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Karlo Blažević

Development of a measurement procedure for the detection of micro and nanoplastic particles in tissue samples

MASTER THESIS

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ABSTRACT

Development of a measurement procedure for the detection of micro and nanoplastic particles in tissue samples

With the mass accumulation of plastic pollution in the environment, problems are present even on the micro and nano scale. Micro and nanoplastic (MNP) particles with a diameter of less than one millimeter, mostly generated by the degradation and wearing down of larger plastic parts, are accumulating in the ecosystem and reaching the human body in different ways. The potential of the particles to adsorb harmful substances poses a big threat to ecosystems and human health.

Analytical chemistry offers essential tools for the detection, quantification, and characterization of small particles, enabling researchers to study their distribution, composition, and impact. The most used methods are different types of microscopy and spectroscopy.

This work provides the process of preparing and analyzing different tissue samples for MNP research. A review of the most common tissue digestion methods was made, along with two analytical methods that were used to detect, quantify, and characterize MNPs: optical microscopy and spectroscopy. MNP particles were found and identified in the tissue samples, along with present contaminants.

Keywords: micro and nanoplastics, tissue, microscopy, spectroscopy

SAŽETAK

Razvoj mjernog postupka za detekciju mikro i nanoplastičnih čestica u uzorcima tkiva

Sve većim rastom plastičnog onečišćenja u okolišu javljaju se problemi čak i u mikro i nano razmjerima. Plastične čestice promjera manjeg od milimetra koje uglavnom nastaju razgradnjom i trošenjem većih dijelova plastike, nakupljaju se u ekosustavu te različitim putevima dolaze i do organizma čovjeka. S potencijalom adsorpcije štetnih tvari, mikro- i nano plastika predstavlja veliku prijetnju ekosustavima i ljudskom zdravlju. Analitička kemija nudi bitne alate za detekciju, kvantifikaciju i karakterizaciju malih čestica, omogućujući istraživačima proučavanje njihovog sastava, distribucije i utjecaja. Najčešće metode koje se koriste su razni oblici mikroskopije i spektroskopije.

Ovaj rad sadrži proces pripreme i analize različitih uzoraka tkiva za istraživanje mikro- i nano plastike. Napravljen je pregled najčešćih metoda digestije tkiva te dvije analitičke metode koje su korištene za detekciju, kvantifikaciju i karakterizaciju mikro- i nano plastike: optička mikroskopija i spektroskopija. U uzorcima tkiva pronađene su i identificirane mikro- i nanoplastične čestice, uz prisutna onečišćenja u obliku fragmenata i vlakana.

Ključne riječi: mikro- i nano plastika, tkivo, mikroskopija, spektroskopija

Content

1. INTRODUCTION

The global use of plastics has caused serious environmental pollution and has become one of the biggest problems in environmental protection. Plastic parts degrade into smaller particles which can be easily spread and accumulated in ecosystems. Plastic parts smaller than 5 mm in diameter are called micro and nanoplastics (MNPs) and their distribution in the environment is concerning. MNPs have been detected in water, earth, air, animals, and humans.[1]

Scientists have been developing methods for detecting and characterization of the particles to study their distribution and impact. The size and shape of the particles can be complex and diverse. [2] The degraded plastic surface can absorb harmful substances which can cause health issues in humans.[1] Morphology, along with the level of degradation and present harmful substances is the main focus of research. Knowing more about the source and distribution of MNPs will lead to a better understanding of the impact, how to prevent the pollution, and deal with the current problem it is causing.

Analytical methods like microscopy and spectroscopy are the main tools that are being used for MNP research.[3] Microscopy is a quick way of MNP detection, while spectroscopy provides information on particle composition. These methods allow researchers to identify and characterize MNPs based on their morphology, size distribution, and chemical composition.[4] However, successful results of these methods require good sample preparation to ensure accurate results.[5] Throughout the whole process of sample preparation and analysis, it is important to minimize outside particle contamination. Researchers follow contamination control protocols, such as the use of clean rooms, filtered air systems, and blank controls, ensuring the results reflect the actual presence of MNPs in the sample.[5]

In this work, a process of sample preparation, particle detection, and identification has been performed. Different types of tissue samples have been digested with combinations of HNO_3, KOH, and H_2O_2 solutions. A more gentle approach has been achieved by enzymatic digestion using enzymes like protease, lipase, and amylase. The quality of tissue digestion was measured visually and quantitatively by weighing the samples before and after digestion and by the ability of the samples to filter. The filtered samples were then further analyzed for MNP particles by optical microscopy and LDIR (Laser Direct Infrared Imaging) spectroscopy. MNP particles were successfully found and identified in the tissue samples, along with present contaminants in the forms of particles, fragments, fibers, and undigested tissue.

2. THEORY

2.1. Plastics

Plastics are types of polymers derived from either natural or synthetic materials massively manufactured by different chemical and physical processes, with different types of additives to give them properties fit for their purpose. The development of the petrochemical industry has been one of the biggest contributors to the massive production of plastics and their wide use nowadays.[6] As a result today, the production and use of cheap plastic materials is worldwide.

Different plastics have various applications due to their different chemical composition. Polyethylene (PE) is made from ethylene monomers through polymerization, comes in various forms and densities, and provides specific properties suitable for different use.[7] It can come as flexible, chemically resistant low-density polyethylene (LDPE) used for making plastic bags, packaging, and film, or as strong, resistant to chemicals and impact, high-density polyethylene (HDPE). Polypropylene (PP) is a thermoplastic polymer with various applications due to its low density, but good chemical and heat resistance, toughness, and durability. PP can be recycled and is often used for packaging, textiles, films, consumer and household goods, as well as in automotive, medical, and other industries. Polystyrene (PS), a light and easy-to-process polymer can come as general-purpose polystyrene (GPPS) used for light packaging and cutlery, high-impact polystyrene (HIPS) used for making toys, household appliances, and electrical insulation, or as expandable polystyrene (EPS) used for insulation panels and disposable food packaging. Polyvinyl chloride (PVC) due to its durability, and mechanical and chemical resistance is widely used in construction, piping, insulation, medical, packaging, and automotive industries. When PVC burns, it releases harmful chlorine substances in the environment.[8] Other plastics are also used for various applications like polyethylene terephthalate (PET) used for plastic bottles, polyurethane (PU) foam, polycarbonate (PC), Acrylonitrile Butadiene Styrene (ABS), Polyvinylidene Fluoride (PVDF), Nylon (Polyamide), Polyethylene Glycol (PEG), Polyethylene Vinyl Acetate (EVA), etc.[7]

Because of the high demand for such products, the biggest polluters of the environment with plastics are textile, packaging, fast food, restaurant, fishing, agricultural, construction, tobacco, and healthcare industries. It is also important to recognize that due to the heavy restrictions and regulations for plastic waste management, industries are making efforts to reduce their use of plastic and implement more sustainable practices. As the awareness of plastic pollution grows, companies and consumers are searching for alternatives to plastic products and developing new

ways of waste management. Government regulations and policies play an important role in the future use of plastic and its impact on the environment.

2.1.1. Micro and nanoplastics

Microplastics are generally considered solid polymers with a dimension of <5 mm that can originate from primary production of plastics or accumulate from fragmentation of larger plastic products, which is considered secondary production.[9] Nanoplastics aren't defined as precise since the detection and analysis are harder than that of microplastics because of their smaller size. They are usually considered as particles with a size less than 100 or 1000 nm and their origin can as well be classified from primary or secondary production. Primary production considers intentional manufacturing or synthesis of the nanoplastics from processes such as nanoprecipitation or emulsion polymerization. Secondary production of nanoplastics is considered degradation from microplastics due to UV-induced and mechanical processes.[10]

Due to their size, MNP particles can easily be transported by wind and water contributing to its pollution. Washing synthetic fabrics in clothing, tire wear from driving, personal care products, plastic litter, microbeads, industrial waste, fishing equipment and weathering of large plastic debris are the most common sources of microplastic pollution.[11] As a result, researchers have found MNPs in different places, such as:

- Water: increase of plastic fragments in the last couple of decades in oceans, seas, rivers, lakes, and their shorelines[12],
- Soil: Occurrence of nanoplastics in plastic-contaminated soil and its' degradation[13],
- Air: accumulation of MNP particles in the air and its' deposition through rainfall[14],
- Food: the presence of MNP particles in food, its' identification, and ways of separation[15],
- Beverages: use of plastic teabags and their impact on MNP contamination[16], and even in living organisms such as:
    - animals: digestion of MNP particles by fish and the presence of it in seafood[17],
    - plants: uptake of nutrients and MNP particles from the soil by vegetables[18],
    - and humans: the presence of MNP particles in breast milk.[19]

2.1.2. Impact on the environment and general health

MNPs can be ingested by humans through various pathways, mainly due to their small size and enormous presence in the environment. From contaminated food or water; to airborne particles

and dust. Nanoplastics are of particular concern due to their incredibly small size, which can potentially enable them to cross cellular barriers and interact with biological systems in ways that larger particles can not.[20]

One of the biggest problems that MNPs carry is the additives used to improve their properties, like plastificators, stabilizers, flame retardants, colorants, UV-stabilizers, lubricants, fillers, and impact modifiers. These additives contain substances that can be harmful to human health.[7] Many chemicals that make plastics are dangerous. Plastics that are used to make household products and food packaging can contain Bisphenol A (BPA), phthalates, and heavy metals which have been proven to be endocrine disruptors if inhaled or ingested.[21]

Specifically, BPA is a plastificator that makes the plastic more resistant to high temperatures, impacts, and collisions. In the early 1930s, scientists found out that it was estrogenic[22] and later research confirmed that it is a substance of concern with hormone-alternating properties and was connected to higher rates of child obesity, anxiety, depression, and cardiovascular diseases.[23] Phthalates on the other hand are used for the improvement of plastic elasticity, flexibility, and pliability, and are the most massively produced synthetic chemical group. Some of them can be endocrine disruptors and can cause cardiovascular damage to the human body.[24] The accumulation of these particles in the environment will have a major negative effect on living organisms. Stronger regulations regarding waste management, removing toxic chemicals from overall use, and funding the research on these topics is crucial so solutions can arise as soon as possible. MNP accumulation will only increase over time if the correct means are not implemented, and so will the problems that come with it.

2.2. Tissue digestion

Before any particle analysis in any tissue sample, a quality tissue digestion protocol must be performed. There are many tissue digestion techniques possible and the key is to find one that suits the type of sample the best. Specifically, while analyzing MNP particles, tissue digestion is achieved by using strong oxidizing acids (e.g. HNO_3), strong bases (e.g. KOH), or combinations of both.[25] Along with the mentioned methods, a more gentle and specific soft tissue digestion protocol is achieved by enzymatic digestion which indicates the potential use of industrial enzymes for the digestion of various living organisms.[25] The primary goal is to eliminate all present tissue and leave the MNP particles intact so they can be properly identified and characterized. The challenge is to digest as much tissue as possible without impacting the

physical and chemical composition of the MNPs.[11] A table comparison of different types of digestion can be found in Table 1.

2.2.1. Oxidative tissue digestion

The oxidative tissue digestion method is based on breaking down biological tissue with the use of strong oxidizing reagents to degrade present organic material like proteins, carbohydrates, and lipids. During the digestion period, the oxidizing agent helps break down complex compounds into simpler ones, like carbon dioxide, water, and salts. Due to the nature of oxidizing agents, it is advised to control the conditions of digestion such as temperature, pressure, and digestion time. Common oxidative agents include different concentrations of potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2), and nitric acid (HNO_3), ranging from 10 to 35%.[26]

2.2.2. Alkaline tissue digestion

Using alkaline reagents and strong bases like sodium oxide (NaOH), 0,5 to 5 M[26], or potassium oxide (KOH), usually around 10%[27] helps break down complex organic molecules into simple compounds. In alkaline conditions, hydrolysis denaturalizes proteins into amino acids, while lipids get saponified into fatty acids and glycerol. The temperature, pressure, and digestion time depend on the used reagent and the type of tissue.[28]

2.2.3. Enzymatic tissue digestion

The enzymatic tissue digestion method is used to break down biological tissue by hydrolyzing proteins or nucleic acids with specific types of enzymes fit for the purpose. Enzymes are selected according to the nature of the sample and the molecules we want to break down. For example, proteases like trypsin, chymotrypsin, or proteinase K are used to break down proteins, while nucleases are used to digest nucleic acids. Proteinase K can be expensive so other commercially available and inexpensive proteinases are available[29], like Protamex. A mixture of pancreatic enzymes was used by W. L. Von Friesen in extracting microplastics from bivalve tissue and proved successful with over 97% of tissue dissolved within a day.[25] This technique is mostly used in molecular biology, biochemistry, and proteomics research to extract specific components of interest from tissue samples while leaving other cellular structures or particles intact.[29]

Table 1. Tissue digestion methods

Digestion type	Chemicals used	Pros and cons	Literature
Oxidative	HNO_3, H_2O_2, HCl	Strong oxidizing agents Can be too strong and affect particle structure and composition.	Extraction of MNPs from mussels using HNO_3 as a solvent for tissue digestion[26]
Alkaline	NaOH, KOH	Can affect particles sensitive to pH change. Cheap, commercially available.	Preparation of biological samples using KOH as agent for tissue digestion, with further detection with Nile-red method[27]
Enzymatic	protease, nuclease, lipase	The gentle process has minimal effect on present particles Enzymes in high concentrations are expensive and can be hard to get.	Extraction of MNPs from bivalve tissue using enzymatic tissue digestion[26]
Combination	A combination of oxidative, alkaline, and enzymatic digestion	For non-homogenous tissue gives many ways for the tissue to dissolve. Can be expensive and take more time. Solutions may react with one another and form unwanted compounds.	Efficient extraction of small microplastic particles from rat feed and feces for quantification using combinations of tissue digestion methods[30]

2.3. Micro and nanoplastic identification and characterization

2.3.1. Microscopy

Microscopy plays a crucial role in MNP research, providing valuable insights into the size, shape, morphology, and distribution of particles in various environmental samples. It is also used for tissue studies, fiber identification, detection of aggregates of MNPs, and in general for environmental research. A number of techniques and types of microscopes can be used to detect MNPs.[31]

2.3.1.1. Bright- and dark-field microscopy

The most common technique is bright, followed by dark-field optical microscopy to detect polymer particles in environmental samples and organisms.[32] In bright-field microscopy, illuminating light through a sample on a bright background and observing through objective lenses of different magnifications provides an image of the sample's structure. It is often combined with staining methods to make the sample analysis easier. In dark-field microscopy, the sample is illuminated with a scattered light that reflects from the sample and the background.

The objective lenses capture only the scattered light from the sample and not the background making the picture distorted with a dark background and bright, illuminated particles. In MNP research, they are mostly used for particle detection and identification of size, shape, and degradation level.[32]

2.3.1.2. Fluorescence microscopy

Fluorescence microscopy is a technique that uses fluorescent dyes to label the sample and observe it by shining light of a certain wavelenght that excites the fluorescent dye molecules which makes them emit fluorescent light. The emitted light passes through filters to get to the detector which provides a bright image of the sample on a dark background. A sensitive method that can provide insight into the molecular structure of samples is used in biology, molecular biology, medicine, and material science, including MNP research.[33]

2.3.1.3. Confocal microscopy

Confocal microscopy is a type of fluorescence microscopy providing high-resolution 3-D pictures by capturing optical sections of thick samples. It is useful for observing transparent and fluorescent samples as it gives insight into sample structure and its 360° view. In environmental research, it is used for analyzing dynamic processes in cells, as well as tracking the degradation of MNP particles.[34]

2.3.1.4. Atomic force microscopy

Atomic force microscope (AFM) consists of a probe scanning the surface of the sample sending the data about vertical movements made across the surface due to deformations or the structure to a computer. AFM is used for topographic imaging, determining mechanical and chemical properties of individual molecules which makes it useful in material science, biology, chemistry, and nanotechnology.[35]

2.3.1.5. Electron microscopy

Another technique is electron microscopy providing high-resolution 3-D pictures like transmission electron microscopy (TEM) and scanning electron microscopy (SEM).[36] While SEM involves scanning the surface of a sample with a focused beam of electrons providing a detailed picture of the sample's topography and morphology, TEM transmits a beam of electrons through an ultra-thin specimen to provide information about the internal structure of

the sample like crystallography, defects, and composition of the sample.[36] The techniques can be automated and coupled with spectroscopic methods to make them extremely viable.[37]

All of the mentioned techniques can be used in MNP research providing different sorts of valuable information about the particles: origin, level of degradation, chemical, and physical composition. As an important part of analytic chemistry, microscopy should always be used when detecting and identifying MNPs due to its versatility and ability to provide quick and meaningful information about the samples.

2.3.2. Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) is a non-destructive spectroscopy technique used to obtain an infrared spectrum (IR) of the emission or absorption of a solid, liquid, or a gas and determine its chemical composition. The instrument shoots a photon of light on the sample, the molecule absorbs the photon and converts to a higher energy state and depending on the vibrations of molecular bonds (*i.e.* bending, stretching, twisting, rocking), identifies the chemical bonds in the sample due to its element-specific IR absorbance peaks.

It is mainly used in material identification in industries for polymers, plastics, chemicals, and pharmaceuticals. In recent times, it found its use in MNP research as well as monitoring air and water pollution. It is a fast and reliable method used in environmental studies, forensics, medicine, pharmaceutic and biological research. Two microscopic approaches for analysis are currently available. One is the preselection of suspected particles by optical microscopy followed by FTIR analysis. The other is chemical imaging without any preselection by using FTIR microscopes with focal plane array detectors. This approach was used by Primpke and others while using focal plane array FTIR microscopy and image analysis for an automated approach to microplastics analysis.[38]

In MNP research, a similar version of the FTIR is commonly used, called micro-FTIR (μFTIR). The purpose of μFTIR is to analyze particles that are too small to be characterized by commonly used FTIR methods. When using a conventional IR source, the beam size of μFTIR typically ranges from 20–100 μm and can be reduced down to 3–5 μm using a synchrotron radiation source. To benefit from the advantages of using μFTIR characterization method, a good sample preparation is necessary: the analyzed samples need to be thin enough to transmit light.[39]

2.3.3. Raman spectroscopy

Raman spectroscopy is an analytical method used to study vibrations in molecules by analyzing the scattering of monochromatic light through the sample. Typically, a laser is directed at the

sample in the visible, near-infrared, or ultraviolet range, depending on the sample and its' application. When the laser interacts with the sample, part of the light undergoes elastic scattering, known as Rayleigh scattering and a small fraction of the light undergoes inelastic scattering, known as Raman scattering. The scattered light shifts in frequency, either to lower energy (Stokes Raman scattering) or higher energy (anti-Stokes Raman scattering). The difference in energy between the incident and scattered light corresponds to the vibrational or rotational energy of the molecule. The Raman spectrum is obtained by measuring the intensity of the scattered light as a function of frequency shift, resulting in a graph of Raman peaks that gives information about the chemical bonds and molecular structure of the sample. It can help identify molecular compositions and structures making it a useful tool in MNP research.[19] It can characterize various types of plastics, estimate the number and size distribution in environmental samples, as well as track composition changes due to weathering and degradation. The method helps to monitor levels of plastic pollution and assess potential risks like toxicity and bioaccumulation in living organisms.

In MNP research a method that analyses small samples with high resolution on a micrometer scale is micro-Raman (µRaman) spectroscopy. A non-destructive technique that enables the identification and characterization of small regions on a sample. µRaman is used for detecting impurities and contaminants in the sample, quality control processes, forensics, and biological and pharmaceutical research.[4]

2.3.4. Laser direct infrared imaging

Laser direct infrared imaging (LDIR) is a fully automated analytical technique that uses a quantum cascade laser as its IR source to detect and characterize particles in a sample. Aqueous samples can be dropped on a low-energy slide, or filtrated through gold-plated filters to perform particle analysis. The laser can emit IR light at a certain wavelength or sweep through a whole spectrum in a short time. Scan mode is used to locate and describe the morphology, while sweep mode is used to characterize particles by comparing their measured spectrum automatically with a built-in library or manually by comparing it with an online library of absorbance or reflectance spectra. LDIR can detect and accurately characterize particles from 10 to 500 µm in size. A built-in attenuated total reflectance (ATR) crystal allows for deeper and more correct particle analysis of smaller particles.

Minimizing contamination and tissue particles in observed samples is key to a good and quick analysis. These factors can make the analysis slower and inaccurate due to the increased number of particles that interfere with MNP analysis.[40] LDIR provides magnified pictures of particles,

as well as identifies their chemical composition, size, and distribution, making it a valuable tool in microplastic analysis. It serves as a better alternative to visual examination by optical microscopy, as it detects more particles accurately in the range of 20 to 500 μm.[41] Depending on the expertise of the researcher, its automation and fast analysis can make it a faster analysis method than FTIR or Raman.[42]

2.3.5. Pyrolysis-gas chromatography-mass Spectrometry

Pyrolysis Gas Chromatography – Mass Spectrometry (Py-Gc-Ms) is an analytical method combining pyrolysis with gas chromatography and mass spectrometry used to identify polymers and other high molecular weight substances. Py-Gc-Ms provides information about the molecular structure of the material, thermal stability, and decomposition time. It is used in microplastic research to detect polymer microparticles in samples.

In the first step, the particle is heated to a high temperature without oxygen and broken into small molecular parts which are then transferred to a gas chromatograph and separated based on their volatility and reaction with the stationary phase. After separation, the particles are then broken down into small ions and enter the mass spectrometer allowing their identification and the analysis of chemical compounds within the observed sample. However, due to a limited sample amount of about 0.5 mg, it is not suited for the analysis of complex sample mixtures.[43] The method provides valuable information about MNPs which makes it a powerful tool in particle analysis and has significant impact and applications in environmental research.

2.3.6. Microfluidics

Microfluidics is a useful method for detecting, separating, and analysis of MNP particles. It involves using small volumes of fluid (usually in micro- and nanoliter scale) in microfluidic chip systems. They are designed to capture MNPs in water, sediment, or soil. The device uses antibodies or aptamers that selectively bind to MNPs enabling their identification and quantification. By using imaging in microfluidic channels, it is possible to determine the physical characteristics of MNPs. The chips can be engineered in a way they can sort MNPs based on their size and shape making them useful for MNP extraction from environmental samples. Researchers use this method to study their transport in different flow conditions in different fluids. It can also be used to study degradation and the effects of factors like pH, temperature, or exposure to UV light and chemicals on the particles.

This method can be combined with spectroscopy techniques to identify plastics in samples. It is currently evolving and provides certain advantages to MNP research like small sample

volumes, control over experimental conditions, the potential for automation, and further research in the technology, making it a method to be used more.[44]

2.3.7. Nile Red staining method

The Nile Red staining method is a fluorescence-based method used to detect and characterize lipids in a sample. It is commonly used in biological and environmental research to identify lipids in substances, including microplastics with adsorbed lipids.

When Nile Red connects to lipids it emits strong fluorescence signals when excited with specific wavelengths of light. Nile red dye is dissolved usually in acetone or ethanol to create a staining solution. The sample is submerged in the solution for some time depending on the type of sample. After staining, the sample is observed with a fluorescence microscope or an imaging system with a blue or UV light to detect fluorescence particles, to analyze their properties, and to quantify the lipid content in the sample.

It is commonly used in biology and lipid metabolism research but it can be used in MNP research as well.[27] It is used to detect lipid-covered MNP particles in environmental samples and helps researchers detect MNPs in samples with adsorbed lipids in aqueous solutions.

2.3.8. Diffractive light scattering

Diffractive light scattering (DLS) is an analytical method used for particle analysis in a liquid suspension. It provides details about the mean and size distribution of nanoparticles, macromolecules, and colloidal particles depending on their Brownian motion.

Brownian motion causes them to move randomly due to collisions with surrounding particles. Smaller particles exhibit faster Brownian motion compared to larger ones and as the particles move, the scattered light intensity fluctuates over time. DLS measures these fluctuations and calculates the autocorrelation function which is then analyzed using mathematical models to get data on particle distribution. Through the calculation of the diffusion coefficient, the hydrodynamic radius of the particle can be measured. This method is commonly used in nanotechnology to measure size and particle distribution in drug delivery systems, medical imaging, protein, and biomolecule analysis. DLS is also used to measure the size distribution of MNP particles in environmental samples, such as water, sediment, and soil. It can help understand how MNPs react to the change in pH, salinity, temperature, and how they form aggregates. It monitors how certain MNPs react to other natural particles in different environments.[45]

Understanding these impacts is vital for MNP research and monitoring of MNP distribution in different ecosystems. When combined with spectroscopy methods like FTIR and Raman, it can help determine the chemical composition of the particles. Overall, DLS is a valuable analytical method in MNP research due to its non-destructive nature makes it suitable for studying MNPs in suspensions, contributing to our understanding of the pollution and its environmental impact.[45]

3. EXPERIMENT

3.1. List of chemicals and instruments

Tissue

The tissue used in the experiment was processed meat in two types, one rich in fat, and the other in protein. The reason for using this kind of tissue for the experiment was its' homogenous nature which provided the same conditions for every piece of tissue sample used for digestion and its' different chemical compositions. Tissue named Type 1 contained 10-15% protein, 20-30% fat, and 2-5% carbohydrates, while other, named Type 2, contained around 70-80% protein and less than 10% fat. The tissue types are presented in Figure 3.1.

Along with the prepared tissue samples, additional samples of digested mice tissue doped with three sizes of polystyrene (PS) particles were provided by Dr. Verena Kopatz at the Medical University of Vienna. Parts of mice tissue included the pancreas, lungs, kidney, brain, heart, liver, stool, spleen, and testicles. The samples were analyzed by the same procedure and instruments used for the processed meat samples.

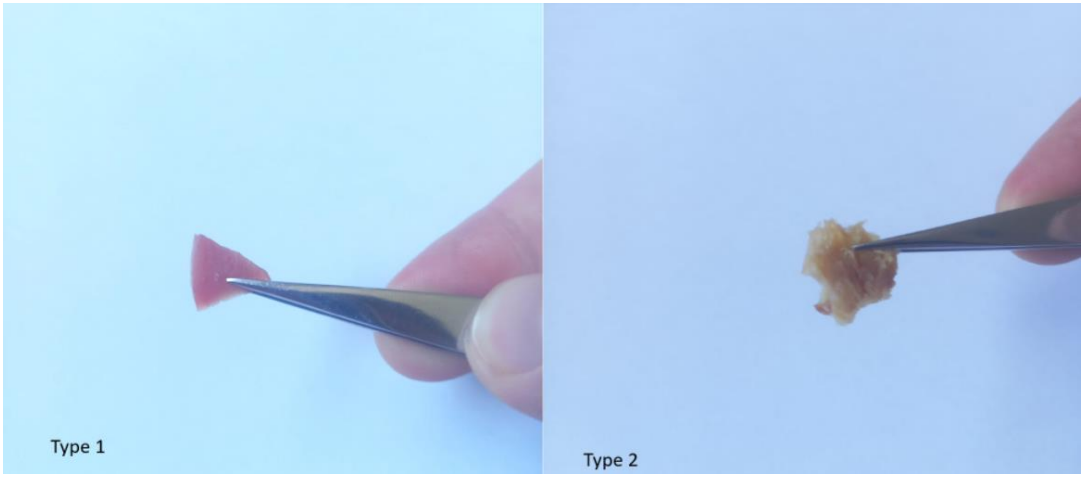


Figure 3.1. Processed meat tissue samples: Type 1 and Type 2

Chemicals

Chemicals used for tissue digestion were as follows and are presented in Figure 3.2.:

- 2 mol/L HNO_3 solution
- 25% KOH solution
- Kreon 25000, a mixture of enzymes such as lipase, amylase, and protease
- Protamex, a commercially available protease enzyme
- Sodium dodecyl sulfate (SDS)

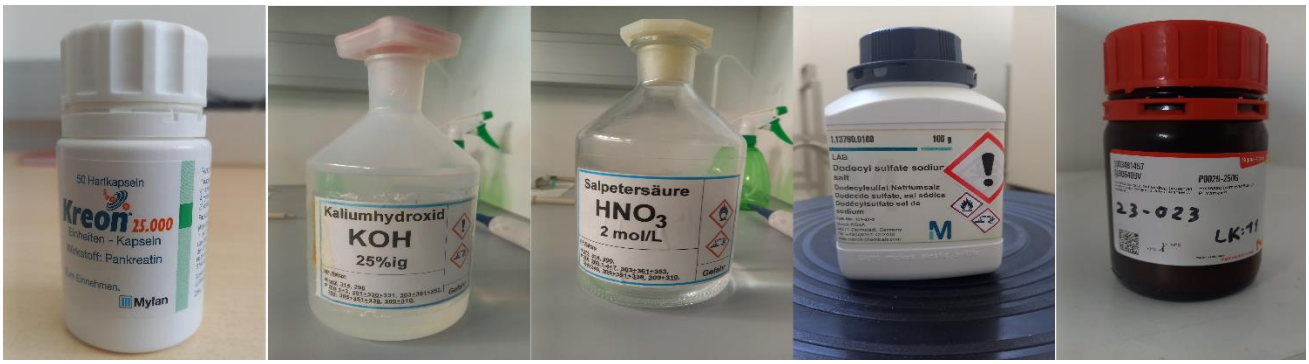


Figure 3.2. Chemicals used in the experiment

Microscopy

For the purpose of this research, an optical microscope Zeiss AX10 Imager M1m was used to visually analyze the samples with bright and dark field methods, and is shown in Figure 3.3.

Figure 3.3. Zeiss AX10 ImagerM1m microscope

LDIR analysis

Agilent 8700 LDIR Chemical Imaging System was used to obtain valuable data about MNP particles present in the sample. Analysis can be done on gold-plated filters and mirrIR low-energy slides. For the filter analysis, a previously prepared sample on a gold-plated filter, or a drop of the sample solution on a slide is fixed on a sample holder and inserted in the instrument. The slides used in this research were from Kevley Technologies and will be further addressed as Kevley slides. The slides have no interfering absorption from 4000 to 400 cm^{-1}. The filter/slide is put on a holder and inserted in the instrument ready to be analyzed. The instrument used in the experiments, as well as preparation for filter and Kevley slide analysis is shown in Figure 3.4. Using the Agilent Clarity software, the particle analysis feature scans the selected area, counts the particles, and provides information about their size and dimensions. Using the QC laser, it characterizes all particles by comparing spectrograms gained from the particle analysis with ones uploaded in the software's spectrogram library. This gives information about their chemical composition.

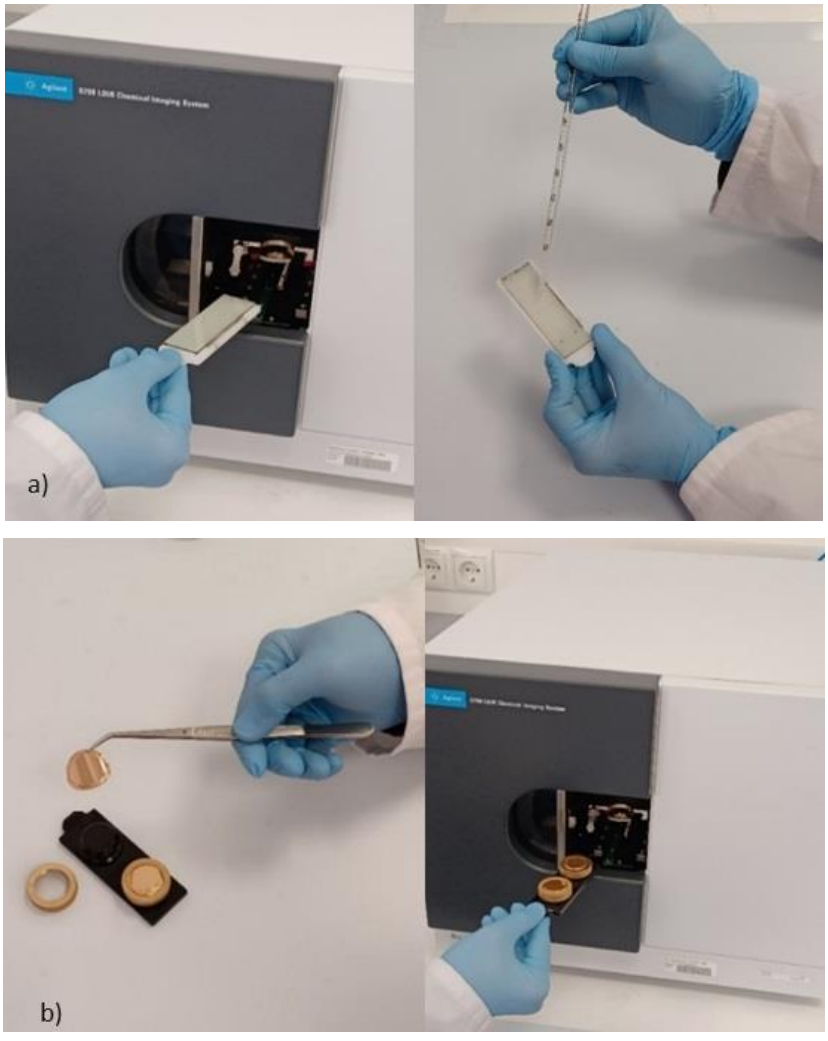


Figure 3.4. Preparing a Kevley slide, a) and a filter, b) for LDIR analysis

3.2. Contamination control

Before conducting any sample preparation and further experiments, an appropriate contamination control is required. Making sure to use glassware anywhere applicable is the key to minimizing risk from outside MNPs coming into the samples. Furthermore, filtrating the solutions in which the samples will be digesting is necessary, as well as doing blank sample tests to determine if there are MNP particles present in the mentioned solutions.

It is important to use freshly prepared and filtered solutions stored in glass hardware. The sample preparation process should be performed in controlled areas like laminar flow hoods to make sure outside particles do not contaminate the solutions or samples. Any use of plastic materials or storage in dusty areas and lead to outside contamination making the research more difficult.

3.3. Sample preparation

Samples Type 1 and Type 2 were placed in glass bottles, filled with 20 mL of prepared solutions of KOH, HNO_3, Kreon 25000, protease, and SDS. They were left to digest under a laminar flow hood at 38°C which is shown in Figure 3.5. The approach to the experiment is shown in Table 2. The samples were visually monitored over the course of 7 days. On the last day of digestion, the samples were filtrated through 25 mm wide, 0.4 µm pore, gold-plated filters. Afterward, they were put in a glass dish filled with 30% H_2O_2. After a full day of H_2O_2 treatment, the solutions were filtered again through the same filters which is shown in Figure 3.6.

Table 2. Samples and tissue digestion methods

Sample	Tissue type	Treatment	***m*(sample)/g**
1	Type 1	Kreon 25000 + 3% SDS solution	306
2	Type 1	5% KOH solution	314
3	Type 1	10% HNO3 solution	324
4	Type 2	Kreon 25000 + 3% SDS solution	109
5	Type 2	5% KOH solution	114
6	Type 2	10% HNO_3 solution	114
7	Type 1	5% Protease + 3% SDS solution	317
8	Type 2	5% Protease + 3% SDS solution	117

Figure 3.5. Tissue digestion

Figure 3.6. Sample filtration

For the mice tissue samples, no sample preparation was needed since the samples arrived already digested by 10% KOH solution in small test tubes. The only thing needed was to put a drop of the solutions on Kevley slides for further analysis on the microscope and LDIR. The entire process of the experiment with all tissue types is shown in Figure 3.7.

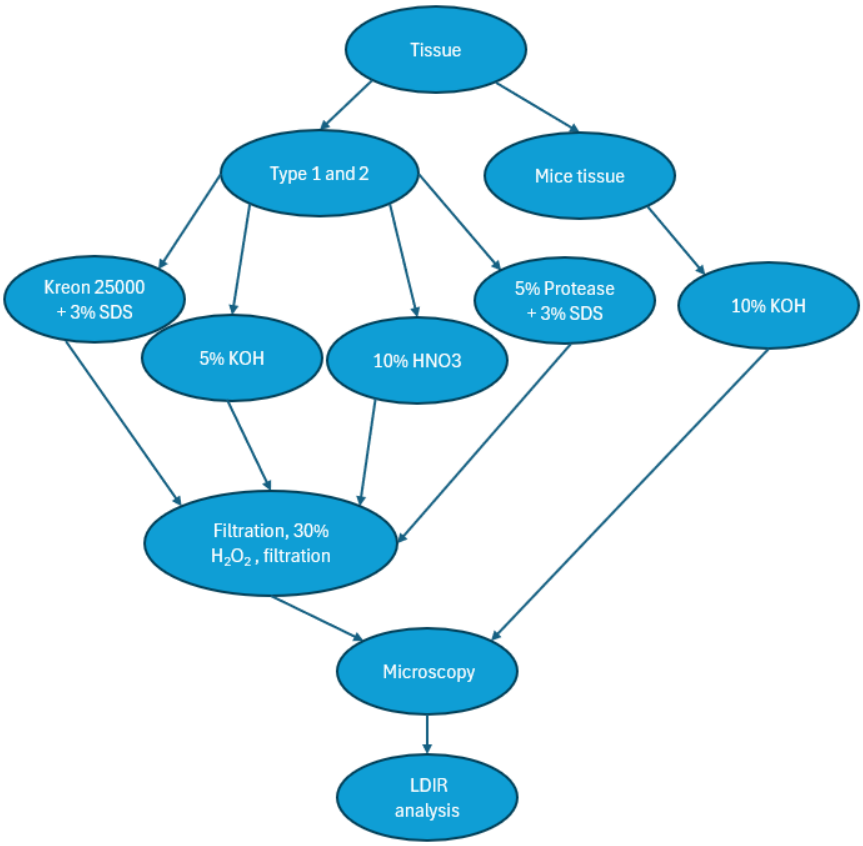


Figure 3.7. Schematic view of the experiment

4. RESULTS AND DISCUSSION

4.1. Tissue digestion

The quality of tissue digestion was judged on the visual observation. According to the visual analysis of the filtration and overall digestion, the digestion quality was ranked at four levels;

1) all of the tissue was digested and the filtration was easy,

2) most of the tissue was digested, but some was still present,

3) difficult filtration due to undigested tissue, and

4) the filter clogged due to too much undigested tissue that made filtration impossible.

The samples that showed less undigested tissue after seven days of digestion were filtered through 0,4 μm gold-plated filters. The filters were then weighed, and a difference between the initial weight of the tissue and after dissolving was calculated by the formula (1):

$$\beta = \frac{m1-m2}{m1} \times 100\% \qquad (1)$$

where m_1 was the mass of the added sample before digestion and m_2 the mass of the sample after digestion which was calculated by deducting the mass of a filter before filtration from the one after filtrating the solution with the digested sample. The evaluation of the tissue digestion process is shown on Table 3.

Table 3. Tissue digestion process evaluation

Sample	Solution	m_1/mg	m_2/mg	β/%	Digestion Quality
1	Kreon 25000+SDS	306	118	68.24	3
2	KOH	314	53	89.75	1
3	HNO_3	324	96	76.79	2
4	Kreon 25000+SDS	109	66	58.53	4
5	KOH	114	51	73.51	2
6	HNO_3	114	47	77.02	2
7	Protease + SDS	317	92	77.54	3
8	Protease + SDS	117	31	91.28	1

As seen in Table 3, tissue digestion was not successful for samples that were digested by Kreon 25000; the filters got clogged by the amount of undigested tissue and filtration was not complete. Samples digested by HNO_3 showed a better outcome, but the solution still did not fully digest the samples. The gold-plated filter proved resistant to the acid and remained unchanged which made it safe and easy to use for LDIR measurements. The same thing happened in an experiment performed by Whiting and O'Connor where they used acidic solutions for their sample preparation. The filters were applicable and suitable for LDIR analysis.[42]

KOH digested the fat-rich Type 1 sample, but did not digest the protein-rich Type 2 sample quite as well. The opposite happened by enzymatic digestion with protease; it almost digested Type 2 tissue completely, and did not digest the Type 2 sample. The digestion process was gentle and did not affect the filter paper after filtration, which made it viable for this type of sample and particle identification. Judging by the composition of tissue Type 1 and Type 2, KOH proved better at digesting fat tissue, while protease did better at digesting protein. The results were similar for Von Friesen, L. W. while digesting bivalve tissue, as enzymatic

digestion proved to be better for the digestion of organic matter, and in a shorter time than digestion with KOH. The process in the article was different from the process used in this research, but the author also suggested adapting the enzymatic digestion process by altering the concentration, temperature, or enzyme type according to the tissue type that requires digestion.[25]

4.2. Microscopy

4.2.1. Processed meat tissue samples

Even though some of the samples did not filter fully, microscopy was performed on all of them. The filtered samples were analyzed on *Zeiss AX10 Imager* using bright- and dark-field microscopy methods. Microscopy pictures of the samples can be seen in Figures 4.1 through 4.8.

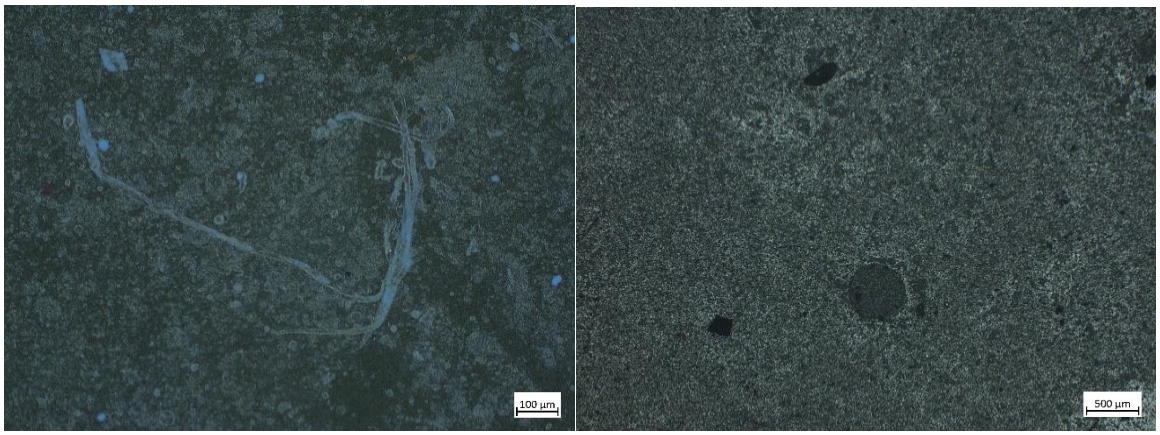


Figure 4.1. Sample 1: digestion with Kreon + SDS

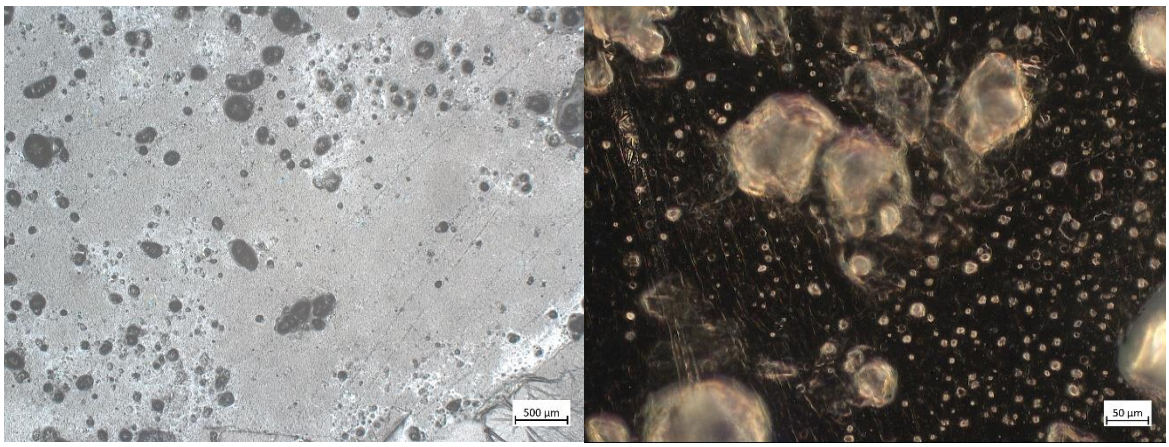


Figure 4.2. Sample 2: digestion with KOH

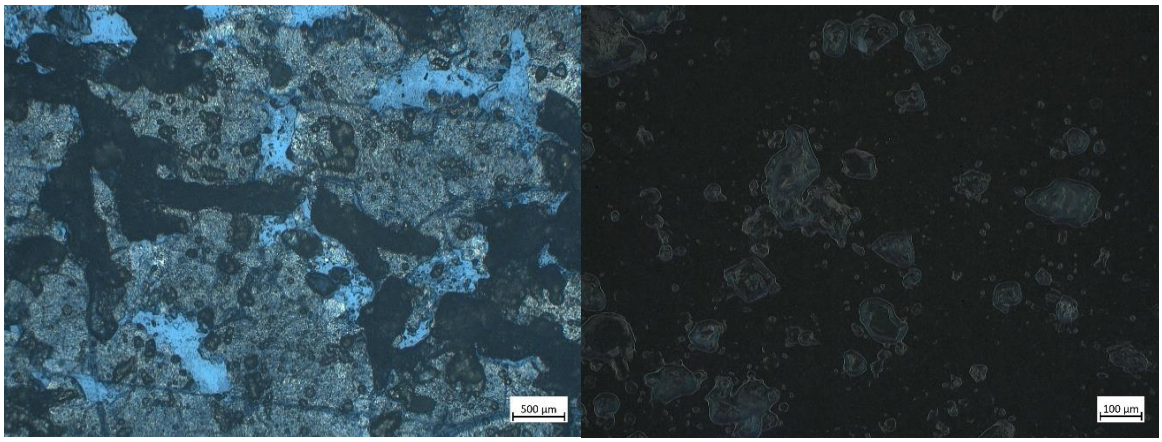


Figure 4.3. Sample 3: digestion with HNO_3

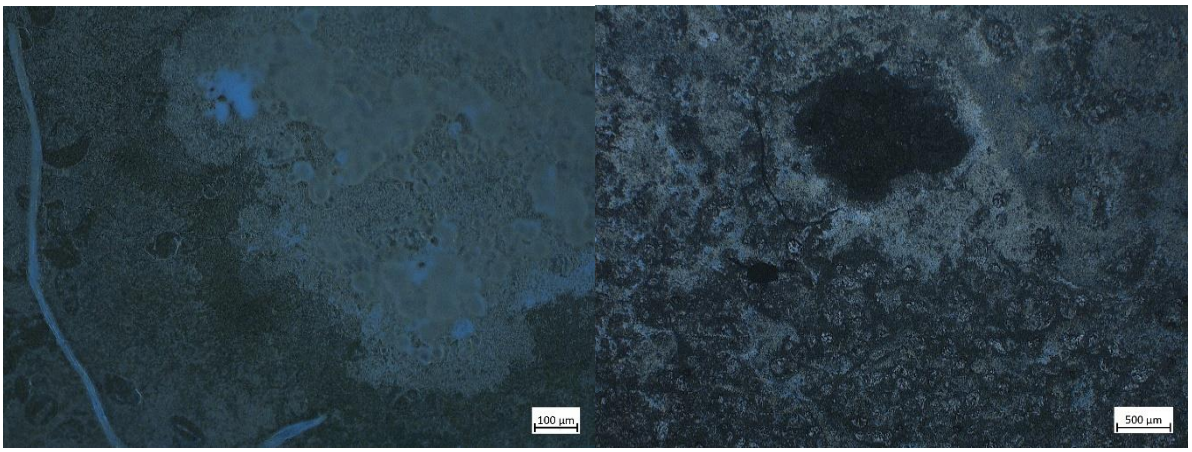


Figure 4.4. Sample 4: digestion with Kreon + SDS

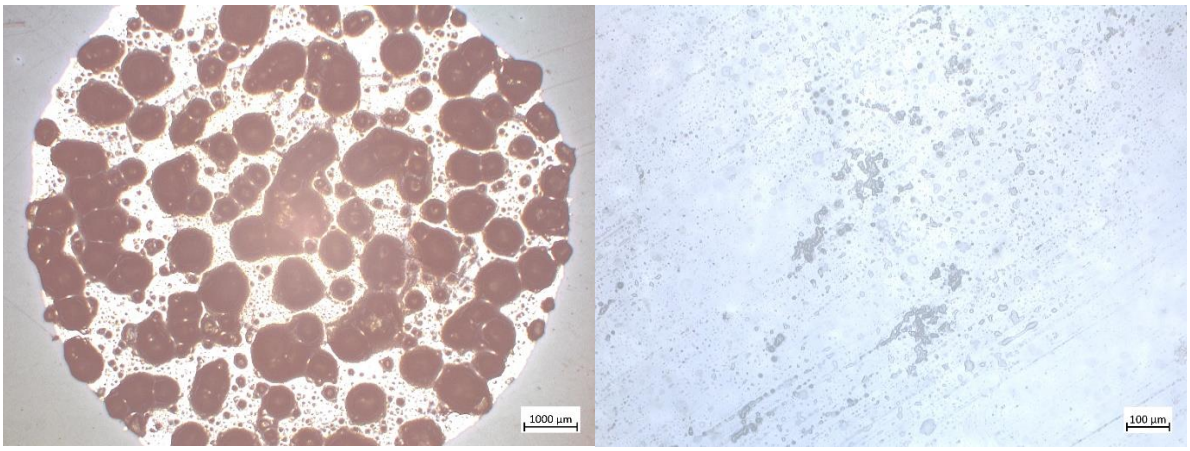


Figure 4.5. Sample 5: digestion with KOH

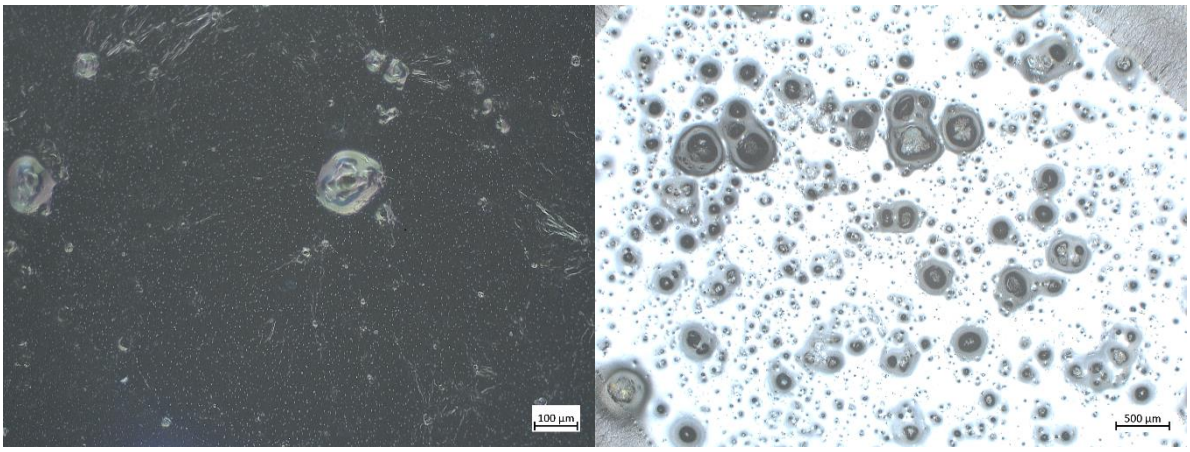


Figure 4.6. Sample 6: digestion with HNO_3

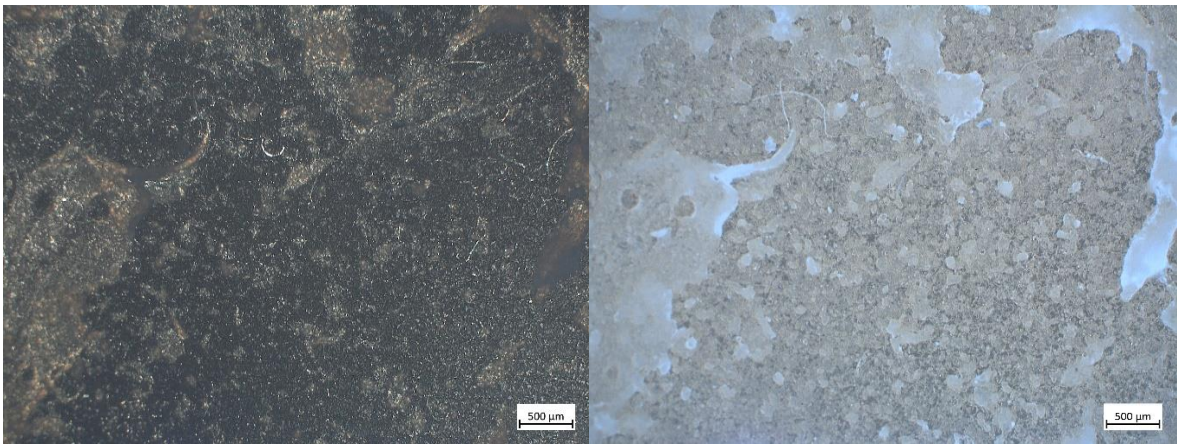


Figure 4.7. Sample 7: digestion with Protamex + SDS

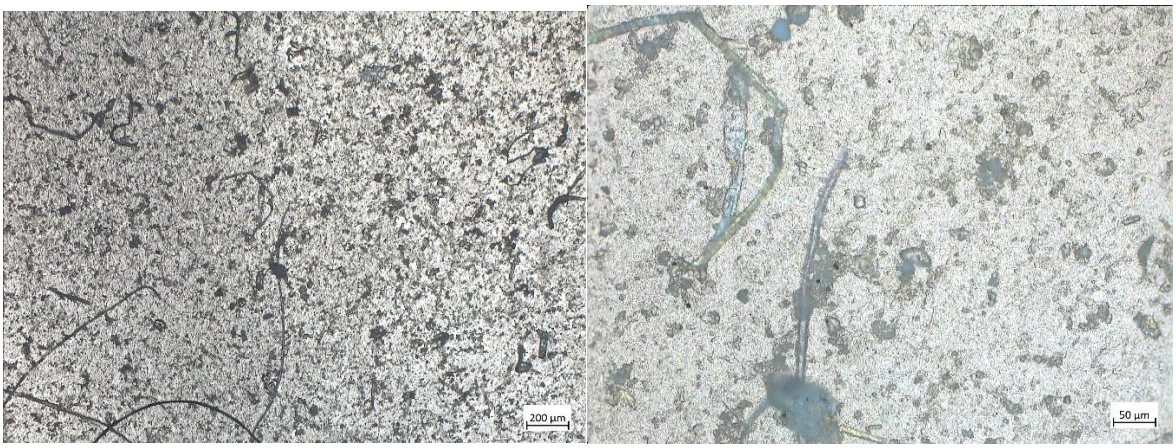


Figure 4.8. Sample 8: digestion with Protamex + SDS

From the presented microscopy figures we can better see the quality of tissue digestion and present contaminants. The MNP particles were mostly trapped in undigested tissue matrixes which made it difficult to differentiate them from other contaminants. The identification of

particles can be difficult if there is a lot of undigested organic matter still present in the sample, and the difficulty of identification magnifies with the smaller size of the particles.[25] particles in the tissue, even through a lot of undigested tissue. In all samples, we could also see a lot of fibers and fragments which required further identification by the LDIR to know their chemical composition and find out the source of contamination. Microscopy is a good method to detect particles, but as in Cheng, Y.-L.'s research paper about the characterization of microplastics in sediment, it was not solely enough to count the particles and be sure of their origin and composition, so further LDIR analysis was performed.[40]

4.2.2. Mice tissue samples

To analyze the received mice tissue samples, a drop of 0.5 µL of the digested solution of each tissue sample was dropped on a Kevley slide. A drop of ethanol was added as well to better disperse the present particles and get a good image. The slides were then put under the microscope and analyzed. In Figure 4.9. we can see a bright-light microscopy picture with all particle sizes visible in the sample, circled and explained what they are. All other microscopy pictures can be seen in Figure 4.10. through 4.17.

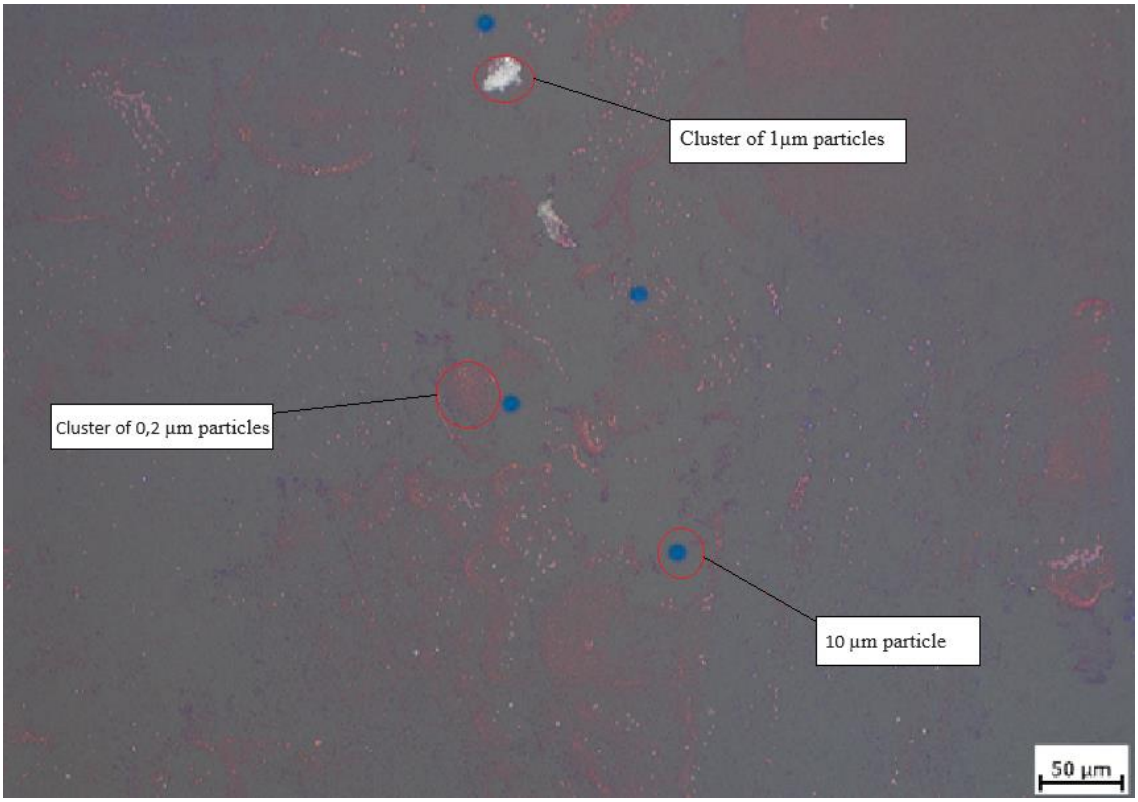


Figure 4.9. Kidney sample

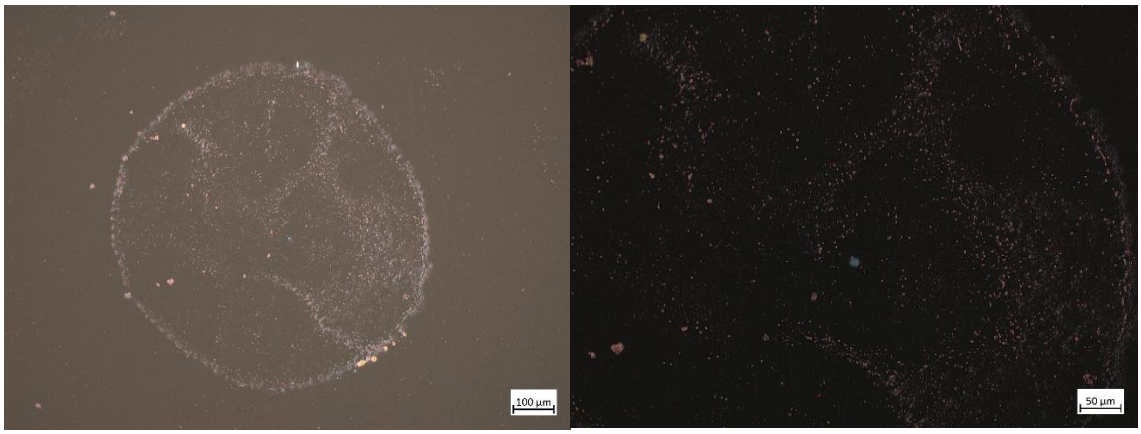


Figure 4.10. Heart sample

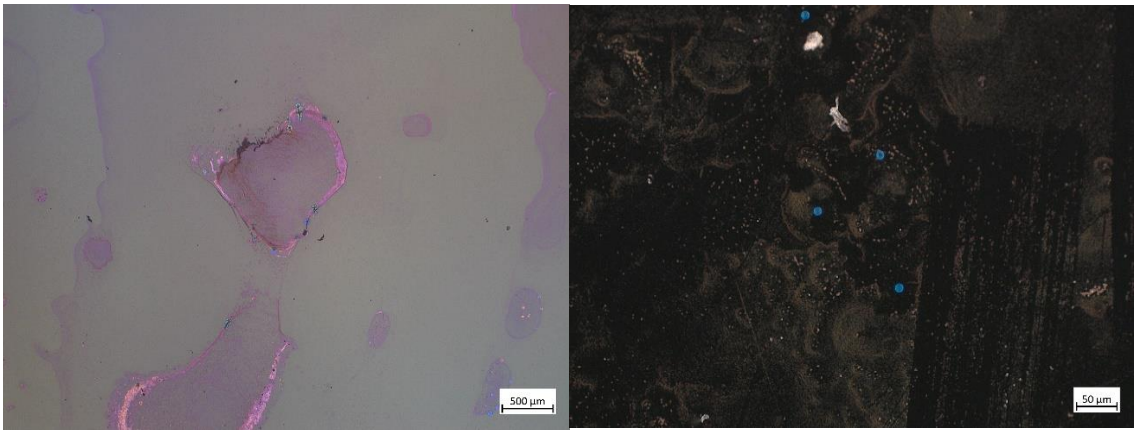


Figure 4.11. Pancreas sample

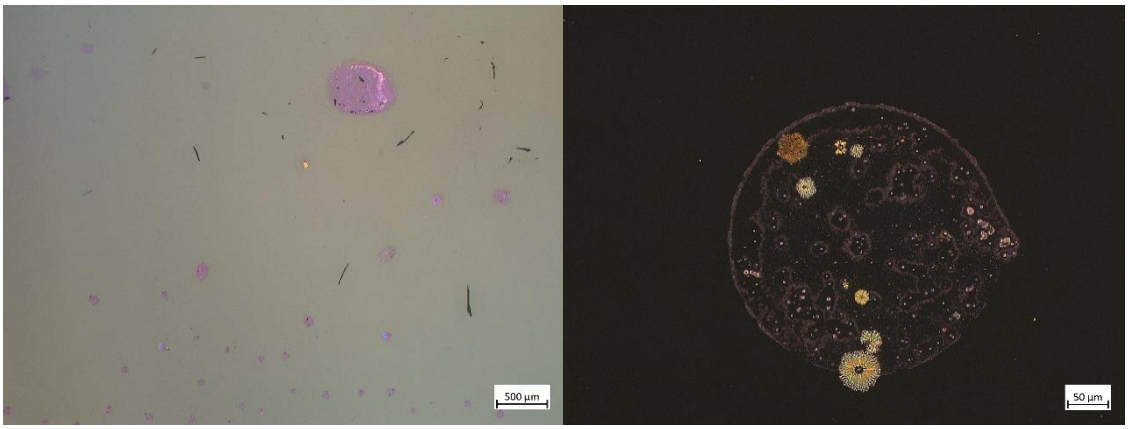


Figure 4.12. Testicle sample

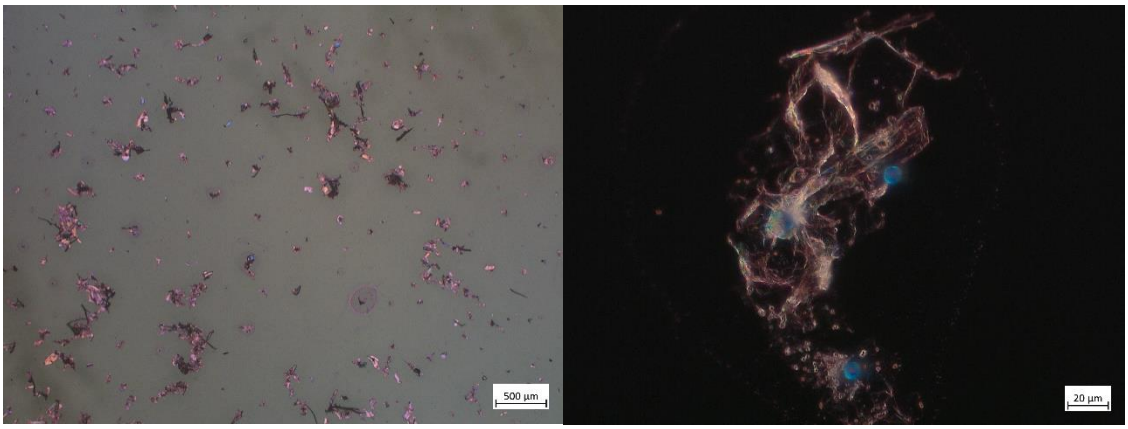


Figure 4.13. Stool sample

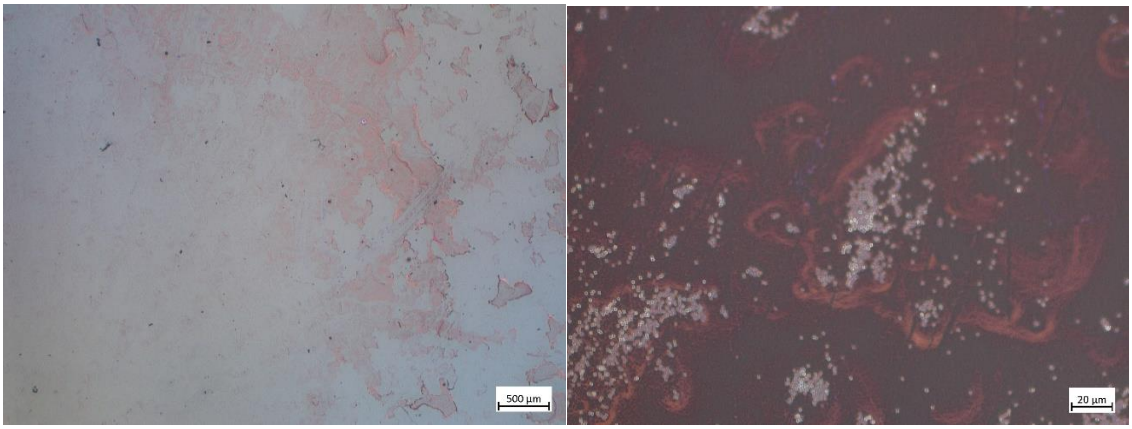


Figure 4.14. Liver sample

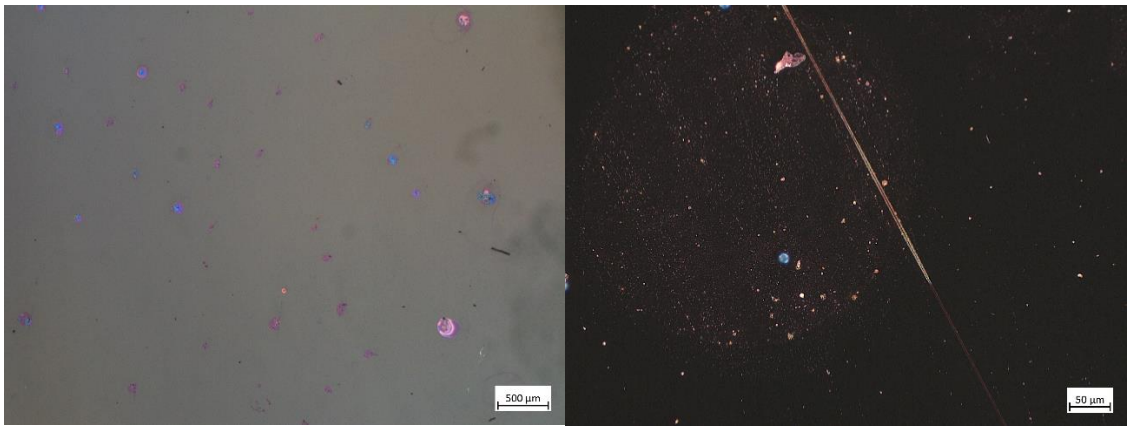


Figure 4.15. Lung sample

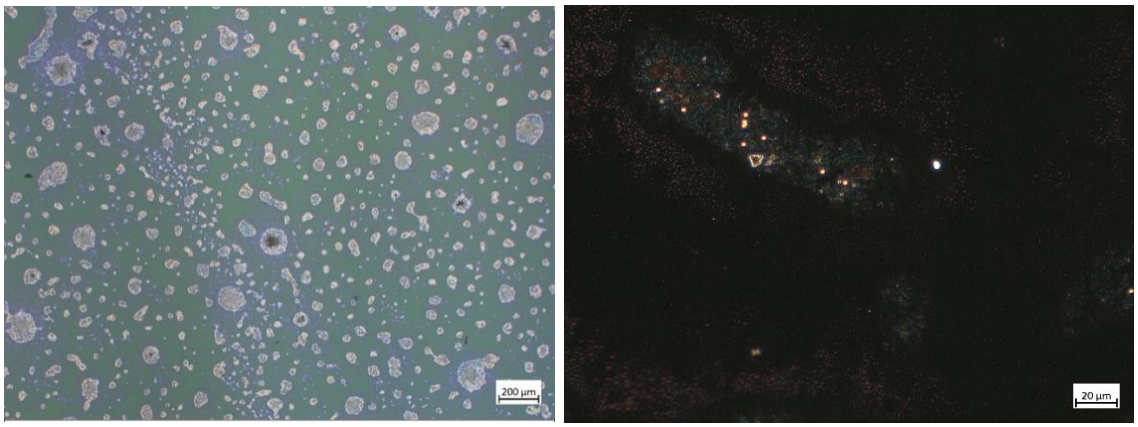


Figure 4.16. Brain sample

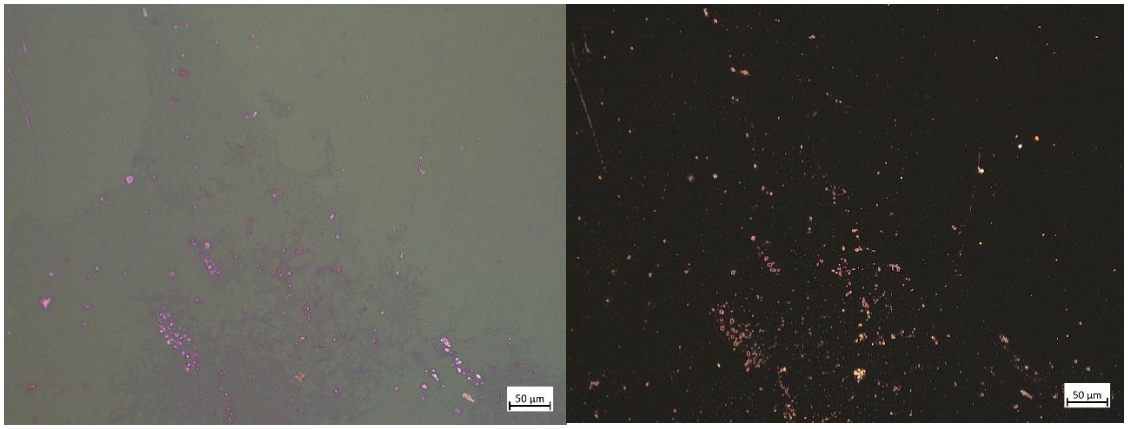


Figure 4.17. Spleen sample

Despite incomplete digestion in some of the samples, all sizes of MNPs were identified. In most of the samples, a small amount of undigested tissue was left. However, where present, undigested tissue interfered with the MNP analysis. MNPs that were trapped inside undigested tissue matrixes made them hard to distinguish. It was not an issue identifying the blue 10 µm particles due to their bigger size, but the 1 µm (white) and 0.2 µm (pink) particles were difficult to see one by one. On the other hand, smaller particles gathered in aglomerates which made the visual identification easier. Along with the MNP particles, some fibers and fragments of unknown chemical composition were present in the sample which required further LDIR measurements for more information.

4.3. LDIR measurement results

4.3.1. Processed meat tissue samples

To count and characterize all the particles in the samples, LDIR spectroscopy was performed. In the case of processed meat samples, the best-digested samples, samples 2 (Type 1 tissue digested with KOH) and 8 (Type 2 tissue digested with protease and SDS) were analyzed by

the LDIR. The gold-plated filters with digested tissue were inserted in the LDIR and further analyzed for particles with Agilent Clarity software. Particle count, detailed pictures of the filter paper, characterization, and respective spectrograms for each particle are presented in Figures 4.18. through 4.25.

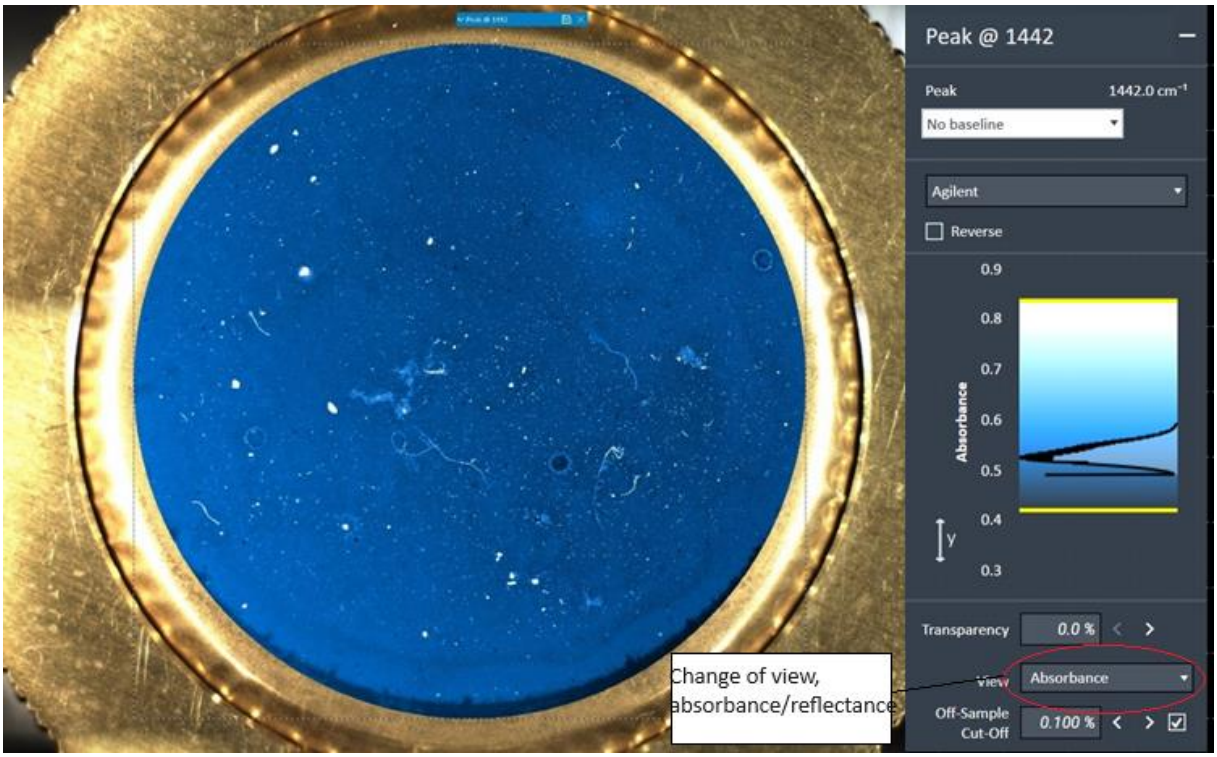


Figure 4.18. Sample 2: Peak analysis

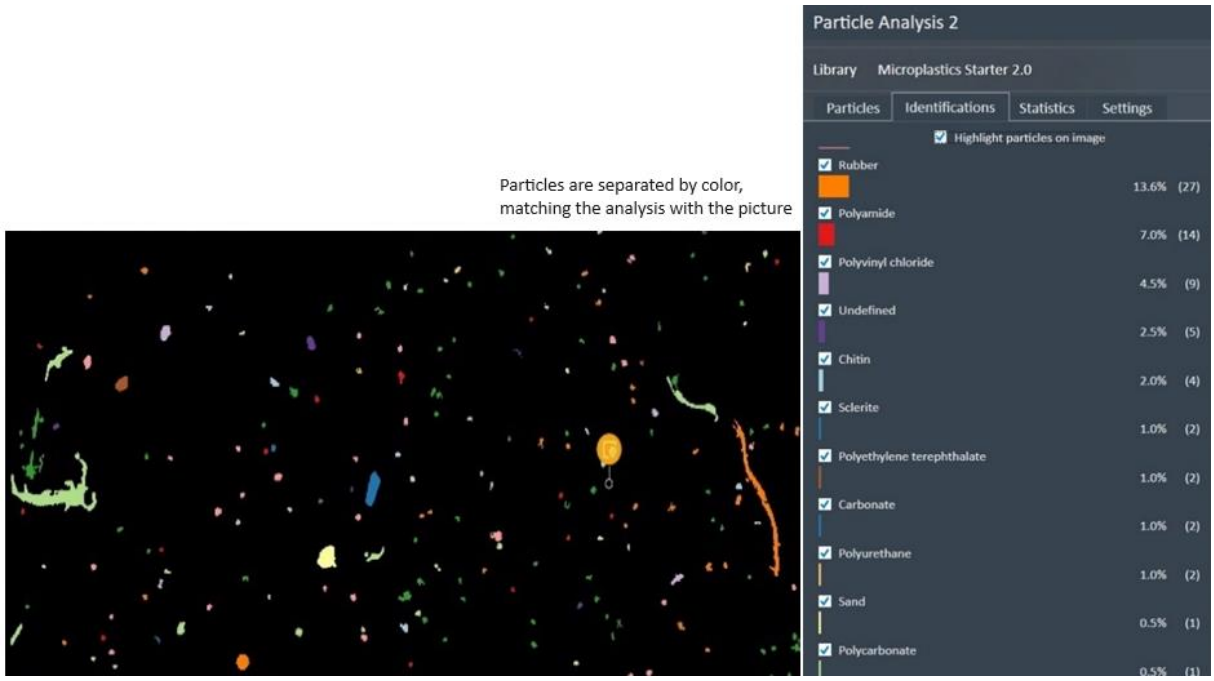


Figure 4.19. Sample 2: particle analysis

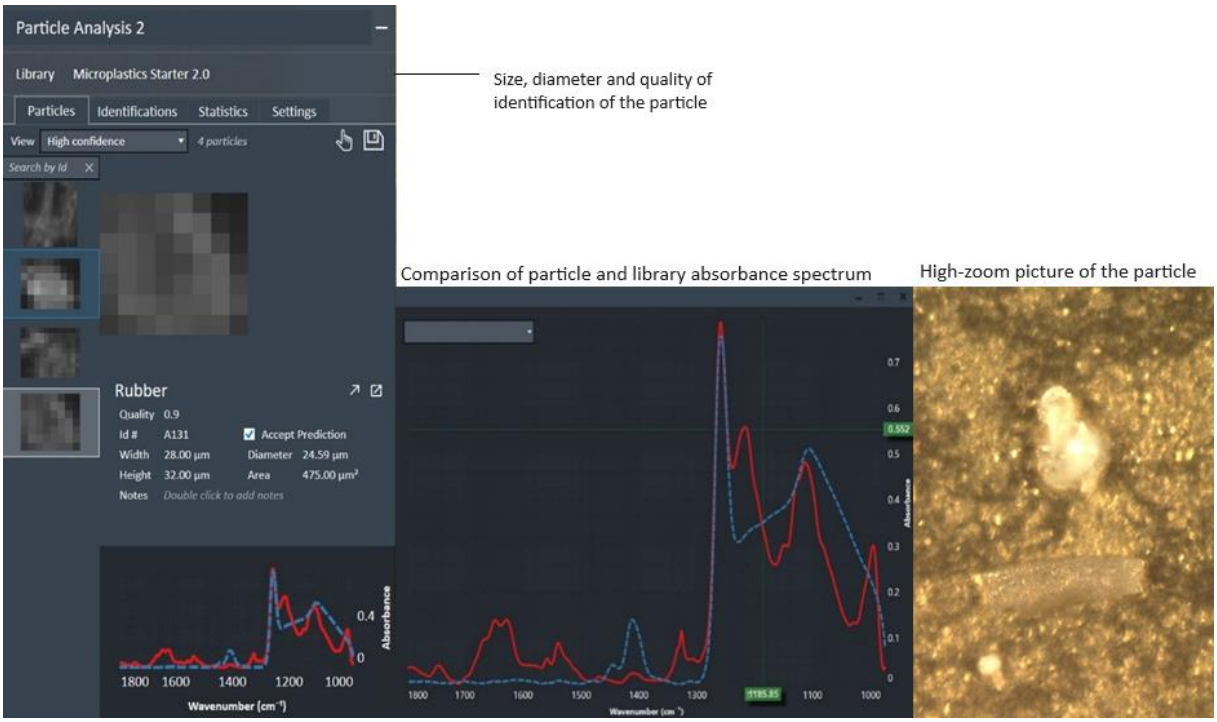


Figure 4.20. Sample 2: rubber fragment

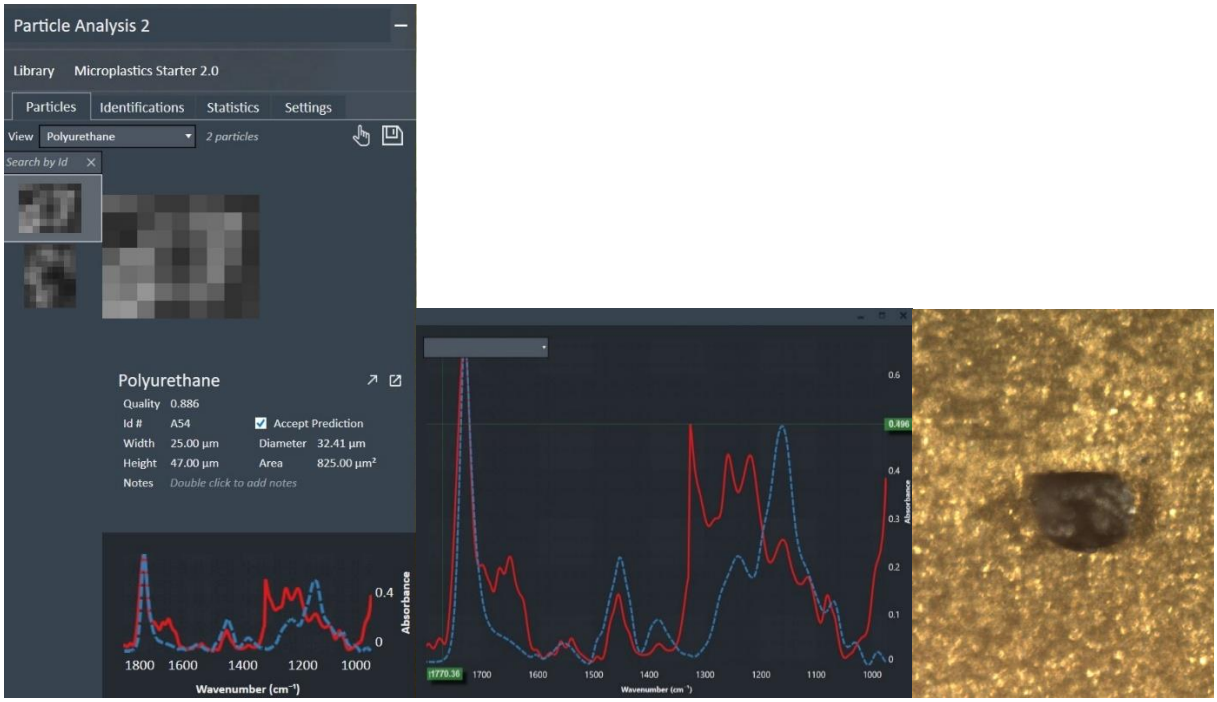


Figure 4.21. Sample 2: polyurethane particle

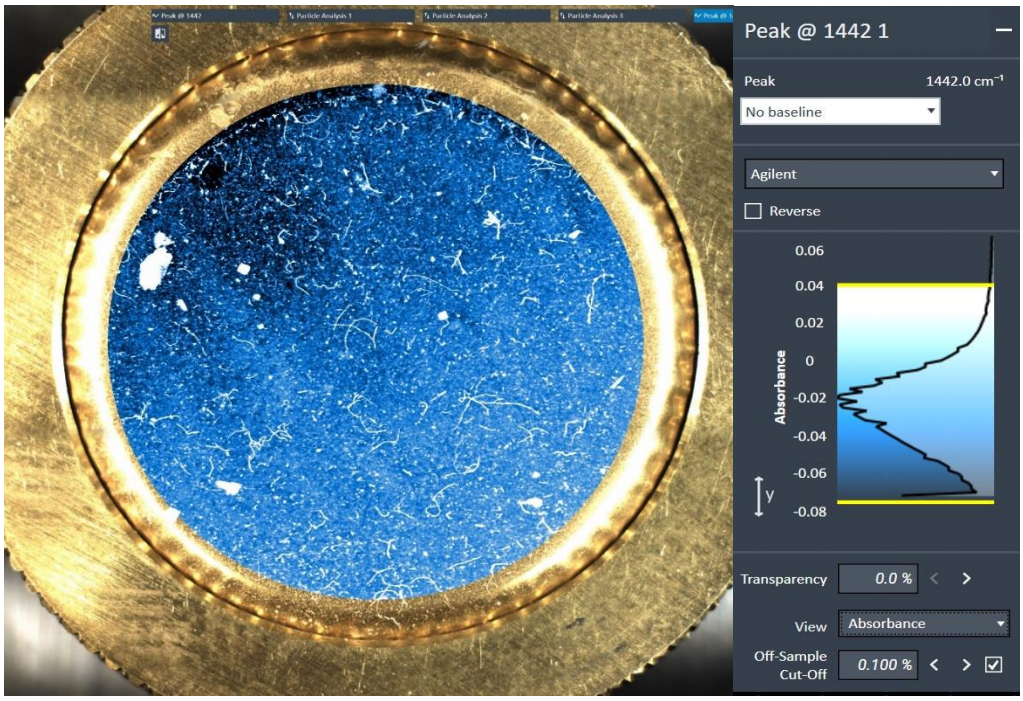


Figure 4.22. Sample 8: peak analysis

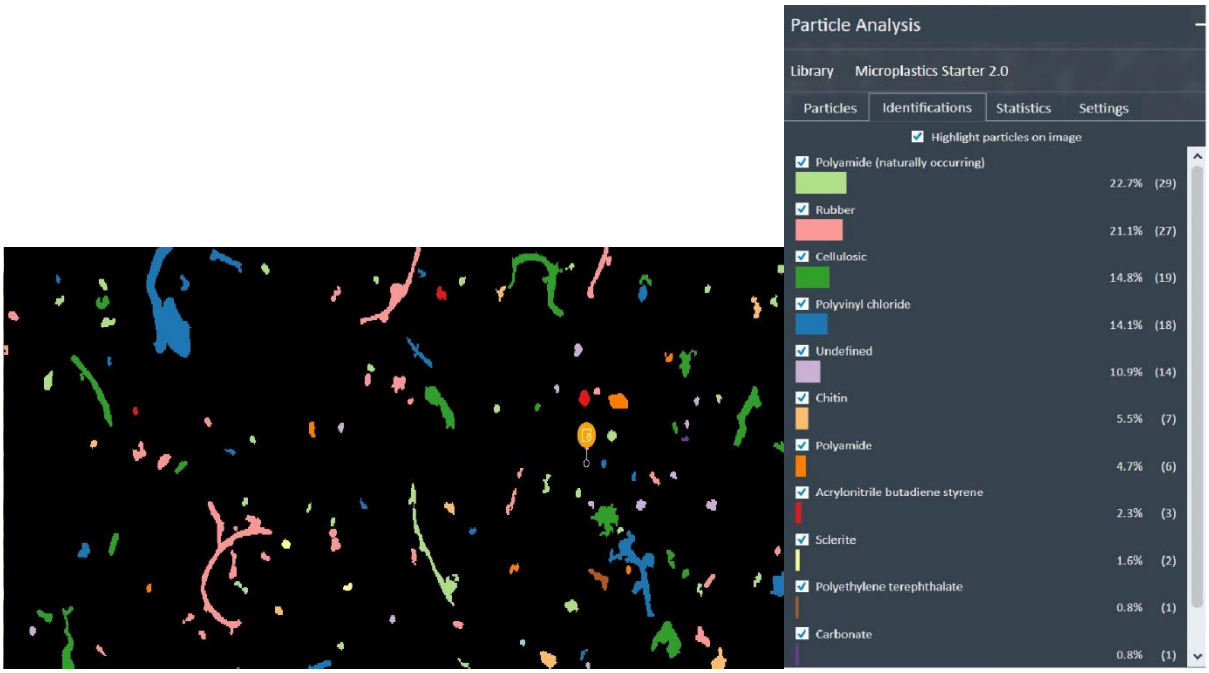


Figure 4.23. Sample 8: particle analysis

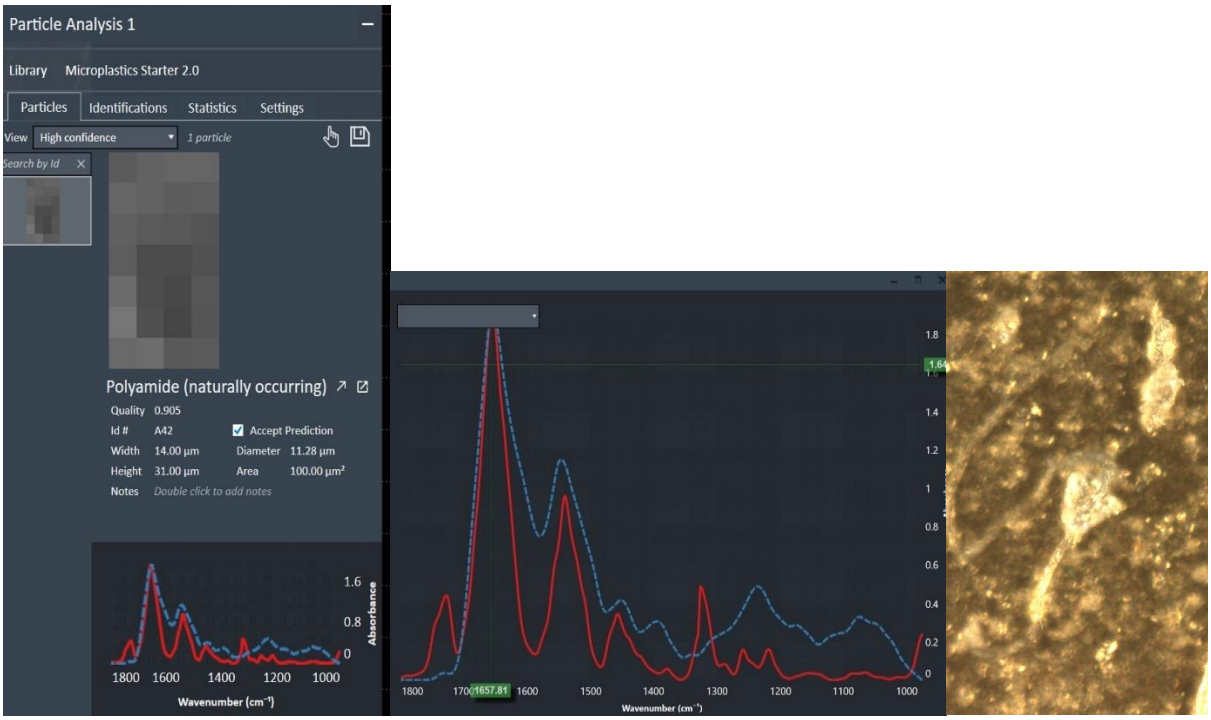


Figure 4.24. Sample 8: polyamide particle

Figure 4.25. Sample 8: celulose fiber

Already from the absorbance spectrum analysis, we could see there were a lot of particles present in the samples, much more in sample 8 than in sample 2. Particle analysis showed many types of particles, fibers, and fragments present on the filter paper, and most of them were identified. The majority of particles greater in size were easier for the LDIR to identify as they

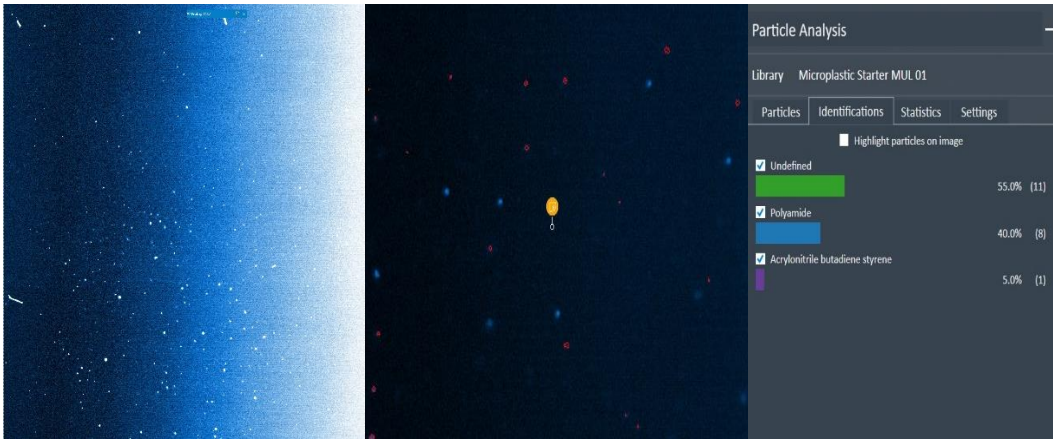
had higher absorbance and a better absorbance spectrum correlation as was the case in Cheng and Zhang's article about the characterization of microplastics in sediment samples using LDIR spectroscopy.[27] Like in Rosales, G. G.'s article about microplastic extraction from edible mussels, only results with a confidence of 75-90% were taken into account.[45]

In both of the samples, we could see many naturally occurring polyamide particles which would indicate a high presence of undigested tissue. Rubber fragments and cellulose fibers were present in both samples which also indicate outside contamination while performing the research or contamination that was already present in the tissue. Detected undefined particles were either too small for the LDIR to identify them, or the software library did not contain any similar spectrograms. With more experience in identification, using the software, and upgrading the spectrogram library, more accurate results can be obtained.[42]

Some MNP particles were identified among all other particles, fibers, fragments, and undigested tissue with high confidence in both samples and were made from rubber, PVC, polyurethane, or polycarbonate. Due to the high amount of particles, fibers, and fragments, it was unclear if their origin was from the tissue itself or contamination from the sample preparation and identification process. A better tissue digestion protocol would leave fewer particles and tissue on the filter paper which would make the characterization faster and easier.[27]

4.3.2. Mice tissue samples

LDIR analysis of the mice tissue samples was performed on Kevley slides, rather than gold-plated filter papers. A similar approach using Kevley slides was done by Whiting and O'Connor while researching MNP's in water sediments. A drop of the sample solution was added to a Kevley slide and further analyzed by the LDIR.[42] Peak and particle analysis was done the same way as for Type 1 and 2 tissue samples. Results of the LDIR analysis are shown on figures 4.26. through 4.34.



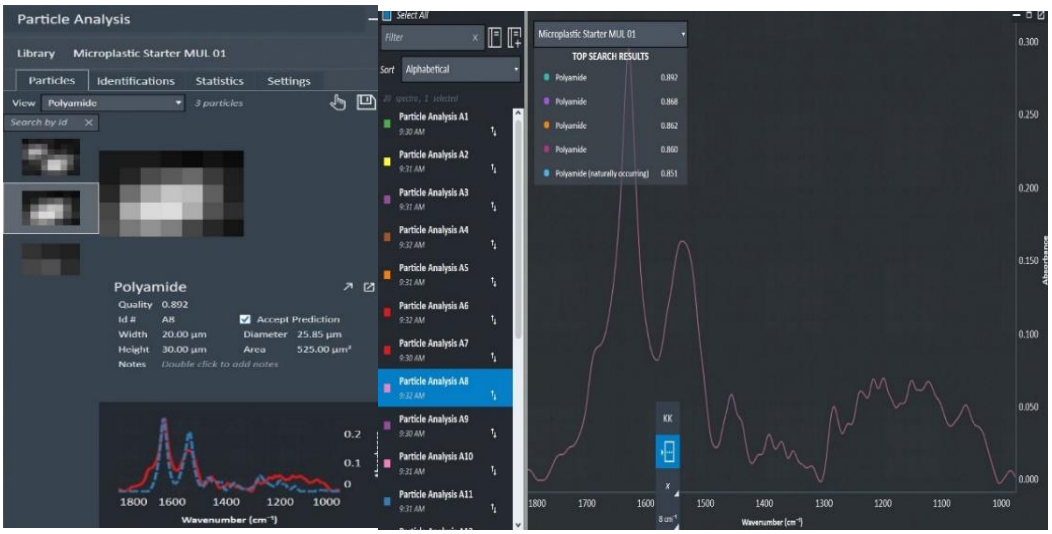


Figure 4.26. Brain sample

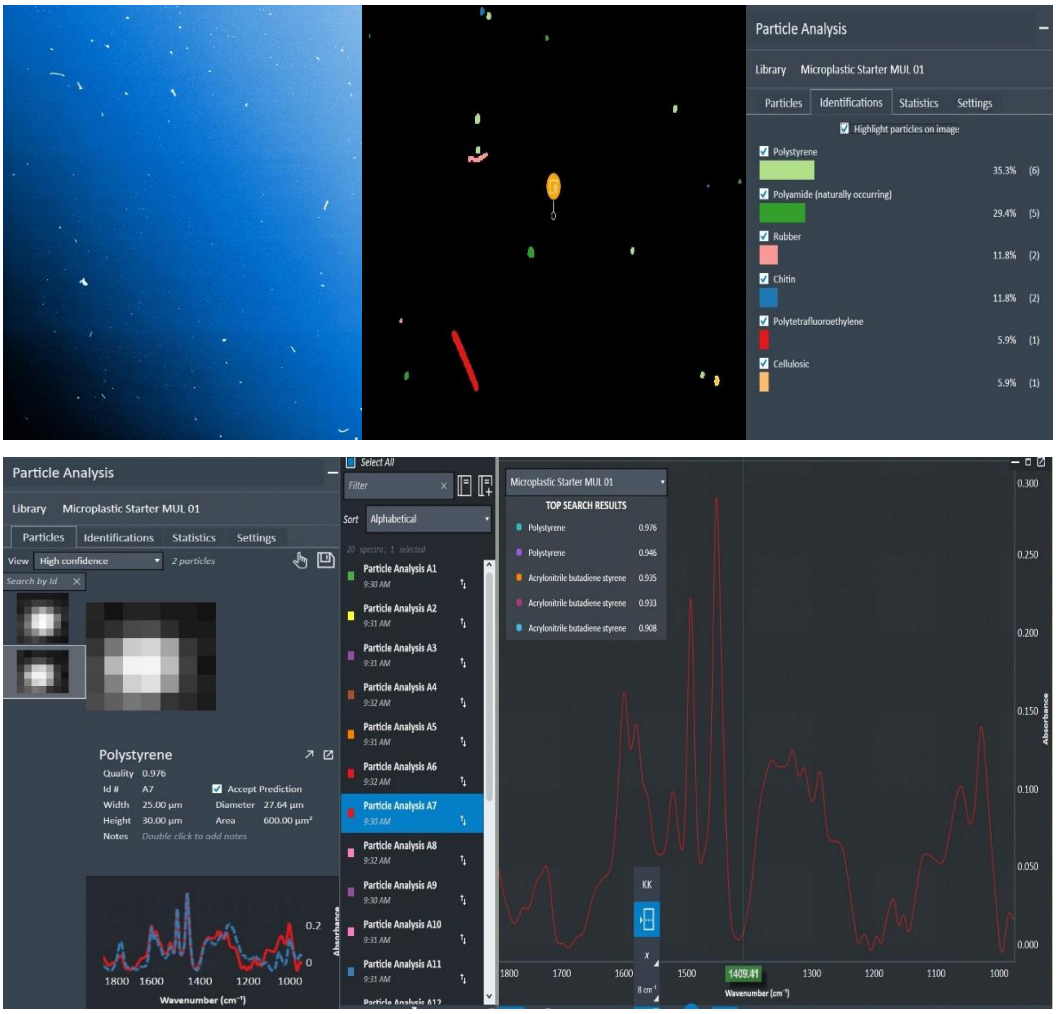


Figure 4.27. Heart sample

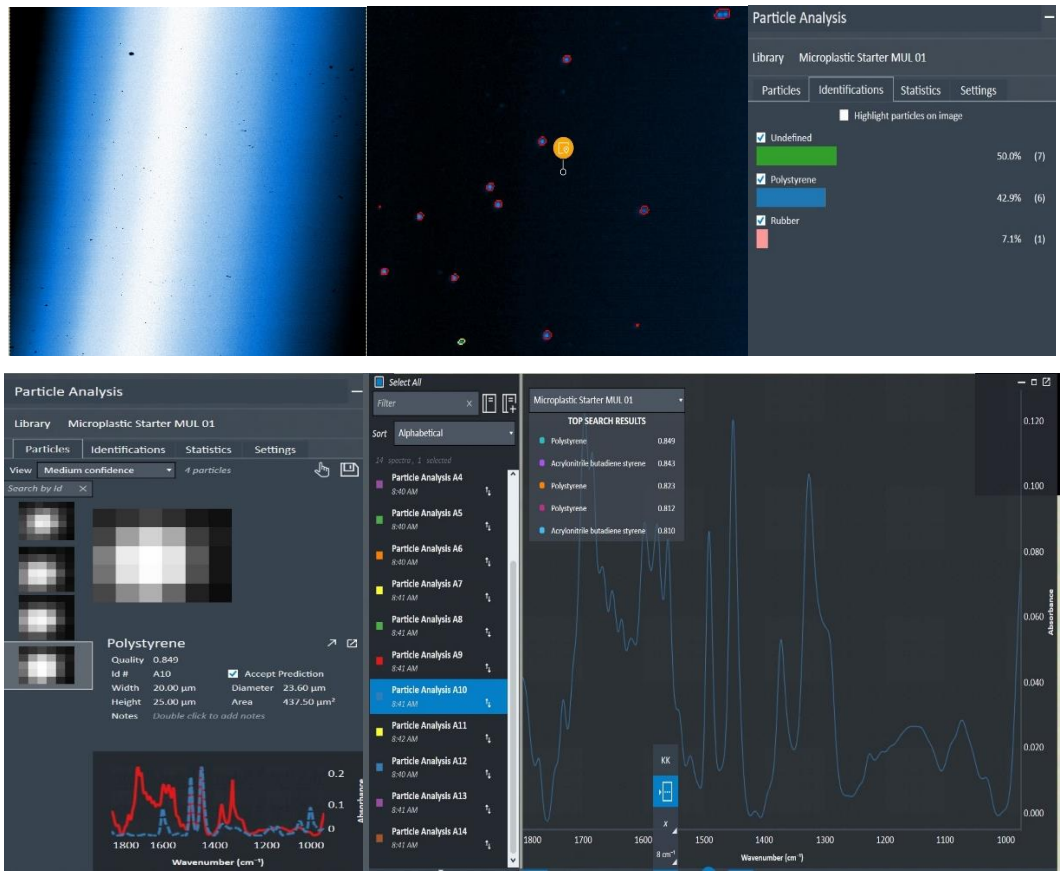
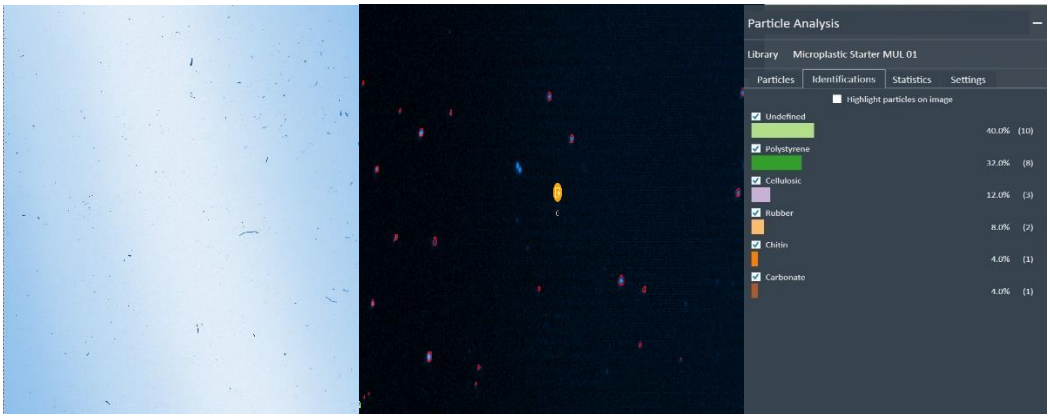


Figure 4.28. Pancreas sample



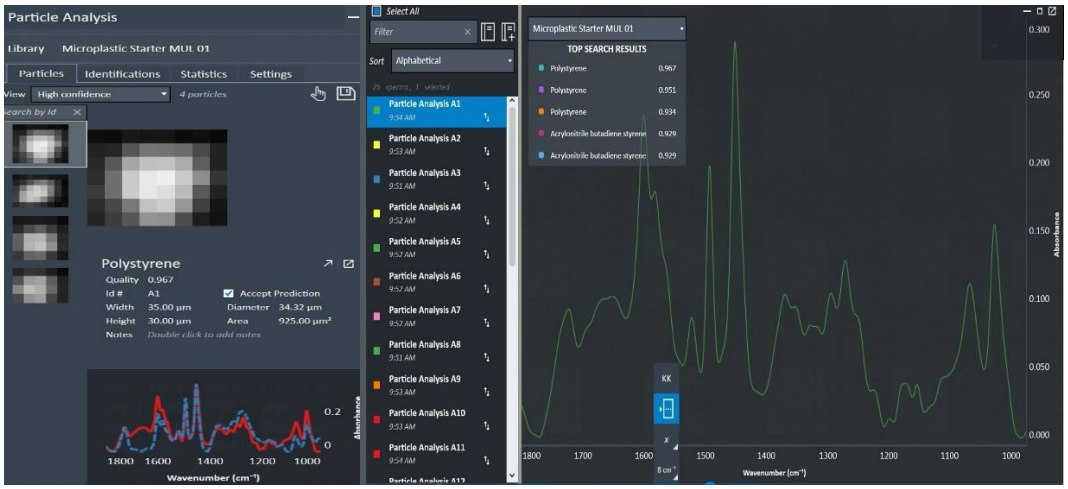


Figure 4.29. Testicle sample

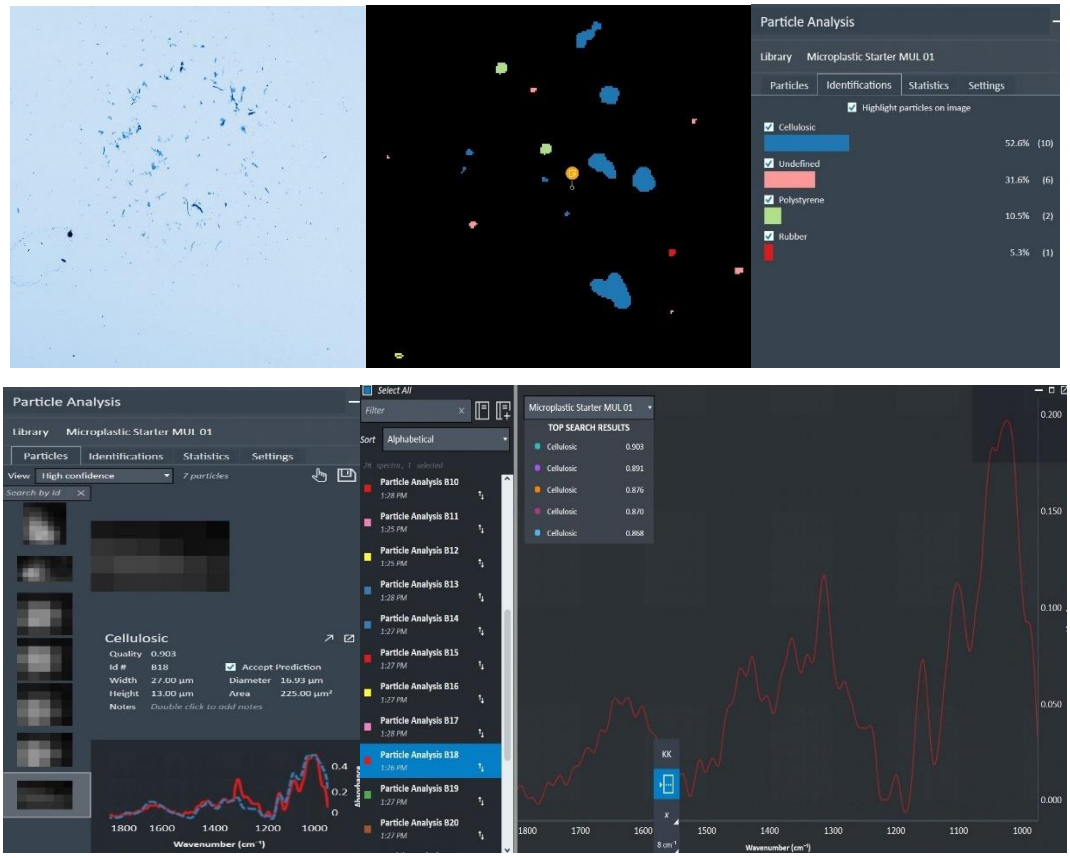


Figure 4.30. Stool sample

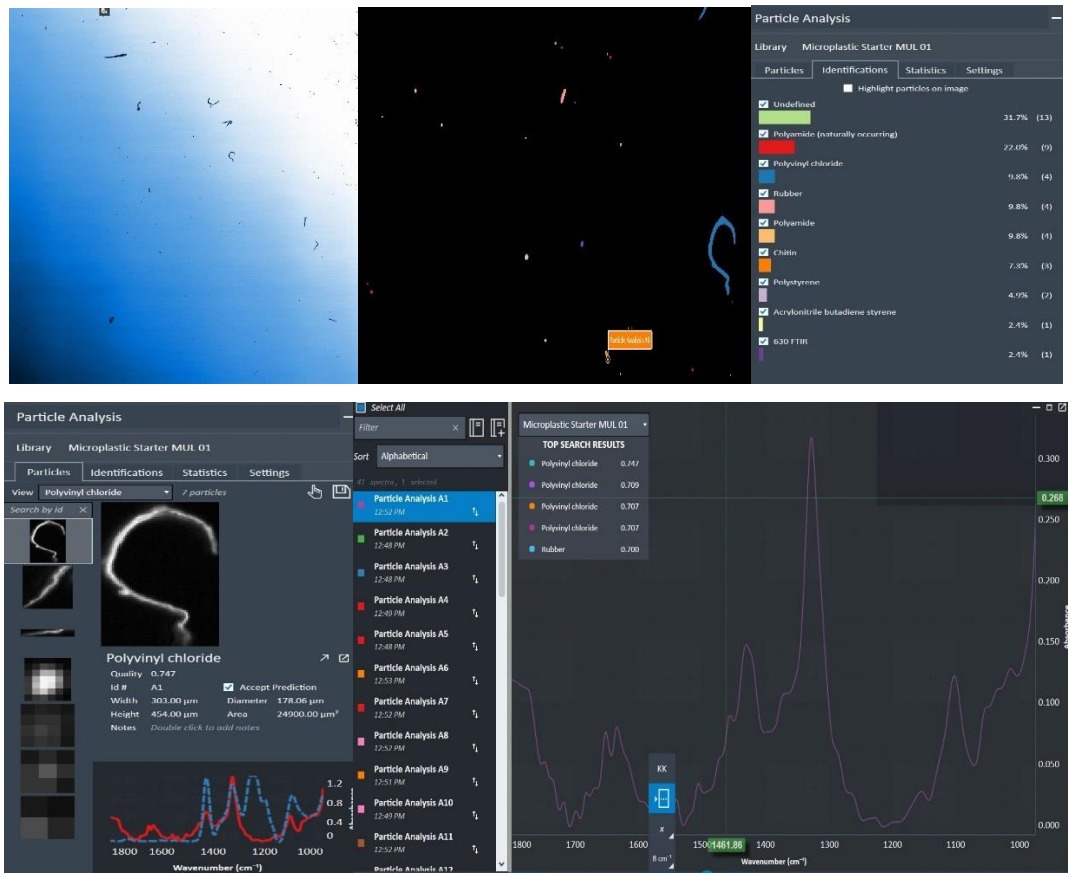


Figure 4.31. Liver sample

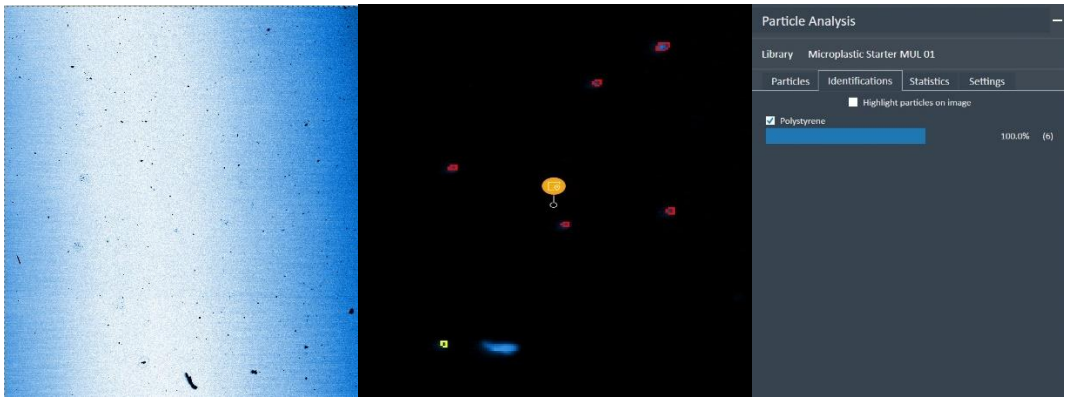


Figure 4.32. Lung sample

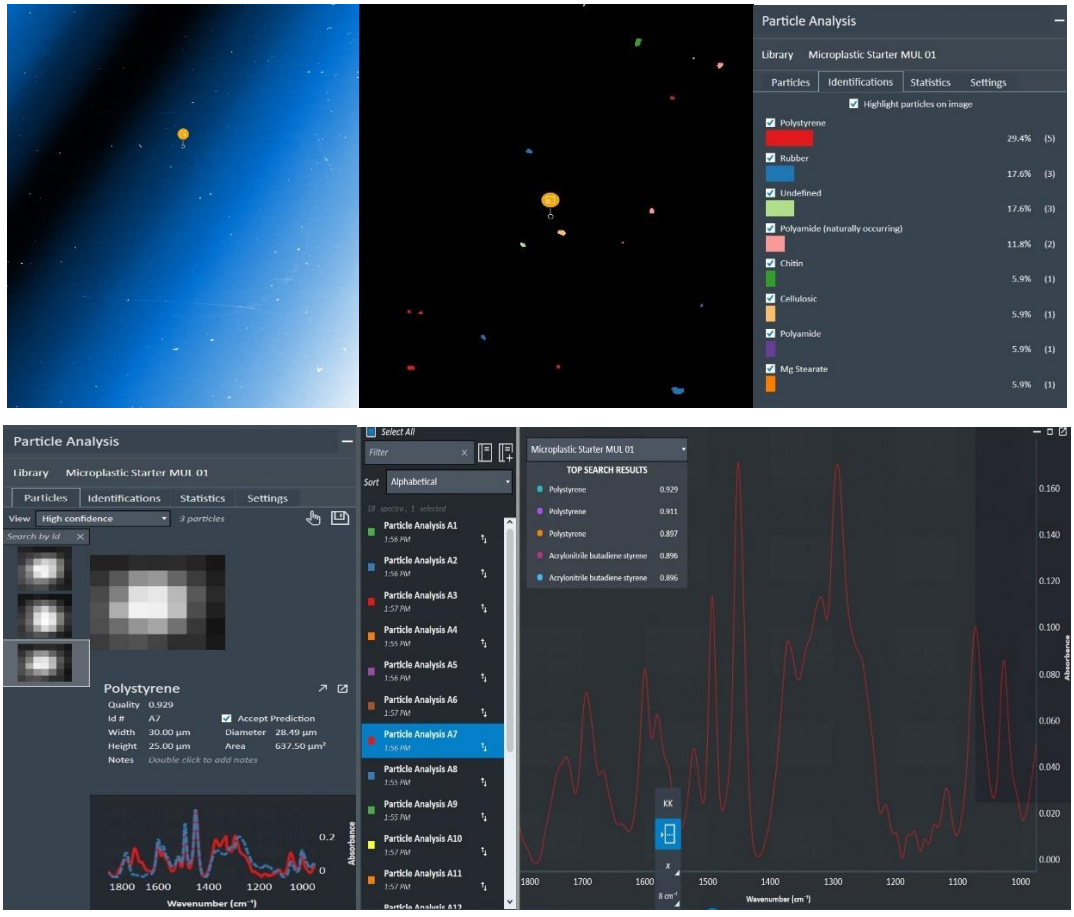


Figure 4.33. Kidney sample

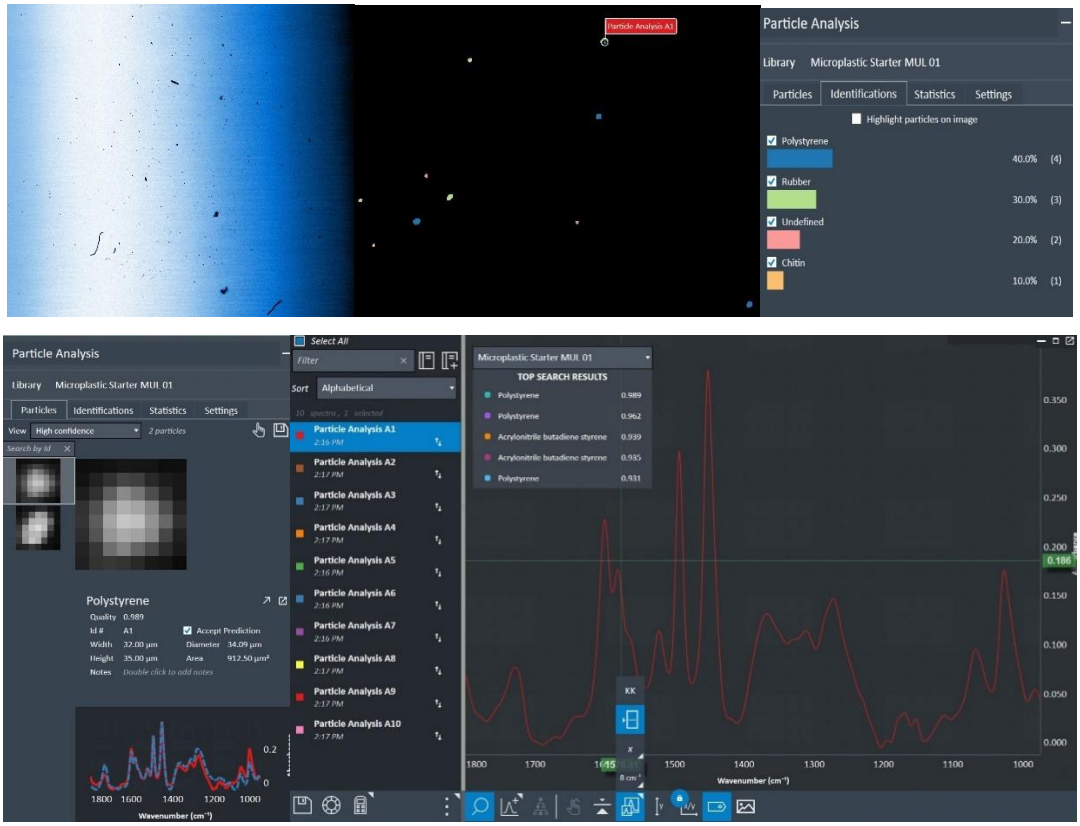


Figure 4.34. Spleen sample

Mice tissue analysis was faster and easier due to less undigested tissue in the samples. The peak analysis of the mice tissue samples already indicated that there are fewer particles present than on the filter papers of processed meat samples. The particle analysis confirmed that in all of the samples, polystyrene particles were present. The biggest blue PS particles were found in all samples and they were easy to detect and were identified with high confidence. The smaller 1 µm and 0,2 µm pink and white particles were detected by LDIR's high-magnification camera. However, they were only identified when gathered in aglomerates and by using the precise ATR crystal. Even then, the absorbance signals were low and the confidence was not high enough for the results to be definite. Formation of agglomerates of particles can lead to unprecise results and wrong identification, so particles less than 20 µm in diameter are hard to characterize precisely by the instrument.[41]

Along with MNP particles, other contaminants were found in the samples like cellulose fibers and naturally occurring polyamide. Like in Lopez-Rosales, A.'s research of airborne microplastics, fibers and fragments were the most occurred contaminants in the samples.[47] Undefined particles were either too small to identify or the spectrogram library did not contain any similar compounds.

5. CONCLUSION

Finding the right tissue digestion method for MNP isolation from tissue samples has proven to be a difficult task. There was a lot of undigested tissue present in most of the samples, and in some, filtration was not even possible due to the clogging of the filter paper during filtration. Tissue digestion with HNO_3 and the Kreon 25000 mix of pancreas enzymes performed the worst and the samples were not suitable for LDIR analysis. Alkaline digestion with KOH proved to be the best in digesting fatty tissue, while the enzymatic tissue digestion method with protease did the best in digesting protein. Different combinations of digestion environment and concentrations could lead to a more quality tissue digestion and easier analysis.

Microscopy was then performed on all sample types. With the bright-field technique, a realistic picture was provided, while dark-field made it easier to distinguish particles from tissue due to the dark background. Switching between these techniques gave us a complete picture of the particles present in the samples and the quality of tissue digestion. From the microscopy pictures, we could see a lot of particles, fibers, fragments, and undigested tissue in the samples, but to identify them, LDIR spectroscopy was performed.

LDIR spectroscopy has proven successful in identifying MNP particles from both processed meat and mice tissue samples. In the case of mice tissue samples analyzed on Kevley slides, the amount of undigested tissue and the number of all particles was much smaller compared to the filter analysis of processed meat samples. This made identification and characterization easier, faster, and more correct as particles didn't get trapped in undigested tissue matrixes. In both sample types, particle analysis showed microplastic presence as particles of rubber, PS, polyamide, and PVC were found on the surface of the filter papers and Kevley slides. Bigger particles gave out bigger absorbance signals and could be identified with greater confidence than smaller particles. Particles smaller than 20 µm were harder to detect and identify without the use of the ATR crystal. Even then, due to low absorbance signals, confidence in their identification was not very high, making the LDIR not viable for nanoplastic identification.

When researching MNP particles in tissue samples, the tissue digestion method is different from one tissue sample to another, but analytical methods like microscopy and spectroscopy are irreplaceable. Showing the presence of microplastics through visual characterization with an optical microscope and its characterization with LDIR spectroscopy proved to be a very quick, automated, and useful tool. In this stage of global MNP research, it is vital to show the presence of MNPs everywhere around us to raise awareness of the problems they are causing. That is the first and most important step in reducing global plastic use and pollution.

6. LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations

MNP – micro and nanoplastics

LDIR – Laser direct infrared imaging

PE – polyethylene

LDPE – low-density polyethylene

HDPE – high-density polyethylene

PP – polypropylene

PS – polystyrene

GPPS – general-purpose polystyrene

HIPS – high-intensity polystyrene

EPS – expandable polystyrene

PVC – polyvinyl-chloride

PET – polyethylene teraphthalate

PU – polyurethane

PC – polycarbonate

PA - polyamide

ABS – acrylonitrile butadiene styrene

PVDF – polyvinylidene fluoride

PEG – polyethylene glycol

EVA – polyethylene vinyl acetate

BPA – bisphenol A

AFM – atomic force microscopy

TEM – transmission electron microscopy

SEM – scanning electron microscopy

FTIR – Fourier transform infrared spectroscopy

ATR – attenuated total reflectance

Py-Gc-Ms – Pyrolysis-Gas Chromatography-Mass Spectrometry

DLS – diffractive light scattering

QC – quantum cascade (laser)

SDS – Sodium dodecyl sulfate

Symbols

m_1 – mass of the added sample before digestion, g

m_2 – mass of the sample after digestion, g

β – digestion efficiency, %

7. LITERATURE

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