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Comparison of cellulolytic enzyme treatment and Fenton oxidation for analysis of microplastics in tire rubber particles

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Abstract

Uncertainties in the quantification of microplastics in various products arise from the applied pretreatment processes. Road dust, a significant source of microplastics, requires precise quantification methods to ensure accuracy. In this study, we examined the impact of pretreatment processes on the accuracy of microplastic quantification in road dust, specifically focusing on tire rubber particles. We compared the effects of cellulolytic enzyme (EZM) and Fenton (FT) treatments by analyzing the changes in particle number, size, shape, and identification accuracy for each treatment. Both treatments increased the number of tire rubber particles, reduced their size, and made them more spherical. Notably, the FT treatment resulted in smaller particle parameters (Feret, Min-Feret, Major, Minor, and Area) compared to the EZM treatment. Identification accuracy also varied, with 89% of tire rubber particles identified after EZM treatment, compared to 51% after FT treatment. Furthermore, microplastic volume was overestimated by 4.5% following EZM treatment and underestimated by 21% after FT treatment. These findings demonstrate that pretreatment procedures significantly influence the accuracy of microplastic quantification. Our study underscores the need for further research to determine whether current microplastic estimates are accurate, as the estimated volume can change due to organic removal processes.

Practitioner Points:

- Pretreatment to eliminate organic materials is necessary for improving the efficiency of microplastic analysis.
- Tire rubber particles (TRPs) are a significant plastic material found in urban surfaces.
- Pretreatment can reduce the size of TRPs and lead to material misidentification of materials.

- Compared to the Fenton oxidation treatment, cellulolytic enzyme treatment results in less particle fragmentation and volume modification.

KEYWORDS
microplastic, misidentification, plastic load estimation, road dust, tire

INTRODUCTION

Microplastics, defined as plastic particles smaller than 5 mm, are pervasive pollutants in various environmental matrices, including in marine, freshwater, and terrestrial ecosystems (Hidalgo-Ruz et al., 2012; Hanvey et al., 2017; Lin et al., 2024; Zarfl, 2019; Zhou et al., 2020). Road dust is a particularly notable source of microplastics, which contains particles from tire wear, road markings, and other vehicular emissions and is continuously deposited (Boucher & Friot, 2017; Burghardt & Pashkevich, 2023; Goßmann et al., 2021; Knight et al., 2020; Parker et al., 2020; Sommer et al., 2018; Yamamoto & Furumai, 2022). These vehicle-traffic-derived particles accumulate on roads, mixing with other matrices, such as with plants and soil carried by rain or wind, resulting in complex road dust (Sugiura et al., 2021; Yamamoto & Furumai, 2022). This complex mixture can then be transported to different environments, posing risks to aquatic organisms and potentially entering the food chain, ultimately impacting human health (Eze et al., 2024; Li, Busquets, & Campos, 2020; López-Martínez et al., 2021).

The microplastics in environmental samples must be accurately quantified to assess their distribution, sources, and ecological impacts (Ahmed et al., 2021; Zhao et al., 2018). When evaluating these microplastics, pretreatment methods involving chemical, enzymatic, or oxidative pretreatments have been designed to remove organic matter and other contaminants from the matrices containing microplastics, facilitating the identification, and quantification of microplastic particles (Akyildiz et al., 2023; Kida et al., 2023; Li, Chen, et al., 2020; Zhao et al., 2018). However, these pretreatments can introduce biases and inaccuracies to the quantification process (Nguyen et al., 2019; Pfohl et al., 2021; Schrank et al., 2022; Tagg et al., 2017; Tsering et al., 2022). For example, Ortiz et al. (2022) observed a 10% decrease in particle weight and substantial changes in the particle sizes of PET, PE, PVC, PP, and EPS after Fenton treatment. Pfohl et al. (2021) reported changes in the tire particle distribution and modifications in the IR spectrum at 1130–975 cm^{-1} and 470–200 cm^{-1} after Fenton treatment; however, the weight distribution was not determined. Considering the physical and chemical changes that may occur during these processes (Kida et al., 2019), the current estimates of microplastic quantities may be over- or underestimated depending on the pretreatment method and material, although the specific effects of these pretreatment processes have not been investigated.

In this context, we aimed to evaluate the impact of two pretreatment methods, enzyme (EZM) and Fenton (FT) treatments, on the accuracy of quantifying the microplastics in road dust. To select target components from the environmental matrix, specific enzymes can be applied to degrade coexisting organic matter (Rocha-Santos et al., 2020). The EZM process in this study combines alkaline hydrogen peroxide (Ho et al., 2019; Mierzejewska et al., 2019) with cellulolytic enzymes to remove plant materials (Paz-Cedeno et al., 2022) present in road dust. The method of organic matter removal using only hydrogen peroxide in samples containing plant material is insufficient for cellulose removal, so other methods to increase oxidative stress (e.g., the use of Fenton's reagent, KOH, etc.) are required (Olsen et al., 2020). The FT method employs a combination of hydrogen peroxide and iron catalysts to achieve oxidative degradation; the FT method is known for its speed and ease of use (Lusher et al., 2020; Pereira et al., 2012). Road dust generally contains plant materials, which reduce the quantitative efficiency of microplastics. Although cellulolytic enzymes can remove plant materials, most studies on road dust have applied only the Fenton treatment. There is a lack of comparative study between FT and EZM treatment from the viewpoint of accurate estimation of microplastics.

Our aim in this study was to elucidate the impact of two pretreatment methods on the accuracy of tire rubber particle quantification. By comparing the effects of the EZM and FT treatments, we aimed to identify the changes in particle number, shape, and volume. Specifically, we focused on assessing how these methods affect the micronization and identification of tire rubber particles to determine a method that minimizes quantification errors and misidentifications. Our findings highlight the importance of selecting appropriate pretreatment methods to increase the accuracy of microplastic analysis; however, further research is needed to refine the identification rates and particle size analyzes for other plastic materials.

MATERIALS AND METHODS

Tire rubber particles

Tire rubber particles were prepared in a laboratory. Regular commercially available tires (Bridgestone, NEXTRY, 145/80R13, 75S) were cut into sawdust samples. The samples passed through a sieve (stainless steel, 500 μm), resulting in tire rubber particles that were of 250 μm in size, which were kept until the experiments. Five replicates (n = 5) of 104 particles were prepared by picking up with tweezers to compare the changes in the particles caused by the two pretreatments. The particles used for the two treatments did not significantly differ in five size parameters (Feret, MinFeret, Major, Minor, and Area) ('before' in Table 1). An example of tire rubber particles is provided in Supplementary Materials Section 4 and Figure S5(c).

Treatments

EZM and FT treatments were compared to evaluate the damage occurring to the tire rubber particles. Pretreatment was performed for three days in both treatments, which is the period used for microplastic extraction from road-derived samples (Sugiura et al., 2021).

The EZM treatment used in this study combined organic removal processes with hydrogen peroxide and cellulolytic enzymes to eliminate plant particles from road dust. We used alkaline-adjusted hydrogen peroxide (AHP), as suggested by Ho et al. (2019), to achieve a faster reaction than that achieved with hydrogen peroxide alone. As an AHP solution, 30% hydrogen peroxide (FUJIFILM Wako Pure Chemical Corporation, Wako 1st Grade, product number 080–01186) was adjusted to pH of 7.0 ± 0.5 using 1 M sodium hydroxide solution to enhance the organic matter degradation. Tire rubber particles were immersed in this 100 ml AHP solution (less than 5% w-sample/v-AHP solution) within a disposable conical tube with a bent cap (polypropylene, VWR) for a three-hour reaction in a water bath at 50°C. The particles were collected by filtration through a 150 μm stainless-steel etching screen (custom-made, Anping Country Bolin Metal Wire Mesh Co., Ltd.) after the incubation. The collected particles were placed into a 30 ml citric acid buffer solution (less than 5% w-sample/v-buffer, pH 4.25, FUJIFILM Wako Pure Chemical Corporation, product number 199–07185) with ×10 diluted Cellic® CTec2 cellulase enzymes (0.15 ml, aqueous solution, Sigma-Aldrich, product number SAE0020-50Ml) (less than 33.3% w-sample/v-enzyme, modified from Paz-Cedeno et al., 2022). The buffer solution containing particles and enzymes was incubated in a conical tube with a bent cap (polypropylene, VWR) a 50°C for three days. The buffer solution was then filtered through a mixed cellulose ester (1 μm, ADVANTEC) to collect the particles the particles on the screen were dried in a 50°C oven.

The procedure described by Sugiura et al. (2021) was adopted for FT treatment in this study. The FT treatment commenced by immersing tire rubber particles in 30% hydrogen peroxide (15 ml, less than 7% w-sample/v-H_2O_2), followed by an incubation period exceeding one day at 35°C. This oxidation procedure was carried out in 50 ml centrifuge tubes (Corning) that had been covered with glass to avoid evaporation. Subsequently, 0.5 ml of 50 mM $FeSO_4{\cdot}7H_2O$ solution was added to achieve the Fenton reaction. The particles in the Fenton reagent were kept in an incubator at 1200 Lux and 35°C for three days in glass beakers. Each sample was filtered with a 150 μm stainless-steel etching screen then the screens were dried in a 50°C oven. The reason to stop sizing the particle at 150 μm is a technical limitation in ATR-FTIR analysis (Cabernard et al., 2018; Simon et al., 2021).

TABLE 1 The measured particle size parameters (average of medians in five replicates) before/after the EZM and FT treatment.

	EZM (n = 5)			FT (n = 5)		
	Before	After	(After-Before) /Before	Before	After	(After-Before)/Before
	Avg. (%CV)	Avg. (%CV)	%	Avg. (%CV)	Avg. (%CV)	%
Feret (μm)	718 (1.63)	664 (9.34)	−7.52	734 (4.11)	571 (14.8)	−22.2
MinFeret (μm)	446 (4.10)	412 (6.26)	−7.62	446 (5.38)	319 (16.1)	−28.5
Major (μm)	617 (2.45)	573 (7.63)	−7.13	634 (3.08)	508 (13.7)	−19.9
Minor (μm)	363 (6.83)	337 (4.33)	−7.16	362 (4.72)	266 (15.5)	−11.6
Area ($\times 10^4$ μm^2)	17.9 (1.50)	16.7 (2.36)	−7.06	18.8 (1.56)	11.2 (3.96)	−40.3
Averaged change			−7.30			−27.5

The %CV indicates the coefficient of variation in percentage ($\frac{standard\ deviation \times 100}{average}$).

Morphological changes

The particles were manually placed on a grid to observe their morphology; a detailed description and picture of the grid are provided in the Supplementary Materials (Section 4 and Figure S5). Imaging was performed using a SHIMADZU STZ-161-TLED microscope equipped with a 3R-DKMC04 camera. An LED light was attached to the photo studio box (ottostyle.jp) to avoid the shadow.

The obtained images were processed using ImageJ software (ImageJ 1.53 k, 64-bit, National Institutes of Health, USA) for binarization. Various parameters, including Feret and MinFeret size (longest and shortest dimension of Feret's diameter), area (projected area), major and minor axes, circularity (4[area/perimeter^2]), and solidity (area/convex hull area), were quantified using ImageJ software. The numbers of particles were compared by the average of five replicates after each treatment, whereas the other five size factors were compared using the median value of all the acquired particles in each replicate. Circularity and solidity were assessed for their proximity to a value of one to determine how close the particles were to being spherical. The pretreatment was expected to cause rounding due to particle damage, so specific shape factors were used to quantify this effect. To test for statistical significance, Prisms 9 and 10 were used. Two-way ANOVA was used, followed by Tukey's test for post-hoc analysis. Normality was assessed using the Shapiro–Wilk test (α=0.05, $p < 0.05$). If the data did not follow a normal distribution, the Kruskal–Wallis test was conducted, and significant differences were further analyzed using Dunn's test (α=0.05).

Material identification

After acquiring images to measure particle size, the IR spectrum of the material was analyzed. An FTIR (Agilent Cary 630 FTIR) equipped with an ATR diamond prism was used to acquire the IR spectra. The scan ranged from 650 to 4000 cm^{-1} with a resolution of 8 and 16 cm^{-1} for tire rubber particles before and after treatment, respectively. All samples were scanned 32 times. The obtained IR spectra were analyzed using triangular apodization and Mertz-phase compensation.

We used four installed libraries (Poly_D, ATR Demo, Polymer Handheld ATR, and Elastomer Oring and Seal Handheld ATR), along with a manual tire library (Yamamoto & Furumai, 2022) to identify the materials. A hit quality index (HQI) of 0.7 (Yukioka et al., 2020) was set as the threshold for material identification. Particles with low HQI (<0.7) were classified as "unidentified". Among the identified particles, particles not identified as tire rubber by the tire library were categorized as other plastic. Particles classified as unidentified here are tires that are not identified as plastics because of spectral alterations, while particles classified as "other plastics" are tires that are misidentified as other plastics due to spectral changes. Particles mistakenly identified as other plastics due to pretreatment, yet not affecting the total microplastic quantification, were classified as "other plastics." Conversely, particles that did impact the microplastic quantification were classified as "unidentified." Fifteen tire particles before treatment were analyzed and confirmed to be correctly identified as tire rubber.

Quantification of microplastic volume

The particle volume was used to assess the pre- and post-treatment changes in the microplastics. The comparison was based on the volume calculated using the particle area, assuming a spherical shape, as described by Simon et al. (2018). The calculation methods for the other three approaches are detailed in the Supplementary Materials (Section 2 and Table S2). The volume was calculated for all the particles with a measured size.

RESULTS

Morphological changes in tire rubber particles

To clarify the morphological changes in tire particles after the two treatments, we compared the number, size, and shape factors (Figure 1). The number of particles increased by 25% and 15% after EZM and FT treatments respectively, but this difference was not statistically significant (two-tailed t-test (unequal variance), $p = 0.188$; Figure 1(a)).

Particle size was calculated by averaging the median values of each replicate. Regardless of the treatment, the five particle size factors (Feret, MinFeret, Major, Minor, and particle area) significantly decreased after treatment as shown in Figures 1(b) and S1(a-d) (Two way ANOVA, Tukey's test [$p < 0.05$]). All the particle size factors were significantly smaller after FT treatment than in EZM treatment (Table 1). The %CVs for all the size parameters of the particles were greater in the FT treatment than EZM treatment. The average factor changes after EZM and FT treatment were −7.30% (−7.06 to −7.62%, Table 1) and −27.5% (−11.6 to −40.3%, Table 1), respectively. The largest change of 40.3% was observed for the particle area after FT treatment (Table 1). The particle size distribution in Feret size (Figure 1(c)) confirms that

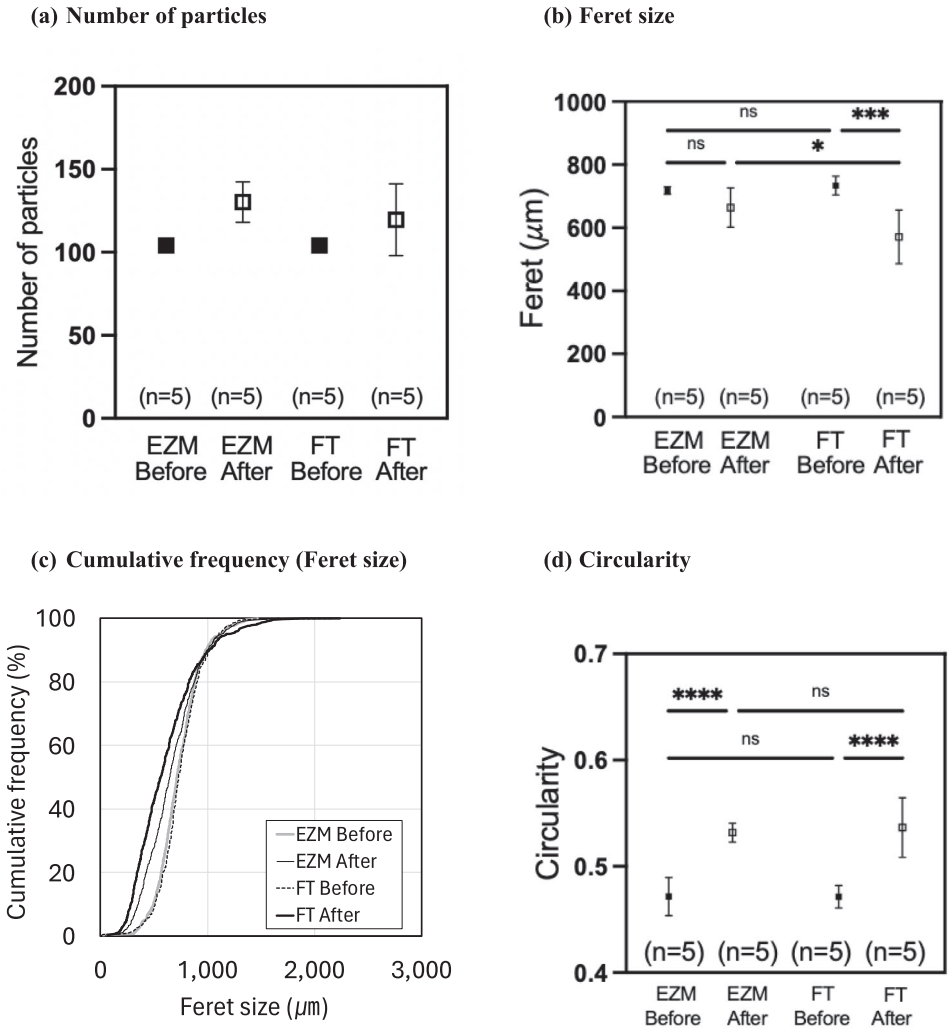

FIGURE 1 Morphological changes before and after EZM and FT treatments. (a) Number of particles, (b) Feret size, (c) cumulative frequency of Feret size for pooled samples of five replicates, and (d) circularity. Plots and error bars in (a), (b), (d) are average and SD for five replicates. Statistical significance (two-way ANOVA, Tukey's test) is indicated as follows: * ($p < 0.05$), *** ($p < 0.001$), and **** ($p < 0.0001$).

the size changes occurred more after FT treatment than after EZM treatment. The sum of the particle areas became less after FT treatment (Figure S1(f)).

The shape parameters, circularity (Figure 1(d)) and solidity (Figure S1(e)), were also assessed after treatment. Both shape parameters increased significantly after both treatments, suggesting that the tire rubber particles became more spherical. However, there was no significant difference in shape parameters between the EZM and FT treatments.

Mis- or unidentified particles after treatments

The material identification results after the treatments are summarized in Table 2. The obtained IR spectra (averaged) are shown in Figure S2. 89 ± 4.4% and 51 ± 5.6% of the particles after EZM and FT treatment were correctly identified as tire rubber. The misidentification rate (to the other plastic materials, not in the tire library) was notably higher after FT treatment (13 ± 5.9%) than after EZM treatment (3.4 ± 2.2%). Particles of which the material could not be identified (HQI < 0.7) were more frequently observed in the FT treatment (36 ± 2.5%). The sum of misidentified and unidentified particles accounted for 10.9% (=3.4% + 7.5%) and 49% (=13% + 36%) of EZM and FT-treated particles respectively (Table 2).

Figure 2 shows the number of particles that were misidentified after the treatments. Six plastic materials were obtained by the library search after the FTIR analysis: EPDM, chlorobutyl, hypalon, neoprene, HNBR, and NBR. The most frequently misidentified material was neoprene both after EZM and FT treatment.

Size–material distribution

To investigate whether the observed increase in misidentification and unidentification rate after the FT treatment in the previous section was influenced by the reduction

TABLE 2 Number of collected tire particles after EZM and FT treatment with material identification results by FT-IR analysis.

		EZM						FT					
		Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Avg. ± S.D	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Avg. ± S.D
Number of collected particles	(a)	144	120	142	127	118	130 ± 12	112	154	112	96	124	120 ± 22
Number of identified materials as	(b)	122	115	129	110	103	116 ± 10	60	84	51	54	54	61 ± 13
Other plastic	(c)	10	2	4	5	2	4.6 ± 3.3	7	15	20	10	25	15 ± 7.3
Unidentified	(d)	12	3	9	12	13	10 ± 41	45	54	41	32	45	43 ± 8.0
Loss	(e)	0	0	0	0	0	0.0 ± 0.0	0	1	0	0	0	0.2 ± 0.4
Tire identified rate (%)	$\frac{(b)}{(a)-(e)}$	85	96	91	87	87	**89 ± 4.4**	54	55	46	56	44	**51 ± 5.6**
Misidentified rate (%)	$\frac{(c)}{(a)-(e)}$	6.9	1.7	2.8	3.9	1.7	**3.4 ± 2.2**	6.3	9.7	18	10	20	**13 ± 5.9**
Unidentified rate (%)	$\frac{(d)}{(a)-(e)}$	8.3	2.5	6.3	9.4	11	**7.5 ± 3.3**	40	35	37	33	36	**36 ± 2.5**

Note: Misidentification was defined for other plastics than the polymers in the tire library. The particles identified with HQI < 0.7 were classified as unidentified.

in particle size, we depicted the relationship between the particle Feret size and the material identification result (Figure 3). The statistical analysis showed that all particles did not follow a normal distribution (Shapiro–Wilk test, $p < 0.05$), so comparisons were made using median values.

Among the enzyme-treated (EZM) particles, the median size of particles classified as "other plastics" (339 μm) was significantly smaller than that of tire rubber particles (663 μm) and unidentified particles (527 μm) (Kruskal–Wallis test; $p < 0.0001$, Dunn's test; adjusted $p < 0.001$). Similarly, in the FT treatment, the median size of particles identified as "other plastics" (357 μm) was significantly smaller than that of tire rubber particles (606 μm) and unidentified particles (597 μm) (Kruskal–Wallis test; $p < 0.0001$, Dunn's test; adjusted $p < 0.001$).

The size difference between tire rubber particles and unidentified particles was relatively small compared to the difference between "other plastics" and tire particles. In the FT treatment, this difference was not statistically significant (Dunn's test; adjusted $p > 0.9999$).

We hypothesized that the treatments applied in this study would lead to polymer degradation and further particle micronization. The observed size differences among the three categories suggest that less damaged tire rubber particles may become unidentified, while more damaged (i.e., smaller) particles are misidentified as other polymers. Generally, smaller particles tend to produce less accurate IR spectra for technical reasons, making material identification less reliable (Lusher et al., 2020; Renner et al., 2018). This could be one of the reasons for the increased misidentification observed after FT treatment. However, size change alone did not always correlate with misidentification. Therefore, we conclude that even relatively mild damage without significant particle size reduction due to FT treatment could lead to an underestimation of tire rubber particle numbers in road dust samples.

Quantification of microplastics

Four calculation approaches were used to estimate the particle volume based on the observed particle size and identified materials (Table 3). The total volume of the tire rubber particles before treatment ranged from 3.5 mm^3 (ellipsoid with major and minor) to 18 mm^3 (sphere with major) for the EZM treatment and 3.4 mm^3 (ellipsoid with major and minor) to 20 mm^3 (sphere with major) for the FT treatment (Table 3-(a)). The geometric mean of the estimated volumes in four different approaches was 7.2 and 7.3 mm^3 for the tire rubber particles before

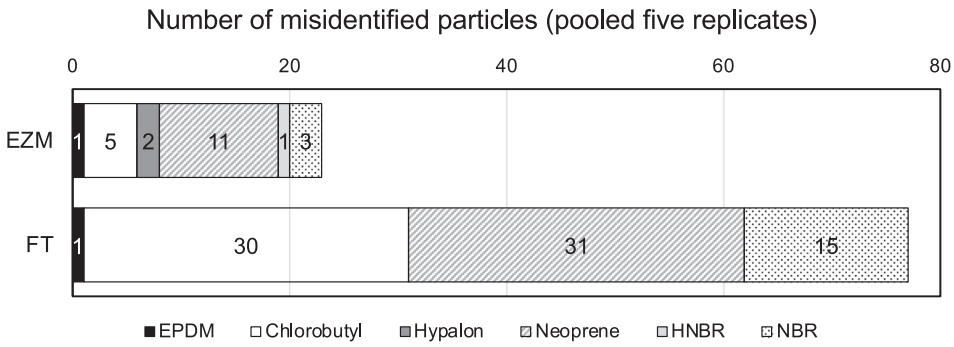

FIGURE 2 Number of misidentified materials after EZM and FT treatment.

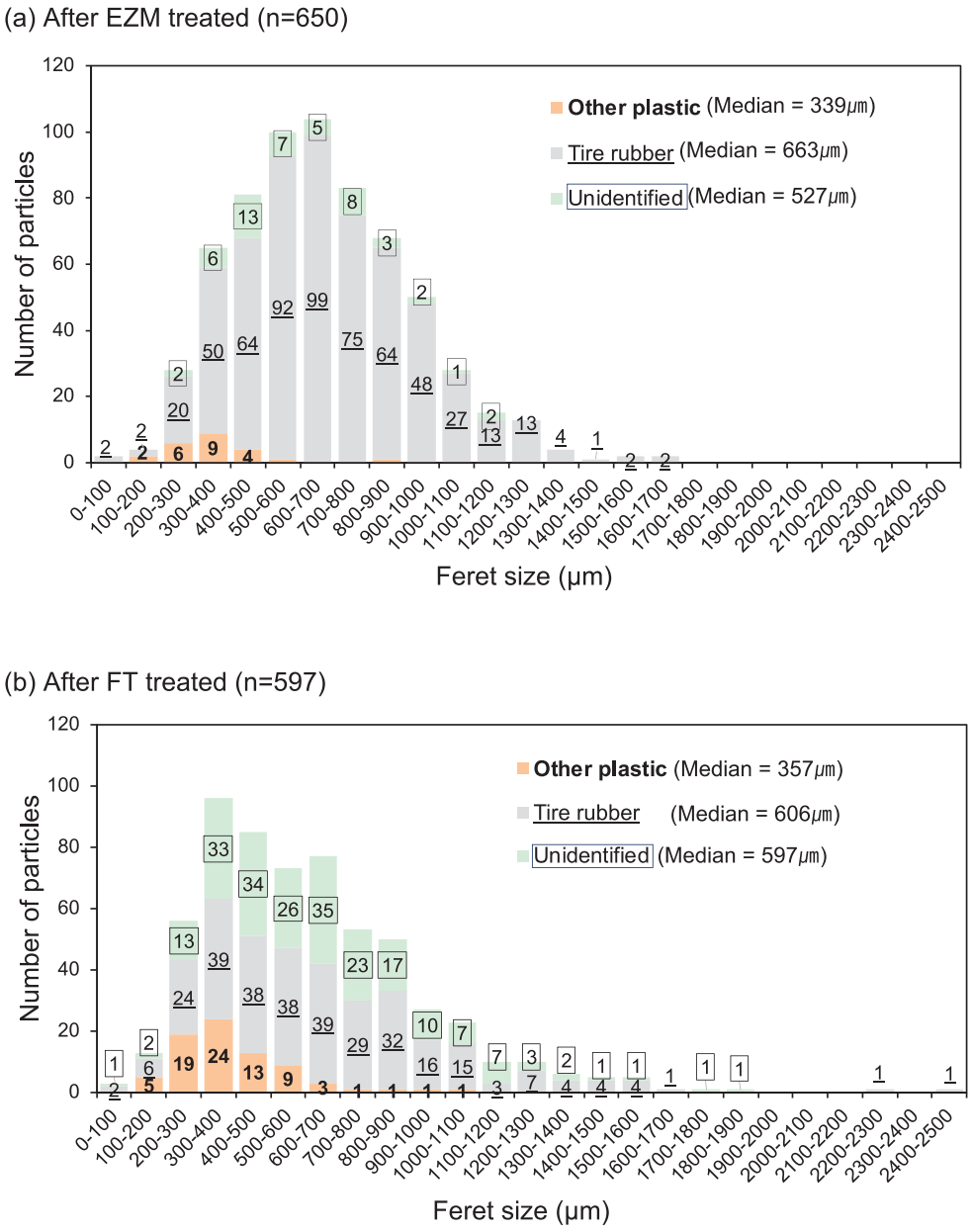

FIGURE 3 Identified material with the size of the particle after EZM treatment (a) and FT treatment (b), pooled for five replicates.

EZM and FT treatments, respectively. The arithmetic mean of the four volumes was 8.6 and 9.1 mm^3 for the particles used for EZM and FT treatments, respectively. The calculation method that assumed a spherical shape and used the area produced the results closest to the geometric and arithmetic means (Table 3-(a), Figure S4).

When the total volume of all particles after the treatments was calculated regardless of the particle material

TABLE 3 Estimated volume of particles by four different calculation approaches.

			(a) Estimated total volume from sum of each particle volume (mm^3)				(b) Estimated total volume from sum of each particle volume using tire-identified particles (mm^3)				(c) Estimated total volume from sum of each particle volume using microplastic particles (mm^3)			
			Ellipsoid		Sphere		Ellipsoid		Sphere		Ellipsoid		Sphere	
			With Feret and MinFeret	With major and minor	With area	With major	With Feret and MinFeret	With major and minor	With area	With major	With Feret and MinFeret	With major and minor	With area	With major
EZM (n = 5)	**Before***	**Avg. (% CV)**	6.1 (0.55)	3.5 (0.40)	6.9 (0.68)	18 (1.3)	6.1 (0.55)	3.5 (0.40)	6.9 (0.68)	18 (1.3)	6.1 (0.55)	3.5 (0.40)	6.9 (0.68)	18 (1.3)
	After	**Avg. (% CV)**	6.4 (1.9)	3.7 (0.88)	7.4 (1.9)	20 (6.3)	6.1 (1.7)	3.6 (0.75)	7.1 (1.6)	20 (5.1)	6.2 (1.7)	3.6 (0.8)	7.2 (1.6)	20 (5.3)
	(After-Before) /Before	**%**	4.4 Average 7.4	6.1	7.1	12	−0.065 Average 2.7	1.4	2.3	7.3	1.7 Average 4.5	3.4	4.2	8.8
FT (n = 5)	**Before***	**Avg. (% CV)**	6.0 (0.64)	3.4 (0.36)	7.0 (0.72)	20 (3.0)	6.0 (0.64)	3.4 (0.36)	7.0 (0.72)	20 (3.0)	6.0 (0.64)	3.4 (0.36)	7.0 (0.72)	20 (3.0)
	After	**Avg. (% CV)**	5.9 (1.4)	3.6 (0.75)	7.1 (1.6)	20 (5.8)	4.3 (1.2)	2.6 (0.74)	5.1 (1.4)	14 (4.4)	4.7 (1.5)	2.8 (0.84)	5.6 (1.7)	16 (5.5)
	(After-Before) /Before	**%**	−0.65 Average 1.4	3.1	1.9	1.4	−28 Average −28	−25	−27	−30	−21 Average −21	−18	−20	−23

For each before-treatment, the tire particle identification assumed 100% accuracy based on the recovery test result*.

identification results, the volume increases of 7.4% by EZM and 1.4% by FT were obtained (Table 3(a)) because of the limitation of the calculation equation. The particles less than 150 μm could be generated within the treatment, but they did not significantly affect our results. The total volume of the identified tire rubber particles increased by 2.7% after EZM treatment and decreased by 28% after FT treatment (Table 3-(b)). Additionally, the volume of microplastics, increased by 4.5% following EZM treatment and decreased by 21% after FT treatment considering both the material misidentification and particle-size changes (Table 3(c)). These results suggest that the FT treatment caused a significant underestimation of tire rubber particles, primarily due to material identification failures rather than size changes.

DISCUSSION

Morphological changes in microplastics and their impacts on microplastic quantification

The FT treatment more significantly influenced the number of particles (Figure 1(a)) and changes in their morphology (Figure 1[b-d] and Figure S1) than the EZM treatment. Two observations in this study support the change of particles is more significant after FT treatment than EZM treatment. First, the FT treatment resulted in greater decreases in five particle size parameters (Feret, MinFeret, major, minor, and area) than the EZM treatment. These morphological changes indicated that the FT treatment may have decreased the size of the original tire rubber. Second, whereas the number of particles noticeably increased in both treatments, the total particle area was reduced after FT treatment (Figure S1(f)). This reduction in particle size after FT treatment is consistent with the observations of Pfohl et al. (2021), further supporting our findings.

The change in particle numbers after treatment underscores the importance of considering multiple factors when quantifying microplastics, particularly those collected from the environment. Microplastic quantification in environmental samples is primarily conducted by assessing the number of particles (Dehghani et al., 2017; Fan et al., 2024; Liu et al., 2022; McNeish et al., 2018; Su et al., 2020; Willis et al., 2017; Zhang et al., 2024). However, this approach may be overestimated due to the increase in particle number after pretreatment. Our research found that using the number of particles for quantifying microplastics resulted in a 15% and 25% overestimation of the microplastics in tire rubber particles regardless of treatment methods (Figure 1(a)). Given that most road dust might be tire rubber particles, the amount of microplastics in road dust in previous research has also been overestimated by fragmentation during pretreatment. Conversely, when both the number and size of particles were considered, the overestimation was markedly reduced to 7.4% for the EZM treatment and 1.4% for the FT treatment (Table 3(a)). This reduction is significant when compared to the estimates based solely on particle count. To increase accuracy in microplastic quantification, we recommend considering not only the number but also the size of microplastics.

Misidentification of FT-treated tire rubber particles due to spectrum changes

In our study, approximately 89 ± 4.4% of tire rubber particles treated with EZM were correctly identified using the tire library, compared to only 51 ± 5.6% of particles treated with FT (Table 2). This discrepancy in identification rates highlights potential issues with FT treatment affecting spectral accuracy. To understand these differences, we compared the averaged spectra for each material (Figure S2) and observed qualitative differences. After both EZM and FT treatments, two peaks around 3000 cm^{-1}, two around 1400 cm^{-1}, and three around 1000 cm^{-1} became less distinguishable, with the 908 cm^{-1} peak disappearing entirely after FT treatment. Table S1 summarizes the wavenumber and functional groups observed in the tire-related samples from the literature. The FT treatment induced more significant spectral changes on tire, potentially due to the alteration on 1130–975 cm^{-1}, consistent with findings by Pfohl et al. (2021). However, interpreting the peaks observed in this range varies depending on the vehicle type of tire or the type of rubber used in the tire (as shown in Table S1), so it is insufficient to discuss the peak changes before and after pretreatment in our study.

Although EZM treatment also caused minor spectral changes around 1000 cm^{-1}, these were less pronounced compared to FT treatment (Figure S3). The more pronounced peak losses around 1000 cm^{-1} in FT-treated particles suggest that the reduction in material identification accuracy may be linked to this spectral range. The light-driven Fenton oxidation method in our study exhibits a much faster reaction rate than non-light reactions (Maezono et al., 2011). Given that OH radicals generated during Fenton treatment degrade organic substances (Zepp et al., 1992), the OH radicals in our method may have caused excessive oxidative stress on components in tire particles. In contrast, the enzyme treatment, primarily acting on cellulose through hydrolysis as its main mechanism (Enari & Niku-Paavola, 1987), likely applied

comparatively lower oxidative stress to components in the tire particles than the Fenton method. Further quantitative studies are needed to determine whether these changes are due to material modifications during the treatments.

Misidentification in FT-treated particles may also result from byproducts of the Fenton reagent. These particles appeared darker than those treated with EZM, likely due to carbon produced during oxidation or other byproducts specific to the Fenton process. Iron oxide in the Fenton reagent likely caused spectral shifts at low wavenumbers, especially below 900 cm^{-1}, due to Fe-O bonds (Gotić & Musić, 2007). Future studies might explore alternative techniques that do not rely on iron oxide. However, the chemical reaction with hydrogen peroxide alone requires longer reaction times and does not efficiently remove organic matter (Olsen et al., 2020), limiting its ability to process natural organic materials like plants. To prevent misinterpretation of results from excessive oxidation or by-products generated during Fenton treatment, it is essential to understand the mechanism of Fenton reaction and implement by-product removal procedures.

Current microplastic analysis using specific algorithms and IR spectra has limitations in identifying aged or mixed samples. Our study found that tire identification rates significantly decreased after FT treatment, even when using a tire-specific library. Further research is needed to determine whether this decrease is specific to tire rubber particles and to address the broader issue of microplastic misidentification in environmental samples. Identification accuracy may be influenced device performance, library selection, and pretreatment type, especially with aged microplastics.

Limitations in estimating microplastics using volume

The volume of tire rubber particles varies significantly depending on the volume estimation method used (Table 3). For example, before EZM treatment, the volume of these particles ranged from 3.5 mm^3 to 18 mm^3, with the maximum volume being approximately 5.1 times larger than the minimum. These variations highlight that the choice of volume estimation method can greatly influence the estimated microplastic volume. While our study did not determine the most appropriate method, we selected the approach that produced results closest to the median among the four estimation methods. The potential range of errors due to differences in volume calculations was −49.2% (3.5 mm^3/6.9 mm^3) to +160.8% (18 mm^3/6.9 mm^3) (Table 3). These discrepancies suggest that the reported amounts of microplastics in other studies may need to be re-evaluated based on the volume estimation method used. To accurately compare microplastic quantities obtained through different methods, it is essential to reassess the chosen volume estimation approach.

It is important to note that the results of our study may be influenced by the specific sample used. For instance, in the previously discussed spherical area approach, the estimated volumes closely matched the geometric mean (Table 3). However, this finding is based on tire rubber particles produced in a laboratory using a saw, which differ from real tire particles. According to Kovochich et al. (2023), tire wear particles found in road dust are closer to a cylindrical shape and often contain surface impurities. In contrast, our rubber particles were more spherical (Figure S5(c)). Therefore, the most suitable volume calculation formula is dependent on the specific shape of the particles, and this must be considered when applying our method to actual environmental samples.

Implications for analysis of microplastics containing tire rubber particles

This study identified greater uncertainty in quantification with the FT method compared to the EZM method (Table 1), as the FT method exhibited lower reproducibility and higher coefficients of variation (%CV) across all size variables. The EZM treatment is recommended for analyzing environmental samples with high concentrations of tire particles due to its greater accuracy. However, it has limitations, including increased time and cost for removing organic substances (see Supplementary Materials, Section 5). Despite its drawbacks in misidentification, the FT method is more efficient for processing large sample sizes or samples with a high content of non-plant organic matter. Therefore, caution is advised when interpreting results, as the FT method may lead to an underestimation of tire particles by as much as 21%.

In this study, we clarify that our research focuses specifically on tire rubber among materials classified as microplastics. Although changes in particle size (Ortiz et al., 2022) and structural alterations (Akyildiz et al., 2023) have been observed for plastic materials other than tires in several studies, our results do not indicate significant quantitative differences for materials other than tires. The quantitative changes by Fenton oxidation were not clarified in other polymers, and further research is needed to verify this.

CONCLUSIONS

We investigated the impact of pretreatment on the accuracy of microplastic particle quantification in tire

rubber particles, focusing on the differences between cellulolytic enzyme (EZM) and Fenton (FT) treatments. Both treatments led to an increase in the number of tire particles, while the particle size decreased. The five size parameters (Feret, MinFeret, major, minor, area) decreased more with FT treatment (11.6–40.3%) than with EZM treatment (7.06–7.62%). Tire rubber particles were identified with greater accuracy following EZM treatment (89 ± 4.4%) than FT treatment (51 ± 5.6%). Our findings suggest that the misidentification after FT treatment was not solely due to the reduction in particle size. When considering both size reduction and misidentification, the EZM method resulted in a 4.5% overestimation of microplastic volume, whereas the FT treatment led to a 21% underestimation. Employing EZM treatment can improve the accuracy of microplastic quantification in road dust. To ensure precise quantification of microplastics in environmental samples, it is essential to address uncertainties related to measurement parameters, weight calculation methods, and the decrease in identification accuracy due to plastic aging.

AUTHOR CONTRIBUTIONS

Soyoung Lee: Methodology; conceptualization; investigation; data curation; validation; formal analysis; visualization; writing – original draft; writing – review and editing; resources. **Kanako Yamamoto:** Investigation; validation; writing – review and editing; formal analysis; software; resources. **Tomohiro Tobino:** Writing – review and editing; supervision. **Fumiyuki Nakajima:** Supervision; funding acquisition; project administration; writing – review and editing; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

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