furthermore, one of the



142 main challenges within this rapidly expanding research field is the diversity of analytical methods and protocols
143 used for OP each assay, which require standardised protocols to support synthesis across the evidence base
144 (Ayres et al., 2008).

145 In 2008, a previous workshop gathered experts on OP and developed consensus statements addressing the
146 importance of standardised samples, the comparison between oxidative potential tests, the formulation of
147 consistent standard test protocols and the establishment of connections between OP tests and epidemiological
148 findings as reliable predictors of adverse health outcome (Ayres et al., 2008). Despite more than 15 years having
149 elapsed since that workshop, whilst protocols have matured, evolved, and proliferated worldwide, little
150 concrete work has been performed regarding the harmonization and standardization of these methods.

151 One of the main objectives of the RI-URBANS European project (<https://riurbans.eu/>) is to bring accessible
152 service tools to enhance air quality monitoring networks, including evaluating air pollution exposure. As OP
153 has been proposed and recommended as a parameter to be measured in the proposal for a new European Air
154 Quality Directive (Council of the European Union, 2024), an international OP interlaboratory comparison (ILC)
155 was launched to assess the consistency of OP measurements between participants that apply different OP DTT
156 protocols, hindering comparison of results obtained worldwide. The main goal of the ILC was to identify
157 potential discrepancies in results (obtained with the OP DTT assay, one of the most common acellular assays
158 used for measuring the OP) that may arise due to differences in experimental procedures, equipment, or
159 analytical techniques. This ILC constitutes a first step to identify potential sources of variability, resulting in the
160 enhancement of the overall accuracy, reliability and comparability of OP measurements.

161 This paper presents the setup and results of this first large ILC study based on the dithiothreitol (DTT) assay,
162 with a large number of participants (20 groups). The first section includes a description of how a simplified
163 protocol was obtained, along with the coordination/management of the ILC. Subsequently, the results are
164 presented in the second section, combined with statistical analysis, both for the harmonized protocol and the
165 “home” protocols of each participant. Finally, some major findings and recommendations are presented.

166 **2. Intercomparison strategy**

167 This ILC was proposed within the RI-URBANS European Project framework to evaluate the discrepancies and
168 commonalities of OP measurements obtained by the different participating laboratories. The setup of the
169 protocol was led by a working group of laboratories with considerable experience in oxidative potential: FORTH
170 (The Foundation for Research and Technology – Hellas (FORTH, Greece), NOA (National Observatory of Athens,
171 Greece), ICL (Imperial College of London, United Kingdom), IGE (Institute of Environmental Geosciences,
172 France) and UoB (University of Birmingham, United Kingdom) (i.e. the “core group”). Considerations regarding
173 sampling techniques of PM filters or monitoring strategic approaches are beyond the scope of this exercise.

174 Multiple OP assays are available; however, following a literature review, it was decided to prioritise the DTT
175 assay for this first ILC due to its widespread adoption and long-term application facilitating broad participation



176 from laboratories. The core group first produced a harmonised and simplified method, detailed in a
177 standardised operation procedure (SOP), that was integrated, implemented and tested by IGE, the organiser
178 for this ILC. This SOP is called the “RI-URBANS DTT SOP” in the following and is presented in detail in Section
179 2.2. Section 2.1 presents the selection procedure for some parameters of the DTT assay according to variations
180 observed in the literature. Sections 2.3 to 2.7 comprise different parts of the implementation of the ILC, along
181 with the procedure for data processing.

182

183 **2.1 Testing the parameters for implementing the simplified RI-URBANS protocol**

184 The simplified RI-URBANS DTT SOP was adapted from the original DTT protocols published in the early 2000s
185 (SOP1: Li et al., 2003, 2009; SOP2: Cho et al., 2005; SOP3: Kumagai et al., 2002; called SOP1, SOP2 and SOP3,
186 hereafter). The principle of the DTT assay relies on the production of superoxide radicals, with DTT acting as a
187 surrogate for cellular reducing agents. This probe contains thiol groups similar to GSH and subjects to oxidation,
188 forming stable cyclic disulphides by donating electrons to oxygen through intermediate redox-active species
189 from PM. In the assay proposed by Kumagai et al. (2002), PM is incubated with DTT and its rate of oxidation
190 over time is assessed through the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with the reaction product
191 2-nitro-5-thiobenzoic acid (TNB), being detected at 412nm. Whilst DTT is in excess, the rate of DTT oxidation
192 is proportional to the concentration of redox-active species in the PM (Cho et al., 2005; Sauvain et al., 2008).
193 The DTT loss over time can then be expressed per unit concentration of PM (usually μg) or by volume of air
194 (m^3) to provide a measure of the intrinsic reactivity of the particles and assess human exposure (Cho et al.,
195 2005), respectively.

196 A review of the pioneer DTT assay protocols revealed differences, and therefore to derive a simplified protocol,
197 some variations of the parameters were examined by the ILC organiser laboratory. These results were
198 evaluated by the RI-URBANS core group to obtain the final harmonized protocol.

199 Three key parameters were tested. First, the necessity for the inclusion of trichloroacetic acid (TCA) was
200 evaluated. In the original DTT protocol by Kumagai et al. (2002), the reaction between samples and DTT is
201 quenched at a specified incubation time by the addition of 1.0 ml of 10% TCA to the incubation mixture.
202 Subsequently, 0.5 ml of the reaction mixture is extracted and combined with DTNB and tris-HCl buffer (pH 8.9).
203 However, more recently, Li et al. (2009) demonstrated that DTNB rapidly reacts with DTT, with the absorption
204 reaching its maximum value immediately and remaining stable for over two hours. In our initial tests, we found
205 that this parameter was consistent showing no differences over all the samples tested with or without TCA
206 (Figure S1). Thus, in the simplified OP RI-URBANS SOP, TCA addition was omitted, and we introduced the DTNB
207 solution directly into the mixture at the prescribed incubation times and recorded the absorption after the 30-
208 minute reaction.



209 Second, EDTA is present in some OP DTT protocols, whether in the buffer of the incubating PM sample with
210 DTT (Kumagai et al., 2002; Li et al., 2009), or in the titration mixture of DTNB (Cho et al., 2005; Kumagai et al.,
211 2002). EDTA is a strong chelating agent which is widely used in biological assays to prevent microbial
212 contamination and to facilitate cell lysis and the extraction of cellular components. Using EDTA is helpful in
213 purifying buffers at a low cost and decreasing a high rate of DTT loss in the blank by scavenging metal ions.
214 However, it can lead to artefacts during the assay. This is especially critical when used in the reaction mixture
215 with the PM, where it could induce complexation with redox changes. Moreover, this is particularly relevant for
216 iron where complexation increases solubility and the resultant EDTA-Fe complex is redox-active (Gao et al.,
217 2024), or in the opposite, EDTA can chelate some metallic species, preventing their reactivity (Charrier and
218 Anastasio, 2012). In addition, EDTA also has antioxidant properties, which may compete in the solution with
219 DTT (Thbayh et al., 2023). Our results on different samples show that the presence of EDTA in the buffer leads
220 to underestimation of the OP DTT loss rates in comparison to without EDTA. An impact was observed in the
221 solutions, mainly the copper reference solution (1 μM) and an ambient PM filter for testing both protocols
222 (Figure S2). That augmentation could also be related to an increase in the blank absorbance without EDTA due
223 to the impurities in the buffer, but this is controlled by subtracting the blank. Little or no impact was shown on
224 the 1,4-naphthoquinone solutions, an organic component. To prevent such undesirable interactions, many
225 laboratories have introduced Chelex® 100, a sodium-form resin to purify the buffers used in the OP DTT assay
226 (Calas et al., 2017, 2018; Charrier et al., 2015; Charrier and Anastasio, 2012), or used commercially available
227 high-purity (e.g. LC-MS/MS grade) water and buffer mixture to create the OP reaction medium (Shahpoury et
228 al., 2019, 2022). Nevertheless, the Chelex® commercial resin comes with a basic pH and requires a pre-
229 treatment that could be an extra source of error for this current first international intercomparison. Finally,
230 EDTA was not included in the SOP to prevent complex ligand chemistries, and because the use of Chelex® was
231 complex to introduce for such first ICL; high-grade buffer powders were sent to all the participants instead.

232 Third, original protocols include the use of a Tris-HCl buffer at pH 8.9 in the solution of titration with DTNB,
233 and this remains widely used. However, Li et al. (2009) showed that the pH of the solution drives both the
234 catalytic redox reaction rate and also the molar extinction coefficient of the product of the reaction, TNB. This
235 report confirmed the previously obtained results by Danehy et al. (1971) that showed that DTNB suffers from
236 alkaline decomposition above pH 8, increasing absorbance values (Figure S3). As a result, for the simplified RI-
237 URBANS DTT SOP, the core group selected a potassium phosphate buffer at a physiological pH of 7.4 to replace
238 tris-HCl in the DTNB solution. This prevents the DTNB alkaline decomposition because the pH is in the
239 favourable range of 5.5-8 where TNB is in the TNB^{2-} form and the DTT+DTNB system does not show significant
240 pH-dependant changes in the absorbance values (Li et al., 2009).

241 Finally, the simplified RI-URBANS DTT SOP also includes a variation considering the instrument used for the
242 measurements. The SOP was elaborated and tested for both cuvette and plate reader spectrophotometers,



243 including the potential application of automatic samplers. However, the simplified SOP remains to be tested for
244 other instruments (such as Liquid waveguide capillary cell instrument (LWCC)).

245

246 **2.2 Simplified RI-URBANS DTT SOP and other measurements**

247 The simplified RI-URBANS DTT SOP is proposed in SI-1. It contains a first step for the preparation of the
248 reagents needed for the analyses included in the ILC. A second step describes how to perform a calibration of
249 the analytical device, using a DTT calibration curve with at least 4 points for a concentration range between 0
250 and 60 μM (titration with 1mM DTNB and reading of TNB formation at 412 nm). In the third step, the SOP
251 defines the performance of the measurements for the test samples provided, including the assessment in
252 triplicates of each test sample and control points (blanks). It should be noted that the DTT protocol also
253 integrates the analysis of control points (blanks), which allows quantifying the inherent DTT background
254 oxidation.

255 The duration of the analyses required for the ILC is variable depending on the instrument used. About 30 min
256 completion time is required, including the assessment of ILC test samples and control points, when performed
257 with a plate reader, and a similar time is needed to perform the calibration curve. When a cuvette-type
258 spectrometer is used, the total analysis time can be at least 2 hours to perform all the triplicates and the
259 corresponding calibration curve.

260 Furthermore, apart from the analytical equipment for monitoring the chemical reactions, the simplified
261 protocol also requires some standard laboratory equipment and conditions, such as access to ultrapure water
262 ($18.2 \text{ Mohm cm}^{-1}$, TOC <5 ppb) for the preparation of reagents, the use of vortex for the homogenisation of
263 samples, refrigerated baths to conserve the reagents and transparent 96-wells plates and dark tubes. The
264 samples also need to be kept under agitation during the experiment and at a constant temperature of 37.4°C .

265 The calculation of the chemical reaction rate of OP DTT during this ILC involves a conversion using a calibration
266 curve. Once the results are obtained, the kinetics of the DTT oxidation can be calculated by subtracting both the
267 intrinsic absorption of each sample (absorption obtained from the samples before the addition of reagents to
268 remove a potential matrix effect between samples) and the inherent DTT auto-oxidation rate (slope of a Control
269 sample) from the DTT consumption rate in the presence of PM.

270 Participants were asked to perform additional OP measurements on the same samples, following the protocol
271 in use in their laboratory ("home protocol(s)") if they wanted to do so. In this case, they had to provide the
272 results in the particular units requested, depending on the applied assay.

273



274 **2.3 Type of samples – test materials**

275 The ILC was performed using three samples (SP1-SP3), including different concentrations of ambient PM and
276 commercial positive control (1,4-naphthoquinone, CAS [130-15-4], Sigma Aldrich), all extracted in ultra-pure
277 water and prepared by the ILC organiser. Providing ultra-pure water extracts - instead of filter fragments - to
278 the participants was selected for the current first ILC to avoid additional uncertainties associated with the use
279 of different procedures of sample extraction, different quality of the ultra-pure water for sample extractions,
280 and changes linked to the processing equipment available. More specifically, the samples sent to the
281 participants included SP1: 1,4-naphthoquinone solution (reference compound, $5 \mu\text{g mL}^{-1}$), SP2: extract from a
282 PM sample influenced by biomass burning emission (obtained from a chamber experiment, at $25 \mu\text{g mL}^{-1}$), and
283 SP3: an urban PM extract highly influenced by traffic emission (from pooled roadside samples obtained from
284 TEOM - FDMS reference samplers, at $25 \mu\text{g mL}^{-1}$). Additionally, a fourth sample, SP4: a sample extracted from a
285 blank/clean quartz filter was sent to the participants, but it was not included in the evaluation since the
286 measured values were close to the instrument limits of detection for most participants. For each of the 4
287 samples, all the sub-samples, distributed to participants, resulted from a unique 1L solution obtained from the
288 original sample substrate. For instance, SP1 and SP3 were powders that were solubilised and homogenised for
289 75 min by vortex agitation in ultra-pure water. SP2 and SP4 were quartz fibre filters subjected to a 75-minute
290 vortex extraction in ultrapure water.

291 Several 5 ml sample aliquots in dark polypropylene tubes were sent to each participant, according to their
292 needs, allowing them to perform triplicate measurements for the RI-URBANS DTT SOP and all the “home” OP
293 protocols implemented in their labs.

294 Solid potassium dihydrogen phosphate (CAS [7778-77-0], Roth), di-potassium hydrogen phosphate (CAS
295 [7758-11-4], Roth), 5,5'-Dithio-bis-(2-nitrobenzoic acid, CAS [69-78-3], Roth) and 1,4-Dithiothreitol (CAS
296 [3483-12-3], Roth) were also distributed to the participants, to prepare the solutions for the RI-URBANS DTT
297 SOP, including the DTT solution, the Dithio-bis-(2-nitrobenzoic acid) (DTNB) solution, and the potassium
298 phosphate buffer solution.

299 **2.4 Transport of samples, ILC performance and duration**

300 Test samples (SP1-SP4) were shipped to all participants on 17th January 2023 via courier in refrigerated and
301 isolated ice packs, and received as chilled liquid samples. The parcels were delivered between 18th January and
302 2nd February 2023. On average, the participants received the samples in 3 ± 2.5 days and performed the analysis
303 in 14 ± 10.5 days and up to 31 days after reception of samples. The recording of these parameters allows their
304 integration to the multiple linear models used in this work.

305 **2.5 Reporting of the results**

306 Participants were asked to report OP DTT results from the RI-URBANS SOP in $\text{nmol DTT min}^{-1} \mu\text{g}^{-1}$ and the %
307 DTT consumed in $\mu\text{g}^{-1} \text{min}^{-1}$, applying three decimal digits for all three replicates of test samples. An Excel
308 spreadsheet with all the calculations pre-included was prepared by the ILC organiser and shared with all the



309 participants to avoid calculation errors and to facilitate the standardisation of results. In addition, participants
310 were invited to report, under the same format, the values for other OP tests, such as OP DTT “home”, and other
311 OP tests like AA, DCFH, OH, ESPR, GSH and RP (routinely applied by each participant) on the same samples.

312 **2.6 Number of participants**

313 It is worth noting that for the first time, a total of 20 research groups participated in the exercise: 14 of them
314 from Europe, including the United Kingdom, Italy, France, Switzerland, Greece, Germany, Serbia, the Czech
315 Republic, the Netherlands and Sweden, 3 participants from the United States, 2 participants from Canada and
316 1 from Australia. The participants were invited for their contribution to the RI-URBANS project, through a
317 public call to participate, or because they contacted the ILC organiser directly and were selected, due to their
318 active role in the OP scientific community. The ILC was performed using anonymous participation; thus, a
319 number was randomly assigned to each participant to present the results. Participant L5 cancelled his
320 participation in the ILC and two participants (L3 and L16) did not send their results for RI-URBANS DTT SOP.

321 **2.7 Data evaluation**

322 The ILC results were analysed by the European Commission Joint Research Centre (JRC), which provides
323 independent, evidence-based science and knowledge support for EU policies and in conducting ILC exercises.
324 The participation of an external independent evaluation was required following the International Global
325 Standard ISO 5725-2, related to the accuracy of measurement methods and results (Part 2: Basic method for
326 the determination of repeatability and reproducibility of a standard measurement method). The data
327 assessment includes statistical evaluation of homogeneity, ageing, and repeatability, as well as the
328 establishment of the assigned values and the related methods.

329 **2.7.1 Estimation of the assigned value and participants’ performance**

330 Different methods can be applied to determine the assigned (consensus) value for a comparison exercise when
331 no reference or certified reference material is available. The first one would be to use a robust mean and
332 standard deviation including all participants, but this could become statistically ineffective if the number of
333 participants is below twenty. Further, the results we obtained were not normally distributed (Figure S4),
334 compromising the accuracy of the robust mean of the samples tested. Thus, three different alternative
335 approaches were evaluated in this ILC until meeting the requirements of Standard ISO 5725-2. The first one is
336 based solely on the results obtained by the ILC organiser, where the samples were prepared, and the
337 homogenisation tests were performed. The assigned values obtained from this approach include a coefficient
338 of variability of 11%, 7% and 6% for SP1, SP2 and SP3, respectively. The second approach applies the Q/Hampel
339 test, a robust algorithm useful for data sets with outliers to calculate the robust standard deviation and mean
340 of the data set. This method is largely applied to the statistical analysis of interlaboratory studies. The
341 Q/Hampel test results (integrating the average of the results from all the participants) present COVs of 60%,
342 61% and 70% for SP1, SP2 and SP3, respectively. However, the values obtained for the dataset were deemed



343 too high to guarantee the good performance of the Q/Hampel test calculation. The third approach relies on
344 calculating the assigned (consensus) value from the average and standard deviation for a specific reference
345 group, including 6 participants selected (without knowing their results) due to their previous pioneering
346 experience in developing OP protocols and measurements, taken as their strong expertise in the domain (>10
347 publications in the field). The participants selected were the L2, L4, L8, L12, L13 and L19. This approach leads
348 to coefficients of variability of 31%, 33%, and 47% for SP1, SP2 and SP3, respectively. Since the first two
349 methods showed a contrasted variability, one with too little and the other with too much variability, the
350 assigned values were calculated using the third approach, integrating the average and standard deviation of the
351 results from this selected experienced OP participants.

352 The individual performance of each group was further evaluated using z-scores, a metric indicating the
353 deviation of each data point from the assigned value as compared with the standard deviation for proficiency
354 assessment σ^* . For each laboratory and test sample, the z-score is computed using the formula: $z = (x_i - X) / \sigma^*$,
355 where x_i is the result from participant i , X is the sample assigned value (0.53, 0.14, 0.07 nmol min⁻¹ µg⁻¹), and σ^*
356 is calculated as the reproducibility standard deviation among the six selected participants (0.16, 0.06, 0.04 nmol
357 min⁻¹ µg⁻¹). An "action signal" is triggered if a participant's entry produces a z-score exceeding +3 z or falling
358 below -3 z, indicating a deviation of more than 3 standard deviations from the assigned value. Similarly, a
359 "warning signal" is raised for a participant z-score above +2 z or below -2 z, representing a deviation between
360 2 and 3 standard deviations. A participant z-score between -2 z and +2 z signifies satisfactory performance
361 concerning the standard deviation for proficiency assessment.

362

363 2.7.2 Analysis of the variability of the results

364 Additionally, the statistical distribution of results was evaluated using multiple linear regression models. In the
365 first model, the effects of the protocol variables on the measured OP were investigated using linear regression
366 models adjusted on the instrument (3-class variables: plate reader, cuvette and LWCC), delivery time (time
367 between sample shipment and reception, continuous), and analysis time (time between reception and analysis,
368 continuous). An additional model (M2) compared the RI-URBANS SOP and the DTT-home protocols was further
369 adjusted based on the protocol (2-class variable: RI-Urbans, DTT-home). Finally, the evaluation of the average
370 performances (3-class variables: low - $0 < |z\text{-score}| < 2$, middle - $2 < |z\text{-score}| < 3$, and high - $|z\text{-score}| > 3$) was
371 added in the M2 model, to assess whether performance affected DTT activity in the same direction (i.e. positive
372 or negative), while considering other protocol variables. Each model was run separately for SP1, SP2 and SP3
373 samples. All analyses were conducted using R (version 4.2).

374



375 **3. Results and Discussion**

376 Out of the group with a total of 20 participants, 18 presented results obtained using the RI-URBANS DTT SOP.
377 Different instruments were used to apply the simplified SOP. Overall, 9 participants (47.5%) used the cuvette-
378 type spectrophotometer, 8 (42%) used the plate reader-type spectrometer, and 2 (10.5%) implemented LWCC
379 measurements (one participant used two instruments).

380 **3.1 Homogeneity of the samples**

381 An initial assessment of the homogeneity of the OP measurements with the 3 test samples was performed by
382 the ILC organiser, using a plate-reader type protocol, deriving the mean and the standard deviation of 10
383 replicate analyses performed on the same day. The results obtained from the coefficient of variations, showed
384 the sample variabilities were up to 12%, 7%, and 9% for SP1, SP2 and SP3, respectively (Figure S5), showing a
385 higher variability for the 1,4-naphthoquinone solution (SP1) compared to the two filter extracts (SP2 and SP3).

386 The overall uncertainty of the OP DTT assay has been evaluated to 18% for PM_{10} and 16.3% for $PM_{2.5}$ by Molina
387 et al., 2020. Despite some differences observed between our samples, the results are deemed acceptable,
388 presenting a variability of around 10%, which indicates a very good performance of the analysis.

389 **3.2 Ageing of samples**

390 To reduce the number of parameters affecting the preparation of the sample solutions liquid solutions of each
391 sample were prepared and sent to the different participants. However, liquid samples can undergo ageing
392 processes, impacting OP levels over time. For this purpose, an ageing test was performed by the ILC organiser
393 to evaluate the potential changes over time. It consisted of regularly implementing the RI-URBANS DTT SOP to
394 obtain the values of each test sample over time. Figure S6 shows these results, where SP2 and SP3 do not show
395 a strong change over time, while sample SP1 presents a pronounced ageing effect. In routine tests of ICL
396 organizer, the 1,4-naphthoquinone mother solution at such high concentration is usually stable in a glass
397 container for weeks but here, potential interaction with the PP tubes' inner surface may have happened.
398 Consequently, ageing could be a variable of importance for the participants who analysed the samples toward
399 the end of the required period, and such parameter (date of analysis) was thus included in the parameters to
400 be tested for the research of critical parameters.

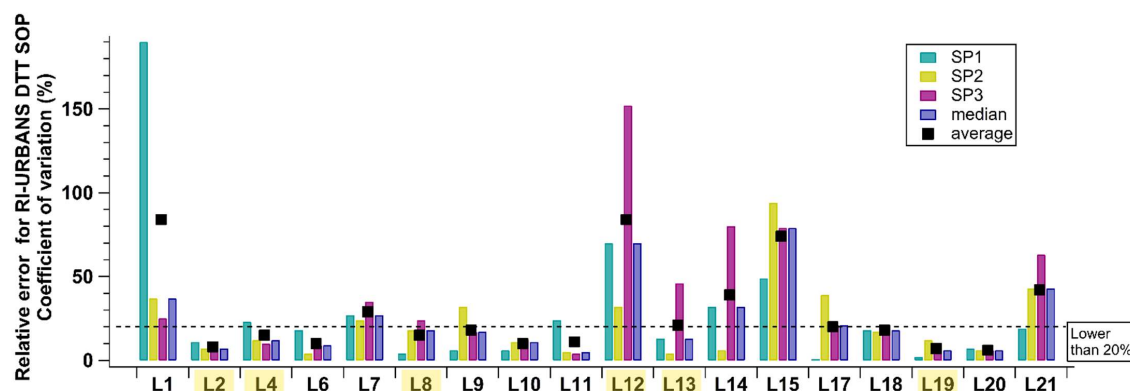
401

402 **3.3 Statistical distribution of results: Participants' variability**

403 In order to assess the intra-laboratory variability of the results, the coefficient of variation (COV = standard
404 deviation/ mean * 100) of the results for each sample and each laboratory are presented in Figure 1, while the
405 standard deviations of the replicates reported for each sample are presented in Figure S7 and table S1. Overall,
406 higher COVs are observed for SP3 and SP1, where most participants (44.4 and 38.9%, respectively) presented
407 higher values for this sample compared to the SP2 sample. Specifically, high average COV values are observed



408 by L1, L12, L14, L15 and L21, with an average variation higher than 40%. Only a few participants (6 groups)
409 presented a variation lower than 10% for the three samples. This is the same pattern for the results obtained
410 by the ILC organiser during the homogeneity test (see 3.1.), where the COV for SP1 was larger than those for
411 SP2 and SP3 but with the highest COV below 15%. These findings confirm more homogenous results for
412 samples SP2 and SP3 compared to SP1 but could also indicate that some participants failed to achieve
413 repeatability observed by the ILC organiser. However, some groups (L2, L10, L19, L20) were able to produce
414 very homogeneous results with COV < 10% for all 3 samples.



415
416 **Figure 1: Coefficients of variation of each participant (L1 to L21) for the three samples in triplicates**
417 **tested using the RI-URBANS DTT protocol and the median and mean repeatability for each laboratory.**
418 **Yellow-highlighted participants are the ones selected for the calculation of the assigned values. The**
419 **dashed line indicates the participant with COV lower than 20%.**

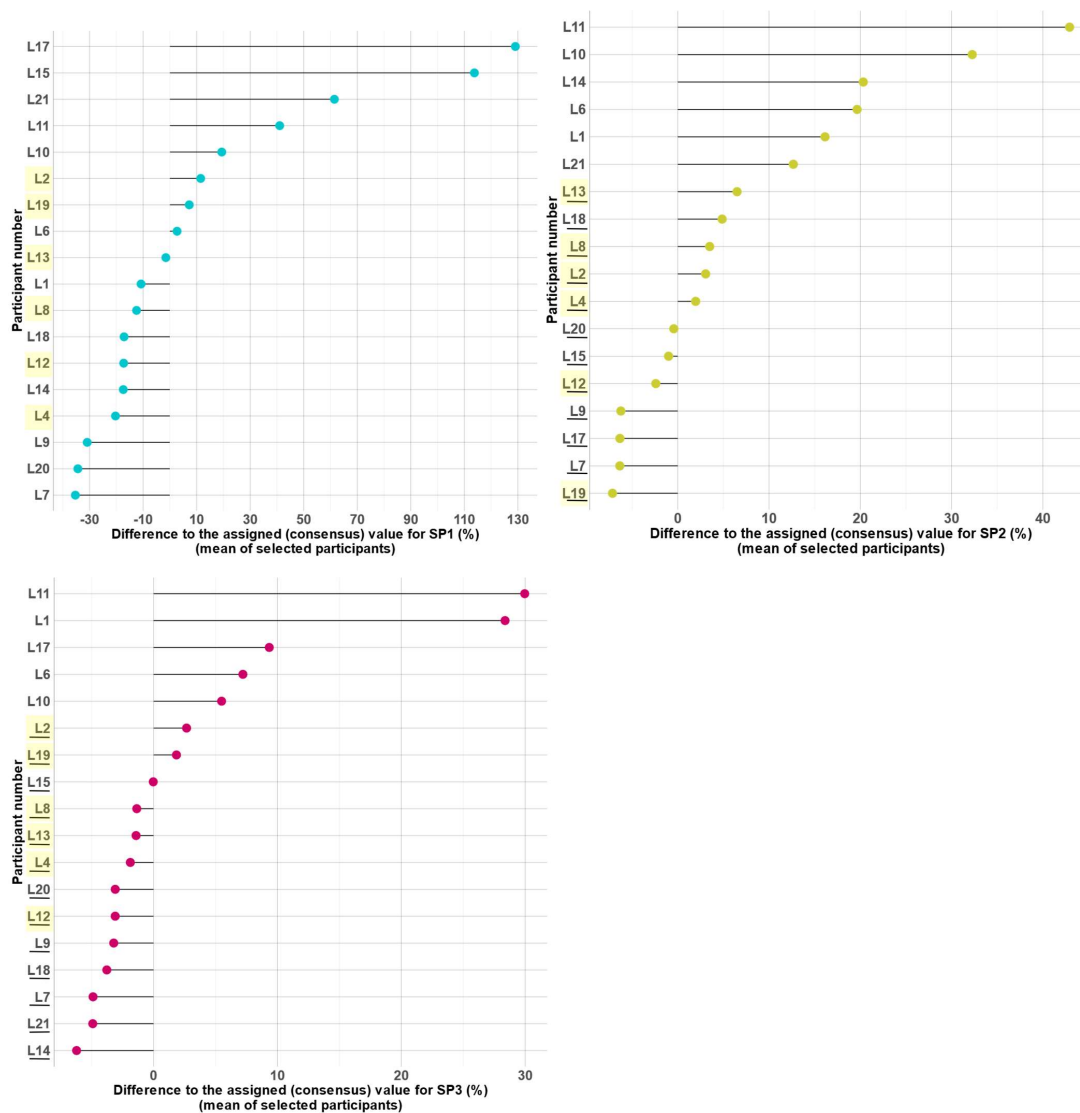
420

421 3.4 Laboratory performances

422 The assessment of laboratory performances first presents the bias in results across participant groups
423 compared to the assigned values and their associated standard deviation for each sample. As illustrated in
424 Figure 2, SP1 exhibited the highest variances, ranging from 130% to -35%, with only five groups displaying
425 differences within $\pm 10\%$. The distribution of results for SP1 indicated a mix of overestimations and
426 underestimations. For SP2, differences are within a narrower range from 43% to -7%, primarily favouring
427 overestimations. For this sample, 12 participants returned results that were within $\pm 10\%$ of the assigned value
428 (see highlighted laboratory numbers in Figure 2). Finally, the results for SP3 demonstrated the least variation
429 among participants, with differences ranging from 30% to -6%, and 16 participants within $\pm 10\%$ of the
430 assigned value, again favouring overestimation compared to the assigned value. In total, 14 laboratories
431 obtained data with $\pm 10\%$ difference to the assigned value for SP2 and SP3 (see highlighted laboratory numbers
432 in Figure 2). These results show again that the reference samples with 1,4-naphthoquinone (SP1) most



433 probably present some characteristics leading to this variability and may be associated with a less stable
434 solution or led to saturation of some detectors regarding the relatively high concentrations, while the samples
435 from filter extractions do not. Additionally, it is interesting to note that there is apparently no systematic
436 pattern where a given participant would obtain out-of-range results for all samples. The results are really
437 diverse, and most participants can obtain “acceptable” results for one or two samples (SP) and larger variability
438 associated with one “unacceptable result” for one of them. In 2020, Molina et al. (2020) explored the total
439 uncertainty of OP DTT of a collection of samples and evaluated it to 18% for PM₁₀ and 16.3% for PM_{2.5}. The
440 leading factors identified were the DTT consumption rate (regression and repeatability of experimental data)
441 and the extraction volume operations (pipette). This underscores the need for further investigations on the
442 experimental causes of the variations observed, possibly in the next ICL.



443

444

445 **Figure 2. Percentage differences from the assigned (consensus) value for each sample (SP1, SP2 and**
 446 **SP3). The results compared the average of the triplicates reported by the participants. Yellow-**
 447 **highlighted participants are the ones selected for the calculation of the assigned values and underlined**
 448 **the ones that obtained data into $\pm 10\%$ difference to the assigned value for SP2 and SP3.**

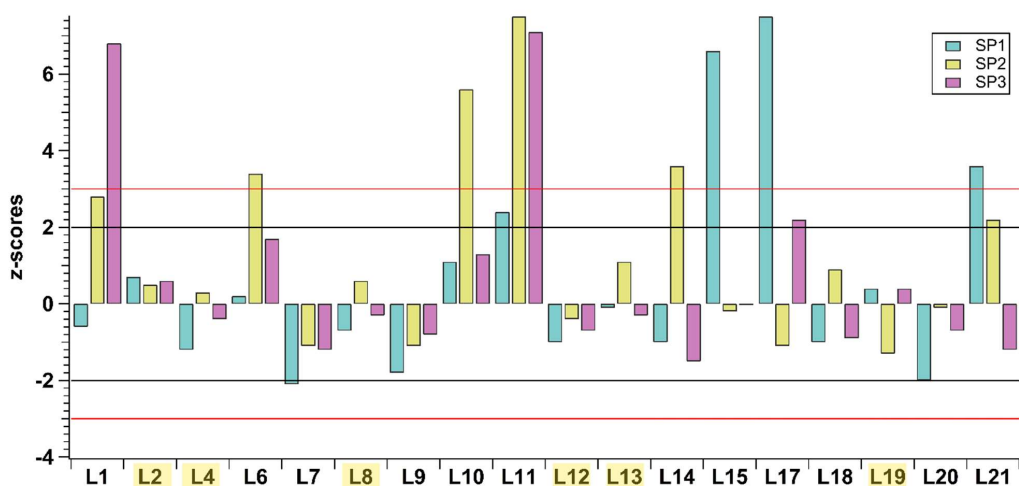
449

450 The individual performance of each group was further evaluated using z-scores. The results are presented in
 451 Figure 3. All underestimations fall within the acceptable range (lower than $-2 z$). Additionally, it is noteworthy
 452 that no laboratory exhibits unsatisfactory performances across all three samples; for almost all participants,



453 while one sample can present poor results, it coexists with two acceptable ones. This has strong implications
454 for spatial and temporal analyses that are often performed for OP. Again, this calls for attributions that there
455 are no systematic biases in the analyses. While factors like sample inhomogeneity may be playing a role
456 (particularly for SP1), some other issues, including variability in the performance of the analysis, may have an
457 impact. Hence, participants exhibiting significant deviations ($|z\text{-scores}| > 2$) for some of their results should
458 thoroughly examine their procedures and possibly implement appropriate corrective actions to avoid similar
459 outcomes in future ILCs.

460 However, half of the participants achieved results within the acceptable limits of this test. Despite disparities,
461 these findings are really promising, especially considering that this is the first intercomparison of its kind. For
462 instance, such results are in the same range to those obtained for some of the first ILCs for PAHs (Grandesso et
463 al., 2012; Verlhac et al., 2014).



464

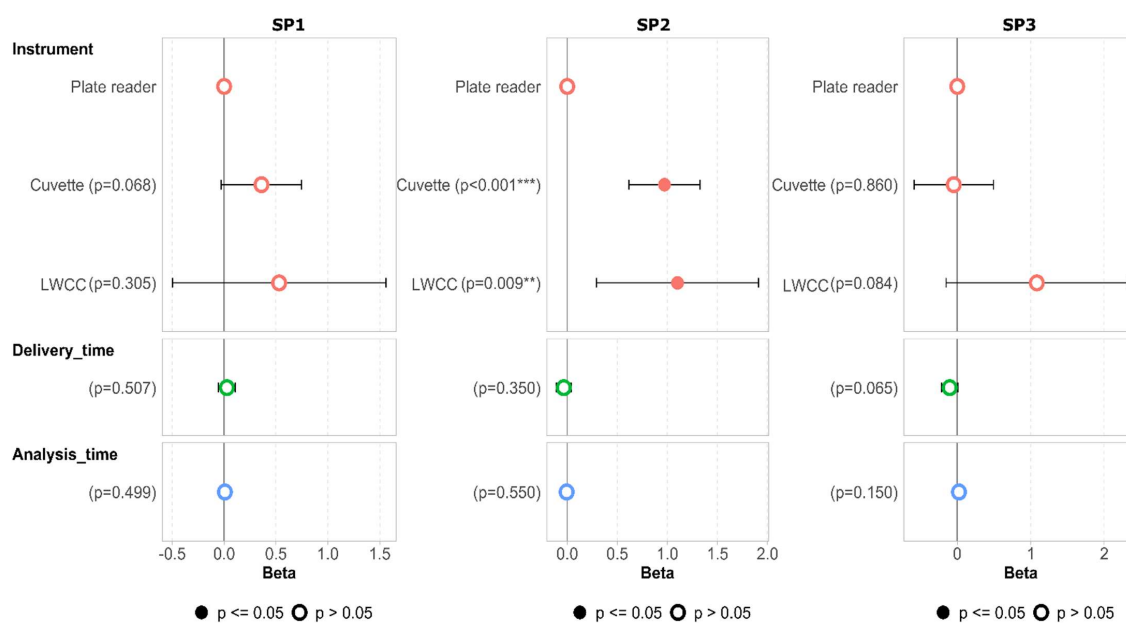
465 **Figure 3. Z-scores were calculated to evaluate each participant's performance in the interlaboratory**
466 **comparison for each sample tested. Yellow-highlighted participants are the ones selected for the**
467 **calculation of the assigned values. Black and red horizontal lines indicate boundaries for triggering an**
468 **action signal as described in section 2.7.1.**

469 To gain more knowledge about the factors causing the variability of the results, we first tried to perform a
470 cluster analysis using the Ward method. This grouped the participants into four clusters (see Figure S8), with
471 the main cluster (in yellow) including the 10 participants encompassing mainly the ones with satisfactory Z
472 scores. The clustering seemed independent of the instrument used and/or the time taken between the sample
473 delivery and analysis (i.e. near the delivery time or later in February).



474 In a second step, a multiple linear regression model was run to evaluate the associations of the results obtained
 475 for the 3 samples, SP1, SP2 and SP3, considering a range of parameters, including the instrument used and the
 476 delivery and analysis time (Figure 4, Table 2). The beta values are shown in Figure 4, representing the
 477 association (effects) between the different parameters evaluated and the OP results obtained. In the model, the
 478 reference variables were the RI-URBANS DTT SOP and the results obtained with the plate-reader instrument.
 479 Regarding the instrument performance, the values provided by the cuvette-spectrometer were higher than
 480 those obtained with plate readers in the case of SP1 and SP2 (showing significant overestimation in the case of
 481 SP2 p -values < 0.05), while the results for SP3 were quite similar. In the case of LWCC, higher variability is
 482 observed when compared to both cuvette and plate reader for all the samples. SP1 LWCC results presented the
 483 highest variability, and SP2 results were significantly overestimated (at a 95 % confidence level) when
 484 compared with those obtained by the plate reader. The RI Urbans SOP was adapted for plate readers and
 485 cuvettes, in order to perform the measurements in similar conditions of concentrations for the reagents. This
 486 was not the case for the LWCC since we did not have all the necessary information concerning the specific
 487 devices used by participants. Figure 4 suggests that the specific conditions of the reaction are probably
 488 important factors for delivering an accurate value of OP. In Figure S6, we showed that SP1's OP activity
 489 decreased over time during storage, but this ageing effect was not found to be significant in the model for either
 490 delivery or analysis time. The storage effect remained consistent for SP2 and SP3, as there was a clear
 491 association between OP and delivery or analysis time (Figure 4).

492



493



494 **Figure 4. Associations (beta in $\text{nmol min}^{-1} \mu\text{g}^{-1}$) between OP DTT values for SP1, SP2 and SP3 using the**
495 **RI-URBANS protocol and technical parameters, including the instrument used and the delivery and**
496 **analysis time obtained by applying an adjusted multiple linear regression model. Full-colour dots**
497 **represent the results with p-values ≤ 0.05 , and white dots represent the results with p-values > 0.05 .**

498

499 A ranking of the samples is also proposed to evaluate the OP activity of the samples tested in this ILC and its
500 relative variability within the participants (only considering the results obtained with the simplified RI-
501 URBANS protocol). For this purpose, SP1 was arbitrarily selected as the one with the highest OP activity with
502 an assigned value of 100, and SP2 and SP3 were evaluated in function of SP1. Figure S12 shows the results
503 obtained for the relative ranking of the samples. It can be noted that most of the participants presented similar
504 relative variability with $\text{SP1} > \text{SP2} > \text{SP3}$. Some exceptions were observed for L1, L17 and L19, which obtained
505 higher ranking for SP3 than SP2. A higher variability in the relative activity is obtained for SP2 than for SP3.
506 Within the participants showing a higher relative ranking for SP2 (higher than 50% compared to SP1), most of
507 them used either cuvette-type or LWCC instruments (except L20), suggesting some overestimation in the
508 results using these instruments. Overall, this similar ranking for the samples achieved by most of the groups is
509 noticeable and very encouraging. In fact, most of the data treatment performed on OP with atmospheric
510 variables or health data relies on associations and regressions where the relative variability of a time series is
511 of utmost importance, more than the absolute value.

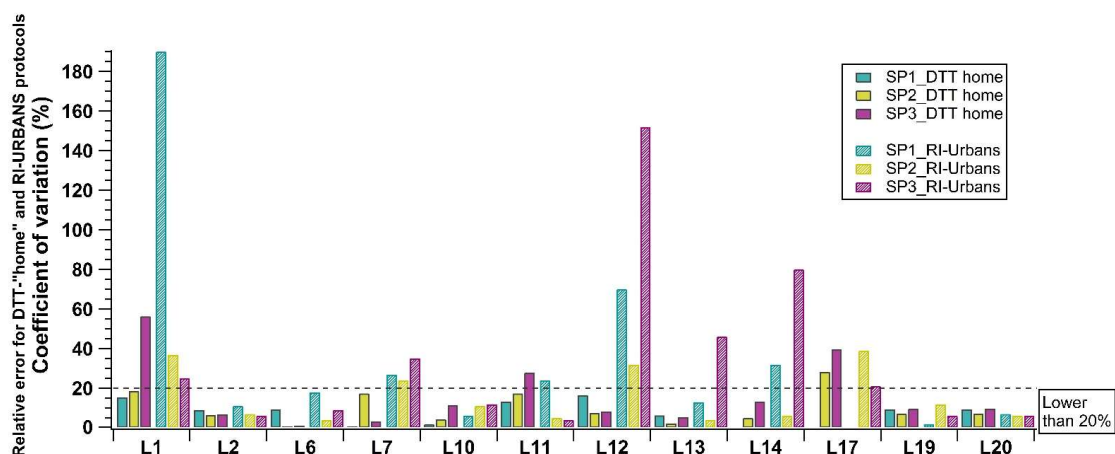
512 3.5 Comparison with other OP tests provided

513 Participants were also invited to report results obtained using other OP assays. Since not all participants
514 submitted results from equivalent "home OP" tests, we exclusively focus on the outcomes obtained through the
515 "DTT-home" protocols involving 13 participants (Table S3, Figure S9). It is important to note that DTT-RI-
516 URBANS protocol was simplified and does not include EDTA, Tris-HCL, TCA neither Chelex®, whereas DTT-
517 "home" protocols are diverse and should exhibit at least one up to 3 of the last mentioned compounds at
518 different step of the reaction; however, this is challenging to evaluate because not all groups have submitted
519 the protocols related to their "home" results.

520 We have first evaluated the COV individually from the results obtained with protocols (RI-URBANS SOP versus
521 all DTT-"home") for each test sample. Figure 5 shows that lower COVs are generally observed in the
522 performance of the DTT-home protocols (more details can be found in Table S3). However, six out of 12
523 participants presented similar COVs (within 20%) for the two protocols. These results could indicate that the
524 use of a simplified OP protocol needs some extent of training and guidance before its application. In addition,
525 some of the participants presented higher COV values (L1 and L13 for DTT-home) when using the LWCC
526 instrument. The lack of a simplified protocol for this instrument did not seem to be a major issue, as the
527 application of the DTT-home protocols was also associated with high COV.



528



529

530 **Figure 5: Coefficients of variation of each participant for the three samples tested from triplicates**
531 **using both the RI-URBANS DTT and the DTT-“home” protocols. The average and standard deviation of**
532 **each participant are also detailed in Table S3.**

533

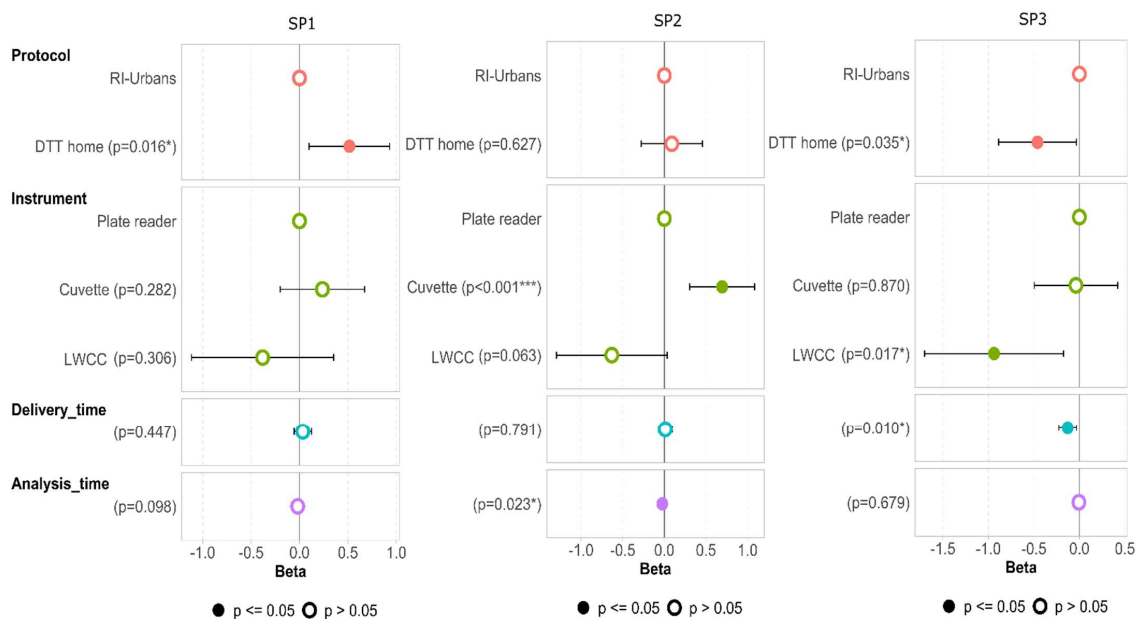
534 Another multiple linear regression model was run to evaluate the main differences in the results obtained
535 between RI Urbans SOP and DTT-“home” protocols (Figure 6). For SP1, a significant overestimation was
536 observed for the DTT-home protocol and the opposite was observed for SP3. In the case of SP2, there was no
537 statistically significant difference between both protocols. More details on the concentration of DTT for each
538 DTT method could provide more insight into this trend. Regarding the instrument performance, the LWCC
539 presents poorer results compared to the cuvette and plate reader for all the samples, which is opposite to the
540 trend observed with the results of the RI-URBANS protocol only (Figure 4). However, the results are
541 significantly underestimated for SP3 only ($p < 0.05$). For the cuvette-based measurements, the results are higher
542 than those obtained with plate readers in the case of SP1 and SP2 (significantly overestimated for SP2) and
543 similar for SP3, which is in line with the direction observed with the RI-URBANS protocol only. The delivery
544 and analysis time show a statistically significant lack of effect for SP2 (analysis time) and an underestimation
545 for SP3 (delivery time) but nothing significant for SP1, although it had undergone ageing in the tests of the ILC
546 organiser. Since the effects are very small compared to the effects of the protocol, or the instrument, these two
547 variables (delivery and analysis time) may cause a greater impact on DTT values when protocols are
548 harmonised, but not to date.

549 The results obtained by the participants (the z-scores evaluation) were also added to the former model to
550 evaluate the effect on OP values while adjusting the protocol variables (Figure S10). The results show a



551 significant OP overestimation of all the samples for the labs with poor performances in SP2 and SP3 samples,
 552 and also a significant underestimation in the OP value obtained for SP2 for the group with intermediate
 553 performance.

554 Hierarchical cluster analysis was conducted, incorporating both the DTT "RI-Urbans" and DTT "home"
 555 outcomes (Figure S11). Because some participants did not implement a DTT "home" protocol, the cluster
 556 analysis involved a reduced set of OP values. The results reveal the presence of four primary clusters, with the
 557 predominant cluster encompassing most participants (8 out of 12 for the DTT- "home"). The participants within
 558 the green main cluster largely align with the results derived from the DTT "RI-Urbans" outcomes, encompassing
 559 the groups in the two primary clusters (Figure S11). This assessment illustrates some consistency of results
 560 obtained across various OP DTT protocols. Some of the participants with more reliable results for the RI-
 561 URBANS DTT SOP maintain their consistency regardless of the protocol used. However, some of those that did
 562 not show an acceptable performance for the simplified protocol (i.e. L1 and L10) presented a better
 563 performance for the DTT-"home", and the opposite was observed for the L19 (almost for SP1).



564

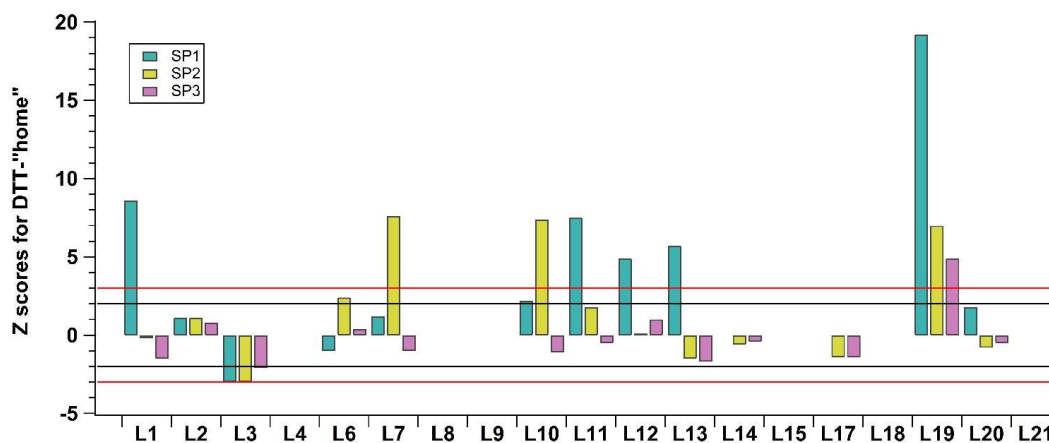
565 **Figure 6. Associations (beta in $\text{nmol min}^{-1} \mu\text{g}^{-1}$) between OP DTT values obtained for SP1, SP2 and SP3**
 566 **and the different parameters of the ILC, including the DTT protocol, the instrument used and the**
 567 **delivery and analysis time obtained by applying a multiple linear model. Full-colour dots represent the**
 568 **results with p-values ≤ 0.05 , and white dots represent the results with p-values > 0.05 .**

569



570 Finally, to assess the performance of the participants in the DTT-"home" protocols, a comparable approach to
571 the simplified RI_URBANS SOP was employed for those participants who supplied OP results. The z-scores were
572 computed using the assigned values of each sample (SP1-SP3), obtained with RI-URBANS SOP application.
573 Figure 7 illustrates the z-scores of the OP results obtained through the application of the DTT-"home" protocols,
574 revealing a significant variation in the outcomes. Only five participants managed to produce satisfactory results
575 for all the tested samples. Despite the fact that the COV of the participants using DTT-"home" protocols showed
576 an improvement over the results of the simplified DTT SOP (Figure 5), the outcomes are still distant from the
577 consensus values of the samples obtained in this exercise. The results indicate a high degree of variability in
578 the OP activity using "home" OP methodologies, underscoring the pressing requirement for standardized
579 methods and harmonised protocols to ensure more reliable OP research.

580



581

582 **Figure 7. Z-scores were calculated for the DTT-"home" protocol results to evaluate each participant's**
583 **performance in reference to the RI_URBANS assigned (consensus) values, for each sample tested. Black**
584 **and red horizontal lines indicate boundaries for triggering an action signal as described in section 2.7.1.**

585

586 4. Strengths and limitations of this first intercomparison

587 The greatest strength of this ILC was the high number of participants (20) enhancing the comprehensiveness
588 and diversity of the study and allowing for a broader range of perspectives and expertise. This also allowed
589 comprehensive collaborative discussions during the preparation phase, promoting knowledge exchange and
590 consensus-building, and contributing to a more robust ILC design. These all show a willingness from the groups



591 to be actively part of the development of the intercomparison and to pursue a harmonisation on the OP
592 measurements.

593 The development of the first simplified OP DTT protocol (available in SI-1) also consolidates the experimental
594 experience of the participants, fostering methodological consistency across different research groups as a first
595 step toward method harmonisation. Finally, the collaboration with the JRC, an independent organisation for the
596 assessment of results, adds credibility and objectivity to the study, ensuring that findings are impartially
597 evaluated.

598 The sharing of liquid samples in this comparison comes with both advantages and limitations. On one hand, it
599 eliminates biases associated with extraction methods and solvent purity. However, some samples exhibited
600 signs of ageing during the interlaboratory comparison duration (though this was not identified as a critical
601 parameter when identifying the main causes of variability). In addition, this approach introduced certain
602 challenges with some of the “home” OP protocols which were designed originally to be used with solid samples.
603 Finally, all liquid extracts should be provided with a similar PM concentration to limit known nonlinearities and
604 to avoid potential saturation issues, as can be the case of LWCC instruments. Next, ICL should, in the future,
605 include the whole chain assessment, including the extraction step.

606 The testing of three samples with different patterns makes it difficult to draw unilateral conclusions. A larger
607 sample size could support a more robust statistical analysis of the results, particularly for the factors
608 determining OP variability. Moreover, the inclusion of samples that are readily accessible worldwide, such as
609 standard reference materials, could facilitate the future adaptation of a simplified OP DTT protocol while
610 allowing comparison with an assigned (consensus) value.

611 The sole focus on the DTT method for the ILC could limit the broader evaluation of OP. The addition of other
612 OP assays, such as those included in previous inter-comparison studies (Ayres et al., 2008; Calas et al., 2018;
613 Shahpoury et al., 2022) could provide a more comprehensive understanding of the performances of the
614 different groups involved in the OP domain of research. There are contrasting reports about the relative
615 sensitivity of DTT assay to various organic and inorganic PM components, with some studies showing higher
616 reactivity towards the organic fraction. Therefore, additional consensus studies would be needed to assess this
617 aspect and the comparability of DTT to other OP metrics that rely on proper lung anti-oxidants and could be
618 considered more physiologically relevant. Such studies could support the identification of chemical species
619 which should be prioritized for future air quality management programs.

620 In conclusion, while this ILC of OP has highlighted considerable variability in the performance of the assay
621 between groups, it has notable strengths and provides a starting point towards the harmonisation of OP
622 measurements.

623

624 **5. Recommendations for standardisation of OP protocols**



625 Based on the findings of this ILC and also on general literature about OP, some recommendations for further
626 standardising OP DTT measurements are proposed (Table 1). These include guidelines for sample and
627 laboratory conditions, instrument type and calibration and the reporting of results. Additionally, a reference
628 material (1,4-naphthoquinone, copper or other solution at a known concentration) should be proposed to
629 facilitate future ILCs. Additionally, since some differences were observed in the results obtained from the use
630 of the simplified SOP compared with the “home-developed” DTT protocols, harmonisation of procedures is
631 needed to ensure data comparability. We describe below the crucial parameters that need to be considered in
632 the move toward greater harmonisation of OP methodologies.

633 *OP assay selection*

634 • To date, it remains unclear which oxidative potential (OP) assay is most effective at predicting health
635 outcomes related to oxidative stress. This uncertainty arises because different assays yield markedly different
636 results for the same particulate matter (PM) samples. Additionally, even OP values from the same assay are
637 often linked to various PM components and sources, depending on the different studies (He and Zhang, 2022).
638 Thus, based on current knowledge and epidemiological evidence, two complementary OP assays (a thiol-
639 based probe (OPDTT or OPGSH) and another one (among OPOH, OPAA, or else)) are recommended to provide
640 a better picture of the potential oxidising damages from PM compounds and to strengthen the power of
641 epidemiological studies. These aspects were previously discussed in a recent work integrating five different
642 OP assays (Dominutti et al., 2023). Finally, the final choice of the best OP test (or combination) must be based
643 on epidemiological evidence, which has begun only recently and needs more hindsight to be determined.

644 *Sampling*

645 • OP can be analysed in filter samples conventionally collected for air quality monitoring using small
646 portions of these if adequately preserved (frozen). Pre-burn quartz filters or Teflon filters are appropriate
647 and blank filters must be measured to remove the background induced by the matrix of the material. A
648 previous study had shown no differences in the OPDTT values observed using Quartz or Teflon filters
649 (Frezza et al., 2022). However, it should be further evaluated when other OP assays are considered.

650 *Sample storage*

651 • Previous studies that evaluated the effect of storage time and conditions did not show a substantial effect
652 on the OP DTT results (Frezza et al., 2022). However, we recommend that PM samples should be
653 immediately transported to the lab after sampling. The filters must be kept cold after sampling (at 4°C if the
654 OP analysis is done within a few days after collection or -20°C if the analysis is delayed).

655 • The lifetime of the ROS may be very short, and measurements of OP on PM-extracted filters are likely
656 affected both by the age of the samples, how they have been sampled and stored, and the nature of the
657 extraction methodology. How all of these processes impact on the ageing of samples and the ultimate



658 quantification of OP needs to be addressed. Ageing studies should be performed for each OP assay in the long
659 term to define the maximum storage time of aerosol filters at low vs ambient temperature conditions.
660 Some OP components might be so short-lived that only online techniques are warranted.

661 *Laboratory conditions*

- 662 • OP assays are “trace” detection assays that require clean ambient conditions and high-quality reagents
663 free of metal contamination. Considerations should be given to the use of certified clean rooms or proper
664 laminar flow bench stations to prevent contamination of the samples.
- 665 • Use of clean material: vials, cones, and spatulas have to be washed before use (5% HNO₃ bath to remove
666 metals and rinse three times in ultra-pure water before drying in laminar flow).
- 667 • Control laboratory temperatures and light exposure by using dark polypropylene tubes at least for
668 reactants

669 *Extraction step*

- 670 • The extraction step may be highly variable according to the procedures used, and several parameters are
671 known to impact OP results, such as the choice of the solvent, the concentration of buffer, the way of agitation,
672 and the quantification of the final extracted mass. Notably, the ultrasonication of PM samples in aqueous
673 solutions generates ROS (Miljevic et al., 2014) and it could introduce artefacts in OP measurement. This effect
674 was also observed in the work of Frezzini et al. (2022), where different extraction methods were evaluated,
675 with ultrasonic baths overestimating the results observed.
- 676 The effect of the solvent used was not evaluated in this ILC exercise. However, we recommend the use of
677 ultrapure water or simulated lining fluid for the sample extraction. Future ILC exercises should include the
678 evaluation on the extraction conditions, including solvent use and methods.

679 *Reaction step*

- 680 • Several aspects in the reaction process affect the OP value, like the initial concentration of reactants (since
681 the DTT test is mass-dependant (Charrier et al., 2016)), ratio of reactant/sample, time of reaction (some
682 compounds present a non-linear reaction over time), the temperature of the reaction (which should be
683 standardised to 37°C), agitation (mixing samples) and the type of measurements (kinetic or end-point value),
684 etc.
- 685 • Current literature mainly addresses extraction or reaction parameters separately. We advise that the
686 whole chain factors should be evaluated together to quantify their relative impact on the results.

687 *Development of a reference material with a certified “OP value.”*

688 The setup of reference material or in-house standard solutions (in collaboration with reference institutions
689 JRC or NIST, for instance) with a known OP value could help laboratories test and train themselves on the OP
690 protocol before testing the unknown ILC samples. This is something to be developed and tested in future ILCs.



691 *Instrument calibration*

- 692 • Investigate the optimal frequency for the calibration of spectrophotometers for such assays.

693 *Report of results/units*

694 The calculation of OP DTT activity during this ILC involved a conversion using a calibration curve. Since the
695 OP activity measures the rate of a chemical reaction and not a concentration, for future comparison exercises,
696 the possibility of exploring alternative methods for OP calculation should be tested. To date, results are mass
697 normalised in nmolAnti-oxidant min⁻¹ µg⁻¹, or volume normalised in nmolAnti-oxidant min⁻¹ m⁻³. The OP per
698 µg refers to the reactivity of one µg of the tested PM, whereas the OP per m³ refers to the exposure of one m³
699 of inhaled air.

700 Table1. Summary of recommendations for future OP measurements on filters

Condition or step	Recommendations
OP assay selection	<ul style="list-style-type: none">• Two complementary OP assays (a thiol-based probe (OPDTT or OPGSH) and another one (among OPOH, OPAA, or else)) are recommended to provide a better picture of the potential oxidising damages from PM compounds
Sampling	<ul style="list-style-type: none">• Pre-burn quartz filters or Teflon filters are appropriate and blank filters must be measured to remove the background induced by the matrix of the material.
Samples storage	<ul style="list-style-type: none">• The PM filters must be kept cold after sampling (at 4°C if the OP analysis is done within a few days after collection or -18°C or -20°C if the analysis is delayed).
Laboratory conditions	<ul style="list-style-type: none">• Clean conditions (including certified clean rooms or proper laminar flow bench stations)• High-quality reagents free of metal contamination• Use of clean material, which must be washed before use (5% HNO₃ bath to remove metals, rinse three times in ultra-pure water, and dry in laminar flow bench stations).• Control laboratory temperatures and light exposure by using dark polypropylene tubes
Reaction step	<ul style="list-style-type: none">• Several aspects in the reaction process affect the OP value, and to minimise their impact, standard conditions should be fixed as the initial concentration of reactants, ratio of reactant/sample, time of reaction, the temperature of the reaction (37°C), agitation (mixing samples) and the type of measurements (kinetic or end-point value), etc.
Instrument calibration	<ul style="list-style-type: none">• Investigate the optimal frequency for the calibration of spectrophotometers for such assays.

701

702 6. Conclusions

703 This study represents an innovative effort as the first interlaboratory OP exercise specifically aimed at
704 harmonising this OP assay. This exercise provides the very first roadmap for refining interlaboratory



705 comparisons of OP, fostering greater confidence in the reliability of OP data and encouraging the scientific
706 community to advance towards global OP harmonisation.

707 This first exercise focused on OP DTT, as it is widely used within the scientific community and has already
708 shown positive associations with health outcomes (Bates et al., 2015; Borlaza et al., 2022; Dabass et al., 2018;
709 Donaldson et al., 2001; Gao et al., 2020; Marsal et al., 2023; Weichenthal et al., 2016b, a, c). Even if there are
710 several crucial points to be evaluated and harmonised in the whole chain of the determination of OP (sampling
711 methods, sample storage, extraction conditions and methods) as well as the use of different OP assays, this first
712 ILC engaging several research laboratories pay the way for future developments towards the standardisation
713 of OP methods. Despite the need to evaluate and harmonize several crucial aspects throughout the entire
714 process of OP measurements (such as sampling methods, sample storage, extraction conditions and methods,
715 and the use of different OP assays), this initial ILC, which involves multiple research laboratories, paves the way
716 for future advancements in the standardization of OP methods.

717 Our findings emphasise both the strengths and challenges associated with the use of the current OP DTT assay
718 for driving a measurement of PM OP. Overall, half of the participants achieved results falling within a
719 satisfactory range of z-scores for this test. The participating group performance levels are comparable to those
720 observed in initial ILCs for PAHs in the 2010s (Grandesso et al., 2012; Verlhac et al., 2014). While notable
721 agreement was observed in certain samples and between several groups, discrepancies and variability were
722 also identified, emphasizing the need for harmonisation in the procedures and conditions. A number of factors
723 may contribute to the underperformance observed in certain samples and participants. The main reasons are
724 not clear, but the analysis conditions in the participating laboratories and the lack of experience in this type of
725 metrological exercise are possible causes. Standardisation of protocols and harmonisation of procedures
726 emerged as critical components to ensure the accuracy and comparability of OP data across laboratories. This
727 collaborative approach fosters a more robust OP science, facilitates data exchange and integration, and will
728 ultimately contribute to a better understanding of the health impacts associated with PM exposure, allowing
729 for more accurate exposure assessments and regulatory decisions.

730

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752 **Data availability**

753 All OP data is published in the SI.

754 **Author contributions**

755 GU designed the study and supervised the OP tests for the development of the simplified DTT RI-URBANS
756 protocol. CR, TM, RE and PAD tested the different steps and prepared the samples and logistics of the
757 intercomparison exercise. GH, R.M.H, A.N, I. M., K. B, N.M and G.U. evaluated and decided the samples to be
758 compared and the protocol content. JPP and FC performed the data analysis and evaluated the performance of
759 each group. PAD and AM developed the multiple regression model. PAD processed the data of the study and
760 wrote the paper together with JLJ and GU before a first review by the RI-Urbans members. All authors
761 participated in the interlaboratory comparison. All authors reviewed and edited the manuscript.

762 **Competing interests**

763 At least one of the (co)-authors is a member of the editorial board of Atmospheric Measurement Techniques.

764

765 **7. References**

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