Supplementary material of

An interlaboratory comparison to quantify oxidative potential measurement in aerosol particles: challenges and recommendations for harmonisation

Dominutti et al.,

SI-1 Simplified DTT RI-URBANS Protocol «Evaluation of acellular oxidative potential of particles by dithiothreitol (DTT) assay»

Method 1 – OP DTT assay using plate readers

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 1mM DTNB and reading of TNB formation at 412 nm) and report the results on the Excel file provided.

Reagents:

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

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.

1.

2. ***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.

3.

4. ***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.

5. ***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.

6. ***Particulate Matter suspension solutions to be tested - samples***

Solution SP1 5.0 µg mL^{-1}, solution SP2 25 µg mL^{-1}, Solution SP3 25 µg mL^{-1}, solution SP4 25 µg mL^{-1}.

Material

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.

The samples need to be under agitation during the experiment time at 37.4°C.

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

Procedure for plate readers automatically injected

DTT Exposure and DTNB analysis:

Set up the temperature of the plate reader at 37,4°C for the duration of the assay.

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).

	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 ultrapure water in $Control_{ox}$ 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 wells
4. Set up the plate reader at 37,4°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^{-1} is obtained by **substracting both** the intrinsic absorption of each sample (to remove a potential matrix effect between samples, the value obtained in step 8) **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^{-1} μg^{-1} 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.
- % DTT consumed μg^{-1} min^{-1} is obtained by the % of DTT lost over the reaction with samples relative to the inherent DTT auto-oxidation and normalised by the reaction time and per µg of PM.

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

Use it to add the results using your participant reference number, the analytical protocol and instrument used and the reference number for each sample.

Once you have reported results for the DTT simplified protocol, feel free to test the samples with your own protocols, filling the other tabs of the Excel file.

Procedure for plate readers without injectors

DTT Exposure and DTNB analysis:

Set up the temperature of the plate reader at 37.4°C for the duration of the assay.

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 sample (inherent DTT background oxidation).
2. Place 20 µL of samples SP1 to SP4 into each well and 20 µl of ultrapure water in $Control_{ox}$ 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 sample 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 37.4 °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 stir 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^{-1} is obtained by **substracting both** the intrinsic absorption of each sample (to remove a potential matrix effect, value obtained in step 8) **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^{-1} μg^{-1} 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.
- % DTT consumed μg^{-1} min^{-1} is obtained by the % of DTT lost over the reaction with samples relative to the inherent DTT auto-oxidation and normalised by the reaction time and per µg of PM.

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

Use it to add the results using your participant reference number, the analytical protocol and instrument used and the reference number for each sample.

Once you have reported results for the DTT simplified protocol, feel free to test the samples with your own protocols, filling the other tabs of the excel file.

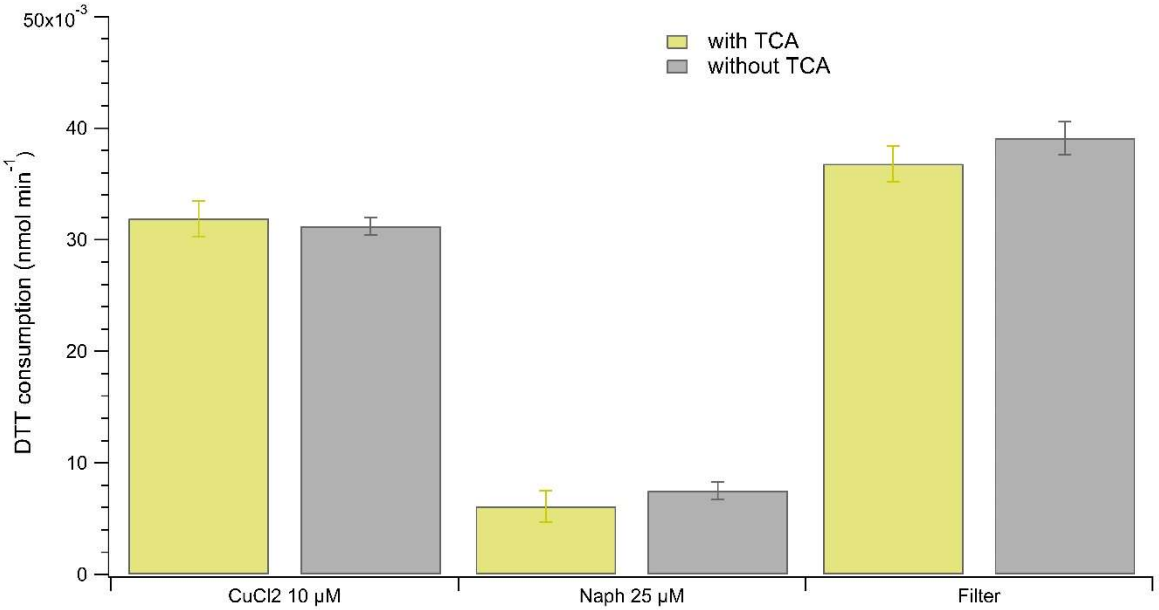


Figure S1. The effect of trichloroacetic acid (TCA; used for quenching the DTT reaction mixture) on the measured OP DTT values (in nmol min^{-1}). The experiments were conducted using CuCl2 (10 µM), 1,4-naphthoquinone (Naph; 25 µM), and aqueous extracts of ambient PM samples (filter).

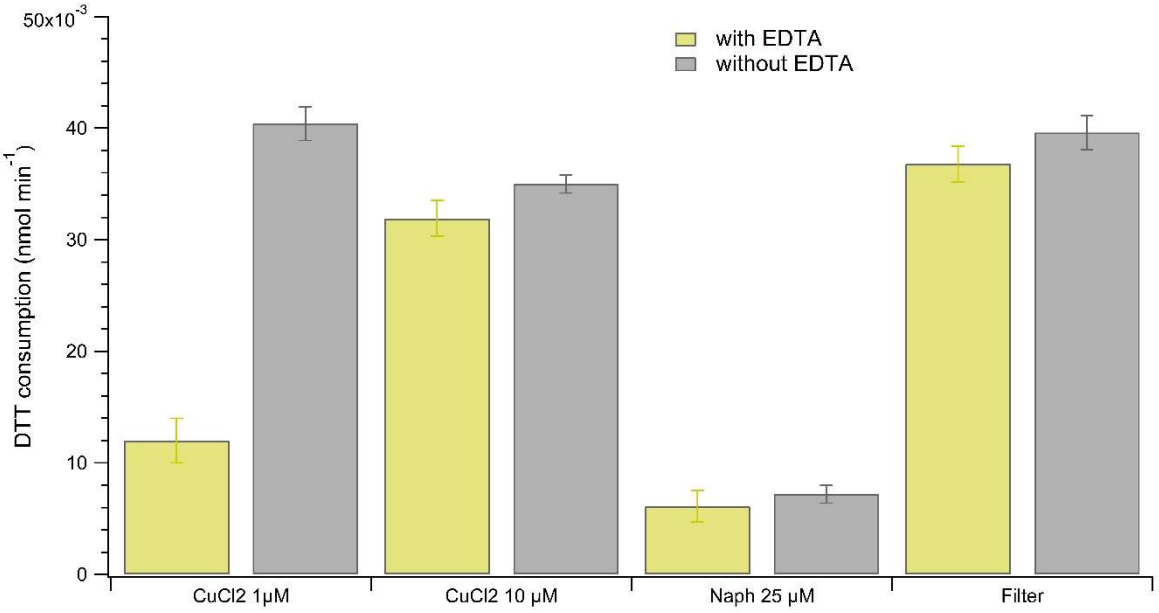
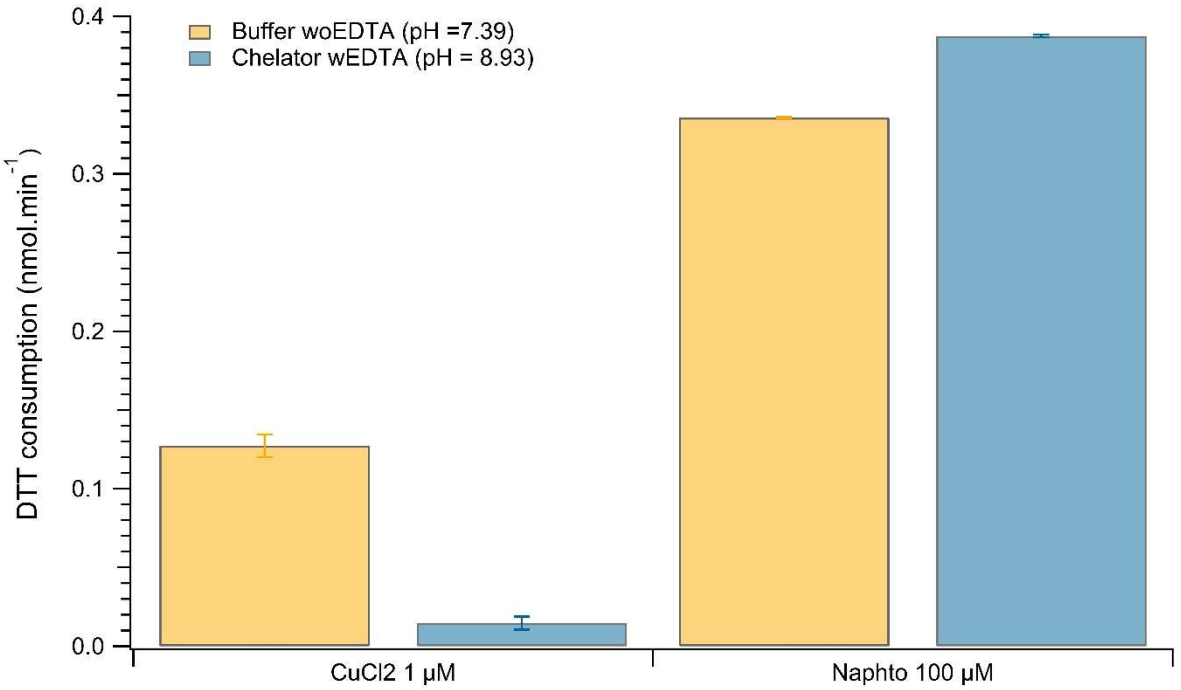


Figure S2. The average effect and standard deviation of EDTA (used for buffer purification) on the measured OP DTT values (in nmol min^{-1}). The experiments were conducted using CuCl2 (1 µM and 10 µM), 1,4-naphthoquinone (Naph; 25 µM), and aqueous extracts of ambient PM samples (filter).



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167 *Figure S3. Comparison of the average effect and standard deviation of potassium phosphate buffer (pH= 7.4)*
168 *without EDTA (yellow bars) and the Tris-HCl buffer with EDTA (pH= 8.9) on the measured OP DTT values (in nmol*
169 *min^{-1}). The experiments were conducted using CuCl2 (1 µM), and 1,4-naphthoquinone (Naph; 100 µM) samples.*

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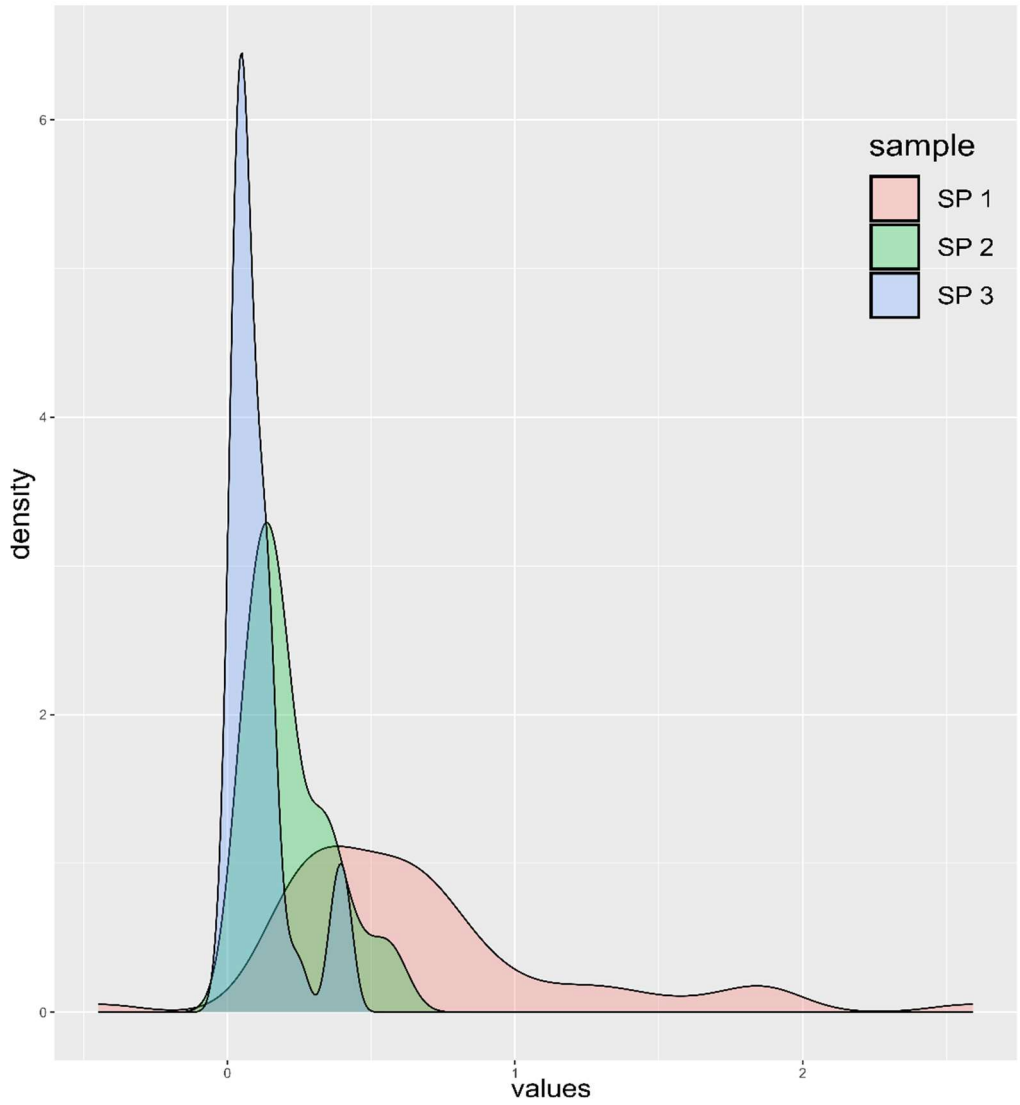
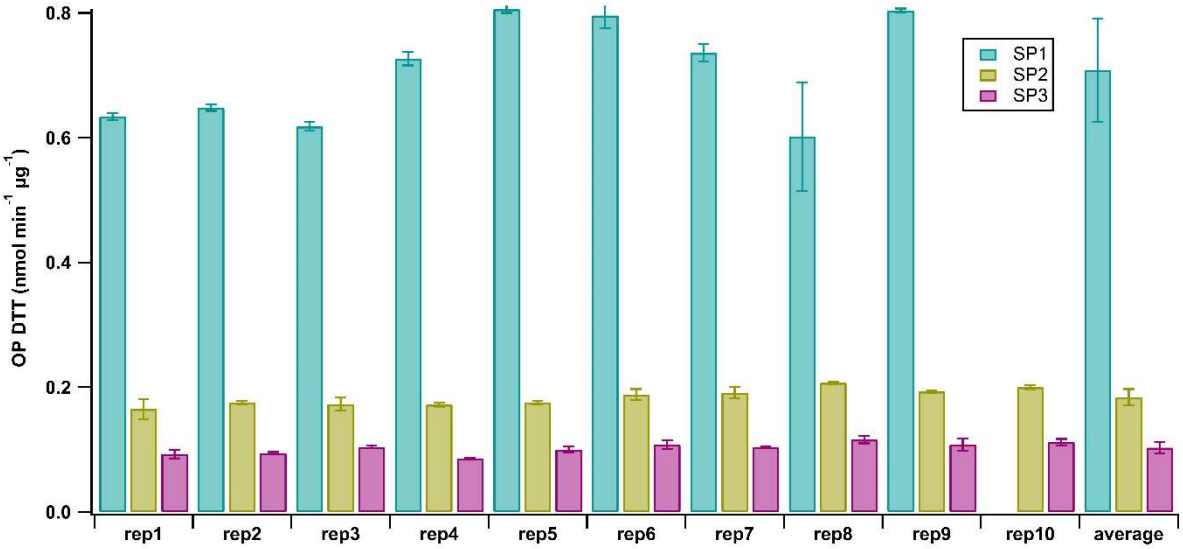


Figure S4. Density plots presenting the distribution of values for each sample applying the RI-URBANS DTT SOP.



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181 *Figure S5. Homogeneity test results (average and standard deviation) obtained from the measurements of each*
182 *sample replicates (n=10 for each sample). SP1 = 1,4 naphthoquinone solution, SP2 = biomass burning emissions*
183 *sample and SP3 = traffic emissions sample.*

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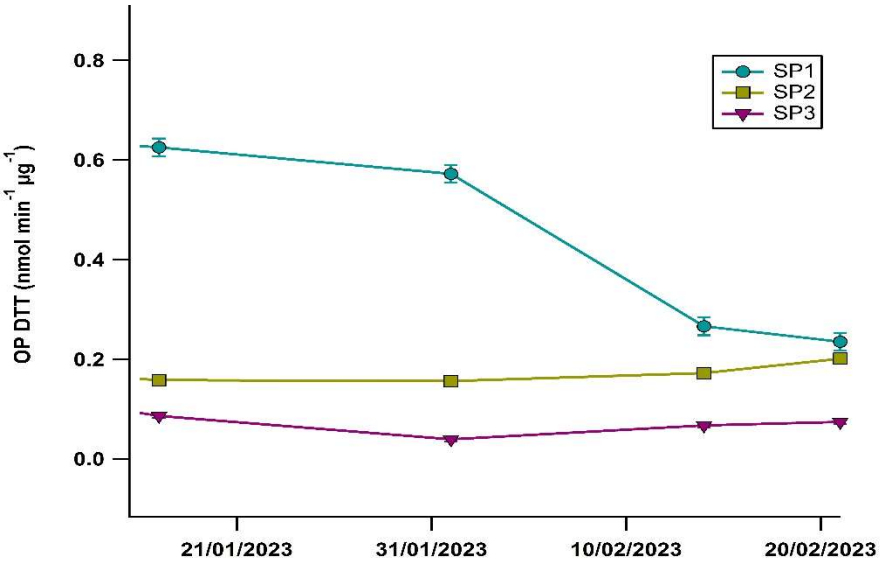

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186 *Figure S6. Results of the OP DTT of each sample (average and standard deviation obtained from the*
187 *homogenization test) over time following the simplified RI-URBANS DTT SOP. The analyses were performed by the*
188 *ILC organiser.*

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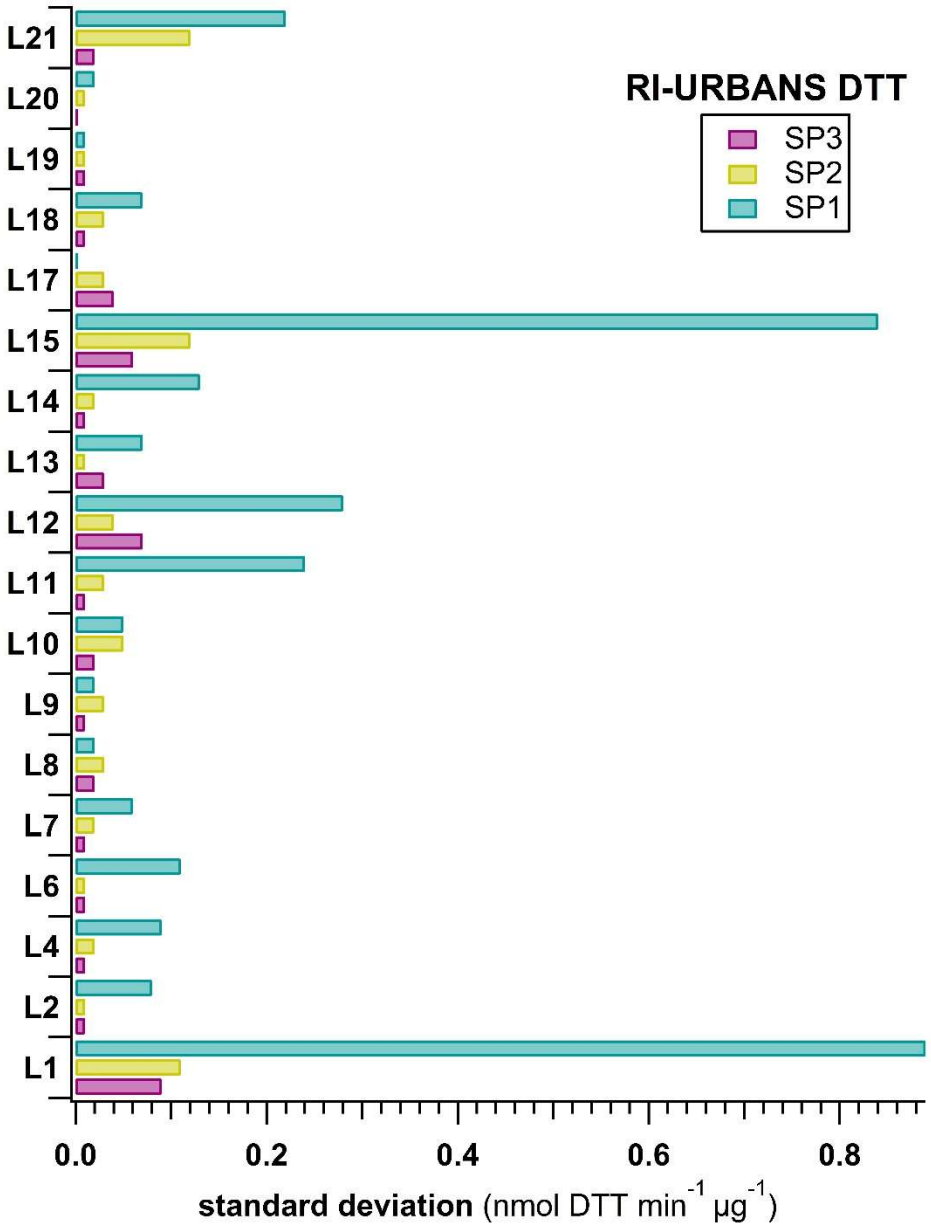

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192 *Figure S7. Standard deviation on the replicates reported for each test sample using the RI-URBANS DTT SOP,*
193 *grouped by participant reference number.*

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Table S1. The average and standard deviation (in nmol min $^{-1}$ μg^{-1}) of each test sample obtained from replicates of each participant applying the RI-URBANS DTT SOP

Participant	SP1_Mean	SP1_SD	SP2_Mean	SP2_SD	SP3_Mean	SP3_SD
L1	0.468	0.888	0.303	0.112	0.363	0.092
L2	0.691	0.078	0.172	0.012	0.106	0.006
L3	--	--	--	--	--	--
L4	0.373	0.087	0.161	0.019	0.061	0.006
L5	--	--	--	--	--	--
L6	0.603	0.109	0.338	0.013	0.152	0.014
L7	0.223	0.059	0.078	0.019	0.031	0.011
L8	0.452	0.020	0.177	0.032	0.066	0.016
L9	0.267	0.016	0.079	0.026	0.047	0.008
L10	0.770	0.050	0.464	0.053	0.134	0.016
L11	0.986	0.238	0.571	0.029	0.379	0.014
L12	0.404	0.281	0.118	0.037	0.048	0.073
L13	0.561	0.071	0.207	0.009	0.065	0.030
L14	0.402	0.128	0.345	0.020	0.017	0.014
L15	1.714	0.844	0.132	0.123	0.079	0.063
L16	--	--	--	--	--	--
L17	1.867	0.005	0.078	0.030	0.173	0.036
L18	0.405	0.071	0.190	0.032	0.042	0.008
L19	0.648	0.013	0.070	0.008	0.098	0.006
L20	0.232	0.016	0.137	0.008	0.048	0.003
L21	1.191	0.222	0.268	0.117	0.030	0.019

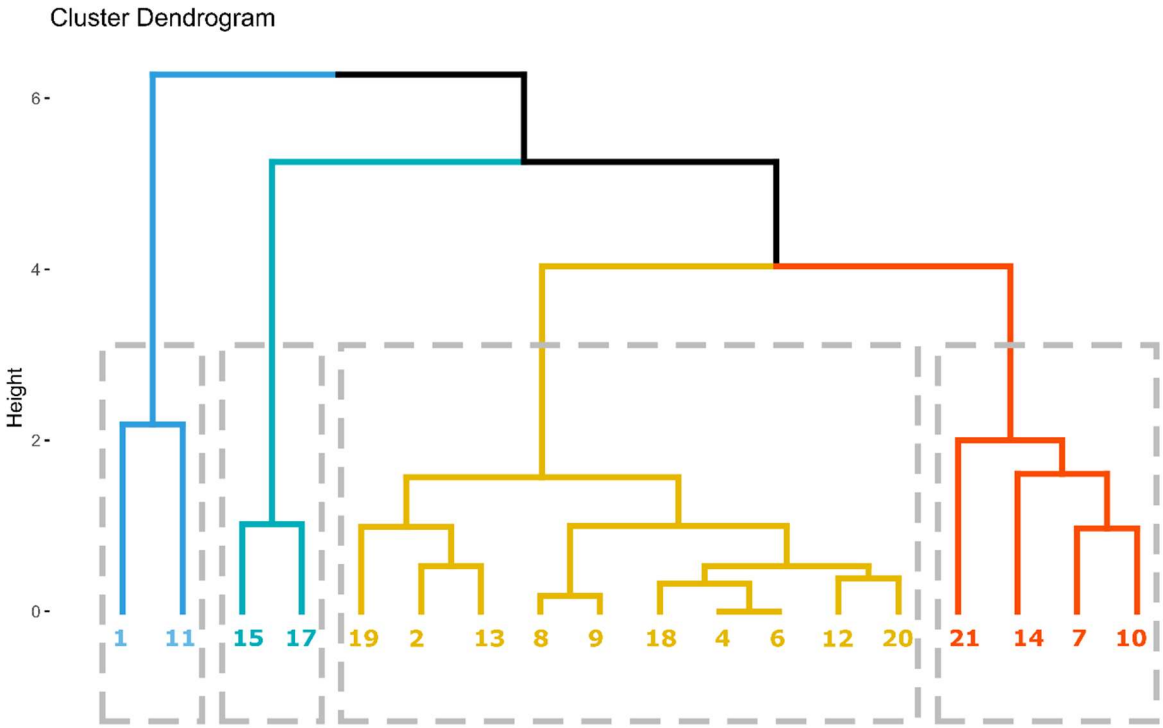

Figure S8. Dendrogram of the hierarchical cluster analysis using the Ward method and applied to the results reported from the simplified OP DTT RI-URBANS SOP.

Table S2. Variables and observations included in the multiple linear models used in this study. Delivery and analysis time variables are not described here

Sample	**Instrument**	**Protocols**	
		M1 = RI-URBANS DTT (n)	*M2 = RI-URBANS DTT + "home" DTT (n)*
SP1	*Cuvette*	27	36
	Plate reader	24	41
	LWCC	3	11
SP2	*Cuvette*	27	39
	Plate reader	24	44
	LWCC	3	11
SP3	*Cuvette*	27	39
	Plate reader	24	44
	LWCC	3	12

Table S3. Average and standard deviation (in nmol min $^{-1}$ µg^{-1}) of each test sample obtained from replicates of each participant applying DTT-"home" protocols

Laboratory	SP1_Mean	SP1_SD	SP2_Mean	SP2_SD	SP3_Mean	SP3_SD
L1	1.934	0.300	0.141	0.026	0.022	0.012
L2	0.702	0.064	0.206	0.014	0.102	0.014
L3	0.027	0.000	0.003	0.001	0.002	0.000
L4	--	--	--	--	--	--
L5	--	--	--	--	--	--
L6	0.350	0.003	0.269	0.002	0.088	0.001
L7	0.721	0.208	0.721	0.092	0.040	0.001
L8	--	--	--	--	--	--
L9	--	--	--	--	--	--
L10	0.885	0.015	0.519	0.022	0.036	0.004
L11	1.747	0.232	0.239	0.041	0.055	0.015
L12	1.327	0.219	0.156	0.012	0.110	0.009
L13	1.452	0.092	0.078	0.002	0.016	0.001
L14	--	--	0.123	0.006	0.059	0.008
L15	--	--	--	--	--	--
L16	--	--	--	--	--	--
L17	--	--	0.081	0.023	0.023	0.009
L18	--	--				
L19	3.677	0.346	0.501	0.036	0.247	0.024
L20	0.814	0.077	0.111	0.008	0.055	0.005
L21	--	--	--	--	--	--

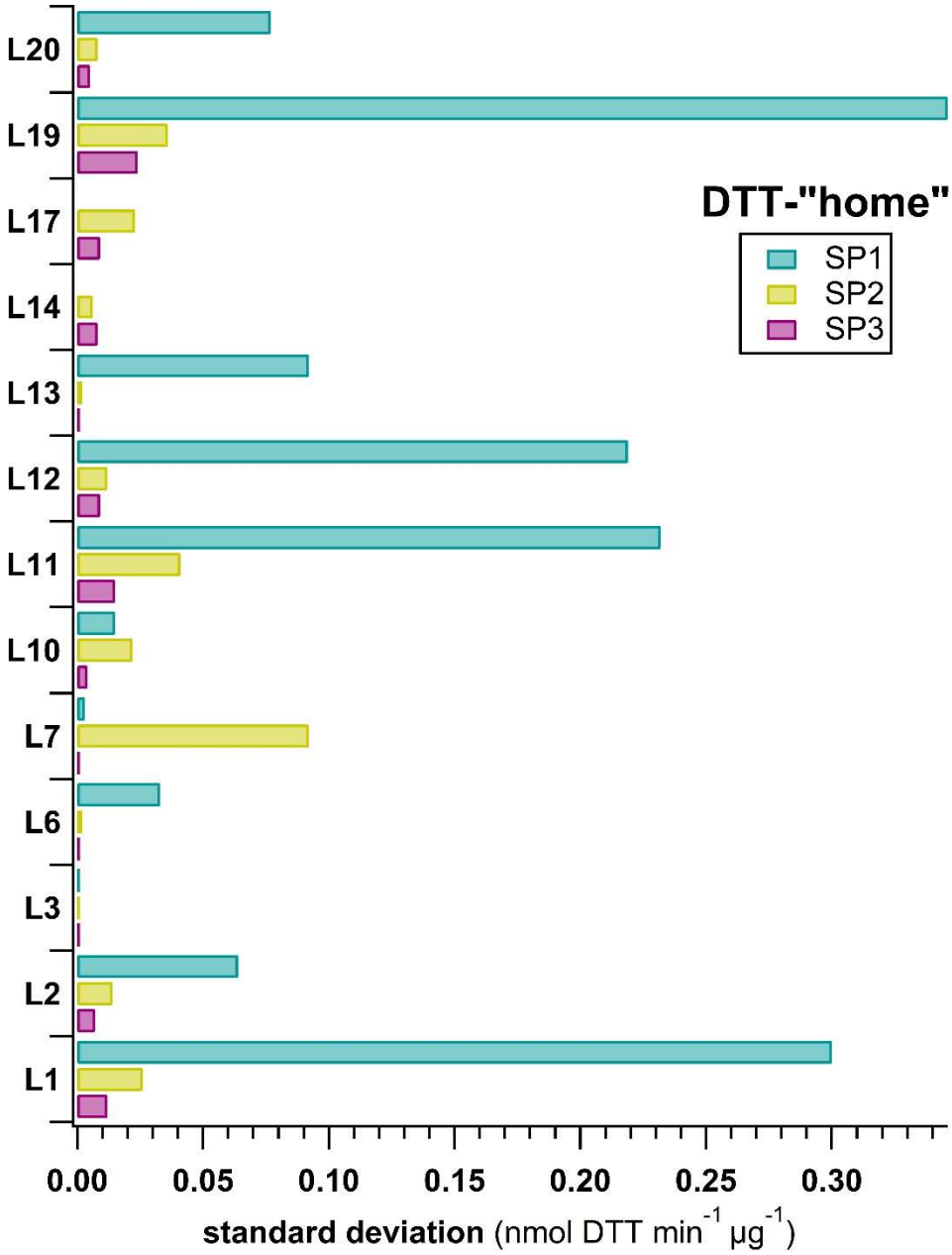

Figure S9. Standard deviation on the replicates reported for each test sample using the DTT-"home" protocols, grouped by participant reference number.

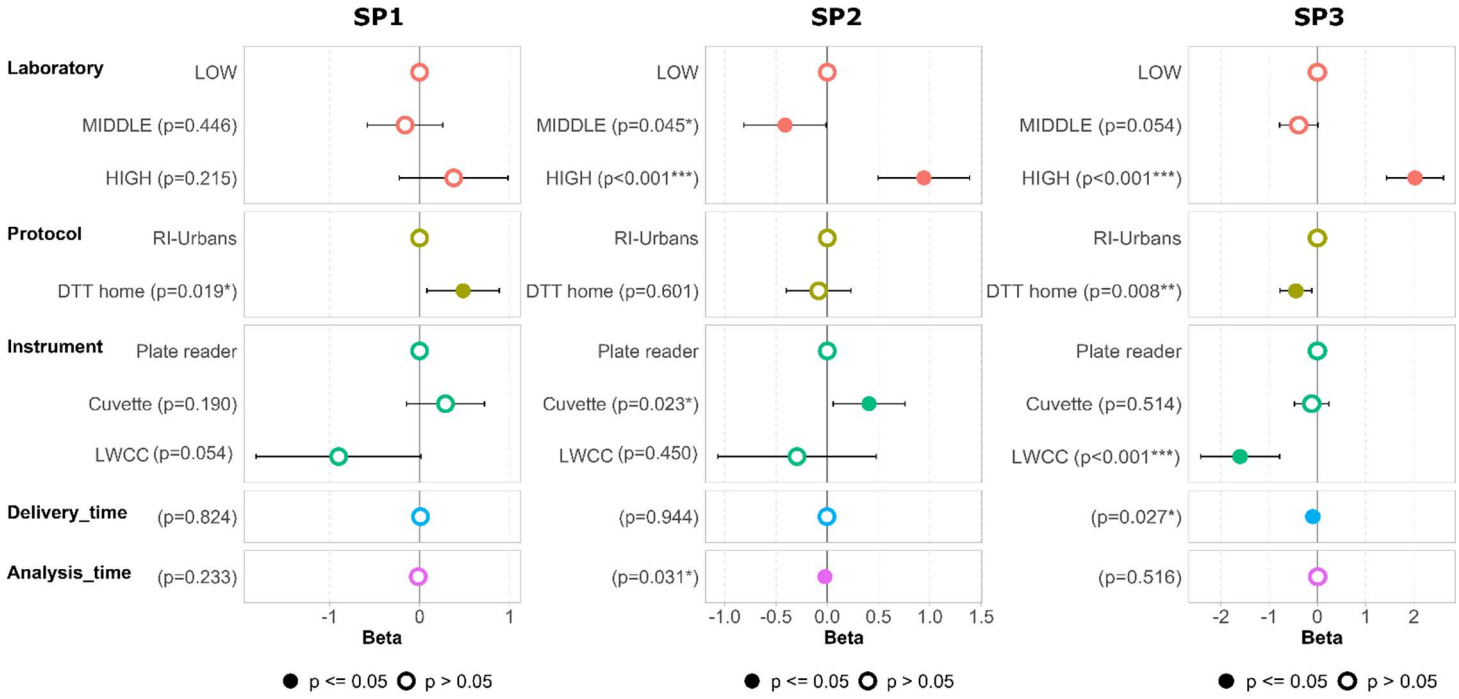

Figure S10. Associations (betas in nmol min^{-1} μg^{-1}) between OP DTT values for SP1, SP2 and SP3 for categorical classification considering the performances of each laboratory grouped into low (0<|z|<2), middle (2<|z|<3) and high (|z|>3) z-scores. The model includes the different parameters of the intercomparison, including the DTT protocol used, the instrument used and the delivery and analysis time obtained by applying a multiple linear regression model.

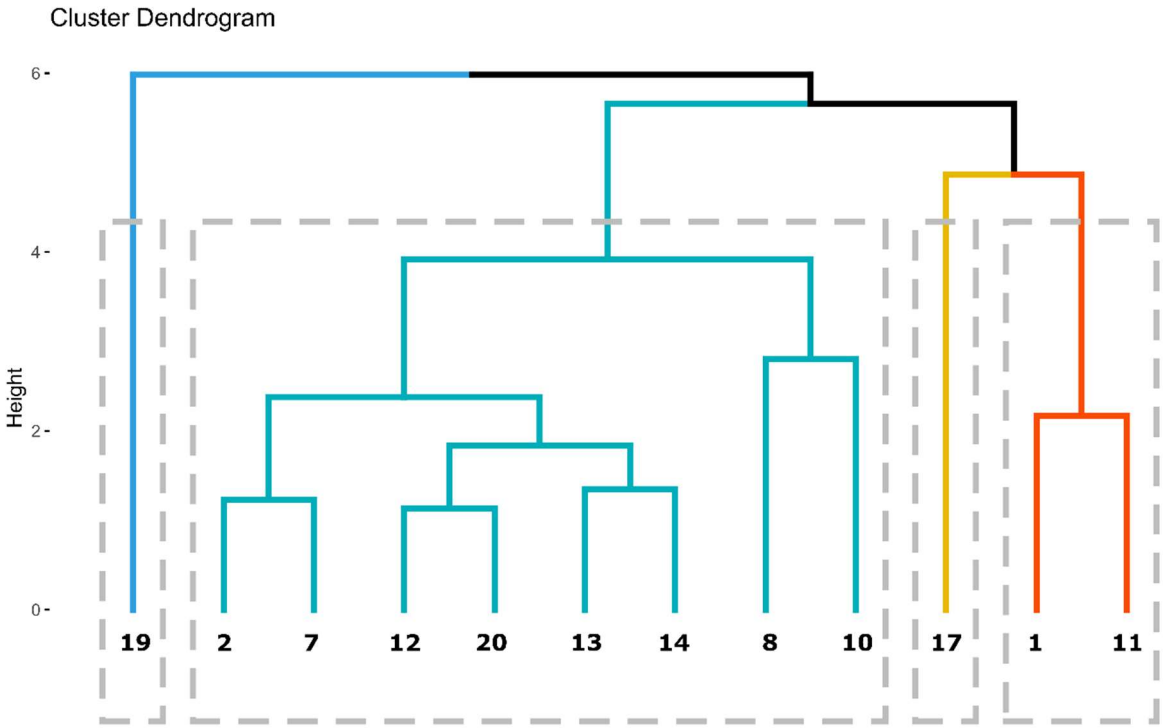

Figure S11. Dendrogram of hierarchical cluster analysis using the results from the groups reporting results of the simplified OP DTT RI-URBANS and DTT "home" protocols (include only the participants that reported results for the two protocols)

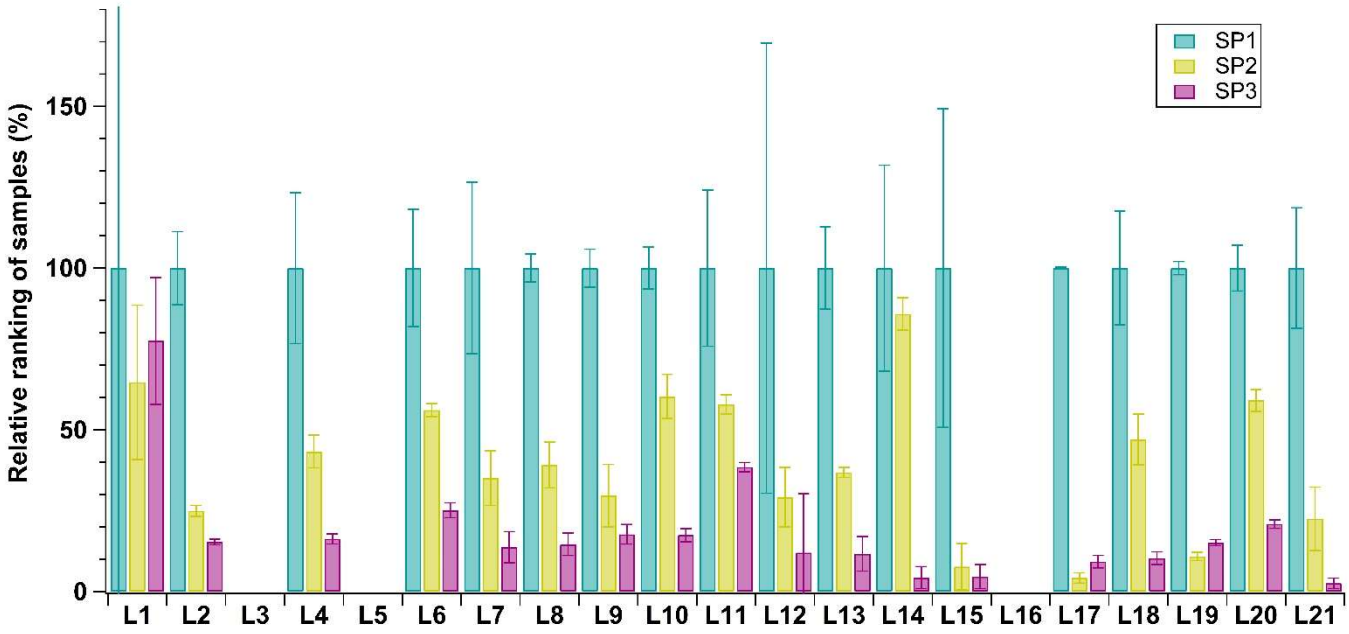

Figure S12. Relative average ranking and standard deviation of the samples evaluated in this ILC, considering SP1 as the reference one (100%).