Harmonizing measurements of oxidative potential of PM: an interlaboratory comparison of the ascorbic acid assay

Supplementary Material

Harmonizing measurements of oxidative potential of PM: an interlaboratory comparison of the ascorbic acid assay

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1. Overview of tests performed for designing the SOP

1.1. Extraction fluid and AA preparation solvent

Several extraction fluid-AA preparation solvent pairs were tested with three samples: one blank, copper sulfate (0.22 µM in the well), and an ambient filter (11 µg/mL in the well). The extraction was tested in ultra-pure water (UP water, 18.2 MΩ cm, TOC<5ppb), potassium phosphate buffer (K-PO_4: KH_2PO_4, K_2HPO_4, 0.1M), Chelex-treated K-PO_4 buffer, and Gamble + 1,2-DPPC. AA was prepared in ultra-pure water (18.2 MΩ cm, TOC<5ppb), K-PO_4 buffer, and Chelex-treated K-PO_4 buffer. Criteria for selecting the extraction fluid-preparation solvent pair were the minimization of the variability and the limitation of time-consuming or error-prone steps. Gamble+DPPC extraction solution was only tested for comparison matter with the organizer's usual protocol, but was excluded from the possible matrices given the complexity of preparation and the potential errors it can induce.

Figure S1 shows that the UP-water/UP-water pair cannot be considered because AA consumption is very low for both samples. The highest values are obtained for the K-PO_4 (extraction)/UP-water (AA) pair, with a very low variability in the results. This pair was therefore selected for the SOP.

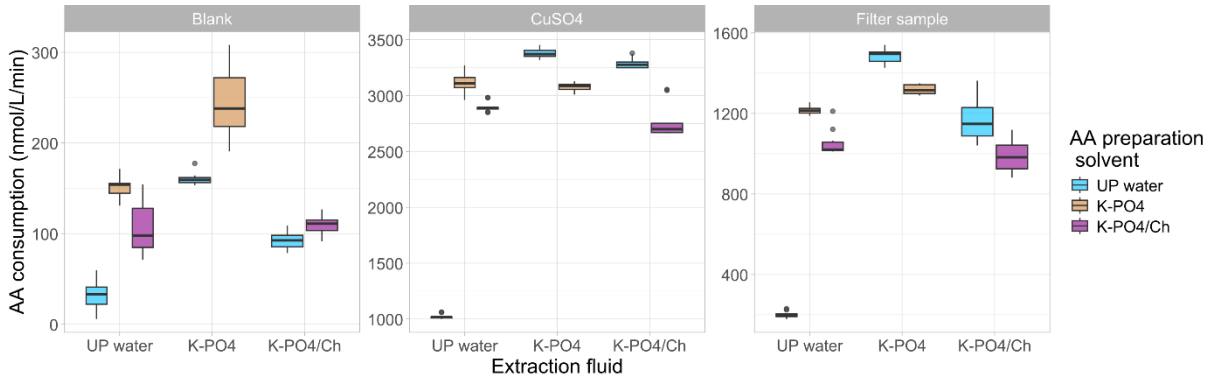

Figure S1. AA consumption slopes for each extraction fluid/AA preparation solvent pair.

1.2. Impact of [AA]/[PM] ratios in the reaction medium

Participants reported [AA]/[PM] ratios ranging from 2 to 60, with some participants working at iso-concentrations (fixing PM concentrations in the wells), while others worked at iso-surface (fixing the filter portion to extract). This parameter was tested by modifying the initial concentration of PM in the well, diluting a 150 µg/mL extract to reach final concentrations in the wells ranging from 2.2 to 66.5 µg/mL and fixing [AA] concentration to 133 µM. Results showed a linear relationship for PM concentrations in the well that flattened for PM concentrations in the well above 20 µg/mL, supporting that extract concentrations should be fixed within this interval.

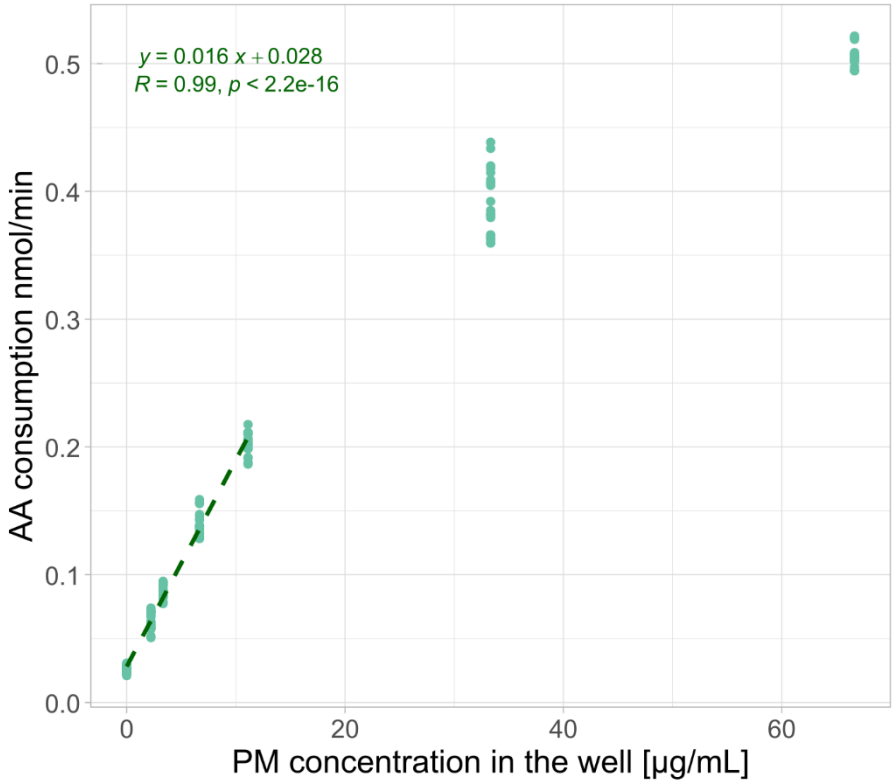

Figure S2. Effect of PM concentration in the reaction medium on AA consumption slope, with fixed initial concentration of AA of 133µM.

1.3. Impact of extract filtration

The impact of the extract filtration prior to their introduction in the well was tested at different PM concentrations (Figure S3). There was no difference in the impact of the filtration step depending on the concentration, with slightly higher AA consumption slopes and lower variability in the results for unfiltered extracts compared to filtered extracts. Not filtering the extracts could lead to more variability in the framework of an ILC, depending on lab workers' difficulty to pipet the samples, however it has to be balanced with the potential contamination that can be induced by the filtration step.

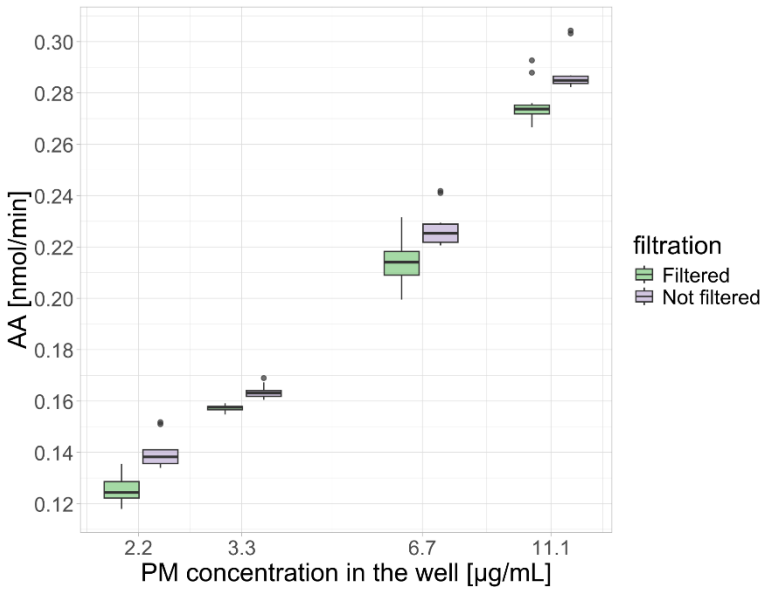

Figure S3. Impact of the extract filtration on AA consumption slope at different PM concentrations.

1.4. pH-dependence of AA's λ_{max}

The pH-dependence of AA's maximum absorption wavelength (λ_{max}) was tested at different AA concentrations, diluting the solutions in UP-water and in K-PO_4. Results showed a shift in λ_{max} when AA was not buffered, caused by the coexistence of both AA and AH^- with pH modifications (pKa_1 = 4.1, pKa_2 = 11.8), therefore impacting the slope of the calibration curve (Figure S4). It was therefore recommended to perform the calibration curve by diluting AA in K-PO_4, with the final volume of each calibration point equal to the total reaction volume for samples.

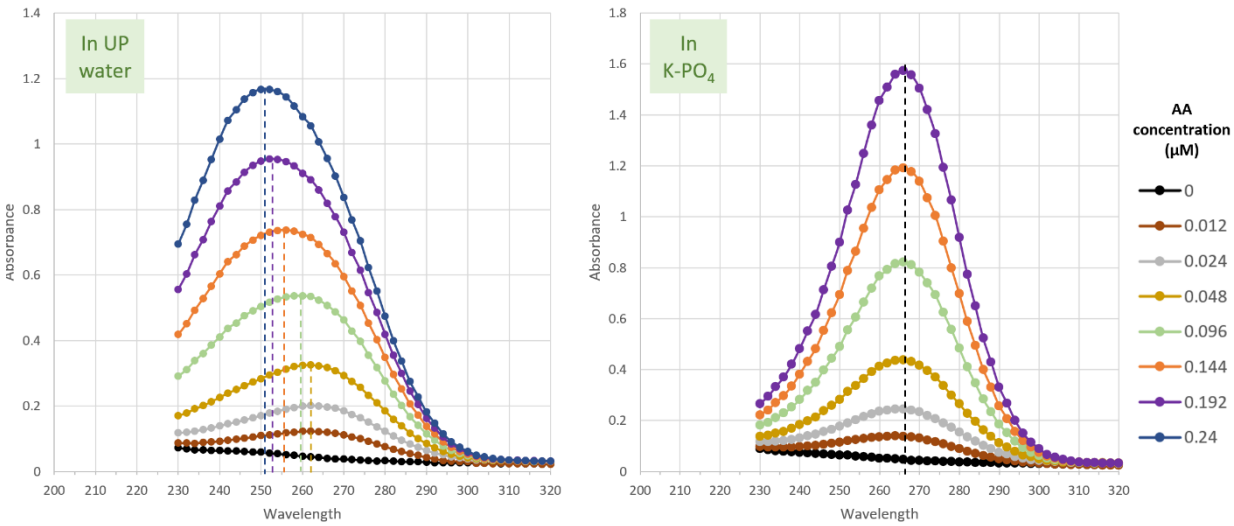

Figure S4. Comparison of AA's maximum absorption wavelength at different concentrations when diluted in ultra-pure water (left) or in potassium phosphate buffer (right).

2. Figures

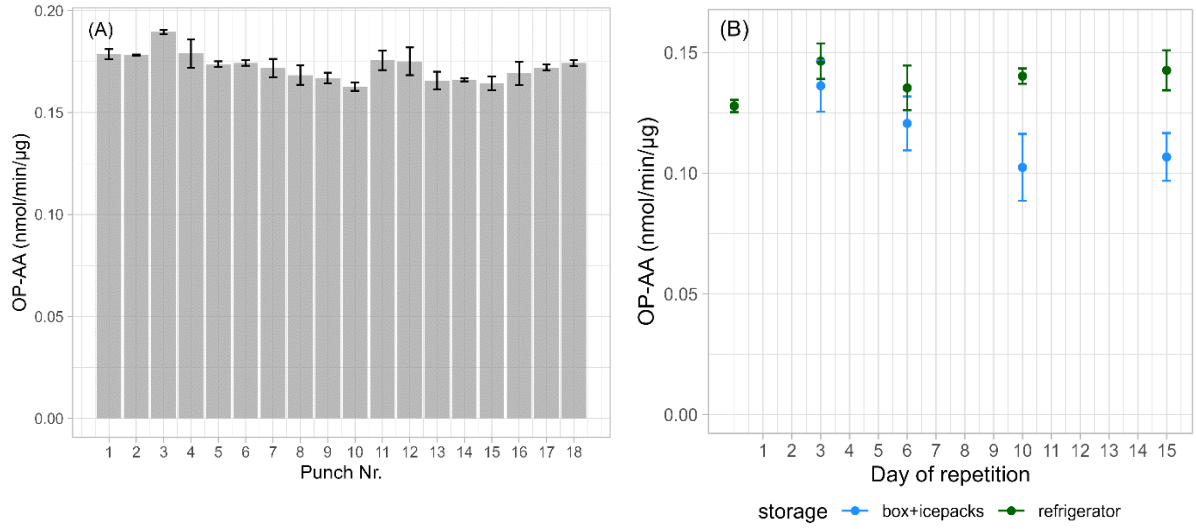

Figure S5. Results of the homogeneity (A) and stability (B) tests

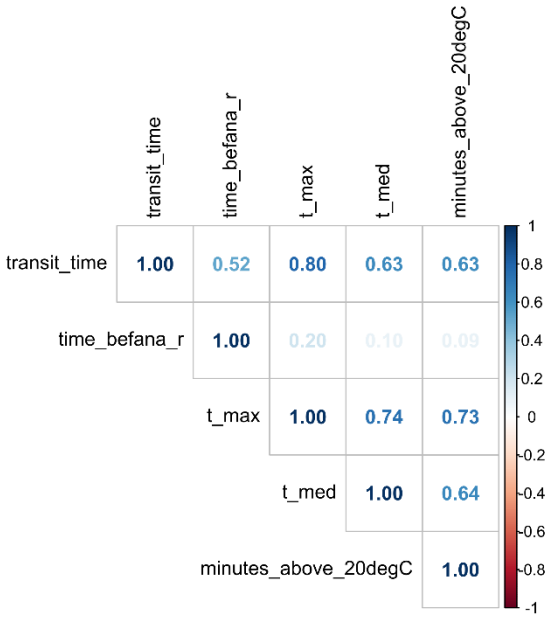

Figure S6. Correlation coefficients between technical variables and OP

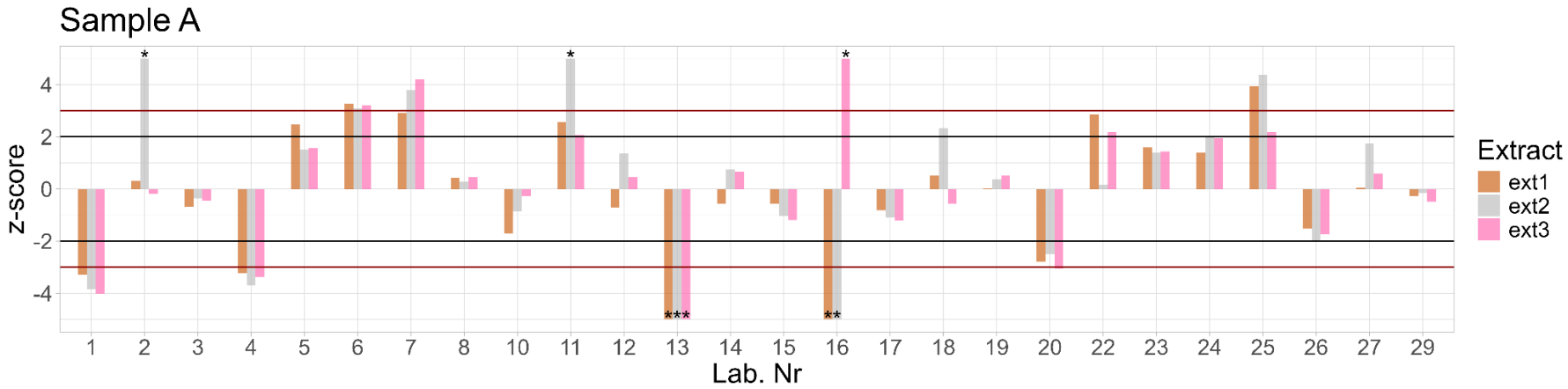

Figure S7. Evaluation of participants' performance in the interlaboratory comparison, with representation of z-score per extract in sample A. Results were cropped for $|z| \leq 5$, and stars represent bars that exceed this value.

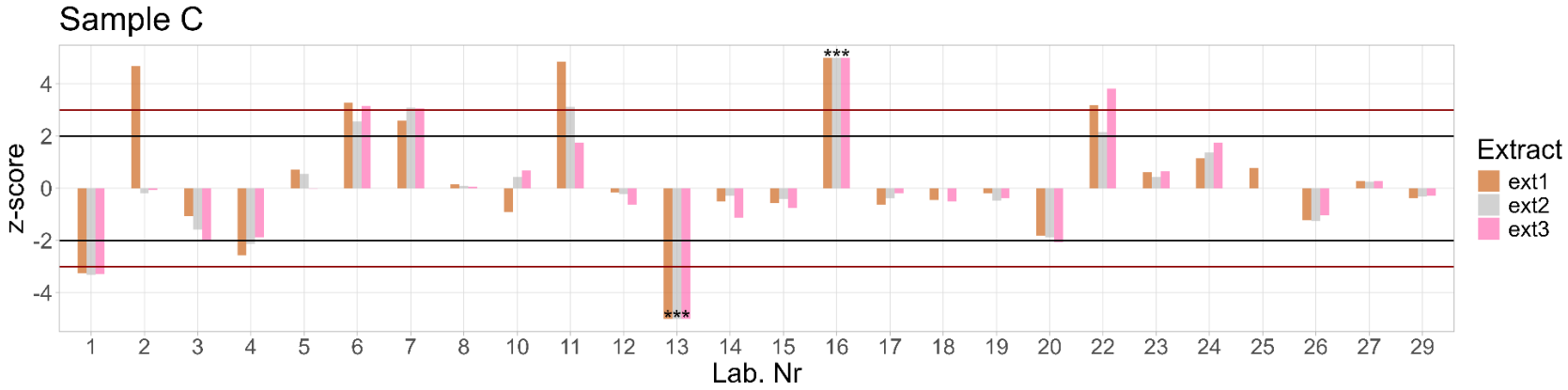

Figure S8. Evaluation of participants' performance in the interlaboratory comparison, with representation of z-score per extract in sample C. Results were cropped for $|z| \leq 5$, and stars represent bars that exceed this value.

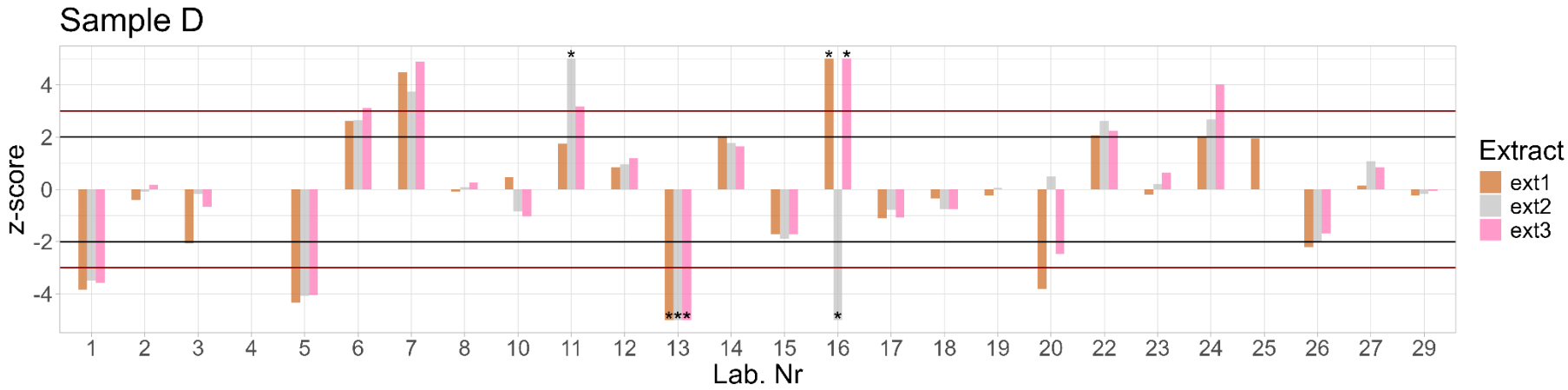

Figure S9. Evaluation of participants' performance in the interlaboratory comparison, with representation of z-score per extract in sample D. Results were cropped for $|z| \leq 5$, and stars represent bars that exceed this value.

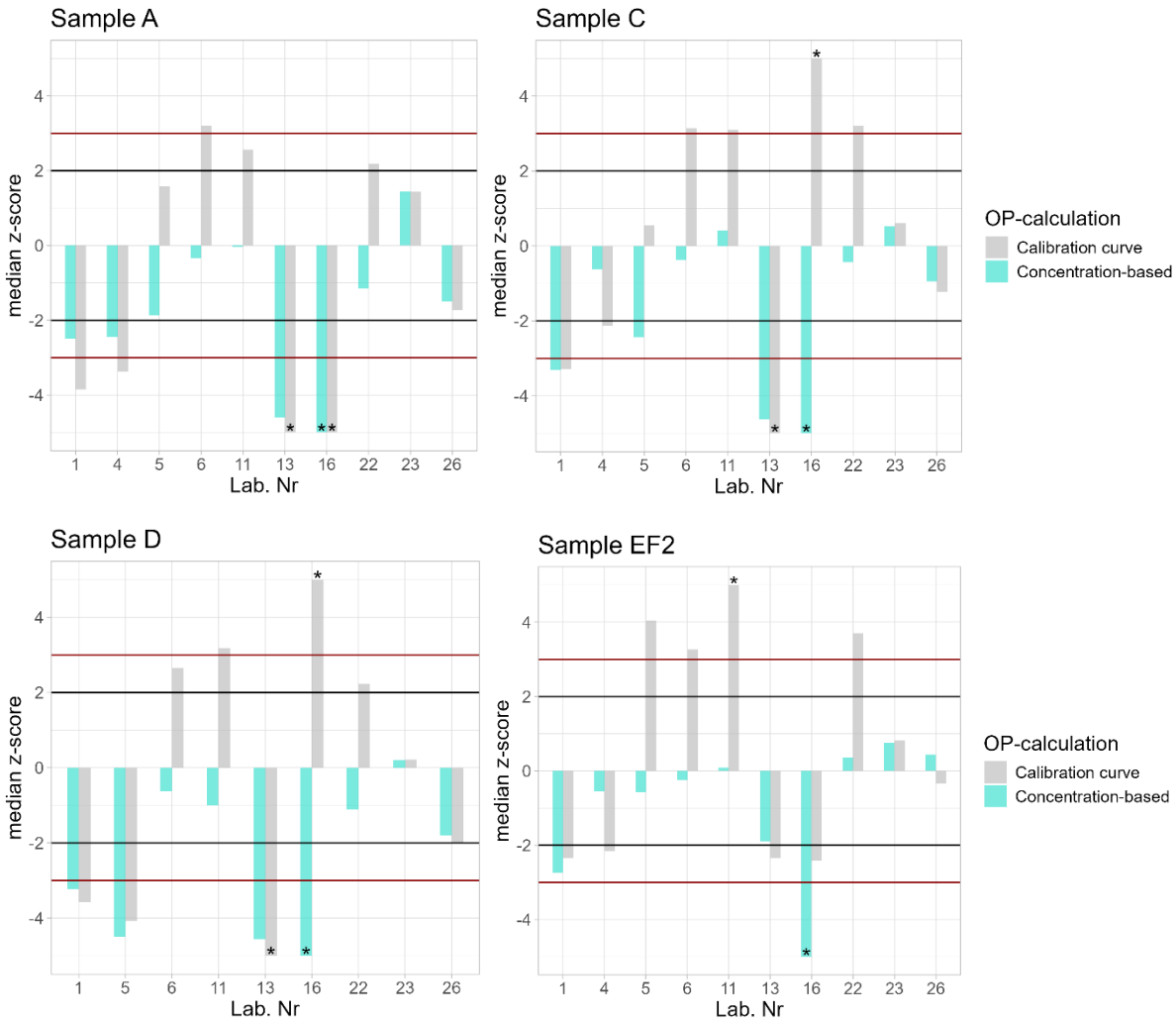

Figure S10. Comparison of participants' performances depending on OP calculation (i.e. using the concentration-based vs. the calibration curve) for participants outside the $[AA]_{th}$ range (i.e. $[AA]_0$ < 119.7 µM or $[AA]_0$ > 146.3 µM)

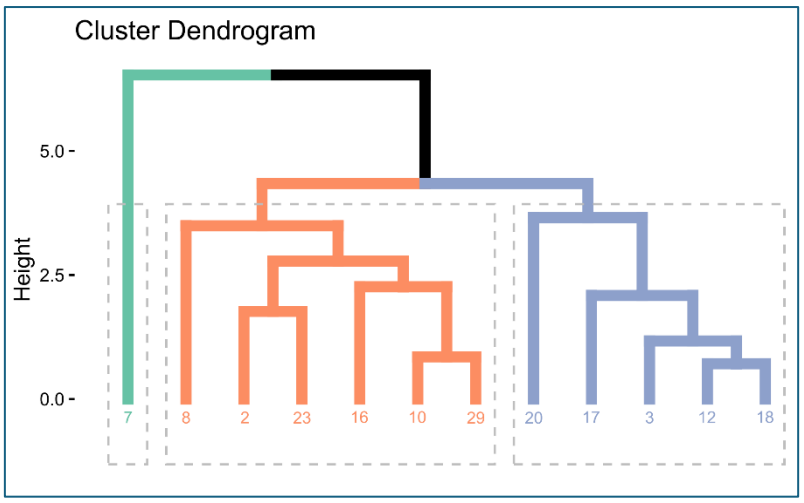

Figure S11. Clustering analysis on the difference between home and SOP results within each laboratory.

3. Tables

Table S1. Summary statistics over all laboratories of the percentage deviations by sample for all extracts (based on the average of replicates per extract).

Sample	Minimum	Mean	Median	Maximum
A	-119.0	5.06	6.09	203.0
B	-1.52	0.25	0.00	3.91
C	-116.0	-1.98	-4.49	97.1
D	-118.0	-1.25	-0.47	114.0
EF2	-46.9	10.70	3.15	146.0

Note: Exclusion of laboratory 16 for statistics calculations.

Table S2. Summary statistics over all laboratories of the z-scores by sample for all extracts (based on the average of replicates per extract).

Sample	Minimum	Mean	Median	Maximum
A	-5.96	0.23	0.29	10.20
C	-5.80	-0.08	-0.20	4.85
D	-5.92	-0.09	-0.06	5.70
EF2	-2.34	0.53	0.16	7.31

Note: Exclusion of laboratory 16 for statistics calculations.

Sample	Minimum	Mean	Median	Maximum
A	-25.7	0.04	0.22	20.3
C	-5.80	0.51	-0.19	25.1
D	-18.8	0.14	-0.06	26.7
EF2	-2.41	0.42	0.05	7.31

Note: with all laboratories

Table S3. Contingency table of z-scores and participation to the OP-DTT ILC1.

Characteristic	N	Did not participate to the OP-DTT ILC1 (n=10)	Participated to the OP-DTT ILC1 (n=16)	*p*-value[1]
Sample A	**26**			**>0.9**
Satisfactory	**16**	**6 (60%)**	**10 (63%)**	
Not satisfactory	**10**	**4 (40%)**	**6 (38%)**	
Warning signal		1 (10%)	2 (13%)	
Action signal		3 (30%)	4 (25%)	
Sample C	**26**			**0.6**
Satisfactory	**15**	**4 (40%)**	**11 (69%)**	
Not satisfactory	**11**	**6 (60%)**	**5 (31%)**	
Warning signal		1	0	
Action signal		2	5	
Sample D	**26**			**0.5**
Satisfactory	**14**	**6 (67%)**	**8 (50%)**	
Not satisfactory	**12**	**4 (40%)**	**8 (50%)**	
Warning signal		2	3	
Action signal		1	5	
Missing		1	0	
Sample EF2	**26**			**0.7**
Satisfactory	**18**	**8 (80%)**	**10 (63%)**	
Not satisfactory	**8**	**2 (20%)**	**6 (38%)**	
Warning signal		1	3	
Action signal		1	3	

[1]*Fisher's exact test for the difference between counts of satisfactory and not satisfactory z-scores in participants who participated to the ILC1 vs. "new" participants.*

Table S4. Description of the physical and analytical parameters evaluated in the multiple regression models.

Characteristics	**N (%)**		
Device			
Cuvette	10 (38%)		
Plater-reader	16 (62%)		
Consumables washed with HNO_3			
Yes	21 (81%)		
No	5 (19%)		
Duration above 20°C (minutes)			
0	19 (76%)		
10	2 (8.0%)		
340	1 (4.0%)		
≥ 680	3		
Unknown	1		
	Minimum	**Median (Q1, Q3)**	**Maximum**
Transit time (days)	0.00	1.60 (0.90, 2.90)	10.60
Time before analysis (days)	2	19 (9, 28)	48
Maximum temperature reached during the transit (°C)	1.6	14.3 (10.0, 19.9)	27.7
Unknown	1	1	1
Median temperature reached during the transit (°C)	-1.3	5.4 (3.1, 8.4)	19.4
Unknown	1	1	1
Average $[AA]_0$ (µM)	-421	130 (123, 137)	12 511
Average slope of the negative control (nM/min)	-3 177	490 (190, 778)	4 058

4. Simplified AA Protocol "Evaluation of acellular oxidative potential of particles by ascorbic acid assay"

Authors: A. Marsal, P. A. Dominutti, S. Houdier, J.L. Jaffrezo, S. Darfeuil, G. Uzu

Object: This is the simplified protocol proposed for the intercomparison of the measurement of oxidative potential of particulate matter by the ascorbic acid assay, supported by a joint ACTRIS-RI-Urbans initiative. **To be included in the intercomparison, you must perform this simplified protocol.** You can optionally perform your AA-home protocols and the simplified OP-DTT protocol from the RI-Urbans first intercomparison.

General recommandations

- Immediately after receiving the package, cold-store its content (fridge or freezer). Turn off the temperature logger in the package and follow the procedure provided in the package to send us the data (xlsx and pdf formats) concerning the shipment.
- To remove any potential contamination, you can wash your lab supplies (tubes, pipette tips etc., except microplates) for 24hrs in a 5% HNO_3 bath, rinse them 3 times with ultrapure water and let them dry in a clean environment (clean room, clean bench, or the most appropriate spot in a lab).
- Water should be of ultra-pure quality (18.2 MΩ cm, TOC<5ppb), if you have any doubt regarding your water quality, we recommend purchasing HPLC grade water.
- If you do not regularly control your pipettes, use a precision balance and report all digits in the Excel results file.
- If it is the first time that you work in UV light with the plates/cuvettes that you are planning to use, make sure that they are sensitive enough (for example by performing an absorbance spectrum).

Material

1. **Provided**

- Ascorbic acid, powder, CAS [50-81-7] in a 15 mL black conical centrifuge tube
- K_2HPO_4, powder, CAS [7758-11-4] in a 50 mL transparent conical centrifuge tube
- KH_2PO4, powder, CAS [7778-77-0] in a 50 mL transparent conical centrifuge tube
- $CuSO_4$, $5H_20$, powder, CAS [7758-99-8] in a 15 mL black conical centrifuge tube
- 4 Petri dishes with filter punches

2. **Lab material needed**

- UP water (18.2 MΩ cm, TOC<5ppb, or HPLC grade)
- pH-meter
- 50 mL black conical tube or amber glass vial
- 15 mL black conical tube or amber glass vial
- 15 mL transparent extraction tubes or transparent glass vial protected with aluminium foil
- Microplates or cuvette that can work in the UV domain
- 1-L volumetric flask
- Iced bath or beaker with ice cubes
- Stopwatch

Timeline

We recommend following the timeline presented in Figure 1. More details on each step are available in the sections below.

	D-1	day of analysis				
K-PO_4 preparation						
Pre-weigh AA powder						
Pre-weigh EM1 powder						
Prepare and label the tubes						
Introduce filter punches in the tubes						
Prepare the program of analysis of the plate reader, optionally check that it works as planned						
Preparation of EM1+EF1+EF2						
Introduce K-PO4 buffer in the extraction tubes						
Start extraction						
Preparation of AA 6mM and 240 µM						
Plate preparation						
Analysis start						

Figure 1. Example of a timeline that can be followed.

Reactant preparation

1. **Potassium phosphate buffer (0.1M)**

Weigh 13.41 g of dipotassium phosphate (K_2HPO_4, CAS [7758-11-4]) and 3.13 g of potassium dihydrogen phosphate (KH_2PO4, CAS [7778-77-0]) and mix them in a volumetric flask of 1000 mL with ultra-pure water (18.2 MΩ cm^{-1}, total organic carbon (TOC) < 5 ppb). Make sure that the powder is fully dissolved. Check the pH using a pH meter, reading must be equal to 7.4 ± 0.1. Buffer preparation can be done the day before the 1st extraction.

2. **Ascorbic acid mother solution (6 mM)**

On the day of analysis, weigh 10.57 mg of the provided AA powder in a 15mL black conical tube or amber glass vial, and add 10.00 mL of UP water. Report the exact mass in the Excel spreadsheet.

3. **Ascorbic acid daughter (240 µM) solutions**

On the day of analysis, prepare the 240µM solution by diluting 2.00 mL of the 6 mM mother solution and completing to a final volume of 50.00 mL with UP water in a 50mL black conical tube or amber glass vial.

Sample extraction

Turn on the temperature of the incubation chamber at 37°C so that it is at the correct temperature when needed.

If the K-PO4 buffer was not prepared on the same day, check that its pH reads 7.4 ± 0.1 using a pH-meter and report the exact value in the Excel sheet. If it does not, prepare a new buffer.

Cuvettes: If your spectrometer cannot process several cuvettes at a time, we recommend limiting each kinetic measurement to 1 negative control + 1 extraction of 1 sample at a time, for example:

- Start extraction 1 with only sample A1. When extraction 1 is over, directly start extraction 2 with only sample A2, and process sample A1 during the second extraction.

This would mean performing 15 different extractions, that could be split in 1 working week.

1. Chemical reference solutions

EM1: On the day of analysis weigh exactly about 13 mg of the sample E (powder in black tube) in a vial and add 20 mL of **UP water**. Use the excel sheet "Sample E preparation" to determine the exact volume of UP water to add, depending on the mass of powder introduced.

EF1: Prepare the **EF1** solution in UP water, by introducing 20 µL of the mother solution in 19.98 mL of **UP water** in a transparent glass vial (exact volumes based on the "Sample E preparation" spreadsheet).

EF2: Prepare the **EF2** solution in K-PO_4 buffer, by introducing 2.00 mL of the daughter 1 solution in 8.00 mL of **K-PO_4 buffer** in a transparent glass vial (exact volumes based on the "Sample E preparation" spreadsheet).

2. Filters

Note: If you perform the extractions one by one, introduce the K-PO_4 buffer right before starting the extraction.

Each filter extraction will be performed in triplicate in K-PO_4 buffer. For each of the 4 filter samples to be analysed, prepare 3 transparent tubes covered with aluminium foil, using the labelling indicated in Table 1.

Table 1. Tube labelling.

Sample A			Sample B			Sample C			Sample D		
A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3

Follow Table 2 and put the corresponding number of filter punches in each tube, depending on the sample type and spectrometer type. Then, add in each tube the volume of K-PO_4 buffer corresponding to each sample type, as indicated in Table 2. For example, in the tube A1, introduce 1 punch of sample A and add 2.59 mL of K-PO_4 buffer if you have a plate-reader, or 2 punches and 5.18 mL of K-PO_4 buffer if you are using cuvettes.

Table 2. Details of number of punches and volume of buffer to introduce in the extraction tubes.

Sample	Corresponding tubes	Plate-reader users		Cuvette users	
		Number of punches to introduce in each extraction tube	Volume of K-PO_4 to add in the extraction tube	Number of punches to introduce in each extraction tube	Volume of K-PO_4 to add in the extraction tube
A (A1-A3) 25 µg/mL	A1-A2-A3	1	2.59 mL	2	5.18 mL
B (B1-B3) blank	B1-B2-B3	1	2.00 mL	2	5.00 mL
C (C1-C3) 10 µg/mL	C1-C2-C3	1	2.19 mL	3	6.58 mL
D (D1-D3) 25 µg/mL	D1-D2-D3	2	2.59 mL	4	5.19 mL

3. Extraction

Depending on the organisation that you decided to follow, perform the extraction by placing the tubes or the EF2 solution that you will extract, along with an aliquot of the K-PO_4 buffer on a multitube vortex mixer in the incubation chamber at 37°C. Turn it on for 75min, at maximum speed (details need to be reported in the Excel sheet).

Plate and plate-reader preparation / using automatic injectors

Set the temperature of the plate reader at 37°C. Since samples are not filtered, we recommend being cautious when pipetting the filter samples, making thorough visual verification that no big portion of the filter is introduced into the well.

1. In the wells A1-A6, prepare a **6-point standard curve** of AA by diluting the 240 µM daughter solution **in K-PO_4 buffer**, directly in the wells (final volume = 180 µL). We recommend performing the 6-point calibration curve on the 0-200 µM range (avoid using the 240 µM solution in your interval). **Directly perform the absorbance measurements of the standard curve** (you can also do it on a separate plate if you prefer).
2. Place 80 µL of each sample, following the configuration below (Table 3). In the C- wells (negative control - **A7, A8, A9**) introduce 80 µL of K-PO_4 buffer.
3. Place the plate into the reader.
4. Place the AA 240 µM solution at one injector, keeping it in an iced bath during the entire procedure. Prime the injector with at least 1000 µL of solution.
5. Shake the plate for 30 seconds using the reader.
6. Wait for 20 seconds.
7. Read the matrix absorbance at 265 nm in wells **A7-A12** and **B1-D12**.
8. Program one injector to dispense 100 µL of 240 µM AA in wells **A7-A12** and **B1-D12**.
9. Immediately read the absorbance at 265 nm in wells **A7-A12** and **B1-D12** (t = 0 min).
10. Program the plate-reader to read the absorbance at 265 nm every 3 minutes during 30 minutes.
11. Program 30 s shaking steps by the reader prior each measurement.

Table 3. Plate configuration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibration curve						C-	C-	C-	EF2	EF2	EF2
B	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
C	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
D	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
E												
F												
G												
H												

Plate and plate-reader preparation / without automatic injectors

Set the temperature of the plate reader at 37°C. Since samples are not filtered, we recommend being cautious when pipetting the filters, making thorough visual verification that no big portion of the filter is introduced into the well.

1. In the wells A1-A6, prepare a **6-point standard curve** of AA by diluting the 240 µM daughter solution **in K-PO_4 buffer**, directly in the wells (final volume = 180 µL). We recommend performing the 6-point calibration curve on the 0-200 µM range (avoid using the 240 µM solution in your interval). **Directly perform the absorbance measurements of the standard curve** (you can also do it on a separate plate if you prefer).
2. Place 80 µL of each sample, following the configuration below (Table 4). In the C- wells (negative control - **A7, A8, A9**) introduce 80 µL of K-PO_4 buffer.
3. Place the plate into the reader.
4. Shake the plate for 30 seconds using the reader.
5. Wait for 20 seconds.
6. Read the matrix absorbance at 265 nm in wells **A7-A12** and **B1-D12**.
7. Manually inject 100 µL of 240 µM AA solution (that was kept in the fridge) in the wells **A7-A12** and **B1-D12**. Make sure to condition your pipette tips by pipetting and discarding 3 times 100 µL of the solution before injecting it in the plate.
8. Immediately read the absorbance at 265 nm in wells **A7-A12** and **B1-D12** (t = 0 min).
9. Program the plate-reader to read the absorbance at 265 nm every 3 minutes during 30 minutes.
10. Program 30 s shaking steps by the reader prior each measurement.

Table 4. Plate configuration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibration curve						C-	C-	C-	E	E	E
B	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
C	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
D	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
E												
F												
G												
H												

Procedure with cuvette-type spectrophotometer

Make sure that your cuvettes can be placed at 37°C and shaken regularly. Since samples are not filtered, we recommend being cautious when pipetting the filter samples, making thorough visual verification that no big portion of the filter is visible in the pipette tip.

If your spectrometer cannot process several cuvettes at a time, we recommend limiting each experiment period to 1 negative control + 1 sample at a time, for example: 1 replicate of negative control and 3 replicates of extraction 1 of sample A (i.e. extraction tube A1).

1. On each day of analysis, perform a **6-point standard curve** of AA by diluting the 240 µM daughter solution **in K-PO_4 buffer**, directly in the cuvette (final volume = 2.7 mL). We recommend performing the 6-point calibration curve on the 0-200 µM range (avoid using the 240 µM solution in your interval). **Directly perform the absorbance measurements of the standard curve**.
2. Prepare 1 cuvette per replicate + 1 cuvette for the matrix absorption measurement. For example, for the 1st extraction of sample A (A1), the reactive mixture will be prepared in triplicates (A1_1, A1_2, A1_3).

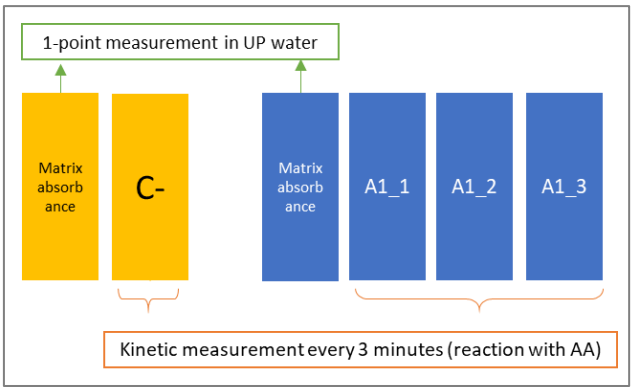

Figure 2. Schematic of the cuvette preparation in the case of the extraction of A1 with a negative control. Yellow represents the cuvettes with the negative control (i.e. 1.2mL of K-PO_4 mixed with UP-water for the matrix absorbance cuvette; 1.2mL of K-PO_4 mixed with AA 240µM for the C- cuvette). Blue represents the cuvette with the extracted sample (i.e. 1.2mL of A1 extract mixed with UP-water for the matrix absorbance cuvette; 1.2mL of A1 extract mixed with AA 240µM for the A1_1, A1_2, A1_3 cuvettes)

3. In each negative control cuvette (yellow on the schematic above), introduce 1.2 mL of heated K-PO_4 buffer.
4. In each sample cuvette (blue cuvettes on the schematic above), introduce 1.2 mL of A1 extract.
5. In the 2 matrix absorbance cuvettes (shown in Figure 2), add 1.5 mL of UP water, shake them gently and measure their absorbance.
6. Introduce 1.5 mL of AA 240 µM solution in the C- cuvette, shake it gently for a few seconds and measure the absorbance as fast as possible, starting a stopwatch at the same time. Once the t = 0 min point is measured for the C- cuvette, proceed the same way for the A1_1 cuvette, and so on for the A1_2 and A1_3 cuvettes. Then, measure the absorbance of each cuvette every 3 minutes during 30 minutes, and keep them at 37°C under gentle agitation between each measurement. **Make sure to write the exact times of absorbance measurements and report them in the Excel spreadsheet**.

Results

Use the Excel spreadsheet provided to calculate the kinetics of AA oxidation as:

- nmol AA/min, by subtracting both the matrix absorbance (plate-readers: step 7 with injectors, 6 without injectors, cuvette: step 5) and the inherent AA-oxidation rate (slope of C- samples) from the AA consumption rate of the samples A-E.
- nmol AA/min/µg, by dividing the nmol AA/min result by the mass of PM that was in the well (provided in the Excel spreadsheet).
- nmol AA/min/m^3, by multiplying the nmol AA/min/µg result by the PM mass concentration of the sample (provided in the Excel spreadsheet) for the filter PM samples.

All these formulae are pre-included in the Excel spreadsheet provided. Use the spreadsheet to report your metadata (participant reference number, analytical protocol, instrument), and your results, using the reference number for each sample.

Once you have reported results for the AA simplified protocol, feel free to test the samples with your own AA-home protocol, or to perform the OP-DTT protocol from the first intercomparison, and fill the other tabs of the Excel file.

5. Simplified DTT RI-Urbans Protocol "Evaluation of acellular reactivity of particles by dithiothreitol assay"

Authors: P. A. Dominutti, S. Houdier, J.L. Jaffrezo, T. Mhadhbi, G. Uzu

Materials

An ice bath is required to keep the DTT and DTNB cold (at least keep the reagent solution fresh in the freezer until use).

The samples need to be under agitation during the experiment time at 37°C.

1. **Plate readers**

One transparent 96-wells plate is sufficient to process all the samples in triplicate. You can use a separate 96-wells for the calibration curve of DTT.

2. **Cuvettes**

Each sample/control involves **twelve** 15 mL falcon tubes in **total**: one incubation tube for each time of titration (t = 0, 10, 20, 30 min) in triplicate (4 times x 3 replicates = 12 tubes) that are preferentially kept in the dark (wrapped up with aluminium foil).

- $Control_{ox}$ measures inherent DTT auto-oxidation (e.g particle-free control) and refers to **tubes A (A1 to A12)**
- Samples (SP1-SP4) refer to **tubes B-E (B1 to B12, C1-C12 and E1-E12)**

Reagents

3. **Preparation of potassium phosphate (0.1M) buffer solution at pH 7.4**

Weight 13.41 g of dipotassium phosphate (K_2HPO_4, CAS [7758-11-4]) and 3.13 g of potassium dihydrogen phosphate (KH_2PO_4, CAS [7778-77-0]) and mix them in a volumetric flask of 1000 mL with ultra-pure MilliQ water. Check the pH using a pH meter reading equal to 7.4 ± 0.1.

4. **Preparation of DTT mother solution (8.3 mM)**

Weight 38.6 mg of 1,4-Dithiothreitol (DTT, CAS [3483-12-3]) and add 30 mL of the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

5. **Preparation of DTT daughter solution (0.25 mM)**

This solution is obtained from 1.20 mL of the 8.3 mM DTT solution and completed to a final volume of 40 mL with potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

6. **Preparation of Dinitrothiobenzoic acid (DTNB) mother solution (10 mM)**

Weight 118.8 mg of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB, CAS [69-78-3]) and add 30 mL of the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

7. **Preparation of DTNB daughter solution (1 mM)**

This solution is obtained by diluting 4mL of the 10mM DTNB solution, completing a total volume of 40 mL with the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

Procedure for plate readers automatically injected

Set up the temperature of the plate reader at 37°C for the duration of the assay.

Before the absorbance measurements of the samples, perform a calibration of your analytical device using a DTT calibration curve for a concentration range between 0 and 60 µM (titration with 1 mM DTNB and reading of TNB formation at 412 nm).

1. Draw up a grid for 96-wells plate, and locate the samples SP1 to SP4 as in the table below, leaving the first 3x4 wells for the $Control_{ox}$ (inherent DTT background oxidation).

Table 5. Plate configuration.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Controlox	Controlox	Controlox	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**t=0**
B	Controlox	Controlox	Controlox	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**t=10**
C	Controlox	Controlox	Controlox	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**t=20**
D	Controlox	Controlox	Controlox	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**t=30**
E	SP4	SP4	SP4										**t=0**
F	SP4	SP4	SP4										**t=10**
G	SP4	SP4	SP4										**t=20**
H	SP4	SP4	SP4										**t=30**

2. Place 20 µL of samples SP1 to SP4 into each well and 20 µL of the extracting matrix in the $Control_{ox}$ wells.
3. Add 220 µL of the potassium phosphate buffer solution (7.4 pH) in the sample wells SP1 to SP4 and in the $Control_{ox}$ wells.
4. Set up the plate reader at 37°C.
5. Place the plate into the reader and incubate for 10 minutes.
6. Shake the plate by the instrument for one minute.
7. **Read the intrinsic absorbance** of the samples/control at 412 nm.
8. At t = 0 min, program the injector A to dispense 50 µL of 0.25 mM DTT in **ALL** wells. Keep the solution under an ice bath or in the fridge until use.
9. At t = 0 min, program injector B to dispense 50 µL of 1 mM DTNB **into the t = 0** wells (lines A and E). Keep the solution under an ice bath or in the fridge until use.
10. Shake the plate by the reader for 30 seconds every minute for 10 minutes.
11. At t = 10 minutes, dispense 50 µL of 1 mM DTNB **into the t = 10** wells (lines B and F) to stop the DTT consumption reaction by the samples.
12. Shake the plate by the device for 30 seconds every minute for 10 minutes.
13. At t = 20 minutes, dispense 50 µL of 1 mM DTNB **into the t = 20** wells (lines C and G).
14. Shake the plate by the device for 30 seconds every minute for 10 minutes.
15. At t = 30 minutes, dispense 50 µL of 1 mM DTNB **into the t = 30** wells (lines D and H).
16. Shake the plate for 60 seconds, wait 10 seconds and read the final absorbance at **412 nm**. The yellow compound (TNB) formed is stable for two hours; only one final absorbance measurement is necessary.
17. Calculate the kinetics of the DTT oxidation as:
    - nmol DTT/min is obtained by **substracting both** the intrinsic absorption of each sample (to remove a potential matrix effect between samples, the value obtained in step 7) **and** the inherent DTT auto-oxidation rate (slope of $Control_{ox}$ sample) **from** the DTT consumption rate in the presence of particles (SP1-4).
    - nmol DTT/min/µg is obtained by subtracting both the intrinsic absorption of each sample and inherent DTT auto-oxidation rate from the DTT consumption rate in the presence of particles and dividing it by the mass of particulate matter in the reaction.

All these formulae are pre-included in the Excel spreadsheet provided.

Procedure for plate readers without injectors

Set up the temperature of the plate reader at 37°C for the duration of the assay.

Before the absorbance measurements of the samples, perform a calibration of your analytical device using a DTT calibration curve for a concentration range between 0 and 60 µM (titration with 1 mM DTNB and reading of TNB formation at 412 nm).

1. Draw up a grid for 96-wells plate, and locate the samples SP1 to SP4 as in the table above, leaving the first 3x4 wells for the $Control_{ox}$ (inherent DTT background oxidation)
2. Place 20 µL of samples SP1 to SP4 into each well and 20 µL of the extracting matrix in the $Control_{ox}$ wells.
3. Add 220 µL of the potassium phosphate buffer solution (7.4 pH) in the sample wells SP1 to SP5 and in the $Control_{ox}$ wells.
4. Introduce the plate into the reader and read the intrinsic absorbance of the solutions at 412 nm.
5. Inject 50 µL of 1mM DTNB **into the t = 0** min wells (lines A and E) (this is done to avoid depletion of DTT with samples at t = 0 with manual injection, which is slower than injectors). Keep the DTNB solution under an ice bath or in the fridge until use.
6. Dispense 50 µL of 0.25 mM DTT in **ALL** wells. Keep the DTT solution under an ice bath or in the fridge until use.
7. Set up the plate reader at 37 °C.
8. Introduce the plate into the plate reader and incubate for 10 mins.
9. Shake the plate by the device for 30 seconds every minute for 10 minutes.
10. At t = 10 minutes, remove the plate from the instrument and inject 50 µL of 1mM DTNB **into the t = 10** wells (lines B and F) to stop the DTT consumption reaction by the samples.
11. Place the plate back on the reader and shake it for 30 seconds every minute for 10 minutes.
12. At t = 20 minutes, remove the plate from the reader and inject 50 µL of 1mM DTNB **into the t = 20** wells (lines C and G).
13. Place the plate back on the reader and shake it for 30 seconds every minute for 10 minutes.
14. At t = 30 minutes, remove the plate from the reader and dispense 50 µL of 1mM DTNB **into the t = 30** wells (lines D and H).
15. Place the plate back into the reader and shake it for 60 seconds, wait 10 seconds and read the final absorbance at **412 nm**. The yellow compound (TNB) formed is stable for two hours; only one final absorbance measurement is necessary.
18. Calculate the kinetics of the DTT oxidation as:
    - nmol DTT/min is obtained by **substracting both** the intrinsic absorption of each sample (to remove a potential matrix effect, value obtained in step 4) **and** the inherent DTT auto-oxidation rate of the blank (slope of $Control_{ox}$ sample) **from** the DTT consumption rate in the presence of particles (SP1-4).
    - nmol DTT/min/µg is obtained by subtracting both the intrinsic absorption of each sample and inherent DTT auto-oxidation rate from the DTT consumption rate in the presence of particles and dividing it by the mass of particulate matter in the reaction.

All these formulae are pre-included in the Excel spreadsheet provided.

Procedure for cuvettes

Recommendation: we suggest performing one triplicate at a time since the whole experiment involves a total of 60 tubes that are difficult to titrate at one time by a single user. For example, test the first triplicate of a Control$_{ox}$ (4 tubes A, that will be used to estimate the DTT auto-oxidation) together with one triplicate of each sample (4 tubes for each sample B, C, D, E). Repeat this experiment three times to complete all triplicates. To do so, follow the next steps:

Prepare 20 falcon tubes of 15 mL **(*Control$_{ox}$* : A1 to 4, Samples: B1-4, C1-4, D1-4, E1-4)**, and introduce 2.2 mL of potassium phosphate buffer solution (pH 7.4) in every tube.

1. In **tubes A**, introduce 0.2 mL of the extracting matrix and in tubes **B, C, D, E,** introduce 0.20 mL of the particulate suspension solutions (SP1 to SP4). Then, add 0.50 mL of 0.25 mM DTT standard solution in all tubes **A to E** (which should be kept in ice until use). All the tubes should remain under gentle agitation for the duration of the tests and heated at 37°C.
2. Start a stopwatch to determine the exact duration of the reaction (should not exceed 30 min) and follow the next steps each time.
3. **Step t = 0 min**. As soon as possible, take the tube t = 0 of each sample (tubes **A1, B1, C1, D1, E1**) and add 0.50 mL of 1mM DTNB solution (kept in ice until use). Stir well. Keep the tubes on ice and in dark conditions to avoid light degradation until the absorbance measurement.
4. **Step t = 10 min. After a 10 min time incubation**, take the tube t = 10 of each sample (tubes **A2, B2, C2, D2, E2**) and add 0.50 mL of 1mM DTNB solution (kept in ice until use). Stir well. Keep the tubes on ice and in dark conditions to avoid light degradation until the absorbance measurement.
5. Perform the procedure as for point 5 for t = 20 min (tubes **A3, B3, C3, D3, E3**) and t = 30 min (for tubes **A4, B4, C4, D4, E4**).
6. At this stage, you have 4 titrated tubes for times = 0, 10, 20, and 30 min for each sample.
7. Before absorbance measurements, perform an autozero of your cuvette-spectrophotomer with a blank (with the extracting matrix of the samples). Then, measure the intrinsic absorbance at 412 nm of each particle suspension alone by mixing 0.20 mL of SPX (1 to 4) with 2.2 mL extracting matrix. **Agitate all the titrated tubes** (Control$_{ox}$/samples) and **measure their absorbance at 412 nm** for each reaction time into a cuvette.
8. Perform steps 1-7 twice again: for the second replicate tubes (A5 to A8, B5-B8, C5-C8, D5-D8, E5-E8) and for the thirs replicate tubes (A9 to A12, B9-B12, C9-C12, D9-D12, E9-E12), to achieve triplicate measurements.
9. Calculate the kinetics of the DTT oxidation as:
    - nmol DTT/min is obtained by **substracting both** the intrinsic absorption of each sample (to remove a potential matrix effect, value obtained in step 7) **and** the inherent DTT auto-oxidation rate of the blank (slope of Control$_{ox}$ sample) **from** the DTT consumption rate in the presence of particles (SP1-4).
    - nmol DTT/min/µg is obtained by subtracting both the intrinsic absorption of each sample and inherent DTT auto-oxidation rate from the DTT consumption rate in the presence of particles and dividing it by the mass of particulate matter in the reaction.

All these formulae are pre-included in the Excel spreadsheet provided.