water

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Supplementary materials

Validation of Sample Preparation Methods for Microplastic Analysis in Wastewater Matrices – Reproducibility and Standardization

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1. Protocols investigated in the study

1.1. Pre-experiments for protocol selection

After conducting an extensive literature review, ten protocols were identified for a round of pre-selection. The pre-experiments were simplified, using only polystyrene spheres (PS) of size 250 µm (BS-Partikel, Germany) and analyzed using a light microscopy (Axioplan 2, Carl Zeiss AG, Germany) to determine negative effects on the microplastics. The pre-experiments were conducted by weighing 1 mg of PS spheres and letting them react for 24 hours at room temperature with the various reagents Table 1. After which, the mixtures were filtered on a 25 mm diameter track etched polycarbonate membrane filter with a pore size of 0.2 µm (Carl Roth, Germany), and rinsed with ultrapure water to remove any remaining reagents from the particles' surface, then the entire filter was scanned by a light microscope to compare changes to the control samples.

Table 1. Protocols investigated in the pre-experiments and the literature source they were adopted from (Bibliography in main paper).

Protocol	Visual effects on PS-spheres (250 µm)	Details
H_2O_2 (30%) [7, 14]	-	No obvious effects observed
H_2O_2 (30%) + H_2SO_4 (98%) [35]	++	Changes to the surface of particles and melting of particles
Fenton reaction [17]	-	No obvious effect observed
HCL 2 M [27]	++	Changes to the surface of particles and melting of particles
HCL 12M [27]	++	Changes to the surface of particles and melting particles
Ultrasound [27]	+	A large number of fractured particles were found
HNO_3 (65%) [21]	++	Changes to the surface of particles and melting of particles. Formation of small (10 µm) particles/bubbles attached to the originals
NaOH (32%) [27]	++	Roughening of the surface (matt appearance). Plus melting and deep scratches to the surface
NaClO (15%) [36]	-	No obvious effect observed

H_2SO_4 (98%) [37]	+	Deep scratches to the surface of the particles

(-) No changes, (+) Smaller changes, (++) Obvious changes

Further experiments were performed on granular PE, PA and PLA (90-125 μm) as well as spherical PS (140 μm) using the same approach described above but for 24 hours and 60 °C for both KOH (10%) as well as NaOH (1 M) [7]. The second round of pre-experiments showed roughening of the PS surfaces (matt appearance) for both alkaline treatments, as well as a complete destruction of PLA particles. Changes on PE and PA were hard to quantify using only a microscope due to their non-uniformly shaped particles. Therefore, the results here were only meant as preliminary results and are not robust.

1.2. Fenton protocol

Chemicals

1. H_2O_2 (30%) (ISO, Stabilized, suitable for Fenton Type I reaction as discussed in the main paper)

2. $FeSO_4$ 7 H_2O (20 g/L)

3. Polyethylene glycol sorbitan monolaurate, or Polyoxyethylenesorbitan monolaurate (Tween 20) as a surfactant to aid in rinsing glassware and filters.

Note: The **K** value in the script is a factor that can be used to scale the procedure up or down, if other volumes of iron sulfate are desired, or when smaller samples are taken. The recommended dose tested in this study was however **K=10 mL** for 2 mL of return activated sludge (RAS).The K value is recommended to be five times the volume of sample.

Procedure:

1. Iron sulfate solution in a concentration of 20 g/L should be prepared. pH should be then set to 3 using 0.5 M sulfuric acid.
   Iron sulfate solutions prepared like this can be used up to four days after preparation. But it needs to be visually controlled for precipitated iron particles, as a result of Iron(II) being oxidized to Iron(III). The solution will then show a slight orange tint and microparticles of rust will start to form in it. When this stage is reached, it is advised to prepare a new solution.
2. Two washing bottles are needed (Made of a material that is not the polymers being investigated, Fluorinated ethylene propylene (FEP) was used in this case). One bottle is filled with ultrapure water, whereas the second is filled with ultrapure water with 0.1 % (v/v) of Tween 20.
   The surfactant bottle helps rinse particles off glass surfaces and the filter membrane.
3. The sludge sample is shaken well to ensure mixing and then 2 mL of sludge is taken with a pipette and placed in a 250 mL conical flask or larger. This provides a safe volume to account for the violent reaction and overflow.
4. Add 10 mL or (**K**) of the Iron sulfate solution prepared in step 1.
5. To start the reaction, 20 ml or (**2 K**) of the Hydrogen peroxide should be added and a timer should be started.
6. after 1 Minute, additional hydrogen peroxide should be added at a rate of 5 ml/min (0.5 **K/Min**) or simply 5 ml (**0.5 K**) at the start of each new minute. This will keep the reaction going.
7. The reaction is exothermic, and the temperature will start to increase in the first minutes, shortly reaching 90 °C. No water bath is needed, and the reaction is violent but controlled. However, wearing a glove that is heat resistant is advised. Shaking or stirring of the flask

might be needed if there is foam build up to prevent it from spilling over. In some extreme cases where boiling is to about to overspill, a washing bottle is used to spray around 1-4 mL of UPW inside the reaction flask to reduce temperatures and bring the boiling under control.

8. At time= 10 minutes, the last 5 ml (**0.5 K**) of peroxide should be added. Then another 10 minutes for cooling is needed. The reaction continues in a weakened state and eventually dies down and cools to below 50 °C.
9. After 20 Minutes has passed, 4 ml (**0.4 K**) of concentrated sulfuric acid (98 %) should be added (That equates to a concentration of 5% in the final reaction volume). The flask should be shaken continuously while adding the acid, this will quickly react with the cloud of Iron (III) particles that have formed during the reaction and clear up the sample within 30 seconds.
10. The solution should now be mostly clear (with a light-yellow color due to dissolved Iron complexes). Quickly adding 10 mL of the surfactant from the washing bottle into the flask to dilute the solution and prevent microplastics from adhering to the glass walls.
11. The content of the flask is then poured into a vacuum filtration unit with a PCTE, 0.2 µm filter. The surfactant bottle can be used here to rinse the contents of the flask into the filtration unit, as well as any particles adhering to the glass walls of the filtration unit.
12. The sample is ready for analysis. Alternatively, it can be stored as a suspension for later, where the same filter from step 11 can be rinsed down into a clean glass test tube using the surfactant bottle and then stored for further analysis or filtration on a different kind of filter.

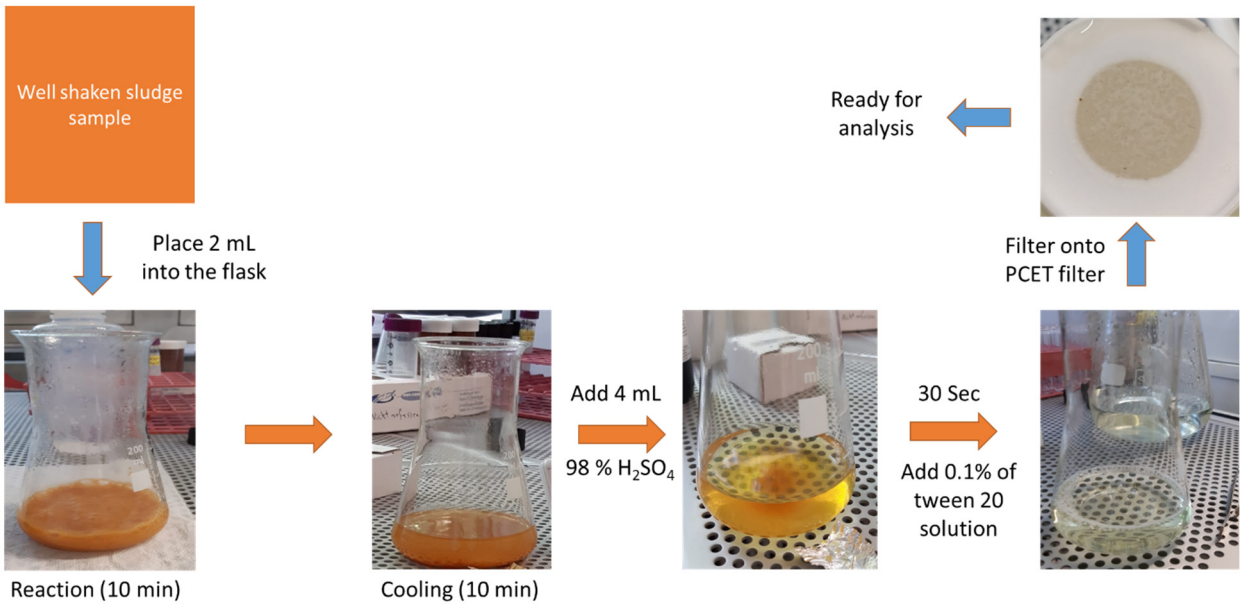


Figure 1. An overview of the Fenton protocol for a 2 mL thickened sludge sample

1.3. Hydrogen Peroxide Protocol

Chemicals

1. Hydrogen Peroxide (30%)
2. Polyethylene glycol sorbitan monolaurate, or Polyoxyethylenesorbitan monolaurate (Tween 20) as a surfactant to aid in rinsing glassware and filters.

Procedure:

1. Two washing bottles are needed (Made of material that is not the polymers being investigated, Fluorinated ethylene propylene (FEP) was used in this case). One bottle is filled with ultrapure water, whereas the second is filled with ultrapure water with 0.1 % (v/v) of

Tween 20.
The surfactant bottle helps rinse particles of off glass surfaces and the filter membrane.

2. The sludge sample is shaken well to ensure mixing and then 2 mL of sludge is taken with a pipette and placed in a suitable test tube or flask (45 mL). Then 20 mL of 30% Hydrogen peroxide solution or (**10:1 ratio**) is added
3. The test tube is sealed and placed in an oven at 60 °C for 24 ± 1 hours.
4. The tubes are removed from the oven, 10 mL of 0.1 % (v/v) Tween 20 is added to dilute the solution and stop further reactions as well as preventing microplastics from adhering to the glass walls of the tubes. Then the sample is filtered in a vacuum filtration unit with a PCTE, 0.2 µm filter. The surfactant bottle can be used here to rinse the contents of the test tube into the filtration unit, as well as any particles adhering to the glass walls of the filtration unit.
5. The sample is ready for analysis. Alternatively, it can be stored as a suspension for later, where the same filter from step 4 can be washed down into a clean tube using the surfactant bottle and then stored for further analysis or filtration on a different kind of filter.

1.4. Potassium hydroxide Protocol

Chemicals:

1. Potassium hydroxide (10% W/W)
2. Polyethylene glycol sorbitan monolaurate, or Polyoxyethylenesorbitan monolaurate (Tween 20) as a surfactant to aid in rinsing glassware and filters.

Procedure:

Exactly the same as the hydrogen peroxide protocol

1.5. Contamination mitigation and cleaning protocol

Material

1. Polyethylene glycol sorbitan monolaurate, or Polyoxyethylenesorbitan monolaurate (Tween 20) as a surfactant to aid in rinsing glassware and filters.
2. Two Fluorinated ethylene propylene (FEP) washing bottles are used, one with ultrapure water containing 0.1 % Tween 20 (v/v) and the other with ultrapure water
3. Additives free washing liquid
4. Small plastic brush
5. Distilled or tap water at higher flow rates for rinsing

Procedure:

1. Using distilled water or tap water, the glassware should be rinsed under running water to remove the visible of the particles (especially if dried up contaminants are found).
2. Using washing liquid and the brush, one should clean the glassware and then rinse them under running water again to remove all visible contaminants.
3. The first and second steps can be eliminated if the glassware in question is free from visible contaminants
4. The final cleaning step uses the Tween 20 washing bottle where the glassware is washed with Tween 20 at least three times and then finally rinsed with the ultrapure water bottle.
5. The glassware can now either be used directly in the next sequence, or it can be dried in an oven at 100 °C and stored till needed.

2. Polymers investigated and their characteristics

Table 2. The reference (control) polymers used for the size distribution analysis

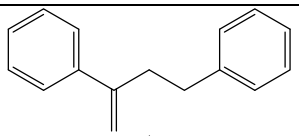
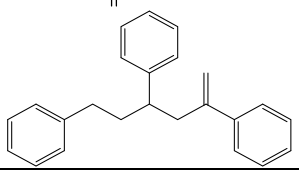
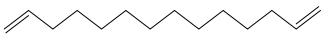
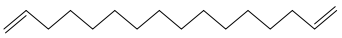
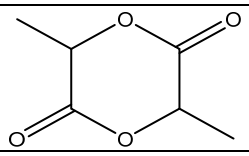
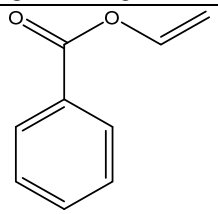
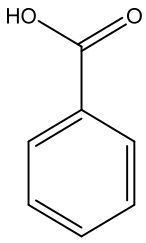
Polymer	Mean diameter μm	D10 D(v,0.1) μm
PA	146.9 ± 3.3	70.6 ± 3.4
PE	115.6 ± 4.4	45.7 ± 11.3
PET	130.9 ± 11.8	48.9 ±11.4
PLA	110.7 ± 1.1	65.9 ±3.2
PS	80.1 ± 1.6	33.2 ± 0.9
PP (KOH analysis)	334.7 ± N.A	105.6 ± N.A
PP (Rest)	190.5 ± 9.4	45.8 ± 6.5
PVC (H_2O_2 analysis)	200.1 ± N.A	125.7 ± N.A
PVC (Rest)	118.8 ± 0.6	54.5 ± 1.8

* There were difficulties in manufacturing PP as well as PVC in the needed quantities. Therefore, two different sizes were used.

3. Results from TD-Pyr-GC-MS

The following characteristic pyrolysis products of the individual polymers were used for identification

Table 3. Characteristic pyrolysis products of the selected polymers for identification

Polymer type	Characteristic pyrolysis products	Formula	m/z (intensity ratio)*	Structure
PS	3-butene-1,3-diyldibenzene (styrene dimer)	$C_{16}H_{16}$	91 (100), 104 (27), 130 (24), 208 (31)	
	5-hexene-1,3,5-triyltribenzene (styrene trimer)	$C_{24}H_{24}$	91 (95), 117 (31), 194 (19), 207 (27)	
PE	1,12-tridecadiene	$C_{13}H_{24}$	55 (52), 81 (44), 67 (38), 95 (26)	
	1,13-tetradecadiene	$C_{14}H_{26}$	81 (42), 95 (27), 109 (13)	
	1,15-hexadecadiene	$C_{16}H_{30}$	55 (63), 81 (50), 96 (45), 69 (37)	
PLA	Lactide	$C_6H_8O_4$	28(75), 45(34), 56(100), 144(1)	
PET	Vinyl benzoate	$C_9H_8O_2$	51 (15), 77(62), 105(100)	
	Benzoic acid	$C_7H_6O_2$	51(18), 77(57), 105(100), 122(99),	

	1,1-Biphenyl	$C_{12}H_{10}$	28 (100), 76 (12), 154(92)
PA	Caprolactam	$C_6H_{11}NO$	55 (79), 67 (11), 85 (61), 113 (100)
PP	2,4-Dimethylhept-1-ene	C_9H_{18}	43 (97), 70 (100), 83 (27), 126 (18)
	2,4,6-trimethyl-1-nonene	$C_{12}H_{24}$	28 (100), 43 (59), 69 (88), 111 (33), 125 (13)

* intensity ratio to largest peak in spectra [%]

4. Images from microscopy

Some of the polymers (especially larger ones like PP) were highly irregularly shaped and determining their sizes only relying on image analysis, especially based on a small number of particles, would be highly error prone. Therefore, this section is meant to only visualize the surface of the particles before and after treatment. For size alterations, please refer to the size distribution analysis.

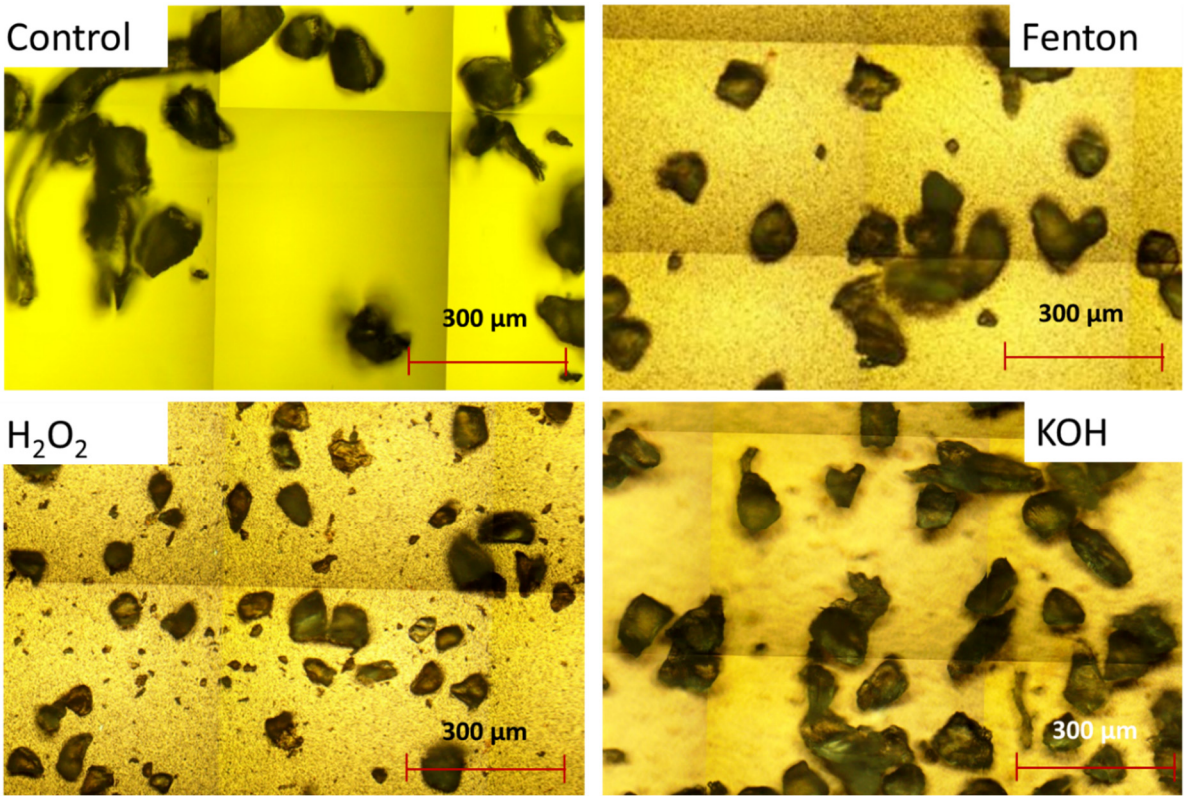


Figure 2. PA: Microscopic images

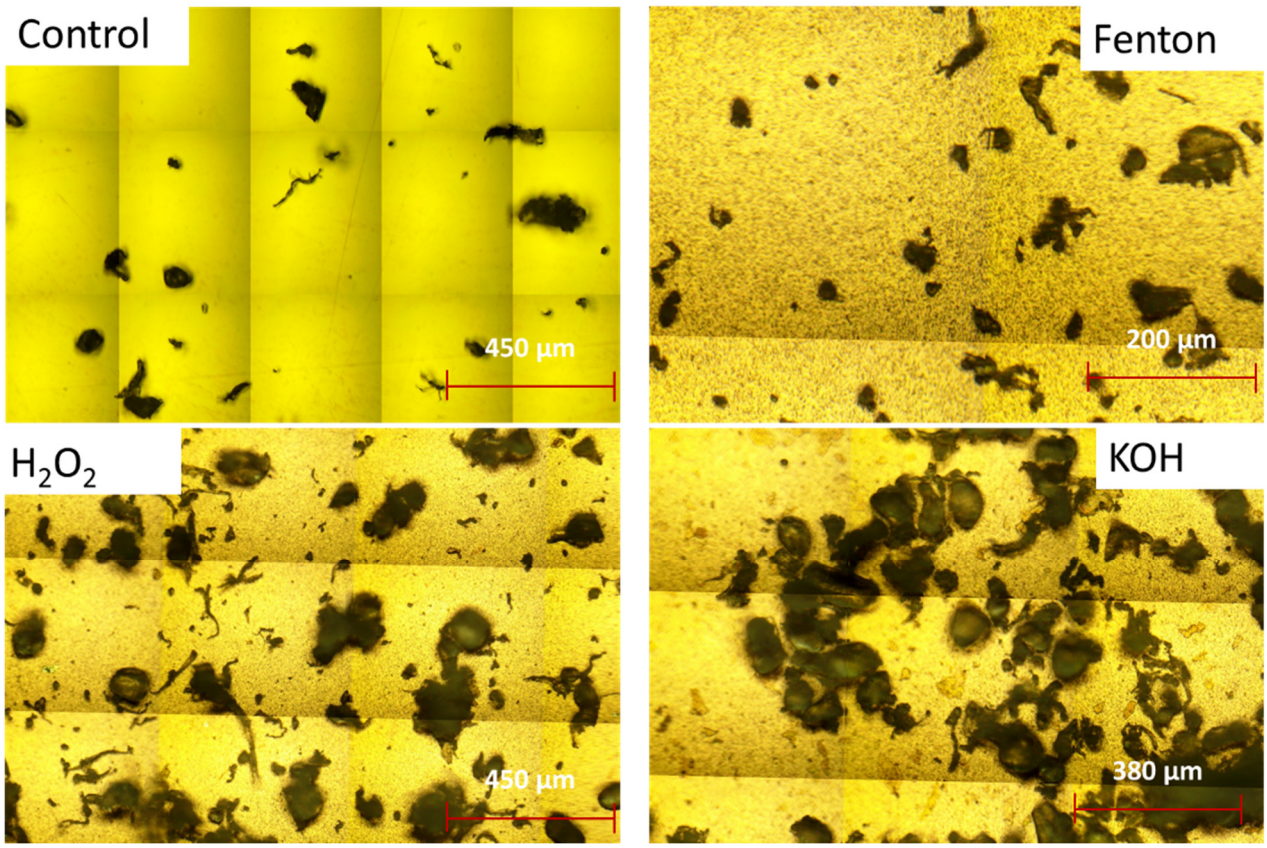


Figure 3. PE: Microscopic images

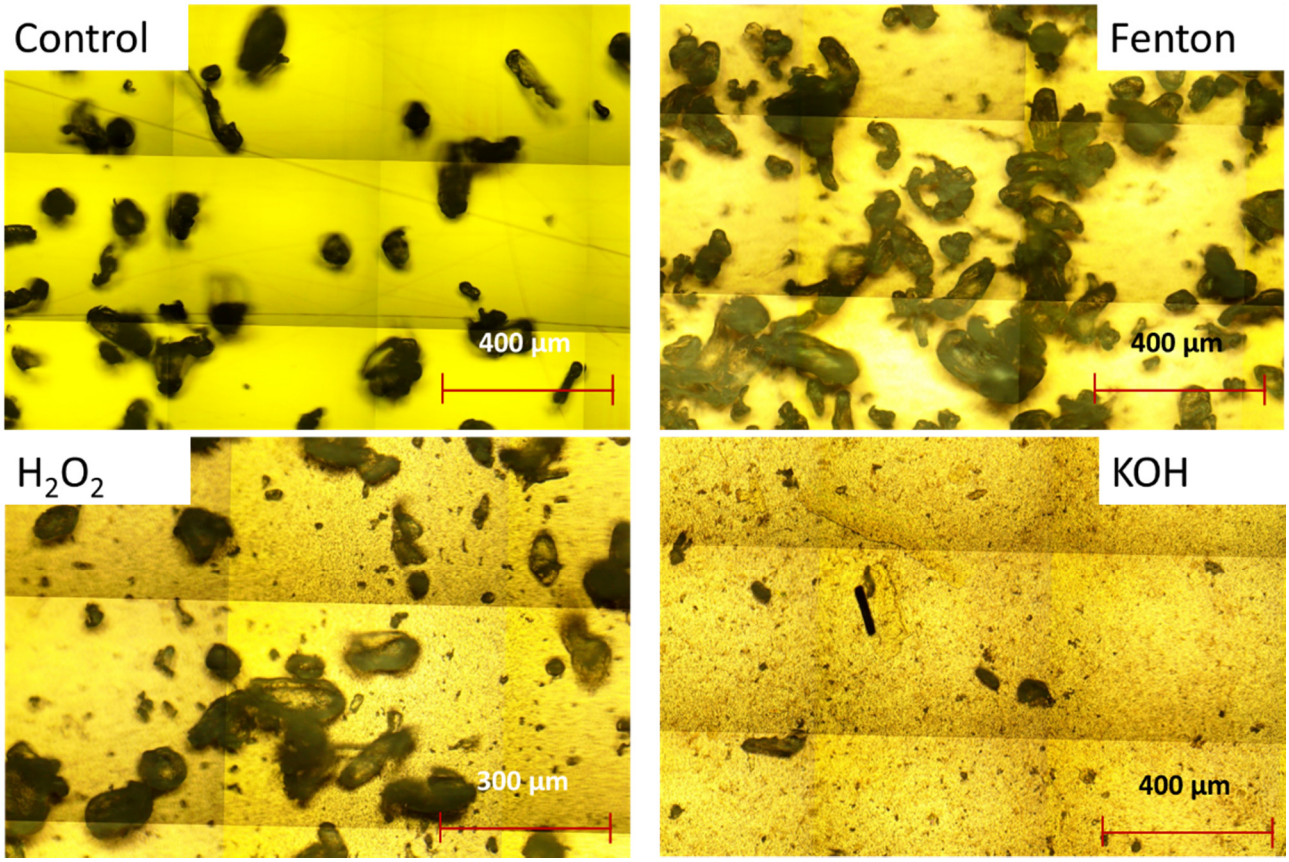


Figure 4. PET: Microscopic images. Most of the particles were destroyed after KOH treatment.

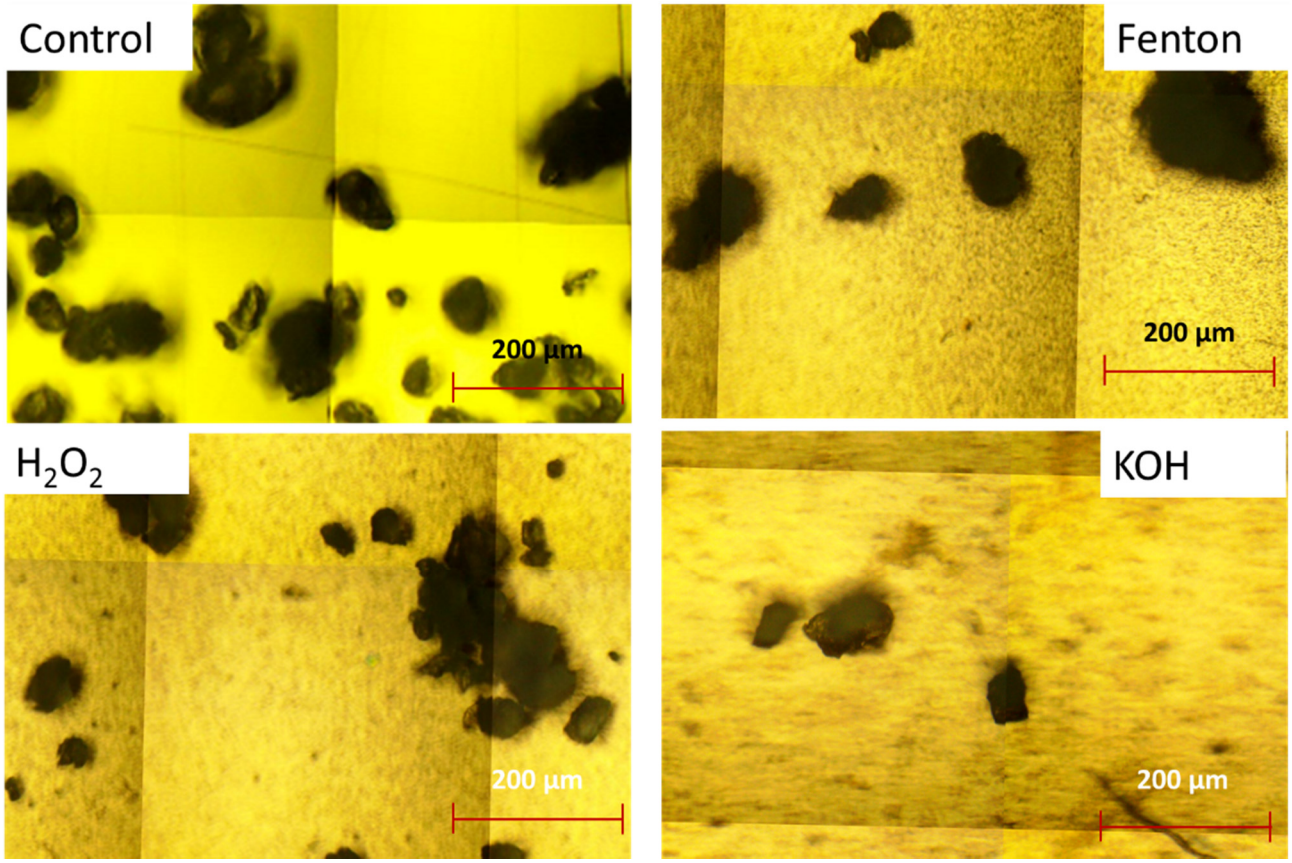


Figure 5. PLA: Microscopic images. Most of the particles were destroyed after KOH treatment.

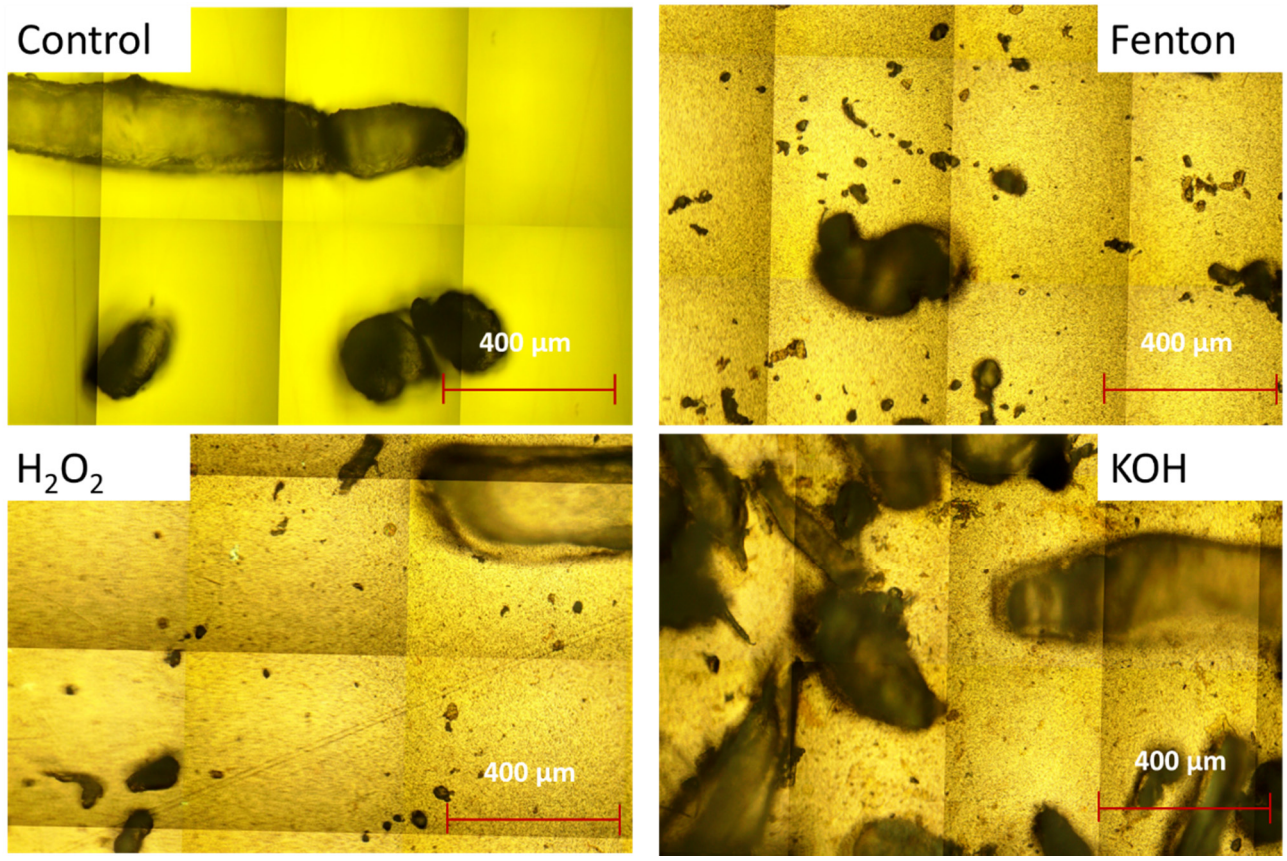


Figure 6. PP: Microscopic images.

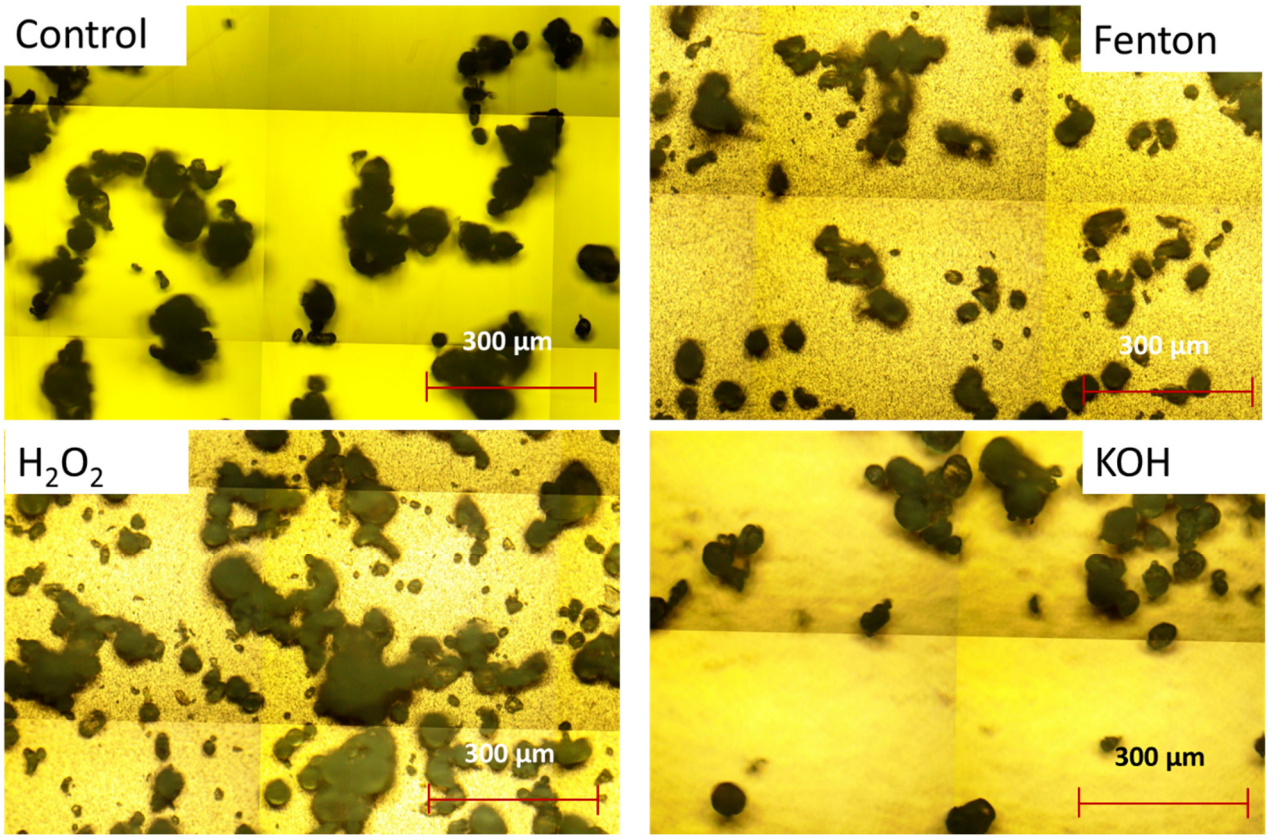


Figure 7. PS: Microscopic images.

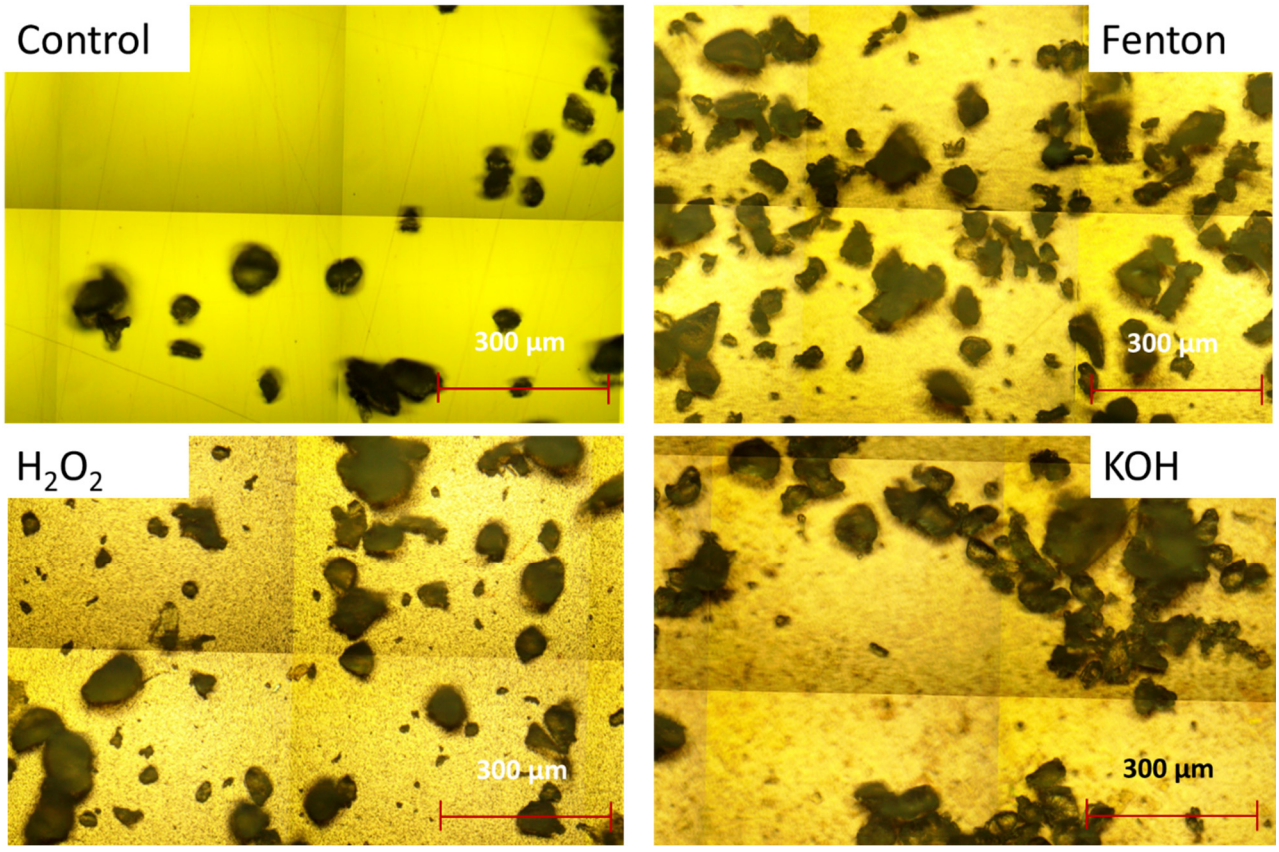


Figure 8. PVC: Microscopic images.

5. μFTIR spectra before and after chemical treatments

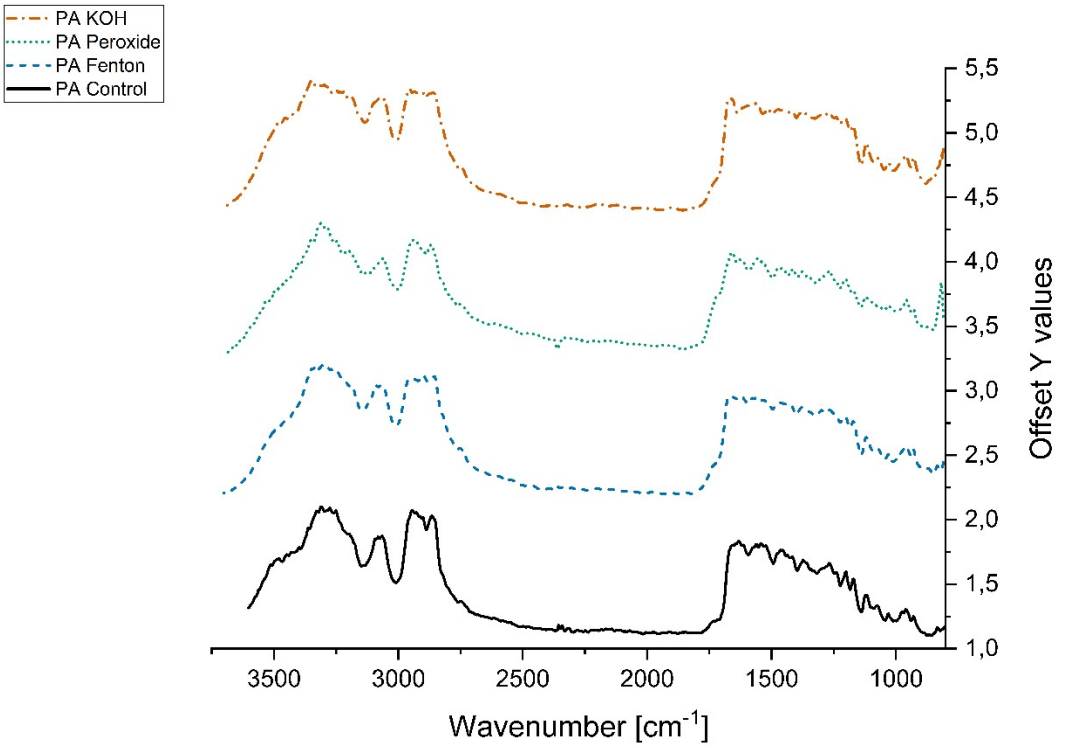


Figure 9. μFTIR Spectra of PA.

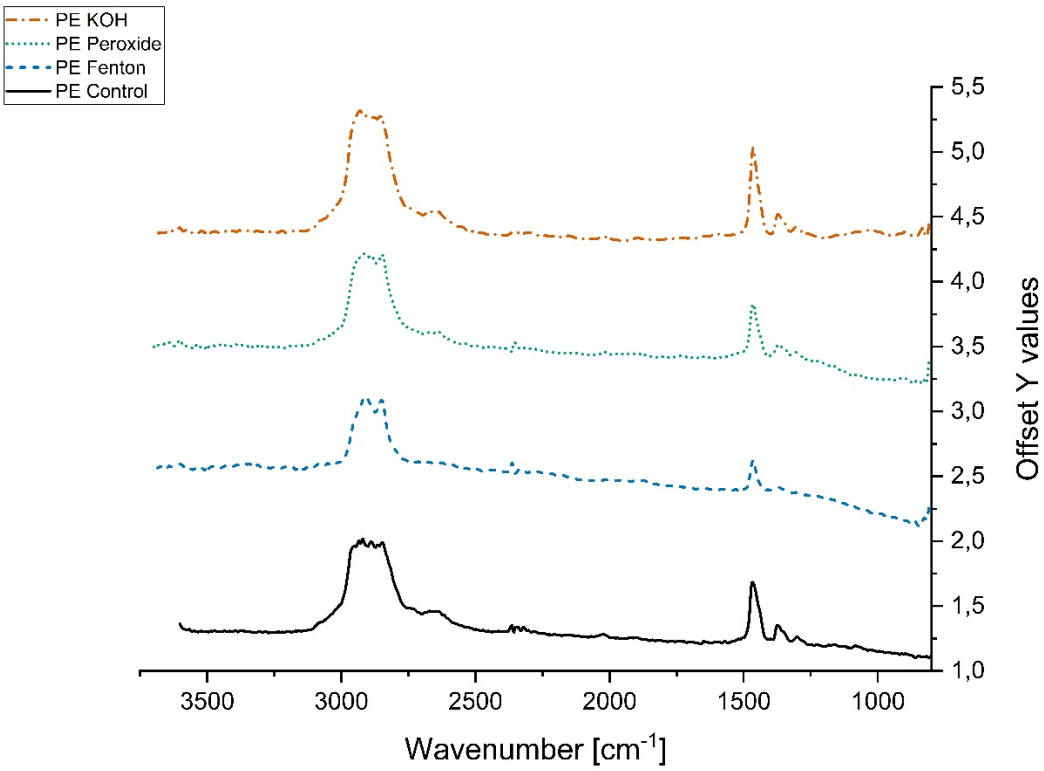


Figure 10. μFTIR Spectra of PE.

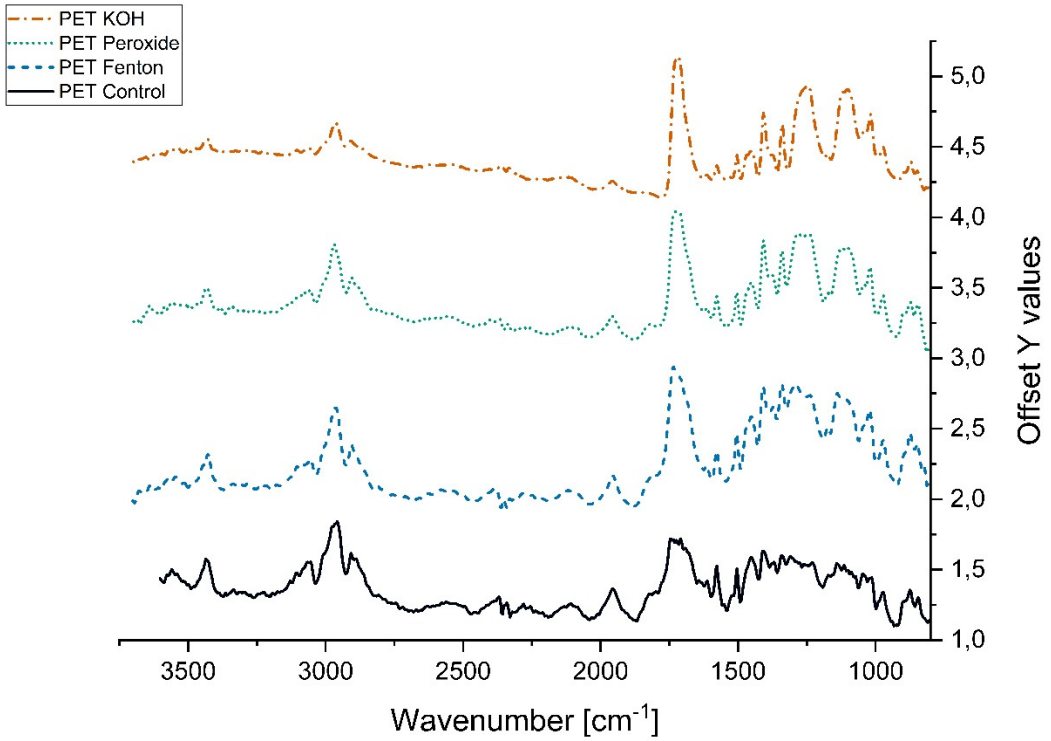


Figure 11. μFTIR Spectra of PET.

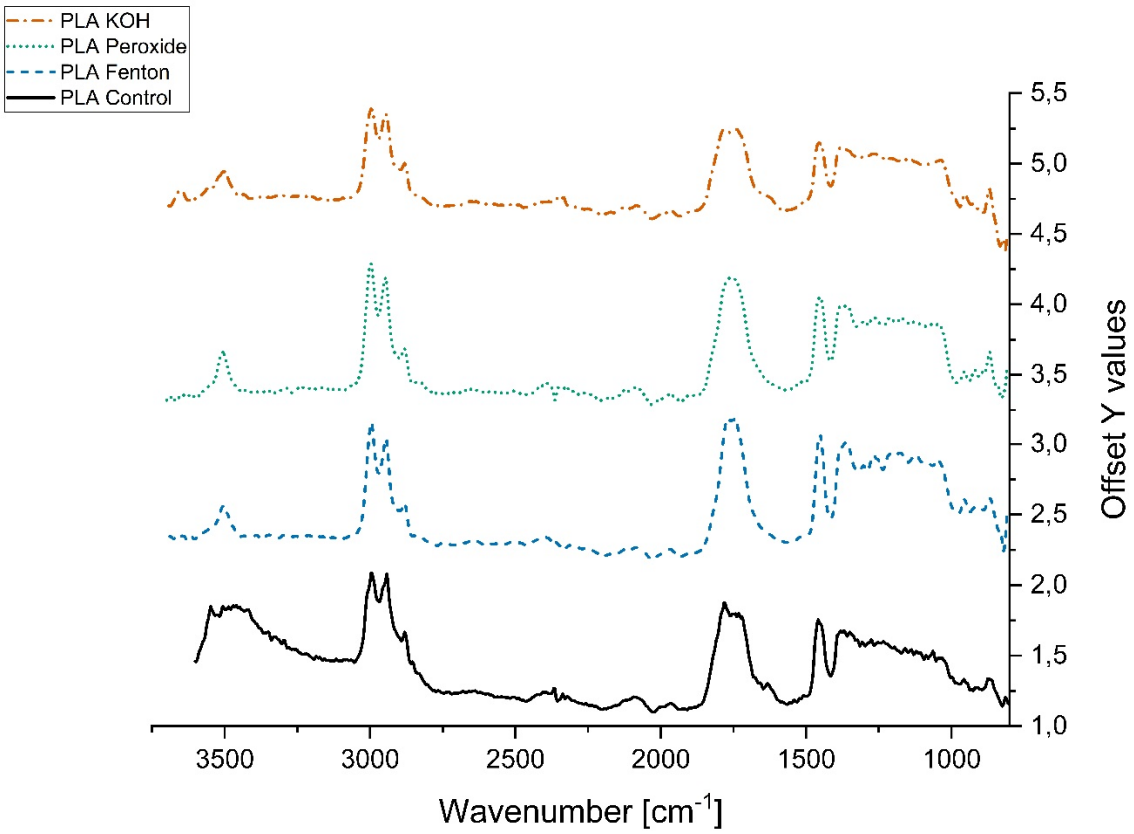


Figure 12. μFTIR Spectra of PLA.

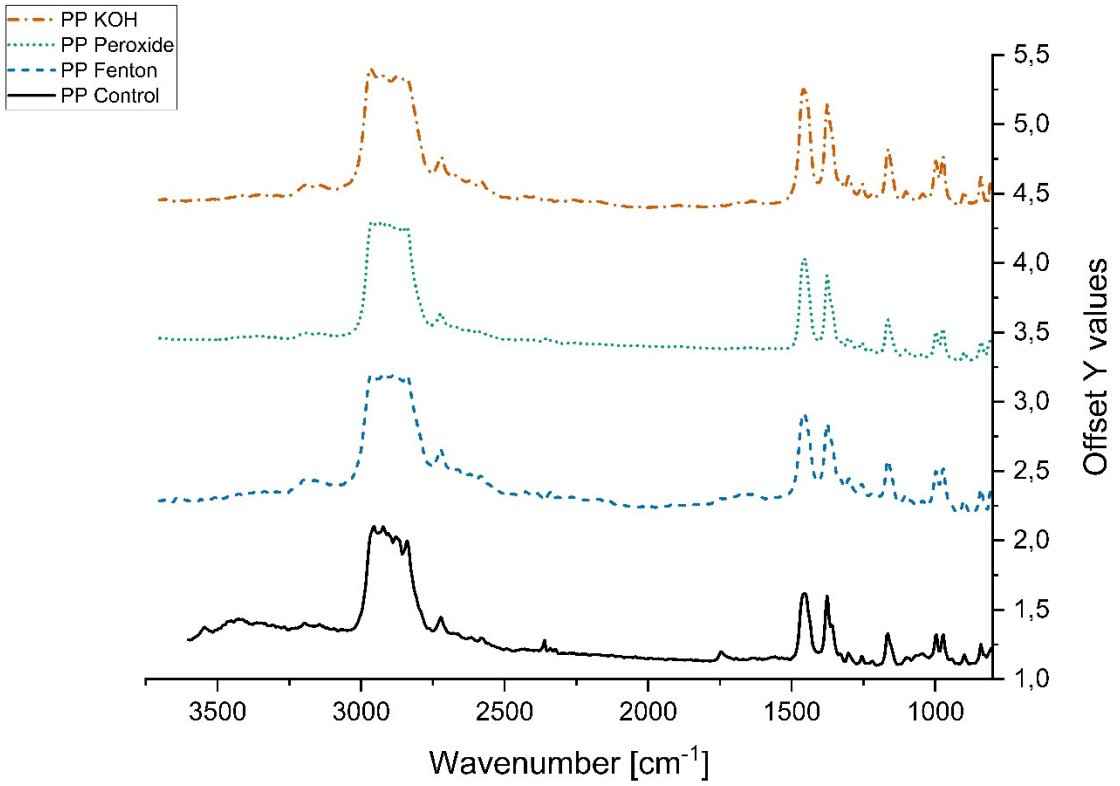


Figure 13. μFTIR Spectra of PP.

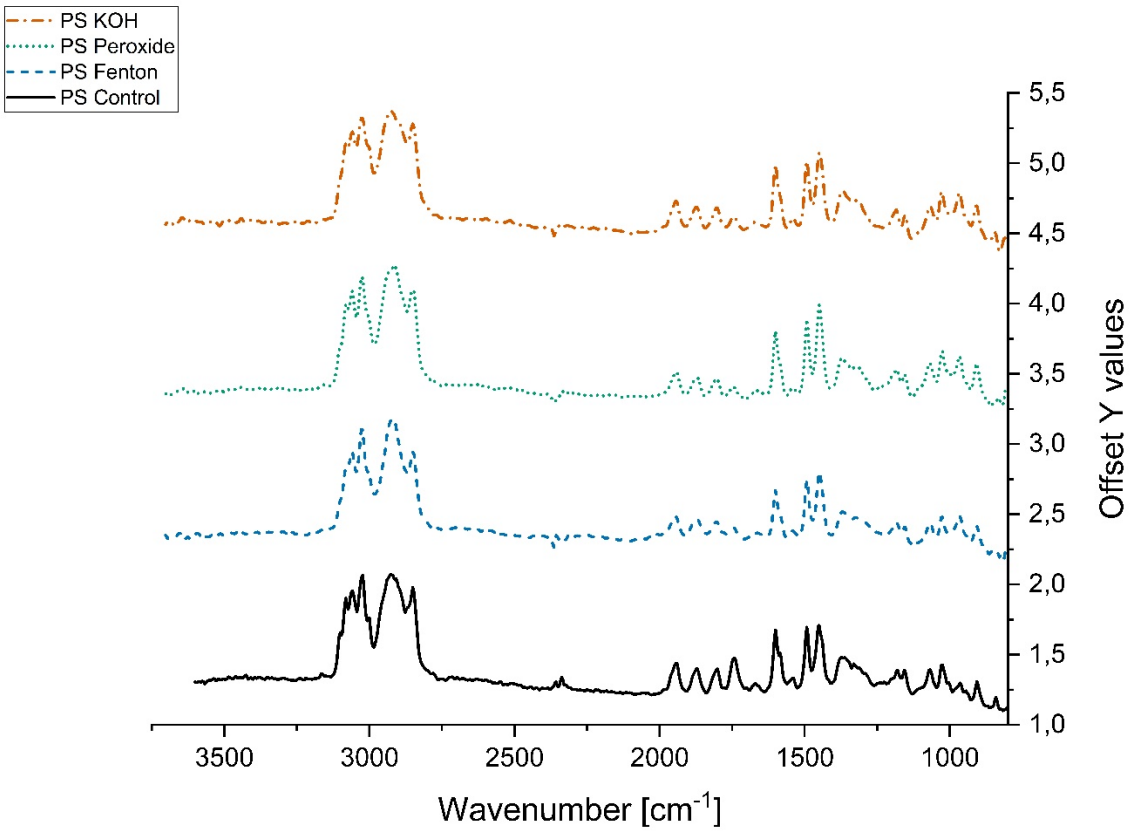


Figure 14. μFTIR Spectra of PS.

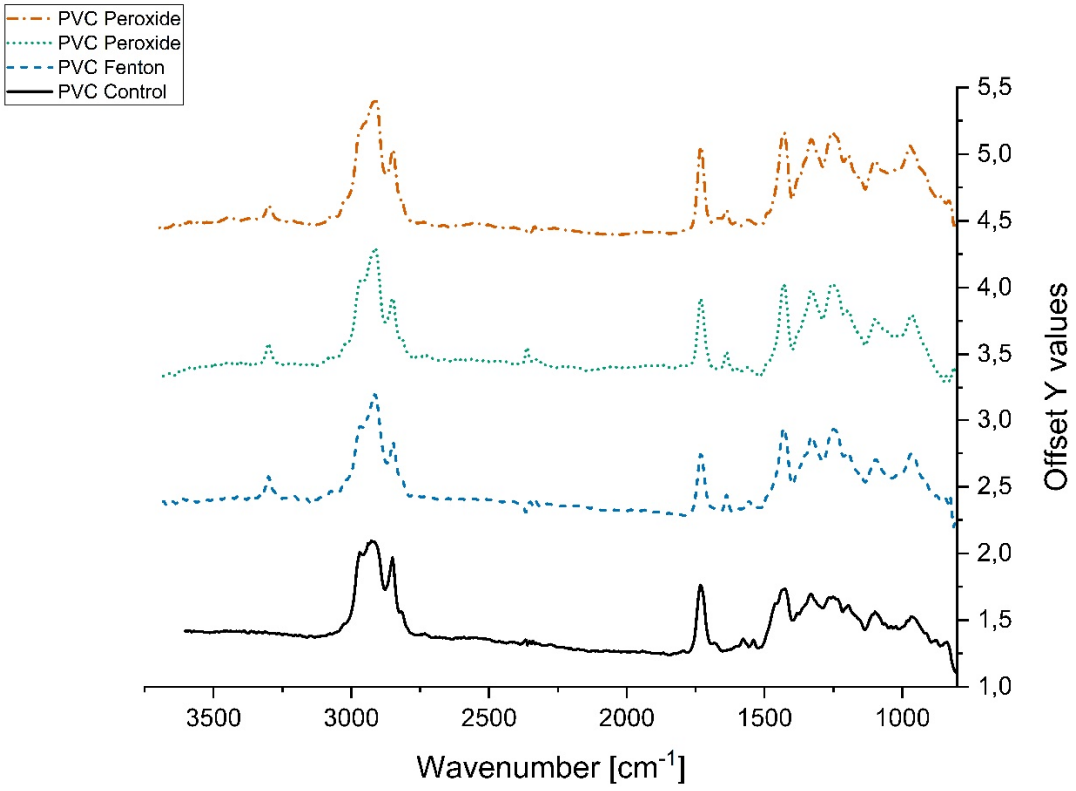


Figure 15. μFTIR Spectra of PVC.

6. Size distribution analysis

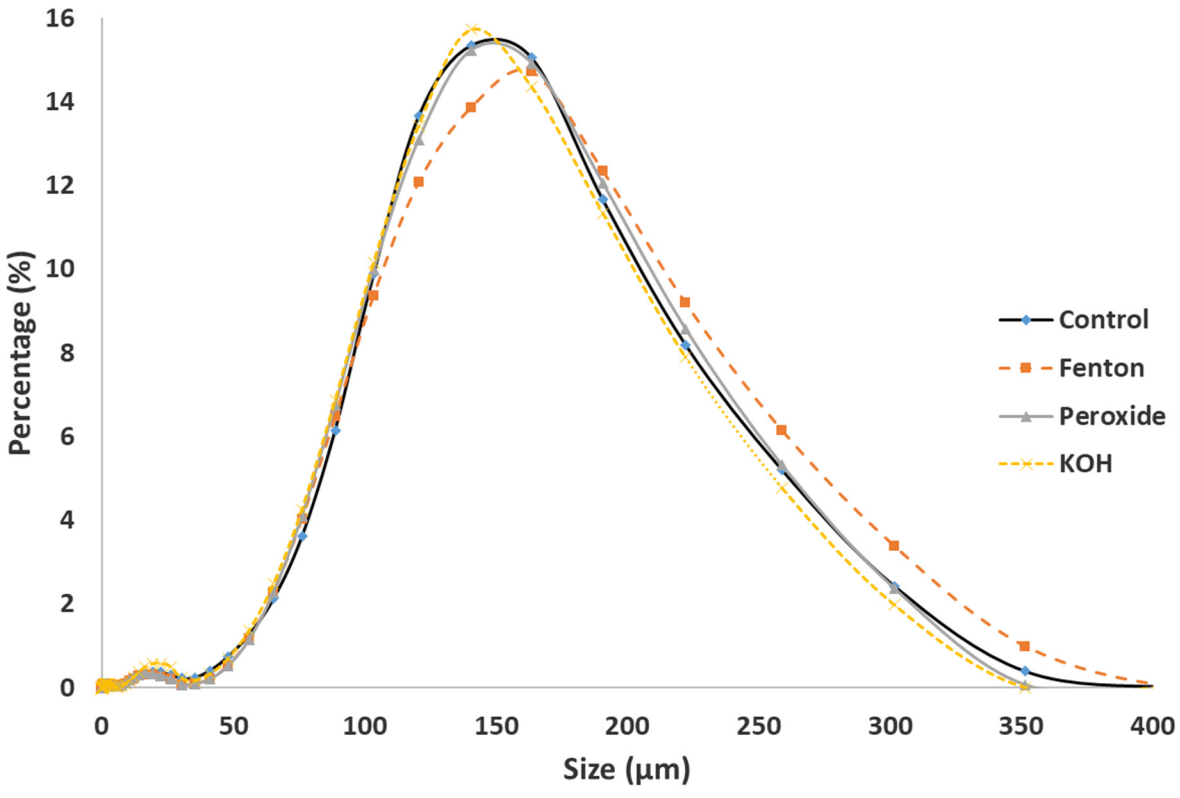


Figure 16. Size distribution analysis (PA).

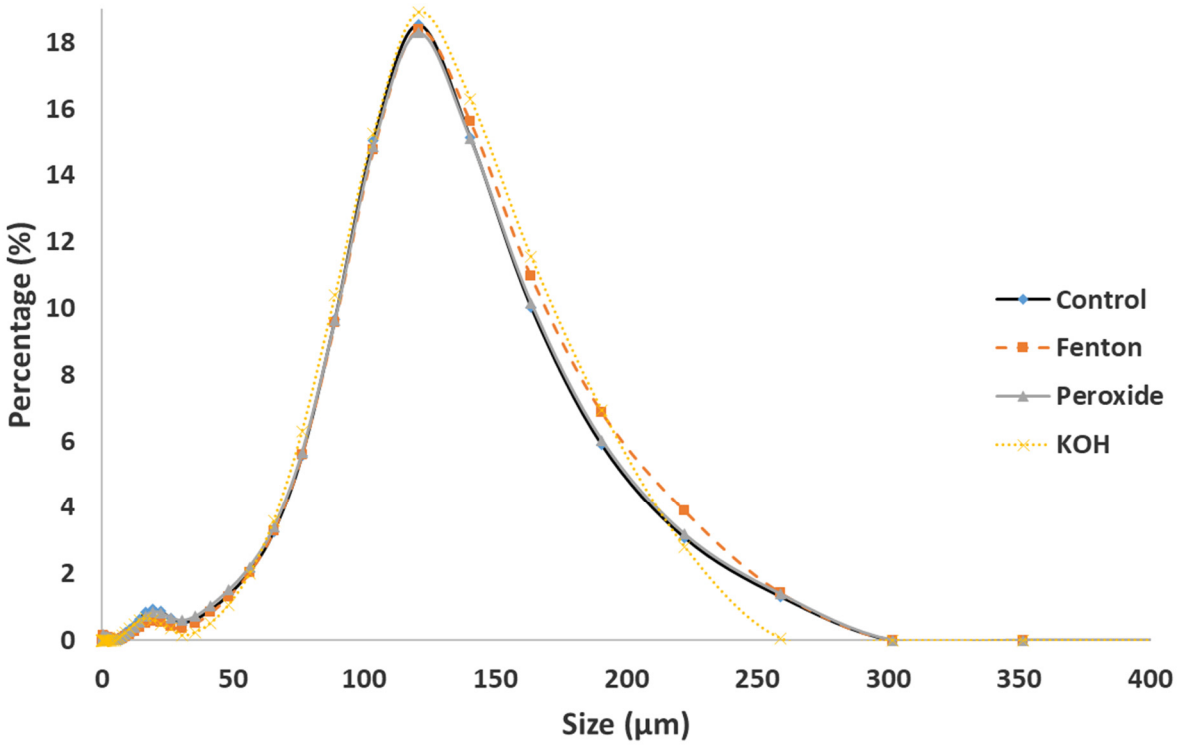


Figure 17. Size distribution analysis (PE).

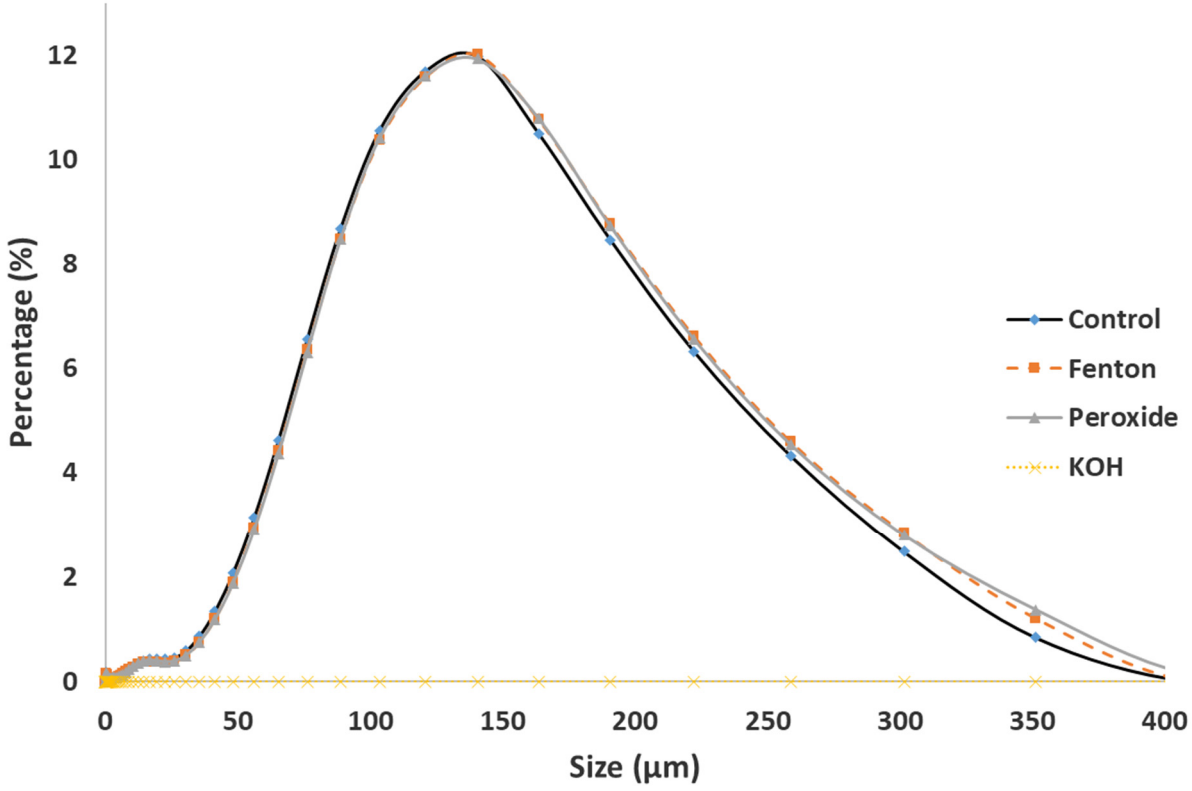


Figure 18. Size distribution analysis (PET). KOH completely dissolved the particles during the tests.

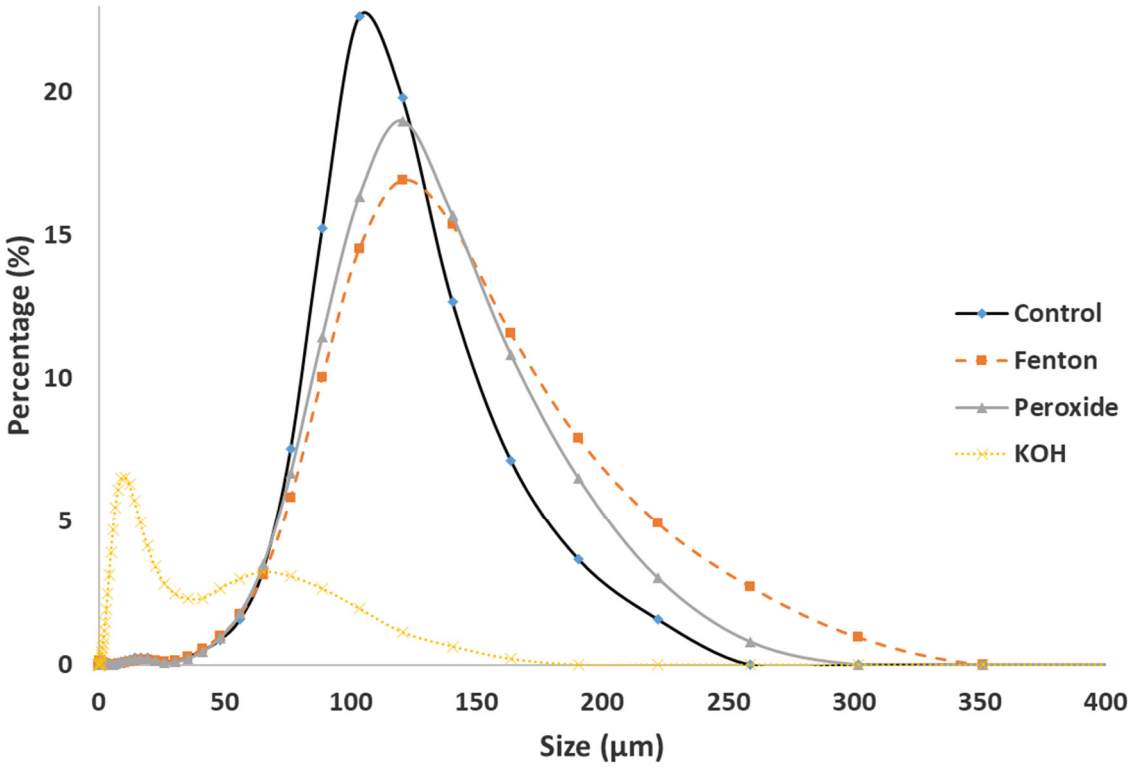


Figure 19. Size distribution analysis (PLA). KOH completely dissolved the particles during the tests.

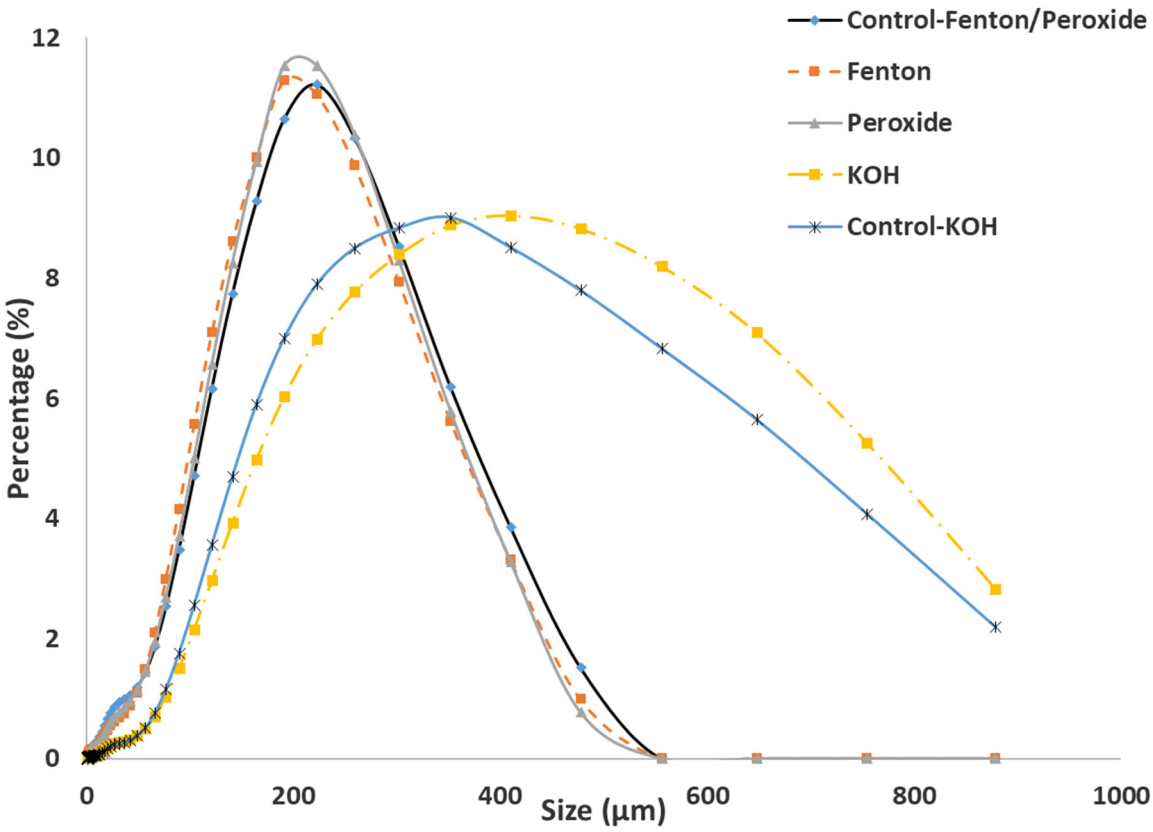


Figure 20. Size distribution analysis (PP). KOH tests were made with a different particle size due to manufacturing difficulties of the microplastics in the needed quantities.

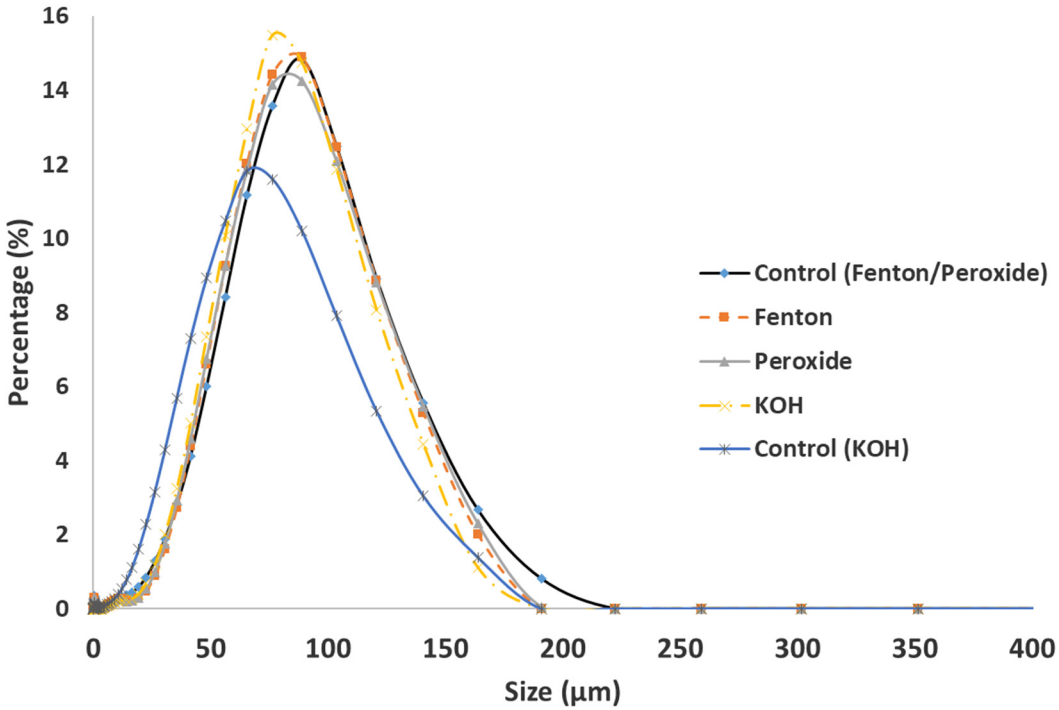


Figure 21. Size distribution analysis (PS). Tests made for KOH were made using a different batch of microplastics that was 18.3% smaller (mean size) than the controls used for Fenton/Peroxide

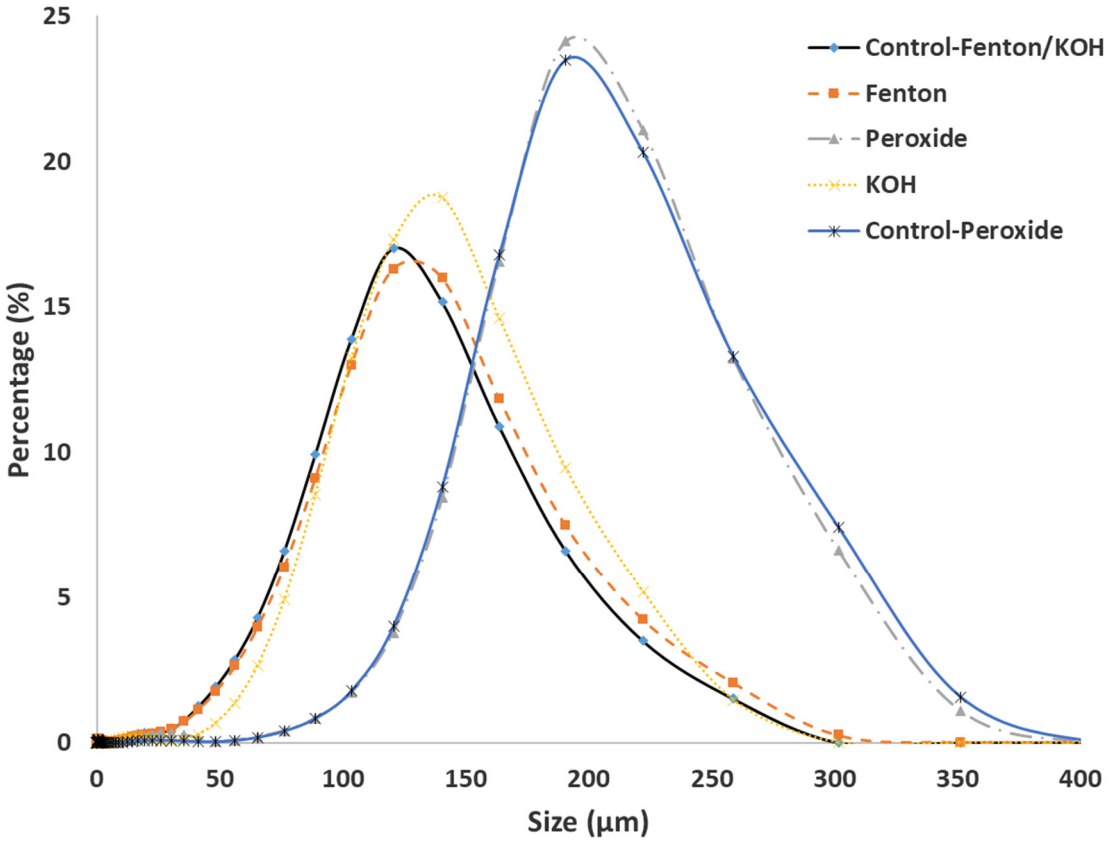


Figure 22. Size distribution analysis (PVC). Peroxide tests were made with a different particle size due to manufacturing difficulties of the microplastics in the needed quantities.

7. Pre-experiments of Organic matter removal efficiency

Figure 23 shows how the sludge did not react very well with Fenton due to it being clumped after drying, resulting in very poor removal visually compared to undried samples.

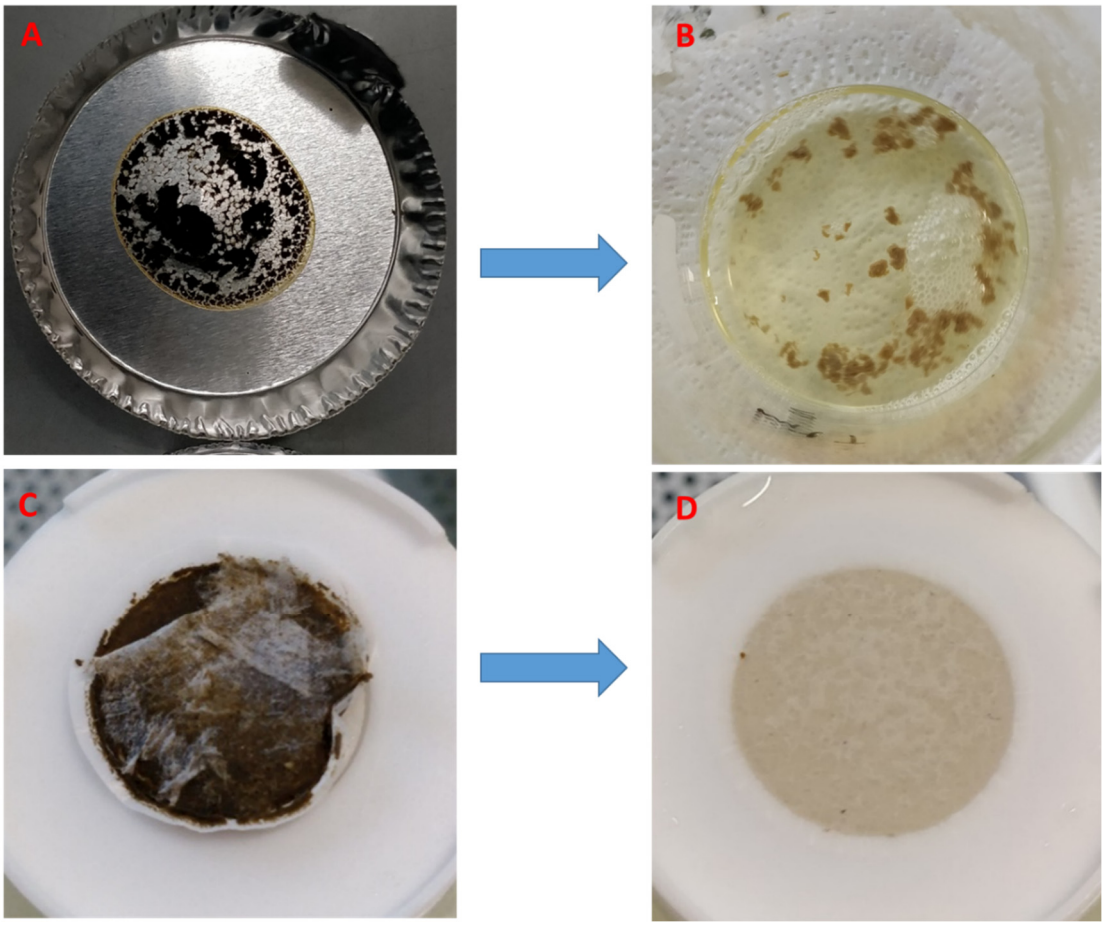


Figure 23. The visual difference between drying 1mL of sludge before treatment Vs. No drying. A: Dried sludge before Fenton's reaction. B: Dried sludge after Fenton's reaction. C: Wet sludge (filtered). D: Wet sludge after Fenton reaction.