

INTER AMERICAN UNIVERSITY OF PUERTO RICO, INC
SAN GERMAN CAMPUS
DEPARTMENT OF SCIENCE AND TECHNOLOGY
GRADUATE PROGRAM IN ENVIRONMENTAL SCIENCE

**DEVELOPMENT AND VALIDATION OF METHODOLOGY FOR MICROPLASTICS
DETERMINATION FROM ANIMAL FEED PELLET**

ZENAIDA J ROSA AROCHO

2025

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Thesis submitted and approved to complete the requirements for the Master of
Science Degree in Environmental Sciences-Chemistry

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December 2025

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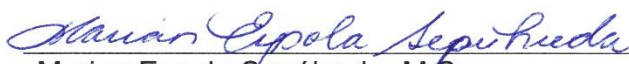
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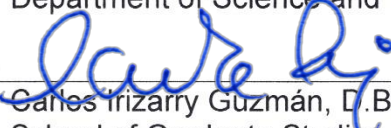
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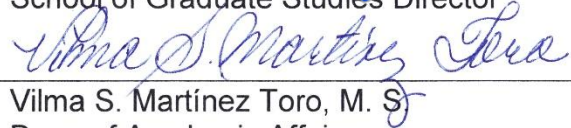
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ACKNOWLEDGEMENTS

This thesis is dedicated to my daughter Camila Victoria, who has been my source of inspiration. Without her unwavering patience, this endeavor would not have been possible. A special thank you to my mom, Miriam, for her inspiration and support through all challenges; to my mentor, Dr. Ángela González, for her exceptional guidance, valuable advice and encouragement; to my esteemed boss, Bryne McDowell, for his constant support and reliability; and to my special friends for being the best cheerleaders. Finally, I dedicate this work to God for providing the guidance and strength to complete this accomplishment.

Table of Contents

ABSTRACT	1
INTRODUCTION.....	2
OBJECTIVES	6
LITERATURE REVISION	7
Plastics	7
Plastics in the environment	8
Microplastics	9
Microplastics in the environment	11
Microplastics in food production	13
Microplastics in animal feed	14
METHODOLOGY	17
Sampling and Sample Preparation	19
1. Sampling	19
2. Processing of Feed Samples	20
Preparation of Solutions.....	21
Optimization of microplastics extraction protocol for homogenized animal feed samples	22
1. Chemical Digestion Process	22
2. Filtration after Chemical Digestion	24
3. Microplastics Density Separation from Sample Solid Residues	25
Classification of microplastics	26
Quality Control.....	26
Validation of Determination of Microplastics in Animal Feed Samples	27
Data Analysis.....	28
RESULTS AND ANALYSIS	29
Processing of Feed Samples	29

Microplastic Extraction - Chemical Digestion Process	30
1. Pre-digestion	30
2. Heating Digestion process	32
3. Cool Down Period After Heating Digestion	34
4. First filtration – Supernatant of Digested Samples	36
Density Separation of Residue	37
1. Optimization of density separation time	38
Application of Developed Methodology to different animal feed samples	41
CONCLUSIONS	46
RECOMMENDATIONS	47
BIBLIOGRAPHY	48

ABSTRACT

Plastic pollution is abundant across aquatic and terrestrial environments, raising concerns due to their persistence and ability to transport and release chemical contaminants. Among those pollutants, microplastics have become a major environmental and health issue, entering food chains through agricultural contamination and/or manufacturing processes. Animal feed is particularly vulnerable to microplastic contamination, from various sources, including feedstocks, equipment, packaging, and handling practices, posing risks to animal health and potential transfer through the food chain. Despite reports of microplastics in animal feed, no standardized extraction method has been established. Therefore, a microplastic extraction methodology from animal feed was developed to maximize recovery of spiked microplastics, while minimizing treatment time and filtration complications. Dry food samples for dogs, cats, chickens, and rabbits were collected and spiked with microplastic fragments of polystyrene (PS), polyester (PES), polypropylene (PP) foam, and fiber of (PE) polyethylene. The optimized protocol achieved 100% for PS, PES, and PP across all animal feed types, while PE recovery ranged between 87 to 93% depending on feed type. During method development, using more than 100 test samples, only one microplastic—a fiber—was detected, suggesting low contamination of microplastics within the size range studied in this research. The developed method demonstrated reliability, reproducibility, and applicability to different feed types, offering a practical approach for monitoring microplastic contamination.

INTRODUCTION

Plastics are synthetic organic polymers, inexpensive to produce either from fossil fuels or biomass. They have a molecular linear structure and may have been produced with additives to provide specific characteristics such as being flexible, durable, lightweight, thermally stable, electronically insulating, and chemically stable. The global market demand distribution is dominated by plastics made of polypropylene (PP) 19.7%, low-density and linear low-density polyethylene (PE-LD-LLD) 17.4%, high-density and medium-density polyethylene (PE-HD-MD) 12.9%, polyvinyl chloride (PVC) 9.6%, polyethylene terephthalate (PET) 8.4%, other thermoplastics 10.7%, polyurethane (PUR) 7.8% and others plastics 7.4% (Plastics Europe, 2021).

In 2019, global plastic production reached 368 million tons (Plastics Europe, 2021). As they persist for centuries in the environment, they are becoming a big problem, contaminating ecosystems. Scientists estimated that in 2010, about 275 million metric tons (MT) of plastic waste was produced in 192 coastal countries, and between 4.8 and 12.7 million tons of it entered the ocean (Jambeck, et al., 2015).

The 60-80% of total marine waste is estimated to consist of plastic waste (Iñiguez, Contessa, & Fullana, 2016; Datu, Supriadi, & Tahir, 2019). As plastics float in the ocean, they gradually break down because of solar radiation, physical processes, and biodegradation. From those processes, smaller pieces of plastic form, known as microplastics (hereinafter referred to as MPs), plastic pieces that have particle sizes of less than 5mm.

Microplastics are classified according to their origin as primary when knowingly manufactured to be microscopic or secondary microplastics when derived from the fragmentation of microplastic items (Wright, Thomson, & Galloway, 2013). The transportation, dispersion, and retention of microplastics across different environments are influenced by their composition, size, shape, and density.

Microplastics have been detected in both aquatic and terrestrial environments. They can be present in the water and sediments of oceans, lakes, and rivers. High-density microplastics, including polyvinylchloride, polyester, and polyamide, have been found in the sediments (Woodall, et al., 2015; Cole, Lindeque, Halsband, & Galloway, 2011), while low-density particles (including microplastics such as Styrofoam) are found on the surface of water bodies since they tend to float (Suaria & Aliani, 2014) (Cole, Lindeque, Halsband, & Galloway, 2011). Because of the small size of microplastics, they are easy to transport and can be carried by currents and waves. Microplastics are one of the top environmental concerns due to their affinity to pollute the environment and enter the food chain, threatening ecosystems and human health. Microparticles may accumulate in agricultural fields, leading to contamination of the food supply and, therefore impact aquatic, domestic, and wildlife animals upon ingestion, causing health disorders (Jeong, Lee, & Redwan, 2024). Microplastics have been reported in many organisms because they can be ingested through the water or by feeding on contaminated prey or food, producing possible chemical, physical, and biological effects (Setälä, Fleming-Lehtinen, & Lehtiniemi, 2014) (Barnes, Galgani, Thompson, & Barlaz, 2009) (Wright, Thomson, & Galloway, 2013).

The presence and quantification of microplastics in both marine and terrestrial ecosystems, along with their impact on living organisms, have been studied worldwide. Most research has concentrated on the marine environment, despite the significant role of terrestrial ecosystems in human and animal exposure. Studies conducted in terrestrial environments have typically focused on plants, with few involving vertebrate animals to assess the adverse effect caused by microplastics (Prata & Dias-Pereira, 2023). Besides the effect of direct consumption of microplastics, additives incorporated into plastics during their manufacturing process to provide certain characteristics or extend their life, often termed plasticizers, may leach out once they are ingested introducing potentially hazardous chemicals to the biota (Cole, Lindeque, Halsband, & Galloway, 2011; Seng, et al., 2020). Known plasticizers (polybrominated diphenyl ethers, phthalates, and bisphenol A) can be bioaccumulated and biomagnified up to the food chain, impacting a variety of organisms (Seng, et al., 2020; Cole, Lindeque, Halsband, & Galloway, 2011; Wright, Thomson, & Galloway, 2013). These chemicals can penetrate the cells and potentially behave as endocrine disruptors (Teuten, et al., 2009; Tosetto, Williamson, & Brown, 2017). Another persistent chemical pollutant used as a plasticizer is polychlorinated biphenyl, which has been reported to cause a variety of biological effects (Neal, 1985). As a result, microplastics pose a risk to food security by disrupting food efficiency and safety, as they are foreign substances present in food (Prata & Dias-Pereira, 2023). Research has also proved the presence of microplastics in animal feed (Maganti & Akkina, 2023) (Mohsen, Lin, Lu, & Wang, 2024). Microplastics in animal feed may come from the plastic packaging and other sources, such as environmental factors or

plastics used in livestock farming. Potential adverse effects have been reported related to the exposure of livestock to microplastics (Prata & Dias-Pereira, 2023).

Microplastics contamination may affect food security, agricultural productivity, economic efficiency, and human health due to microplastic accumulation and additive leaching. Animal feed is an area where microplastics have also been reported, using different sample treatment procedures, which make it difficult comparing the data. Therefore, this study developed a method to determine the presence of microplastics in commercial animal feed, which can be used for dogs, cats, hens, and rabbit dry pellets. This research provides a methodology that can be used to increase research in these areas, since limited reports are available. Identifying the microplastics collected helps predict the types of plastics presents in the produced animal feed, assess the contribution of biomass and identify and correct possible sources. This information can aid in developing strategies to reduce its presence and possible effect on domestic animals.

OBJECTIVES

This study aims to develop and validate a standardized procedure for determining microplastics in dry animal feed.

To achieve this goal, the following objectives were defined:

1. Develop and optimize an efficient extraction procedure to determine microplastics in animal feed.
2. Validate the method developed for determining microplastics in animal feed samples.
3. Evaluate if the developed method of microplastics determination applies to different types of animal feed (dry dog, cat, rabbit, and chicken feed).

LITERATURE REVISION

Plastics

The production of plastic began in the early 20th century and was used for manufacturing industrial products like automotive parts, medical applications, building, construction materials, cosmetics, and packaging (Geyer, Jambeck, & Law, Production, use, and fate of all plastics ever made, 2017). Plastic is mainly used for the packaging system of edible products since it provides safety, extends freshness, and helps to protect the product from contamination (Hester & Harrison, 2019). They are also used for the manufacturing of cosmetics, personal care products, and medical applications (Hester & Harrison, 2019). The single-use container was introduced to minimize contamination risk, with plastics being the most versatile material choice (Geyer, Jambeck, & Law, Production, use, and fate of all plastics ever made, 2017).

Plastics are synthetic materials chemically formed by the polymerization of organic (carbon-based) substances and are classified by their chemical structure of the polymer base and side chains. They are large molecules made of a chain of smaller molecules, called monomers, which are chemically bound together. They are classified by the chemical process used in their synthesis, such as cross-linking, condensation, and polyaddition. They can also be classified in accordance with their structure (physical properties), including tensile strength, hardness, heat resistance, density, and glass transition temperature, as well as their reactions to various chemicals and processes, such as ionizing radiation, oxidation, and organic solvents. Other categories depend on relevant characteristics related to the product design and manufacturing process

(Rodriguez, 2025). There are two major groups of plastics: thermoplastics and thermosets. Thermoplastics become soft when heating is applied and can be shaped in a mold, re-melted, and recycled. They are synthesized under the polyaddition process and do not undertake a chemical change in their composition when heated. This type of plastic includes polyvinyl chloride (PVC), nylon, Teflon, polypropylene (PP), polyethylene (PE), and polystyrene (PS). On the other hand, thermosets are rigid and cannot be exposed to re-melting since they remain hard once they have been produced (irreversible chemical reaction), e.g., some resins, polyurethane, and rubbers (Rodriguez, 2025).

Plastics in the environment

Synthetic polymer/plastic production has increased worldwide since the middle of the 20th century (Hidalgo-Ruiz, Gutow, Thomson, & Thiel, 2012). Over the next 70 years, the annual production of plastics increased nearly 230-fold to 460 million tons in 2019 (Ritchie, Samborska, & Roser, 2022). The era of plastics is marked by their ease of production, durability, and versatility, thanks to their hydrophobic nature and classification as biochemically inert materials. However, global waste generation increased at an accelerated rate affecting the terrestrial and aquatic environments. There are considerable and increasing quantities of plastic contamination in the environment, and the plastics entering the oceans, primarily from land-based sources, are expected to continue to rise over the next decade (Geyer, Jambeck, & Law, Production, use, and fate of all plastics ever made., 2017; Lebrenton & Andrady, 2019), becoming an environmental hazard (Cole, Lindeque, Halsband, & Galloway, 2011; Moore, 2008).

One of the concerns about plastics in the environment is their lifetime. Several studies have estimated plastic degradation rates by suggesting the lifetimes of different types of plastics in the environment (Chamas, et al., 2020). The biodegradation time of plastics in the environment depends on seawater hydrolytic properties, atmospheric conditions, and sunlight radiation exposure (Moore, 2008). The estimated time for plastic degradation in the environment can often vary as new findings from recent studies become available (Chamas, et al., 2020).

Another concern about the presence of plastics in the environment is their affinity with organic molecules. Hydrophobic molecules such as persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are adsorbed and accumulated by plastics due to their high affinity (Hester & Harrison, 2019). These organic contaminants raise concerns due to their persistence in the environment, toxicity, and bioaccumulation in the food chain, causing ecosystem and human health issues.

Microplastics

Microplastics are defined as particles smaller than five (5) mm (Cole, Lindeque, Halsband, & Galloway, 2011; Andrady, 2011; Bosker, Guaita, & Behrens, 2018; Lots, Beherens, Vijver, Horton, & Bosker, 2017). There are two types of microplastics: primary and secondary. Primary microplastics are particles usually produced as resin pellets or bead forms designed for commercial use (Bosker, Guaita, & Behrens, 2018; Ziccardi, Edgington, Hentz, Kulacki, & Dricoll, 2016; Rochman, et al., 2015). Secondary microplastics come from larger pieces of plastic that break down due to

exposure to environmental factors, such as ultraviolet radiation wind and ocean waves, becoming brittle and fragmenting into smaller pieces of plastics (Andrady, 2011; Bosker, Guaita, & Behrens, 2018; Cole, Lindeque, Halsband, & Galloway, 2011; Lebrenton & Andrady, 2019).

Microplastics exist in many shapes, including nurdles (pellets), fragments, fibers, foams and microbeads (Cole, Lindeque, Halsband, & Galloway, 2011; Bosker, Guaita, & Behrens, 2018; Wright, Thomson, & Galloway, 2013; Hidalgo-Ruiz, Gutow, Thomson, & Thiel, 2012; GESAMP, 2019; Hidalgo-Ruiz, Gutow, Thomson, & Thiel, 2012). Microplastic fibers are often found as the most abundant shape collected in the marine environment (Bosker, Guaita, & Behrens, 2018), probably from clothing and household textiles, mainly composed of synthetic materials such as polyester, polyamide, and acrylic. (Henry, Laitala, & Grimstad Klepp, 2019; 652).

Microplastics may have been produced by the addition of other chemical compounds such as plasticizers, colorants, phthalates, and fillers, which are used to enhance the product's longevity, structural integrity, and UV resistance. There has been microplastics reported made of polyethylene, polypropylene, polyvinyl chloride, polystyrene, among others (Andrady, 2011; Bosker, Guaita, & Behrens, 2018; Browne, Galloway, & Thomason, 2010; Kedzierski, et al., 2022).

Microplastics density is an important characteristic that may affect the final resting place of a microplastic in the environment. Microplastic chemical composition can cause some microplastics to potentially have a greater density than that of seawater (1.025 g/cm^3) (Andrady, 2011). Making them sink towards the seafloor. Low-density microplastics such as polyethylene and polypropylene can be found at the sea

surface due to their positive buoyancy (GESAMP, 2019; Datu, Supriadi, & Tahir, 2019). Similar phenomena occur in fresh water.

Microplastics in the environment

Microplastics are one of the most common debris in the marine environment and they have been found in air, soil, and water ecosystems (Cole, Lindeque, Halsband, & Galloway, 2011; Ivar do Sul & Costa, 2014; Bosker, Guaita, & Behrens, 2018). Plastics are extremely resistant to fast degradation, therefore microplastics, due to their specific design for longevity and strength, potentially persist for a long time in the environment (Hidalgo-Ruz, 2012; Barnes, Galgani, Thompson, & Barlaz, 2009).

Attention has focused on the presence and accumulation of microplastics in the ocean because of the increase in contamination levels throughout both sediment and water of the marine environment, and their impact on this ecosystem. Different factors influence the segregation of plastics in the marine environment, such as polymer density and hydrodynamic conditions like wind and ocean currents (Stafella, López-Correa, Pyko, Teichert, & Gomiero, 2020).

It has been proven that microplastics can absorb and accumulate persistent bio-accumulative and toxic compounds (PBT) present in the seawater because of their high affinity with these, which include persistent organic pollutants (POPs) (Hidalgo-Ruiz, Gutow, Thomson, & Thiel, 2012). PBT substances are chemicals that do not degrade easily in the environment and are slowly metabolized and typically accumulated in the fatty tissue of the organisms. This group additionally includes trace

metals and organo-metal compounds. POPs are synthetic organic chemical compounds that are stable and persistent in the environment. These can cause high toxicity with serious effects on humans (like cancer, allergies/hypersensitivity, damage to central and peripheral nervous systems, and disorders of the immune and reproductive systems) and disrupt the endocrine system of some organisms in the marine ecosystem. Various studies stated the ecotoxicological effect of microplastics on the health of aquatic organisms, including plankton, benthic, mollusks, crustaceans, fishes, among others, when ingested (Nanthini Devi, Raju, Santhanam, & Perumal, 2022) and could be transferred to the coastal predators via food webs (Ouyang, Panti, Canicci, Li, & Tam, 2023).

In the terrestrial environment, microplastics affect soil health, which has consequences for agriculture and overall ecosystem function. Most research has focused on detecting microplastics and their effects on human and animal exposure in the marine environment, despite that reports indicate that approximately 25% of the world's soil is severely affected by pollutants, such as microplastics, while an additional 44% is mildly impacted (Rai, Pant, & Pant, 2023) (Prata & Dias-Pereira, 2023). They negatively affect the terrestrial environment by altering soil properties, impacting plant growth, harming soil organisms, and acting as vectors for other pollutants (Zeb, Liu, & Ali, 2024). Microplastics alter soil texture, temperature, and water-holding capacity (Zeb, Liu, & Ali, 2024). Some plastics can increase soil temperature, which can lead to water loss and changes in chemical properties of soils, such as pH, electrical conductivity, and cation exchange capacity (Mendez, 2021). Additionally, the presence of microplastics can increase the availability of heavy

metals (Mendez, 2021). The most found plastics in land-based ecosystems include polypropylene, polyethylene, polystyrene, polyvinyl chloride, and polyethylene terephthalate. (Rai, Pant, & Pant, 2023) (Geyer, Jambeck, & Law, Production, use, and fate of all plastics ever made, 2017). Soils containing plastics may contaminate the biomass components used for animal feed production, because they may be absorbed by plants. The physical effects of plastics on plants include reduced ability to absorb water and nutrients, toxicity, and disruption of photosynthesis. Once animals ingest the plants, the absorbed pollutants may be transferred to the respective organisms' upward food chain (Teuten, et al., 2009).

Microplastics in food production

The food industry is regulated and monitored for contamination through systematic programs to ensure food safety and prevent contamination from entering the food. The presence of microplastics in food does not breach regulations unless it poses a health risk. Studies have detected microplastics in food, and regulatory agencies continue to monitor their presence to assess the potential impact of microplastics in food and drinking water from packaging and other sources on human health, based on scientific evidence. (Food and Drug Administration, 2024). Microplastic contamination may be found in food products due to raw materials, packaging, and processing equipment. These particles are introduced through contaminated water, sewage sludge, and atmospheric deposition. Additional sources of plastic contamination during manufacturing include processing aids and equipment such as conveyor belts, sieves, and containers.

Regulatory agencies in the US and EU have set guidelines and maximum limits for food contaminants, but methods for evaluating microplastics risks are still under review (Sharma, 2024). Some mitigation actions have been identified in the industrial food sector, including implementing manufacturing practices to reduce the use of additives and chemicals in plastic production, modifying equipment, and improving plastic waste management. (Sharma, 2024).

Studies have reported the presence of microplastics in various types of foods and have identified packaging plastic residues as a potential source of contamination. Due to the concern about the impact that these pollutants could have on environmental integrity and human health, actions have been implemented as part of the mitigation plan.

Microplastics in animal feed

There is limited data on microplastics in animal feed. A data review for microplastics levels in livestock farming in Bangladesh was conducted in 2024, confirming the presence of this pollutant in animal feed and animal and human blood (Akanda, Kawsar, & Sadik Sabuj, 2025).

Studies on microplastic levels in various types of feed, especially aquafeed, were performed between 2021 and 2024. A higher average of 2.85 particle g⁻¹ microplastics in chicken by-products for aquafeed ingredients supplied by China was reported (Mohsen, Su, Lin, & Li, 2024). Two other studies were performed to detect microplastics in fish feed. Samples of fish meal were collected from various countries, including China, Peru, Denmark, Russia, Thailand, and others (Xing Liu, Wang, &

Sun, 2021). The highest concentrations of microplastics were detected in fish and shrimp from China meal, with film microplastics being the most prevalent type in both samples. Another study determined microplastics in five brands of commercial fish feed collected from the market. Microplastics were detected in all collected commercial fish feed samples in Bangladesh. The average particle size found was about 1mm, including various types such as fibers, films, fragments, foam, pellets, microbeads, and nurdles. Fiber was the most encountered type of particle. Variations in microplastic content across market brands indicate differences in ingredient quality and/or manufacturing processes (Shraboni, Shuvo, & Uddin, 2024).

There are limited reports of microplastics in animal feed serving as a source of microplastics in the terrestrial environment. Studies for feed of terrestrial animals were performed in 2025. The presence of microplastics was determined in two types of diet (balanced feed and alfalfa) for equines at two sites in Guayas province, Ecuador (González-Puetate, Valle-Mieles, & Yañez-Avalos, 2025). Microplastics were detectable in all samples, with a concentration of 66% of the balanced feed and 34% of the alfalfa. Fibers and fragments of microplastics were quantified and classified by different colors; fibers were more abundant. One additional study was found for microplastic contamination in ruminant feeds of Northwestern Italy (Patruco, Abid, & Rivoira, 2025). The used feed types in three (3) dairy farms are: corn silage (CSG), hay (HAY), and high-protein feedstuff (HPF). The CSG resulted in the most significantly polluted feed (37.9 ± 7.94 items/g), followed by HAY (16.4 ± 3.81 items/g) and HPF (16.3 ± 3.76 items/g). Transparent fiber-shaped microplastics were the most abundant items in all the feed (Patruco, Abid, & Rivoira, 2025). Further research and

the development of a microplastics database are necessary to monitor their impact on the terrestrial environment and the food chain transfer. These investigations could help identify potential sources of microplastics in animal feed. Research on microplastics in animal feed for terrestrial domestic animals is limited.

Several studies use varying extraction methods, which makes it difficult to compare the data. Various methods have been used in studies to extract microplastics from animal feed, including organic degradation steps such as digestions, Fenton reactions, and Amplified Spontaneous Emission spectra (ASE), followed by density separation. (Acosta-Coley & Olivero-Verbel, 2015) These studies involved a series of steps, including digestion with reagents such as nitric acid, potassium hydroxide, and sodium hydroxide, followed by Fenton reactions with hydrogen peroxide (Patruco, Abid, & Rivoira, 2025) (Siddique, Tahsin, & Hossain, 2023) (Mohsen, Lin, Lu, & Wang, 2024). Other studies used the ASE method with tetrahydrofuran and methanol extraction methods, followed by oxidation reaction, adding hydrogen peroxide (Van der Veen, Van der Mourik, & Van Velzen, 2022) (Maganti & Akkina, 2023). Sodium iodide, sodium chloride and zinc chloride solutions were used for the final step of the extraction method, density separation of microplastics in the digested solutions (Siddique, Tahsin, & Hossain, 2023) (Muhib & Rahman, 2023) (Mohsen, Lin, Lu, & Wang, 2024). This study aims to develop a versatile extraction method applicable to various feed types, enabling the monitoring of microplastics in commercial animal feed.

METHODOLOGY

A microplastic extraction method was specifically developed and validated for animal feed pellets. Validation was performed by evaluating the recovery percentage of spiked microplastics in real feed samples to warrant that the process extracted all the microplastics for good reproducibility. This methodology is an adaptation of those reported by Mohsen, et al (2024) and Muhib et al (2023). In summary, several commercial samples from different brands were collected, homogenized, and prepared for extraction. Before digestion, the samples were spiked with a known number of plastic pieces. The spiked samples were exposed to a cool pre-digestion with sodium hydroxide and hydrogen peroxide and then proceeded with chemical-heating digestion. Supernatant from the digested samples was filtered to extract the microplastics. Solid residues were mixed in a saturated sodium chloride solution and performed another filtration to extract the microplastics. The filters with the extracted material were stored in a Petri dish for further microplastic counting and classification. Figure 1 presents a summary of the procedure.

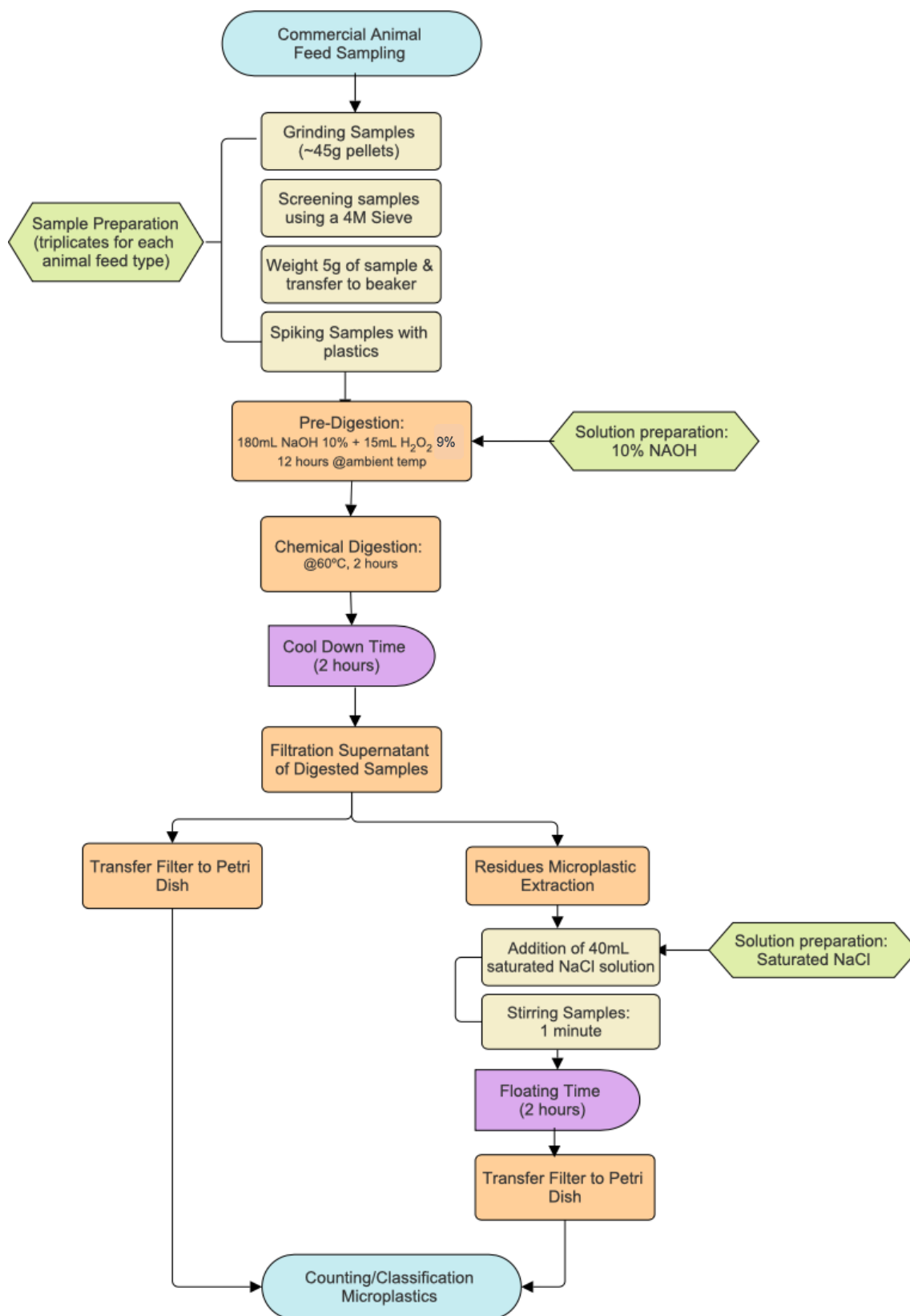


Figure 1: Microplastic Extraction Methodology for Commercial Pellet Animal Feed.

Sampling and Sample Preparation

1. Sampling

All animal feed pellets (dog, cat, chicken, and rabbit) samples were collected from different stores and Agri-centers located on the east side of Puerto Rico Island (between the towns of Caguas, Juncos, Las Piedras, and Humacao). A total of twelve (12) bulk samples (three samples for each type of feed) were obtained and stored in a dry place until further analysis. Samples for dog and cat were obtained from different brands, and their protein and fat content were used as a reference for the development of the extraction process. The details of the source, protein, and fat composition of feed samples are given in Table 1 below.

Animal	Source	Composition
Dog	A Store Caguas	Crude Protein (min) 21.0% Crude Fat (min) 10.0%
	B Store Caguas	Crude Protein (min) 26.0% Crude Fat (min) 16.0%
	C Store Caguas	Crude Protein (min) 25.0% Crude Fat (min) 15.0%
Cat	D Store Caguas	Crude Protein (min) 31.0% Crude Fat (min) 11.0%
	E Store Caguas	Crude Protein (min) 30.0% Crude Fat (min) 12.0%
	F Store Caguas	Crude Protein (min) 26.0% Crude Fat (min) 9.0%
Chicken	Agri-center Juncos	Not available
	Agri-center Las Piedras	Not available
	Agri-center Humacao	Not available
Rabbit	Agri-center Juncos	Not available
	Agri-center Las Piedras	Not available
	Agri-center Humacao	Not available

Table 1: Sampling information for animal feed pellets.

2. Processing of Feed Samples

The feed samples used were in pellet form. The animal feed sample was removed from the original packaging using a stainless-steel spoon, previously rinsed with saturated NaCl solution and dried. The samples were ground and passed through the stainless-steel sieves (Alvarez-Zeferino, Ojeda-Benitez, Cruz-Salas, Martínez-Salvador, & Vázquez-Mirillas, 2020; Pérez-Alvelo, et al., 2021). An electric grinder was chosen for the grinding and homogenizing process (Figure 2).



Figure 2: Example of the grinding of dog food (taken by Zenaida Rosa).

Then, the grinded samples were passed through a stainless-steel sieve (4 mesh) with a pore size of 4.75mm to remove large-sized coarse particles that might obstruct the vacuum filtration process (Maganti & Akkina, 2023).

To simulate microplastics in feed, twenty pieces of microplastics were added to each sample, to evaluate the recovery percentage. Each sample was spiked by adding five (5) pieces of the following microplastics: polystyrene (PS) from a drinking disposable cup, polyester (PES) sewing thread, polypropylene (PP) foam from a

packaging container, and (PE) polyethylene fish line. Polystyrene samples were cut into approximately five (5) mm pieces, and the thread and fish line were no longer than two (2) cm, as shown in Figure 3.



Figure 3: Homogenized dog feed samples spiked with plastics (taken by Zenaida Rosa)

During the examination of the spiked samples, plastic types were counted, classified, and documented. Recovery percent was calculated as follows:

$$\% \text{ recovery} = \frac{\text{number of pieces recovered}}{\text{number of pieces added}} * 100$$

The number of pieces recovered refers to the total number of pieces after treatment. All the tests were done in triplicate.

Preparation of Solutions

A solution of 10% NaOH was prepared as a digestion reagent to break down organic matter and to facilitate microplastic particles isolation. Solution with a 1:10 w/w ratio was prepared using sodium hydroxide (Sigma Aldrich), 99% pure powder, and deionized water. A saturated sodium chloride solution was prepared by adding thirty six (36) grams in 100 mL of deionized water (Shütze, Thomas, & Kraft, 2022) to be used for the extraction/separation process of microplastics and stirring

for three (3) min (Bosker, Guaita, & Behrens, 2018; Pérez-Alvelo, et al., 2021). Then, the solutions were filtered using a 47 mm diameter mixed cellulose ester membrane with a pore size of 0.45 μm (Bosker, Guaita, & Behrens, 2018) (Silburn, et al., 2022), to warrant the absence of microplastics in the digestion and extraction solutions. A glass volumetric cylinder was used to measure the water and acquired 9% hydrogen peroxide solution. The solid chemicals (NaOH and NaCl) were weighed using a 4-digit balance. Solutions were maintained in the glass volumetric flask until used for the digestion process for no more than two (2) days.

Optimization of microplastics extraction protocol for homogenized animal feed samples

Since the high content of protein and fat in dog and cat feed, microplastics extraction was developed and optimized, including two phases: chemical digestion and density separation (Muhib & Rahman, 2023).

1. Chemical Digestion Process

The chemical digestion used a mixture of 180 mL of 10% NaOH (sodium hydroxide) and 15 mL of H_2O_2 (hydrogen peroxide) 9% at room temperature for twelve (12) hours and then at 60°C (Mohsen, Lin, Lu, & Wang, 2024). Solutions and liquid chemicals were measured using a glass volumetric cylinder and added to the samples in the digestion vessel (500 mL beaker). Chemical digestion occurred in two phases: pre-digestion followed by heated digestion at 60°C. A pre-digestion of spiked samples was performed at room temperature for twelve (12) hours (Figure

4). Pre-digestion time was optimized by testing recovery percentages at pre-digestion durations from zero (0) to twelve (12) hours.

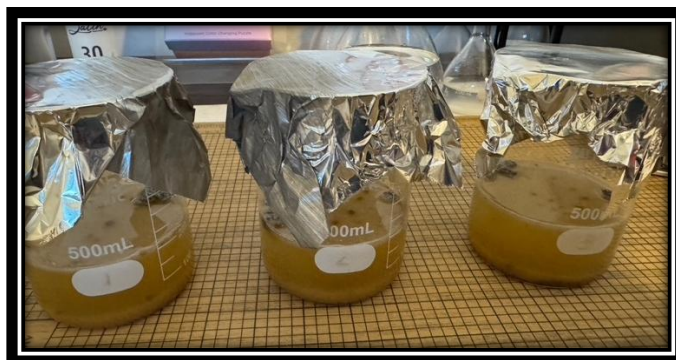


Figure 4: Samples before and after pre-digestion process (taken by Zenaida Rosa).

After the pre-digestion period, the digestion vessels were transferred to a preheated furnace set at 60°C for six (6) hours.

After the pre-digestion and heating digestion process, the samples were sprayed with 70% isopropyl alcohol to reduce the foam contained in the digested animal feed samples (Figure 5).



Figure 5: Samples after heating digestion and sprayed with 70% isopropyl alcohol (taken by Zenaida Rosa).

Samples were left to cool down for an optimized period of two (2) hours. Samples were covered with aluminum foil during the pre-digestion and cooling-down period after the heating digestion to prevent air contamination (Mohsen, Su, Lin, & Li, 2024).

2. Filtration after Chemical Digestion

Digested sample solutions were filtered using an 8-micron Whatman filter (90mm) (Mohsen, Lin, Lu, & Wang, 2024) (Jeyasanta, Sathish, & Patterson, 2024). A vacuum filtration system was connected to the vacuum pump and was used to process the samples. A Buchner porcelain funnel (90mm) was used and adapted to the vacuum filtration system. After filtration, the filters containing microplastics were transferred to a Petri dish for further evaluation. Some spiked plastics in the samples were extracted at this stage. The solution that passed through the filter and was contained in the flask was discarded.

3. Microplastics Density Separation from Sample Solid Residues

A density separation was performed after chemical digestion to recover microplastics from the solid residual material. After filtering the digested samples, 40 mL of saturated sodium chloride (NaCl) solution was added to the residuals, stirred for one (1) minute, and kept for two (2) hours for density separation (Figure 6) (Muhib & Rahman, 2023) (Mohsen, Lin, Lu, & Wang, 2024) (Shütze, Thomas, & Kraft, 2022).



Figure 6: Stirring and density separation of microplastics in solid residuals (taken by Zenaida Rosa).

The supernatant solution was filtered through an 8 μ m Whatman filter (90mm) (Mohsen, Lin, Lu, & Wang, 2024) (Jeyasanta, Sathish, & Patterson, 2024). The filters containing the extracted microplastics was transferred to a petri-dish until examination (Figure 7). The solution that passed through the filter and was contained in the flask was discarded.

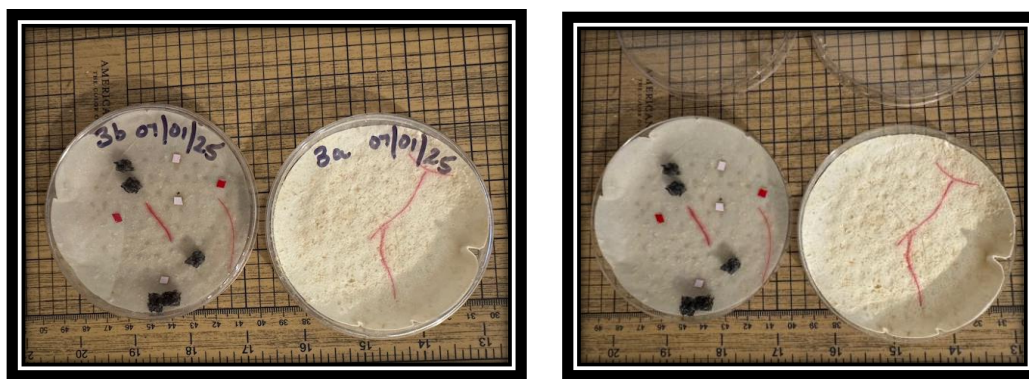


Figure 7: Filters with extracted microplastics transferred and stored in petri dishes (taken by Zenaida Rosa).

Microscopic analysis of the isolated particles was conducted using a Unitron ZST No. 804999 microscope with a magnification of 10X (Shraboni, Shuvo, & Uddin, 2024).

Classification of microplastics

The microplastics can be classified by size, shape, color, and chemical composition (Alvarez-Zeferino, Ojeda-Benitez, Cruz-Salas, Martínez-Salvador, & Vázquez-Mirillas, 2020). The shape and color categories were identified visually and with a microscope, particularly for the transparent fishing line and other microplastics.

Quality Control

Quality control samples were included and treated as samples of the chemical digestion and density extraction process. One blank sample was prepared by adding a mixture of 180 mL of 10% NaOH (sodium hydroxide) and 15 mL of 9% H₂O₂

(hydrogen peroxide) in a 500 mL beaker used for chemical digestion without samples. A second blank was prepared for the saturated saline solution by adding thirty-six (36) grams of sodium chloride (NaCl) to 100 mL of deionized water. Both quality control samples were filtered using a 0.45 µm membrane filter. This was made to verify that the chemicals (powders and DI water) did not introduce microplastics into the samples. There was no plastic particles observed in the solutions used to treat the samples.

Because some food samples were collected in new/clean zip-lock bags, a blank sample was prepared by adding saline solution to new bags, used for collecting animal feed samples, and then filtering the solution as a sample. No presence of microplastics in the samples' zip-lock bags used for storage was observed after filtering.

To minimize contamination from the air and/or clothing, precautions were taken, including rinsing and covering all equipment with aluminum foil, and the use of cotton laboratory coats during sample processing (Zobcov & Esiukova, 2017). Sieves were cleaned with deionized water prior to performing the separation process.

Validation of Determination of Microplastics in Animal Feed Samples

The validated method was used to evaluate the percentage of recovery of spiked microplastics in different animal feed samples, using the digestion/extraction procedure established previously and summarized in Table 2.

Pre-digestion Time room temperature	Digestion @60°C	Precipitation/Cool Down Time	Stirring Time of Digested Solution Saturated NaCl	Floating Time
12 hours	2 hours	2 hours	1 min	2 hours

Table 2: Summary of parameters validated for microplastic extraction methodology.

Samples of dog, cat, rabbit, and chicken feed were processed in triplicate following the above parameters and process were conducted, resulting in a total of 36 samples of 5 grams each being evaluated. All samples were spiked by adding five (5) pieces of the following: polystyrene from a drinking disposable cup, polyester sewing thread, polypropylene from a packaging container, and polyethylene fishing line.

Data Analysis

Statistical analysis was performed using GraphPad Prism version 10.5.0 and Microsoft Excel, version 16.78.3 software. The average and standard deviation of microplastic content in animal feed samples were calculated of the triplicate samples of feed type and source. A student t-test was conducted to assess the statistical differences in the recovered percentage of microplastic content in spiked animal feed sample solutions at various stages of methodology development (Saley, et al., 2019).

RESULTS AND ANALYSIS

The methodology was optimized and validated for the extraction of MP from animal feed pellets. The procedure involves two phases: a digestion step, and a density extraction procedure. Dog pellet food was initially used due to its higher fat content compared to other animal feeds, as shown in Table 2, and the efficiency was also evaluated with rabbit, cat, and chicken feed.

Processing of Feed Samples

Samples commercially acquired were dry pellets, therefore they needed to be homogenized to speed up the digestion procedure. During sample preparation, pellet-shaped samples were first ground and homogenized with a mortar and pestle (Figure 8), as reported in a previous study (Maganti & Akkina, 2023).

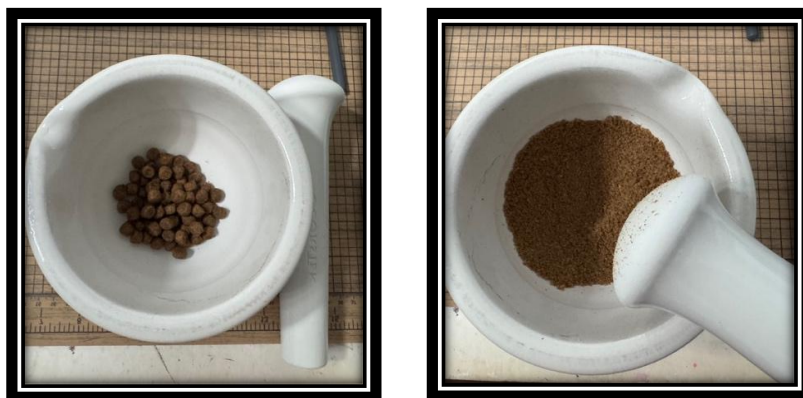


Figure 8: Mortar and pestle used for the grinding of pellet-shaped samples (taken by Zenaida Rosa).

However, with this procedure, homogeneous and reproducible grinding was difficult to reach. Therefore, an electric grinder was chosen to achieve reproducible homogenization of the samples. To evaluate the effect of the grinder on the samples,

several dog pellets were spiked with 20 pieces of microplastic and grounded as shown in Figure 9. After the grinding, the microplastic pieces were evaluated, and no visible fragmentation was observed. Therefore, grinding did not affect the microplastic pieces added to study recovery, at least for the materials used in this study.



Figure 9: Testing the homogenization of dog feed with an electric grinder (taken by Zenaida Rosa).

Microplastic Extraction - Chemical Digestion Process

Before the microplastics extraction, chemical digestion was used to remove as much organic matter as possible, which might hinder the final extraction. The developed method included a pre-digestion step and a final digestion step.

1. Pre-digestion

Samples were treated with sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) to reduce organic matter. Most studies utilize potassium hydroxide (KOH, 10%) to digest organic matter and extract microplastics, but sodium hydroxide (NaOH), a similar strong base, was used because it is more readily available.

A pre-digestion at room temperature was added to improve recovery percentages and reduce the greasy residues observed in the supernatant after the digestion process.

Pre-digestion times were optimized by testing zero (0), six (6), eight (8), ten (10) and twelve (12) hours were tested. A higher recovery percentage was observed when twelve (12) hours of pre-digestion was used compared to samples without pre-digestion. (Figure 10 and Table 3).

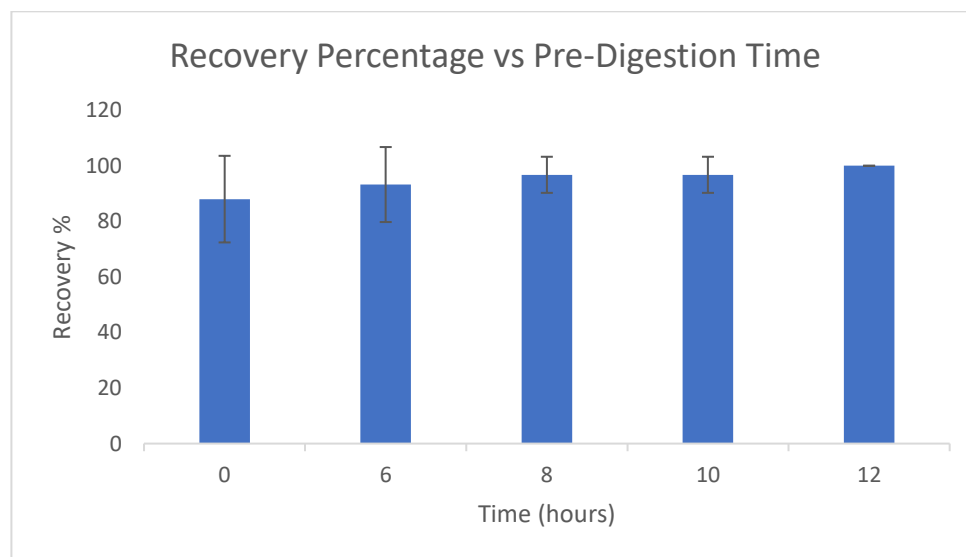


Figure 10: Recovery Percentage vs Pre-Digestion Time tested during methodology development.

Pre-Digestion Time (hours)	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
0	87	100	100	67	88	16
6	100	100	100	73	93	14
8	100	100	100	87	97	7
10	100	100	100	87	97	7
12	100	100	100	100	100	0

Table 3: Recovery Percentage during optimization for pre-digestion time using a mixture of sodium hydroxide and hydrogen peroxide, heated at 60 °C, 2 hours of cool down after digestion, 1 minute of stirring time and two hours for density separation.

A two-tail t-test analysis with 95% confidence, was performed between recovery percentage at zero (0) and twelve (12) hours. There was no significant effect on percent of recovery of the pre-digestion time, $t(6) = 1.5$, $p = 0.2$, despite a 100% recovery for all the types of microplastics when using a twelve (12) hours pre-digestion, while an average of 88% was obtained when no pre-digestion was used. However, it was found to be less fatty material floating on the surface when a longer predigestion time was used. Therefore, a twelve-hour pre-digestion time at room temperature was chosen because minimal greasy residues were observed in samples.

2. Heating Digestion process

After the pre-digestion treatment, a heated digestion at 60°C for two (2) hours was implemented instead of six (6) hours as recommended by (Mohsen, Lin, Lu, & Wang, 2024). Different heating times were tested, aiming to get 100% recovery in

the shortest time. A higher recovery percentage was observed for triplicate samples after two (2) hours, compared to samples digested longer, as shown in Figure 11.

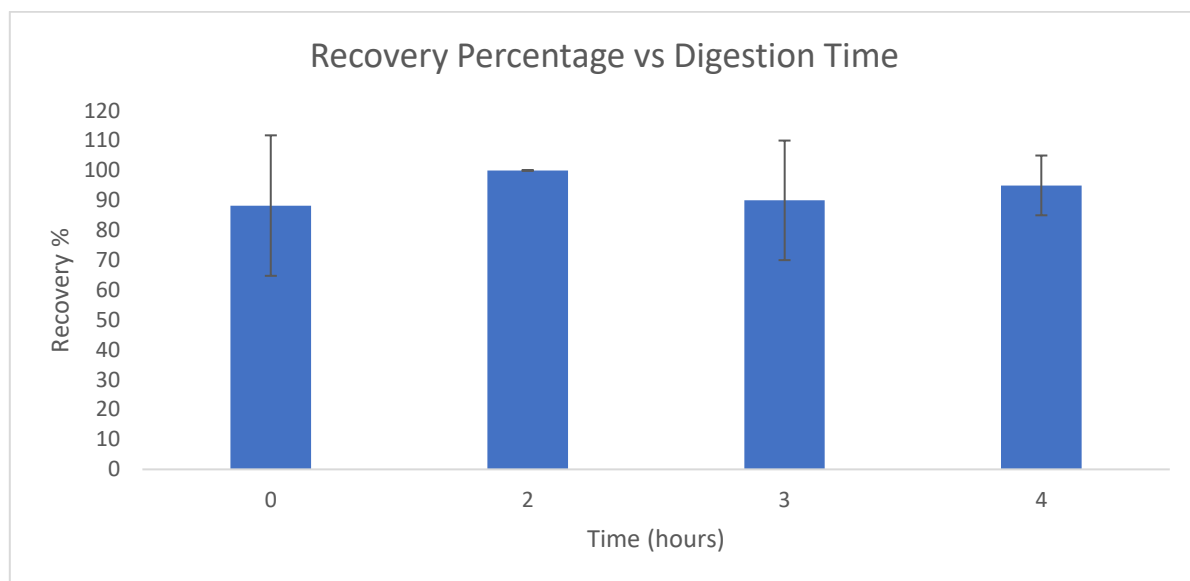


Figure 11: Recovery percentage vs heating digestion time tested during methodology development.

Heating Digestion Time (hours)	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
0	100	100	100	53	88	24
2	100	100	100	100	100	0
3	100	100	100	60	90	20
4	100	100	100	80	95	10

Table 4: Recovery Percentage during optimization for chemical digestion time using a mixture of sodium hydroxide and hydrogen peroxide, heated at 60 °C for 2, 3 and 4 hours, pre-digestion of 12 hours, 2 hours of cool down after digestion, 1 minute of stirring time and two hours for density separation.

The time for the chemical-heated digestion at 60°C was evaluated, including scenarios where the heating time ranged from 0 to 4 hours, as seen in Table 4. A two-tail t-test analysis with 95% confidence was performed between different

digestion times. There were no significant differences in recovery percentage when using zero (0) to four (4) hours heating digestion time, $t(6) = 1$, $p = 0.4$, despite obtaining 100% recovery for all types of microplastics when using a 2-hour heating digestion, while an average of 88% recovery was obtained when no heating digestion was used. Even though results were not statistically different, two (2) hours of heating digestion was chosen to ensure better reproducibility of microplastics extraction, especially for the fishing line units.

3. Cool Down Period After Heating Digestion

Samples were removed from the furnace after heating digestion and allowed to cool down to minimize the presence of bubbles, as performed in a previous study for microplastic extraction in wastewater and sludge (Cunsolo, Williams, & Hale, 2021). Samples were cooled for two (2) hours, rather than overnight as the referenced method suggests, since the recovery percentage of microplastic at this time is shown in Table 5.

Even though there are no differences observed between recovery percent at zero (0) hour and four (4) hours, the cool down period was maintained for two (2) hours since 100% of microplastics were recovered. Reduction of bubbles and agglomerations was observed after the addition of 70% isopropyl alcohol and a cooling-down time.

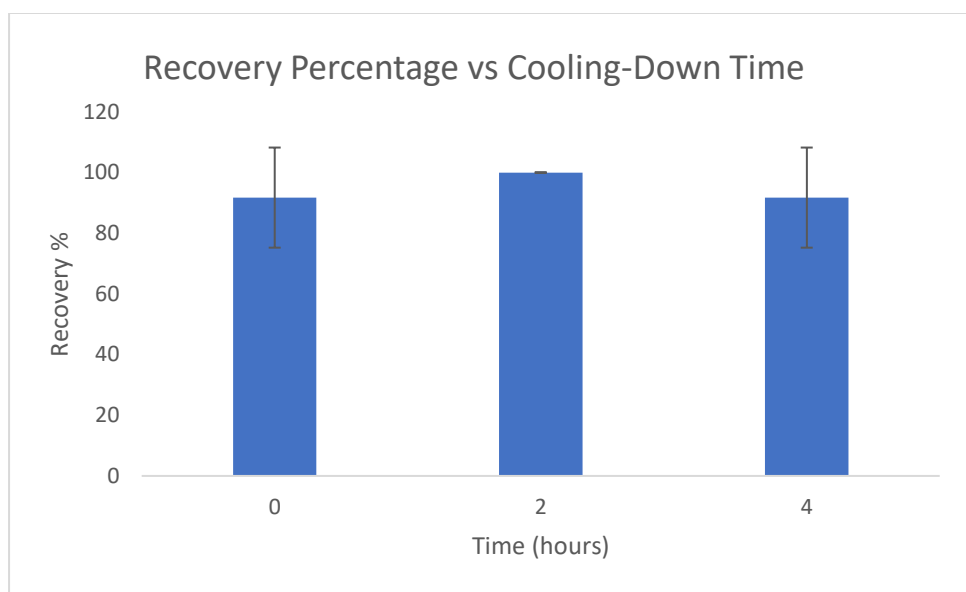


Figure 12: Recovery percentage vs cool-down time tested during methodology development.

Cool Down Time (hours)	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
0	100	100	100	67	92	16.5
2	100	100	100	100	100	0
4	100	100	100	67	92	16.5

Table 5: Recovery percentage during optimization for cooling time after heating digestion using a mixture of sodium hydroxide and hydrogen peroxide, heated at 60°C for 2 hours, pre-digestion of 12 hours, 1 minute of stirring time and two hours for density separation.

There was no significant effect for the cool down time, $t(6) = 1$, $p = 0.4$, despite a 100% recovery for all the types of microplastics was obtained when using two (2) hours heating digestion, while an average of 92% was obtained when zero (0) and two (2) hours of cooling were used. Even though these are not statistically different, two (2) hours of cooling period was chosen to ensure better reproducibility of microplastic extraction, especially for the fishing line units.

4. First filtration – Supernatant of Digested Samples

Once the digestion was finished, the supernatant solution was filtered, to remove any floating microplastic in solution, and reduce the liquid volume to facilitate the extraction in the subsequent step.

Filter pore size was optimized to improve filtering speed, without affecting results. Initially, supernatant of digested sample solutions were filtered using a mixed cellulose ester membrane with a pore size of 0.45 μ m, as reported in previous studies (Bosker, Guaita, & Behrens, 2018) (Muhib & Rahman, 2023). However, since the filtration was observed to be too slow during the first three (3) trials, an 8-micron Whatman filter (90mm) was used for the filtration during the microplastic extraction process (Mohsen, Lin, Lu, & Wang, 2024). Once the supernatant was filtered, the liquid was discarded. Microplastics were counted after completing the filtration of the supernatant and performing density separation from the residues.

Density Separation – Residues Microplastics Extraction

Once the samples were digested, and most of the supernatant liquid filtered, sodium chloride solution, was used on the solid residue to extract the microplastics. The added sodium chloride solution was stirred and left for several hours to allow for proper separation of the microplastics. Sodium chloride solution was used for density separation. Stirring time and setting time were optimized.

Density Separation of Residue

Once the sample was digested and cooled down, the sodium chloride solution was added, and the mixture was stirred. The stirring process helps to prevent the formation of clumps that can trap microplastics and warrant the interaction of the pieces with the solution. Microplastics with less densities than the sodium chloride solution (1.2 g/cm^3) will float (Figure 13). A period of one (1) minute was chosen for the stirring of the solution. Some methods suggest stirring for 30 seconds to one minute to suspend microplastics in solutions (Mohsen, Lin, Lu, & Wang, 2024). Additional stirring time may not be crucial for separating microplastics in a sodium chloride solution. This is because density separation relies on the density difference between the microplastics and the salt solution, rather than the actual stirring time. However, prolonged stirring could lead to increased biofilm formation and make separation more complicated by altering the particle mass (Tu, Chen, & Zhou, 2020).



Figure 13: Samples stirred and left settling down for two (2) hours (taken by Zenaida Rosa).

1. Optimization of density separation time

After stirring, a settling period was included to help reduce suspended particles, promote the flotation of microplastics, and help with further filtration. Samples were left to settle for two (2) and seven (7) hours, and the recovery percentage was evaluated. Results showed that recovery was not affected by the additional time; therefore, a 2-hour setting time was chosen.

To optimize the methodology, additional samples were processed with a density separation time of seven (7) hours, and the recovery percentage for microplastics was compared to that achieved with a two-hour period. Results are shown in Figure 14 and Table 6.

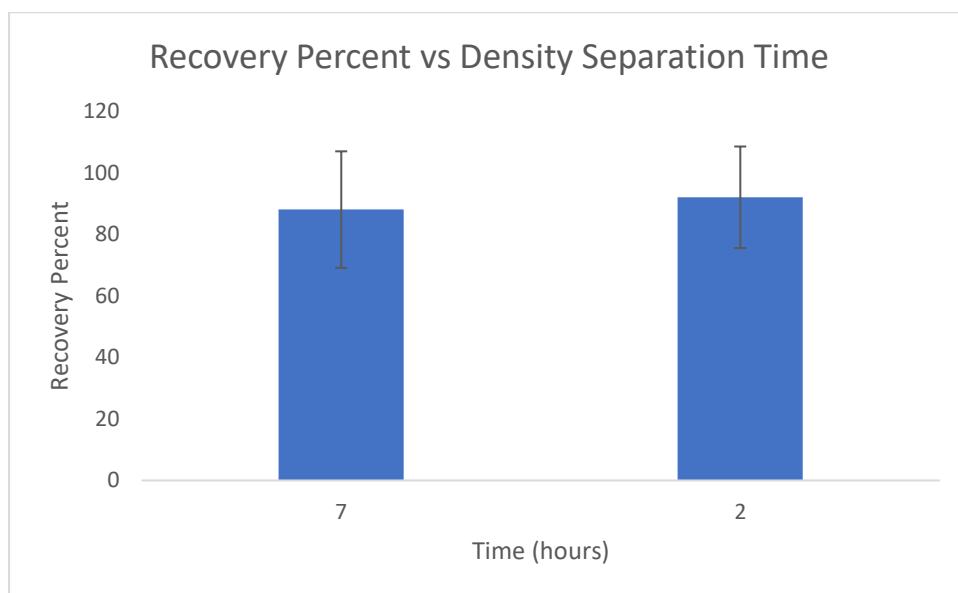


Figure 14: Recovery percent vs density separation time tested during the methodology development.

Density Separation Time (hours)	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
7	100	100	90	60	88	19
2	100	100	100	67	92	16

Table 6: Recovery percentage for 2- and 7-hours period of density separation.

A student t-test with a 95% confidence analysis was performed to compare average recovery percentage when using 2- or 7-hours density separation time. There was no significant difference in the density separation time, $t(6) = 0.3$, $p = 0.8$. When using two (2) or seven (7) hours of density separation time. Even though these are not statistically different, two (2) hours of heating digestion was chosen to ensure better reproducibility of microplastic extraction with a shorter time.

When compared with Mohsen (2024), they reported that samples were left to settle after mixing with the zinc chloride solution; however, they did not specify the time allocated for this process (Mohsen, Lin, Lu, & Wang, 2024). On the other hand, Schütze (2022) recommended a twenty-four (24) hour settling time, for high content of organic matter (compost), when using saturated sodium chloride as a density separation solution, they reported recoveries over 90% of PE and PP microplastics (Schütze, 2022).

For the current study, the method developed yielded recoveries between 88% and 100% of added microplastics, with good reproducibility. The new methodology added a pre-digestion step, reducing the heating time from six (6) hours as reported by Mohsen (2024) to two (2) hours, added a cooling period and proved that a NaCl saturated solution was effective as a density separation solution for these kinds of samples.

Recovery percentages in this study were greater than 90%, which compared with the ones reported by Patrucco (2024) who reported a microplastic extraction protocol for ruminant feeds. By using a heated KOH digestion for 24 hours, and followed by two/three cycles of Fentone reactions to reduce organic matter, they reported extraction recoveries of 100% for high-density, low-density polyethylene, polyamide particles, and polystyrene, and higher than 85% for polyethylene terephthalate and polyamide fibers. Those results are comparable to ours, where we recovered 100% of microplastics of PS, PES-polyester, which contains polyethylene terephthalate, and PP, and higher than 91% for PE. However, the total digestion time of fourteen (14) hours used in our study is shorter than the twenty-four (24) hours reported by Patrucco et al (2024). In our study we included a density separation step since some solid residue was still present after the chemical digestion process, like what Mohsen (2024) reported. The density separation was not required in Patrucco's (2024) work probably because the redox reaction they used was more aggressive, and therefore more efficient removing all residues after digestion. (Patrucco, Rivoira, Bruzzoniti, Barbera, & Tassone, 2024)

In summary, this study demonstrated that the proposed methodology offers practical advantages over existing approaches by using milder digestion reagents, shorter heating times, and an overall reduced processing duration, while still achieving recovery rates comparable to those reported in the literature. These improvements make the method efficient and suitable for routine monitoring of microplastics in animal feed.

Application of Developed Methodology to different animal feed samples

Once the methodology and parameters were optimized for the dog feed samples, similar validation was applied to other commercially available dog feed and others animal feed (cat, chicken, and hen) to determine if this methodology was feasible to extract microplastics, based on the recovery percentage. Twelve (12) commercially available animal feeds were evaluated, for which each sample was spiked by adding five (5) pieces of the following microplastics: polystyrene from a drinking disposable cup, polyester sewing thread, polypropylene from a packaging container, and polyethylene fish line. Polystyrene samples were cut into approximately 5 mm pieces and the thread, and fish line were no longer than 2.5 cm. The plastic types were counted, classified, and documented after extraction of microplastics from spiked-digested samples. The recovery percentage average and standard deviations were calculated for data from triplicate samples. Three experiments for different commercial animal feeds were made for dog, cat, rabbit, and chicken pellet feed in triplicate as shown in Table 7.

Feed Sample	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
<i>Dog pellets 1</i>	100	100	100	100	100	0
<i>Dog pellets 2</i>	100	100	100	87	97	6.5
<i>Dog pellets 3</i>	100	100	100	100	100	0
<i>Cat pellets 1</i>	100	100	100	93	98	3.5
<i>Cat pellets 2</i>	100	100	100	87	97	6.5

Feed Sample	RECOVERY PERCENTAGE (%)				Average	Std. Dev.
	Polyester	Polystyrene	Polypropylene	Polyethylene		
Cat pellets 3	100	100	100	100	100	0
Rabbit feed 1	100	100	100	100	100	0
Rabbit feed 2	100	100	100	93	98	3.5
Rabbit feed 3	100	100	100	93	98	3.5
Chicken feed 1	100	100	100	87	97	6.5
Chicken feed 2	100	100	100	93	98	3.5
Chicken feed 3	100	100	100	93	98	3.5

Table 7: Summary of recovery percentage data for three experiments performed using different commercial animal feeds were made for dog, cat, rabbit, and chicken pellet feed in triplicate.

ANOVA analysis was performed to compare recovery percentage on a standardized test across four different animal feeds. Since the F-statistic was less than the critical p-value, the differences in recovery percentage for all microplastics across all animal feed samples were not statistically significant, $F(35,105) = 2.332$, $p = 0.0005$.

When comparing if there was any difference in the recovery because of the microplastic type, the average recovery percentage for each kind of microplastic used: polystyrene (PS), polyester (PES), polypropylene (PP), and polyethylene (PE) was compared with a 95% confidence. There was no statistically significant difference in the recovery percentage between microplastic types used in this study (PS, PES, PP, PE), $F(3, 44) = 0.2435$, $p = 0.8655$.

Recovery percentage was close to 100 % for most of the microplastics used in this study, except for the fishing line made of PE with an 87% recovery rate. Even

though there were no statistical differences, these low results may indicate that the proposed methodology may have a limitation for this kind of material. Those results may be due to the transparent color of fishing line plastics, making it challenging to see or that some fishing line might get stuck in the funnel or beaker walls during filtration. Figure 15 presents average recovery percentage of the polyethylene for all kinds of animal feed used in this study.

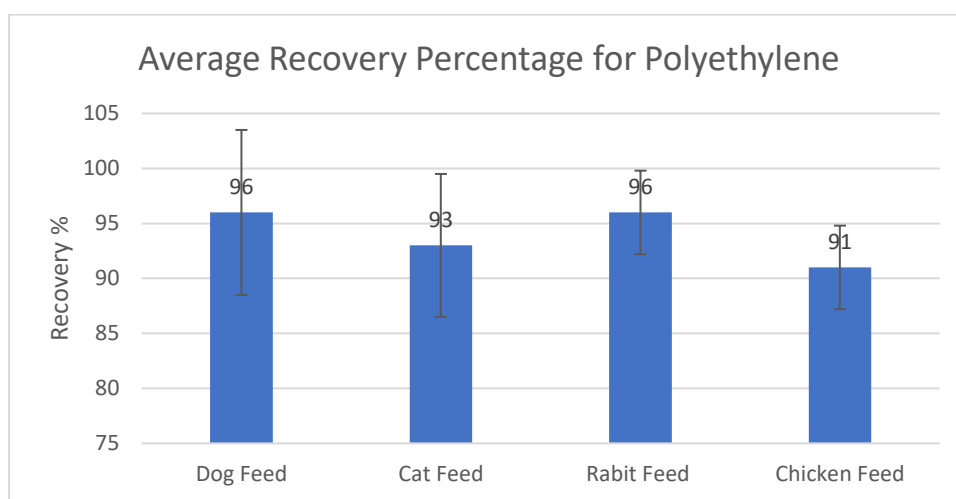


Figure 15: Recovery percent of fishing line for all kinds of animal feed.

During this work, where more than 100 samples were analyzed, only one of the samples presented a microplastic that was not added as part of the spiking. One of the dog feed samples had green fiber as shown in Figure 16. No other particles were observed in other samples, which shows low microplastic contamination with the type of samples used in this study and within the size range used.

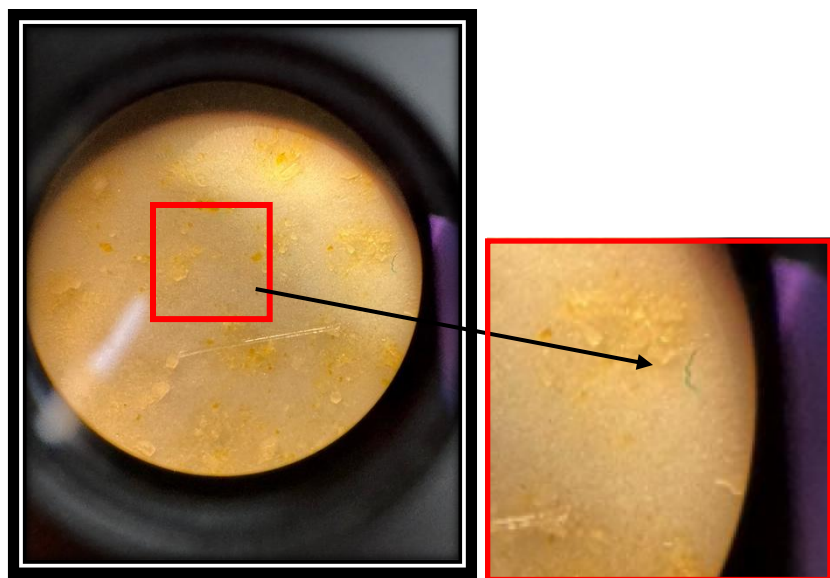


Figure 16: Green fiber observed from dog feed samples.

Reported number of microplastics in animal feed varies depending on the type of food, for example fish feed samples, collected from Bangladesh, contained microplastics with an average of 0.5-2.2 particles per gram (Shraboni, Shuvo, & Uddin, 2024; Siddique, Tahsin, & Hossain, 2023), which is a higher content of particles when compared with our study. Other study analyzing feed samples found microplastic contamination ranging from 0.050 to 0.16 items per gram in shrimp feeds, 0.06 to 0.23 items per gram in fish feeds, and 0.09 to 0.33 items per gram in chicken feeds; these were comparable with microplastic reported for dog feed in our study (Jeyasanta, Sathish, & Patterson, 2024). While Patruco (2025) reported between 16 and 38 items of microplastics per gram in ruminant feed samples, the fibers being the most abundant items in all feeds. (Patruco, Abid, & Rivoira, 2025)

Microplastics detected in our study were larger than 0.1 mm, since it is the smallest visible particle size under a the 10X stereoscope used. The average of

microplastic size found by Mohsen (2024) was 1294 μm (1.294 mm) and the most abundant size ranged from 100 to 1000 μm . Siddique (2023) reported microplastic sizes ranging from 100 to 1500 μm (0.1-1.5 mm) are highly prevalent in fish feed. Our developed method should effectively detect microplastic particles larger than 0.1 mm in animal feed, therefore, different detection methods, such as fluorescent microscopes should be incorporated into our method to detect smaller particles.

Despite the limitation in detecting microplastic particles smaller than 0.1 mm, the developed methodology proved to be reliable for identifying microplastics in dog, cat, rabbit, and chicken feed pellets. It achieved high recovery rates and consistent reproducibility, confirming its suitability for monitoring microplastics in commercial animal feed.

CONCLUSIONS

The purpose of this study was to develop and optimize an efficient method for determining microplastics in animal feed. Methods and parameters suggested in other studies and literature were used as a reference to develop and optimize a feasible and efficient methodology for microplastics extraction from commercial animal feed. Time parameter was evaluated and validated for the two microplastic extraction phases, chemical digestion, and density separation, from animal feed samples. Pre-chemical digestion using a mixture of sodium hydroxide (10%) and hydrogen peroxide (9%) at room temperature for twelve (12) hours, followed by a two-hour heating digestion at 60°C, effectively removed organic material from animal feed samples, reducing the interference during microplastic separation. Using a saturated sodium chloride solution for density separation, spiked microplastics were effectively isolated from digested commercial animal feed samples. A validated extraction method of microplastics was applied to different types of dry dog, cat, rabbit, and chicken feed, and recovery percentages larger than 90% were obtained. The spiked polystyrene (PS) from a disposable drinking cup, polyester (PES) sewing thread, and polypropylene (PP) foam from a packaging container were entirely recovered, while over 87% of polyethylene (PE) from fish line was recovered from all types of animal feed described above.

RECOMMENDATIONS

This work has developed a determination method of microplastics in animal feed pellets, however more research may be needed, for example:

1. Evaluate the effect of different reagent concentrations (NaOH and H₂O₂) on the efficiency of microplastic extraction in animal feed.
2. Incorporate a treatment using microwave oven to improve analysis time and evaluate its impact in microplastics stability.
3. Reduce treatment time to make the procedure more streamlined and faster.
4. Evaluate conditions to improve the digestion of organic matter and eliminate the density separation step.
5. Use a stereo microscope with high resolution to confirm the presence of microplastic particles smaller than 0.1mm.
6. Test the developed microplastics determination method for other forms of animal feed, such as powders, flakes, etc.
7. Apply this developed method for the microplastic extraction of animal feed from livestock and other feed samples.
8. Identify microplastics found in animal feed to determine potential sources and their impact.

BIBLIOGRAPHY

- Acosta-Coley, I., & Olivero-Verbel, J. (2015). Microplastic resin pellets on an urban tropical beach in Colombia. *Environmental Monitoring Assessment*, 187, 435.
- Akanda, R., Kawsar, A., & Sadik Sabuj, S. (2025). Microplastic pollution in livestock farming in Bangladesh: a critical review of its key findings and mitigation strategies to address public health. *Environmental Monitoring Assessment*, 197, 732.
- Alvarez-Zeferino, J., Ojeda-Benitez, S., Cruz-Salas, A., Martínez-Salvador, C., & Vázquez-Mirillas, A. (2020). Method for Quantifying and Characterization of Microplastics in Sand Beaches. *Revista Internacional de Contaminación Ambiental*, 151-164.
- Andrady, A. (2011). Microplastics in the marine environment. *Marine Pollution Bulletin*, 62, 1596-1605.
- Barnes, D., Galgani, F., Thompson, R., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environment. *Philosophical Transactions of the Royal Society, Series B*, 00, 1-14.
- Bosker, T., Guaita, L., & Behrens, P. (2018). Microplastics pollution on Caribbean beaches in the Lesser Antilles. *Marine Pollution Bulletin*, 133, 442-447.
- Browne, M., Galloway, T., & Thomason, R. (2010). Spatial patterns of plastics debris along estuarine shorelines. *Environmental Science and Technology*, 3404-3409.
- Chamas, A., Moon, H., Zheng, J., Qui, Y., Tabassum, T., Jang, J., Abu-Omar, M., Scott, S., Suh, S. (2020). Degradation Rates of Plastics in the Environment. *ACS Sustainable Chemistry and Engineering*, 8, 3494-3511.
- Cole, M., Lindeque, P., Halsband, C., & Galloway, T. (2011). Microplastics as contaminants in the marine environment: a review. *Marine Pollution Bulletin*, 62, 2588-2597.

- Cunsolo, S., Williams, J., & Hale, M. (2021). Optimising sample preparation for FTIR-based microplastic analysis in wastewater and sludge samples: multiple digestions. *Analytical and Bioanalytical Chemistry*, 413, 3789-3799.
- Datu, S., Supriadi, S., & Tahir, A. (2019). Microplastics in *Cymodocea rotundata* Seagrass Blades. *International Journal of Environment, Agriculture and Biotechnology*, 4(6), 1758-1761. doi:doi.org/10.22161/ijeab.46.21
- Food and Drug Administration. (2024). *Microplastics and Nanoplastic in Food*. Obtenido de fda.gov: <https://www.fda.gov/food/environmental-contaminants-food/microplastics-and-nanoplastics-foods>
- Foundation, P. S. (s.f.). *Monomers and Polymers*. Obtenido de <https://www.plasticsoupfoundation.org/en/plastic-problem/what-is-plastic/monomers-and-polymers/>
- GESAMP. (2019). *Guidelines for the Monitoring and Assessment of Plastic Litter in the Ocean*. United Nations Environment Programme.
- Geyer, R., Jambeck, J., & Law, K. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, 3, 1-5.
- Geyer, R., Jambeck, J., & Law, K. (2017). Production, use, and fate of all plastics ever made. *Science Advances*.
- González-Puetate, I., Valle-Mieles, E., & Yañez-Avalos, D. (2025). Chapter 2: Microplastic Contamination in Horse Husbandry. En D. Pinela, E. Valle-Mieles, & I. González-Puetate, *Presence of Microplastics in Livestock Production: A Challenge for Animal Health and Sustainability* (págs. 22-43). doi:DOI: 10.70593/978-93-7185-104-6_2
- Henry, B., Laitala, K., & Grimstad Klepp, I. (2019; 652). Microfibres from apparel and home textiles: Prospects for including microplastics in environmental sustainability assessment. *Science of the Total Environment*, 483-494.

- Hester, R., & Harrison, R. (2019). *Plastics and the Environment*. United Kingdom - England: The Royal Society of Chemistry.
- Hidalgo-Ruiz, V., Gutow, L., Thomson, R., & Thiel, M. (2012). Microplastics in the Marine Environment: A Review of the Methods Used for Identification and Quantification. *Environmental Science and Technology*, 46, 3060-3075.
- Hidalgo-Ruz, V. G. (2012). Microplastics in the Marine Environment: A Review of the Methods Used for Identification and Quantification. *Environmental Science and Technology*, 46, 3060-3075.
- Iñiguez, M., Contessa, J., & Fullana, A. (2016). Marine debris occurrence and treatment: a review. *Renew* 64, 394-402.
- Ivar do Sul, J., & Costa, M. (2014). The present and future of microplastics pollution in the marine environment. *Environmental Pollution*, 352-364.
- Jambeck, J., Geyer, R., Wilcox, C., Sigler, T., Perryman, M., Andrady, A., Narayan, R., Law, K. (2015). Plastic waste inputs from land into the ocean. *Science*, 768-777.
- Jeong, E., Lee, J., & Redwan, M. (2024). Animal Exposure to microplastics and health effects: A review. *Emerging Contaminants*, 10, 1-11.
- Jeyasanta, I., Sathish, M., & Patterson, J. (2024). Microplastics contamination in commercial fish meal and feed: a major concern in the cultured organisms. *Chemosphere* 363, 142832.
- Kedzierski, M., Palazot, M., Soccalingame, L., Flacou-Préfol, M., Gorsky, G., Galgani, F., . . . Pedrotti, M. (2022). Chemical composition of microplastics floating on the surface of Mediterranean Sea. *Marine Pollution Bulletin* 174 (9):113284.
- Lebrenton, L., & Andrady, L. (2019). Future scenarios of global plastic waste generation and disposal. *Palgrave Communications*.

- Lots, F., Beherens, P., Vijver, M., Horton, A., & Bosker, T. (2017). A large-scale investigation of microplastic contamination: abundance and characteristics of microplastics in European beach sediment. *Marine Pollution Bulletin*, 123, 219-226.
- Maganti, S., & Akkina, R. (2023). Detection and Characterization of Microplastics in Animal Feed. *Online Journal of Animal and Feed Research*, 13(5), 348-356.
- Mendez, L. (2021). Microplastics Effect in the Terrestrial Environment. *Handbook of Microplastics in the Environment*, 1-30.
- Mohsen, M., Lin, J., Lu, K., & Wang, L. (2024). Microplastic pollution in aquafeed of diverse aquaculture animals. *Heliyon*, 10, 1-9.
- Mohsen, M., Su, F., Lin, J., & Li, X. (2024). Microplastic Contamination in Aquafeed Ingredients Used as Protein and Carbohydrate Sources. *Bulletin of Environmental Contamination and Toxicology*, 113, 41.
- Moore, C. (2008). Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. *Environmental Research*, 108, 131-139.
- Muhib, M., & Rahman, M. (2023). Microplastics contamination in fish feeds: Characterization and potential exposure risk assessment for cultivated fish of Bangladesh. *Heliyon*, 9, e19789.
- Nanthini Devi, K., Raju, P., Santhanam, P., & Perumal, P. (2022). Impacts of microplastics on marine organisms: Present perspectives and the way forward. *Egyptian Journal of Aquatic Research*.
- Neal, R. (1985). Mechanisms of the Biological Effects of PCBs, Polychlorinated Dibenzo-p-dioxins and Polychlorinated Dibenzofurans in Experimental Animals. *Environmental Health Perspective*, 60, 41-46.

- Ouyang, X., Panti, C., Canicci, S., Li, R., & Tam, N. (2023). Editorial: Impact of Marine Debris on Marine Ecosystem and Organisms. *Marine Pollution, a section of the journal Frontiers in Marine Science*.
- Patruco, S., Abid, K., & Rivoira, L. (2025). Microplastics contamination in ruminant feeds of Northwestern Italy: a preliminary assessment. *Science of the Total Environment*, 1001, 180479.
- Patruco, S., Rivoira, L., Bruzzoniti, M., Barbera, S., & Tassone, S. (2024). Development and application of a novel extraction protocol for the monitoring of microplastic contamination in widely consumed ruminant feeds. *Sci Total Environ*, 947:174493. doi:doi: 10.1016/j.scitotenv.2024.174493
- Pérez-Alvelo, K., Llegus, E., Forestier-Babilonia, J., Elías-Arroyo, C., Pagán-Malavé, K., Bird-Rivera, G., & Rodríguez-Sierra, C. (2021). Microplastic pollution on sandy beaches of Puerto Rico. *Marine Pollution Bulletin*, 164, 112010, 1-9.
- Plastics Europe. (2021). *Plastics - The Facts 2021: An analysis of European plastics production, demand and waste data*. Obtenido de Plastics Europe.
- Prata, J., & Dias-Pereira, P. (2023). Microplastics in Terrestrial Domestic Animals and Human Health: Implications for Food Security and Food Safety and Their Role as Sentinels. *Animals*, 13(661), 1-12.
- Rai, M., Pant, G., & Pant, K. (2023). Microsplastics Pollution in Terrestrial Ecosystems and Its Interaction with Other Soils Pollutants: A Potential Threat to Soil Ecosystem Sustainability. *Resources*, 1-14.
- Ritchie, H., Samborska, V., & Roser, M. (April de 2022). *Plastic Pollution*. Obtenido de Our World in Data: <https://ourworldindata.org/plastic-pollution>

- Rochman, C. M., Kross, S. M., Armstrong, J. B., Bogan, M. T., Darling, E. S., Green, S. J., . . . Veríssimo, D. (2015). Scientific evidence supports a ban on microbeads. *Environmental Science and Technology*, 10759-10761.
- Rodriguez, F. (November de 2025). *Plastic*. Obtenido de Britannica: <https://www.britannica.com/science/plastic/The-polymers>
- Saley, A., Smart, A., Bezera, M., Burnham, T., Capece, L., Lima, L., Smyth, A. R., Morgan, S. (2019). Microplastic accumulation and biomagnification in a coastal marine reserve situated in a sparsely populated area. *Marine pollution Bulletin*, 146, 54-59.
- Seng, N., Lai, S., Fong, J., Faiqu Saleh, M., Cheng, C., Cheok, Z., & Todd, P. (2020). Early evidence of microplastics on sea grass and macroalgae. *Marine and Freshwater Research, CSIRO Publishing*, A-D.
- Setälä, O., Fleming-Lehtinen, V., & Lehtiniemi, M. (2014). Ingestion and transfer of microplastics in the planktonic food web. *Environmental Pollution*, 185, 77-83.
- Sharma, P. (2024). Microplastic Contamination in Food Processing: Role of Packaging Materials. *Food Science and Engineering*, 5(2), 271-287.
- Shraboni, F., Shuvo, T., & Uddin, S. (2024). Bangladesh's Commercial Fish Feed Industry: A Source of Microplastic Contamination. *International Journal of Advance Research in Biological Sciences*, 11(11), 1-23.
- Shütze, B., Thomas, D., & Kraft, M. (2022). Comparison of different salt solutions for density separation of conventional and biodegradable microplastic from solid sample matrices. *Environmental Science and Pollution Research*, 29, 81452-81467.
- Siddique, M., Tahsin, T., & Hossain, I. (2023). Microplastic contamination in commercial fish feeds: A major concern for sustainable aquaculture from developing country. *Ecotoxicology and Environmental Safety*, 267, 115659.

- Silburn, B., Bakier, A., Binetti, U., Russell, J., Kohler, P., Preston-Whyte, F., Meakins, B., Hoytema, N., Andrews, G., Carrias, A., Maes, T. (2022). A baseline study of macro, meso, and micro litter in the Belize River basin, from catchment to coast. *ICES Journal of Marine Science*, 1-14.
- Stafella, P., López-Correa, M., Pyko, I., Teichert, S., & Gomiero, A. (2020). Distribution of Microplastics in the Marine Environment. *Handbook of Microplastics in the Environment*, 1-35.
- Suaria, G., & Aliani, S. (2014). Floating debris in the Mediterranean Sea. *Marine Pollution Bulletin*, 494-504.
- Teuten, E., Saquing, J., Knappe, D., Barlaz, M., Jonsson, S., Bjorn, A., Rowland, S.; Thompson, R.; Galloway, T.; Yamashita, R.; Ochi, D.; Prudente, M.; Boonyatumanod, R.; Zakaria, M.; Akkhavong, K.; Ogata, Yuko; Hirai, H.; Iwasa, S.; Mizukawa, K.; Hagino, Y.; Imamura, A.; Saha, M.; Takada, H. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society, Series B, Biological Science*, 2027-2045.
- Tosetto, L., Williamson, J., & Brown, C. (2017). Trophic transfer of microplastics does not affect fish personality. *Animal Behaviour* 123, 159-167.
- Tu, C., Chen, T., & Zhou, Q. (2020). Biofilm formation and its influences on the properties of microplastics as affected by exposure time and depth in the seawater. *Science of The Total Environment*, 734, 139237.
- Van der Veen, I., Van der Mourik, L., & Van Velzen, M. (2022). *Plastic Particles in Livestock Feed, Milk, Meat and Blood (Pilot Study)*. Obtenido de Plastic Soup Foundation: https://www.plasticsoupfoundation.org/rapporten/Livestock-mps-study-KEY-messages_final_2022-07-05.pdf

- Woodall, L., Gwinnett, C., Packer, M., Thomson, R., Robinson, L., & Paterson, G. (2015). Using a forensic science approach to minimize environmental contamination and to identify microfibers in marine sediments. *Marine Pollution* 95 (1), 40-46.
- Wright, S., Thomson, R., & Galloway, T. (2013). The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution* 178, 483-492.
- Xing Liu, C., Wang, H., & Sun, X. (2021). Occurrence of Microplastics in Fish and Shrimp Feeds. *Bulletin of Environmental Contamination and Toxicology*, 1-9.
- Zeb, A., Liu, W., & Ali, N. (2024). Microplastic pollution in terrestrial ecosystems: Global implications and sustainable solutions. *Journal of Hazardous Materials*, 461, 132636.
- Ziccardi, L., Edgington, A., Hentz, K., Kulacki, K., & Dricoll, S. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. *Environmental Toxicology and Chemistry*, 35 (7), 1667-1676.
- Zobcov, M., & Esiukova, E. (2017). Microplastics in Baltic bottom sediments: quantification procedures and first results. *Marine Pollution Bulletin*, 724-732.