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InPlasTwin

Increasing expertise in micro- and nanoplastics analysis through twinning action

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D6.2 Knowledge exchange on protocols for analysis of plastic additives

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Abstract:	This deliverable presents harmonized protocols developed for the leaching and migration testing of plastic additives from agricultural mulching films and their corresponding micro- and nanoplastics (MNPs). The protocols were designed to ensure comparability between experiments conducted with intact mulching films and those using biodegradable MNPs by applying consistent material-to-medium ratios, controlled experimental conditions, and compatibility with advanced analytical techniques. Through knowledge exchange activities within Tasks 6.1 and 6.2, as well as virtual training under Task 6.3, researchers from JSI and AUA gained practical experience in designing and implementing experiments to assess additive release under environmentally and food-contact relevant conditions. The resulting protocols and methodological insights provide a robust framework for future studies on plastic additives, contributing to improved comparability, consistency, and reliability of data generated within the project and in related research contexts.

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List of Abbreviations and Acronyms	
ACN	Acetonitrile
ASE	Accelerated solvent extraction
AUA	Agricultural University of Athens
BBP	Benzyl butyl phthalate
BFRs	Brominated flame retardants
BPA	Bisphenol A
CRM	Certified reference material
DBP	Dibutyl phthalate
DEHA	Hexanedioic acid, bis(2-ethylhexyl) ester
DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DMP	Dimethyl phthalate
DNOP	Di-n-octyl phthalate
DTU	Danmarks Tekniske Universitet
DTU Food	Technical University of Denmark, National Food Institute
EU	European Union
FID	Flame ionization detector
GC-qTOF-MS	Gas chromatography coupled with quadrupole time-of-flight mass spectrometry
HFIP	Hexafluoroisopropanol
HNO_3	Nitric acid
H_2O	Water
HRMS	High-resolution mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IMR	Institute of Marine Research
IS	Internal standard
JSI	Jožef Stefan Institute
LC-qTOF-MS	Liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

LDPE	Low-density polyethylene
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
MNPs	Microplastics and nanoplastics
NaCl	Sodium chloride
OM	Overall migration
PAEs	Phthalate esters
PBAT	Polybutylene adipate terephthalate
PBDEs	Polybrominated diphenyl ethers
PFAS	Per- and polyfluoroalkyl substances
PTV	Programmed temperature vaporization
QqQ-MS	Triple quadrupole mass spectrometry
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
rpm	Revolutions per minute
SOP	Standard operating procedure
SPE / SPME	Solid-phase (micro)extraction
UHPLC / UPLC	Ultra-(high)-performance liquid chromatography
UPW	Ultrapure water
UV	Ultraviolet
WP	Work package
(μ)ECD	(Micro) Electron capture detector

1. Introduction

Plastic mulching films, including both conventional polymers and biodegradable alternatives, incorporate a wide range of intentionally added substances designed to optimise material performance throughout agricultural application. These plastic additives, including plasticizers, antioxidants, UV/thermal stabilizers, and processing aids, are essential for ensuring adequate flexibility, durability, and resistance to environmental stressors during field use. As these compounds are not chemically bound to the polymer matrix, they can migrate from the material into the surrounding soil during application, ageing, and subsequent degradation processes. The fragmentation of mulching films into micro- and nanoplastic particles (MNPs) may further facilitate the release and distribution of these additives in agricultural environments. Given that some of these substances possess hazardous or bioactive properties, their environmental fate and potential transfer into crops represent emerging issues relevant to soil quality, ecosystem exposure, and long-term food safety considerations in the context of agricultural plastic use.

Sample preparation procedure for studying the release of plastic chemicals from polymers used for food application is harmonized under the plastic regulation (EU10/2011) where details on the testing conditions are provided. However, such harmonized approach does not exist for studying plastic additives in / released from mulching films and plastic additives in fruits.

Previous studies analysing plastic additives, phthalates, PFAS, pesticides, and brominated flame retardants in food matrices have employed a wide variety of extraction techniques (Table 1), reflecting both analyte diversity and matrix complexity. QuEChERS is one of the most commonly used approaches, particularly for fruits and vegetables, and has been applied to freeze-dried strawberries (Scopetani et al., 2026), cranberries (Genualdi et al., 2017), strawberries and mixed fruits (Gordan et al., 2025; Christia et al., 2015), and various foods for PBDEs and BFRs (Babalola et al., 2018; Fernandes et al., 2020). More exhaustive extraction techniques such as Soxhlet have been used for phthalates in freeze-dried or dried vegetables (He et al., 2020; Li et al., 2020), sometimes followed by SPE cleanup. ASE combined with SPE has been applied to more complex matrices such as seafood, enabling efficient recovery of hydrophobic phthalate esters (Hidalgo-Serrano et al., 2020). Targeted analyses have also used SPE alone, for example for BPA in vegetables (Shaaban et al., 2022), while headspace SPME has been selected for more volatile phthalates (Yue et al., 2020). Overall, the literature illustrates that extraction method selection is highly dependent on analyte properties and matrix type, with QuEChERS dominating fruit/vegetable applications, Soxhlet and ASE favored for hydrophobic contaminants, and SPE or SPME used in more targeted workflows. A total dissolution procedure of mulching films using dichloromethane followed by sonication, and analysed using GC-FID and GC-MS has been reported (Monkley et al., 2025).

Analyte	Matrix	Sample preparation	Analytical technique	Reference
Plastic additives	Freeze-dried strawberries	QuEChERS	GC-MS	Scopetani et al., 2026
Phthalate esters	Freeze-dried vegetables	Soxhlet (extraction) - SPE (clean-up)	UPLC-TOF-MS	He et al., 2020
BPA	Vegetables	SPE	UPLC-QqQ-MS	Shaaban et al., 2022
Phthalate esters	Vegetables (dried)	Soxhlet	GC-MS	Li et al., 2020
Phthalate esters	Seafood	ASE - SPE(clean-up)	UHPLC-HRMS	Hidalgo-Serrano et al., 2020

Phthalate esters	Vegetables	Headspace SPME	GC-QqQ-MS	Yue et al., 2020
PFAS	Cranberries	QuEChERS	LC-MS	Genualdi et al., 2017
Pesticides	Strawberries	QuEChERS	UHPLC-MS/MS	Gordan et al., 2025
Pesticides	Fruits	QuEChERS	UPLC-MS/MS	Christia et al., 2015
PBDEs	Foods (incl. vegetables)	QuEChERS	GC-µECD	Babalola et al., 2018
BFRs	Chili peppers	QuEChERS	GC-ECD	Fernandes et al., 2020
Plastic additives	Food simulants	SPE	GC-MS	Fasano et al., 2012
PFAS	Freeze-dried strawberries	QuEChERS	LC-MS/MS	Scordo et al., 2020

Table 1. Overview of sample-preparation methods reported in the literature for the analysis of plastic contaminants in various food matrices

In work package 6 (WP 6), different sample preparation protocols to analyze plastic additives in mulching films and strawberries were tested. The activities carried out in WP6 focused on enhancing the capacity of researchers of JSI and AUA to investigate the release of potentially toxic organic and inorganic plastic additives from MNPs into the environment and into food.

This collaborative effort builds upon the experience gained in extracting plastic additives from mulching films during the hands-on trainings conducted in Tasks 6.1 and 6.2, as described in Deliverable 6.1. It also builds on online trainings and consultations conducted under Task 6.3 (Virtual consultations and training: Enhancing knowledge exchange on leaching of plastic additives from MNPs), where researchers from DTU, and IMR shared their expertise with JSI and AUA. During these efforts, extraction procedures were tested and evaluated, particularly for plastic additives (organic and inorganic) in MNPs, and adapted to ensure compatibility with specific analytical methods. This work represents an important step toward enabling the developed extraction procedures to be applied to the analysis of plastic additives expected in strawberry samples arising from mulching film degradation during strawberry cultivation. These applications will be addressed in subsequent phases of the InPlasTwin project through hydroponic (WP7) and field-scale (WP8) experiments.

2. Sample preparation protocols for mulching films and MNPs

A series of sample preparation procedures (dissolution, extraction, leaching, and migration testing) for extracting plastic additives from MNPs were tested during the stay of JSI and AUA researchers at DTU (as described in Deliverable 6.1).

2.1. Total dissolution tests

Total dissolution using HFIP: The method was adapted from Bhattarai et al. (2025). For the total dissolution of biodegradable mulching film, 0.2 g of the sample was cut into small pieces to increase the surface area and transferred into pre-burnt glass vials. Subsequently, 2 mL of hexafluoroisopropanol (HFIP) was added, and the mixture was left overnight at room temperature to ensure complete dissolution of the polymer, allowing the release of additives, oligomers, and other low-molecular-weight compounds embedded in the polymer matrix into the solvent phase. HFIP is effective for dissolving aromatic–aliphatic polyesters such as polybutylene adipate terephthalate

(PBAT), the main polymer used in biodegradable mulching films, because it can interact with the ester groups present in the polymer structure. In contrast, non-polar polymers such as polyethylene (PE) do not contain these functional groups and are therefore not soluble in HFIP. For this reason, this protocol was applied only to biodegradable mulching film samples. Samples were prepared in triplicate, and three procedural blanks containing only the solvents were included.

After dissolution, 4 mL of methanol was added to induce precipitation of the polymer matrix. The samples were then stored in a refrigerator for 1 h to facilitate complete polymer precipitation. Following precipitation, the samples were centrifuged at 4000 rpm for 10 min to separate the precipitated polymer from the liquid phase. Prior to instrumental analysis, the samples were filtered using Mini-Uniprep G2 syringeless filters equipped with a 0.45 µm glass membrane (Whatman, GE Healthcare Life Sciences, Buc, France) to remove any remaining particulate matter. The filtered extracts were subsequently analysed using LC-qTOF-MS.

Two extraction protocols (Soxhelt extraction & Reflux-based extraction) were tested to dissolve LDPE based mulching films.

Soxhlet extraction: Initially, a Soxhlet apparatus was tested to achieve the total dissolution of conventional mulching films, based on an existing SOP at DTU Food developed for similar polymer extraction procedures. Three specific types of materials were analyzed: a conventional black film, a transparent film, and a black film that had been exposed in the field. Approximately 0.25 g of the mulching film sample was cut into small pieces and weighed accurately. These pieces were placed directly into the solvent bottle rather than the standard sample container to create a continuous reflux system. Before the extraction began, the sample was spiked with a small amount of an internal standard - deuterated benzophenone, to account for any material lost during the procedure. Toluene was used as the solvent, with 20 mL added to the bottle to ensure sufficient volume for the cycle. The toluene was heated until it started boiling. At the condenser toluene was cooled, and dripped back down to dissolve the plastic. Once the extraction was complete, 10 mL of acetone was added to the mixture to initiate the re-precipitation of the polyethylene. This step turned the dissolved plastic back into a solid so it could be removed, ensuring that the polymer would not contaminate or damage the analytical instruments. After the plastic had completely solidified, the clear liquid was carefully collected and filtered through 0.45 µm Mini-UniPrep™ G2 syringeless filters to remove any remaining particulates.The final filtered extracts were transferred into amber glass vials and stored at ≤-18°C until they were analyzed on GC-qTOF-MS analysis. A schematic representation of the procedure is shown in Figure 1.

Two main problems were observed with this specific configuration. First, the extraction process was slow, requiring approximately 2 hours to complete for each cycle, and the number of samples that could be processed at once was limited by the number of available Soxhlet equipment. Second, the process left behind a residue in the bottle that was extremely difficult and tedious to clean, which created a risk of losing the analytes being studied. Because of these limitations, it was decided that a simpler and faster sample preparation method was needed, leading to the development of the optimized 20-minute reflux-based extraction protocol.

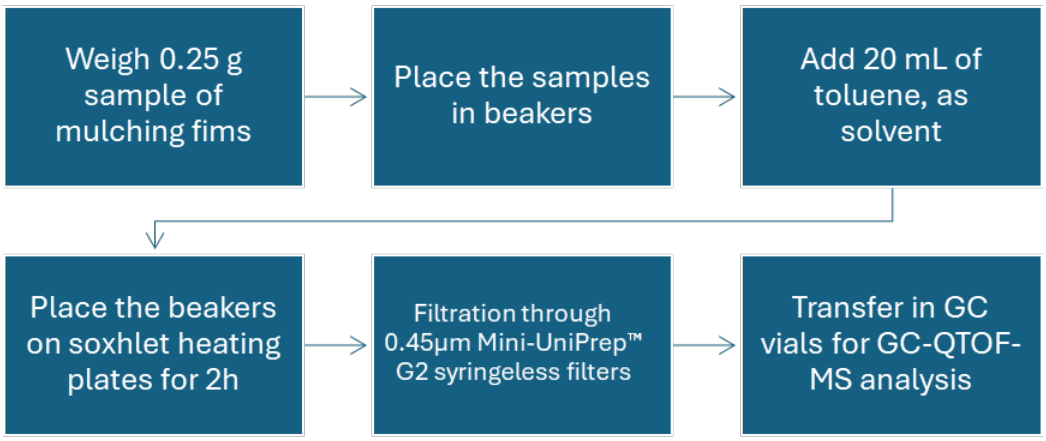

Figure 1. Steps for Soxhlet Extraction of mulching films

Reflux-based extraction: The extraction of additives from LDPE mulching films was performed using an optimized reflux method designed for total dissolution. Three specific types of materials were analyzed: a conventional black film, a conventional transparent film, and a conventional black film that had been exposed in the field. For each material, approximately 0.25 g of the film was cut into small pieces to increase the surface area and weighed accurately on an analytical balance. These pieces were transferred into pre-burnt glass beakers to ensure the apparatus was clean and free from contamination. To account for any material lost during the process, a small amount of an internal standard - deuterated benzophenone, was spiked directly onto the sample before the solvent was added. For the extraction, 10 mL of toluene was added to each beaker using a glass graduated cylinder. The beakers were then placed on a heating plate at 90°C for 20 minutes. Since a standard condenser was not available, a round-bottom flask filled with ice was placed on top of each beaker to act as a reflux system, catching the solvent vapors and dripping them back down to maintain a constant volume. It was critical to keep the temperature at 90°C, which is below the boiling point of toluene (110°C), to ensure the plastic stayed dissolved throughout the extraction period. This process was performed in triplicate for each sample type to ensure the results were consistent.

Once the polymer was fully dissolved, the samples were allowed to cool to room temperature. To prevent the dissolved plastic from clogging the chromatography columns during analysis, 10 mL of acetone was added as an anti-solvent to initiate the precipitation of the LDPE. The mixture was stirred thoroughly and left to stand for 10 minutes to ensure the plastic had completely solidified. Finally, the clear supernatant was carefully collected and filtered through a 0.45µm Mini-UniPrep™ G2 syringeless filters to remove any remaining particulates. Plastic-housed filters were avoided to prevent any extra additives from the filter housing from leaching into the sample. The filtered extracts were then transferred into amber glass vials and stored at ≤-18°C until they were ready for GC-qTOF-MS analysis. A schematic representation of the Reflux-based extraction workflow is shown in Figure 2.

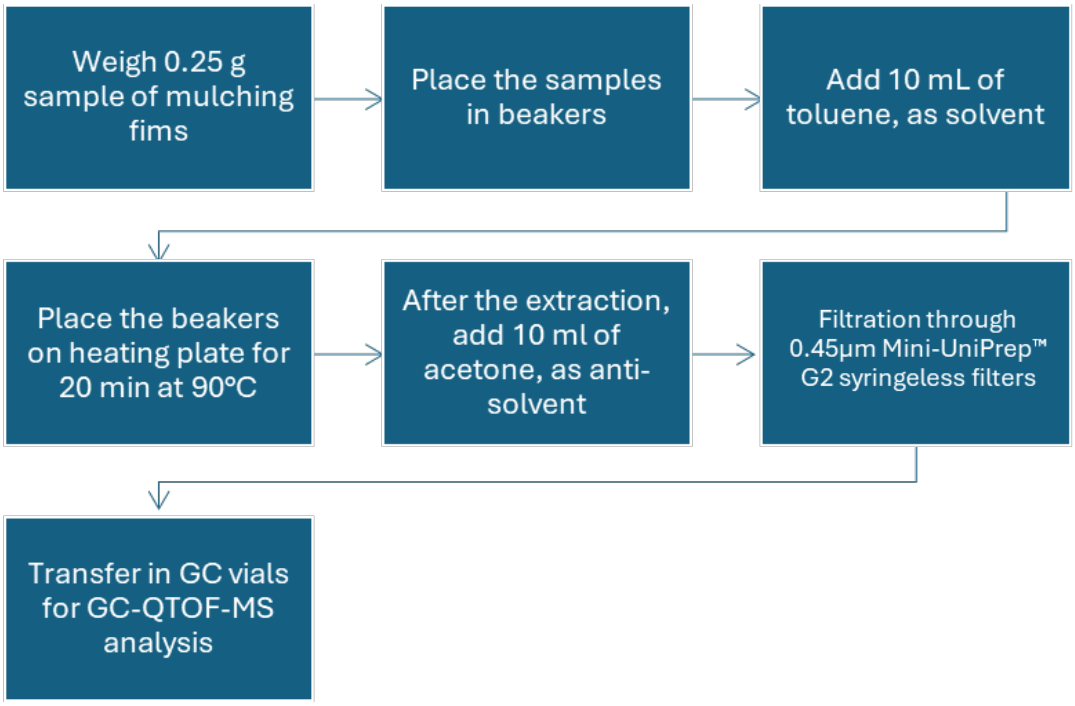

Figure 2. Steps for Reflux Extraction of mulching films

2.2. Extraction testing

Accelerated Solvent Extraction (ASE): The method for ASE extraction was based on Li et al., 2014 is schematically shown in Figure 3. Four samples of mulching films: a low-density polyethylene conventional black mulching film, a low-density transparent mulching film, a low-density conventional black mulching film exposed in the field and a biodegradable (PBAT) mulching film were selected. First, approximately, 0.5 g of the intact mulching film (or similar amount of MNPs) cut in small pieces and weighed. Then, they were mixed thoroughly with pre-burned Ottawa sand in a 36 mL accelerated solvent extraction (ASE) cell, ensuring the complete filling and uniform packing of the cell. The extraction was performed using acetonitrile (ACN), as the solvent, under the following conditions: oven temperature 100°C, pressure 1500 psi, heat time 5 min, static time 5 min (two static cycles), flush volume 50%, and nitrogen purge time 60 s. After the extraction, 10 mL of the extract were collected in a 15 mL glass tube and evaporated to ~0.5 mL under a gentle stream of nitrogen at 45°C. The residue was filtered through 0.45µm Mini-UniPrep™ G2 syringeless filters and transferred to amber LC vials prior to LC–qTOF-MS analysis.

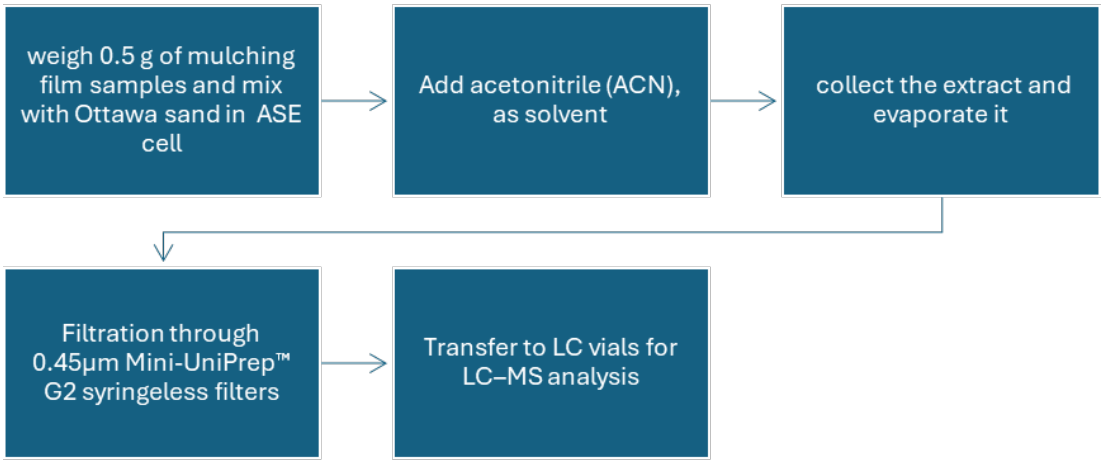

Figure 3. Steps for ASE of mulching films

2.3. Leaching testing

Given the focus of Task 6.2 on MNPs, particularly those originating from biodegradable mulching films, leaching experiment was primarily conducted using MNPs produced from biodegradable mulching films, specifically the fraction <63 µm. In addition, leaching tests were performed using intact mulching films to provide a broader basis for comparison and to enable critical evaluation of potential differences between the two material forms. The intact film experiment included all available film types: conventional black, conventional transparent, field-exposed conventional black, and biodegradable mulching films.

2.3.1. Leaching testing conditions summary

Leaching testing was performed using ultrapure water (UPW) as the extraction medium to simulate the release of substances from mulching films under environmental conditions. Leaching tests were conducted under the following conditions:

- Leaching medium: Ultrapure water (UPW)
- Contact time: 20 days
- Temperature: 20 °C
- Agitation: Continuous shaking at 200 rpm
- Light conditions: Dark

2.3.2. Leaching testing protocol

2.3.2.1. Intact mulching films

Mulching films were cut into single pieces with a mass of approximately 0.2 g, and the dimensions of each piece were recorded. Each sample was placed into a pre-burnt glass bottle, after which 20 mL of

UPW was added. Samples were placed on a shaker and agitated at 200 rpm for 20 days at 20 °C in the dark. After the leaching period, the mulching film pieces were removed. An aliquot of the leachate was filtered using glass membrane filters (0.45 µm Mini-UniPrep™ G2 syringeless filters) for LC-MS analysis, and an internal standard mixture containing d_4-BBP, d_4-DBP, and d_4-DEHP was added to all samples to obtain a final concentration of 200 ng mL^{-1}. Another aliquot was acidified to 2% HNO_3 for ICP-MS analysis and stored in the refrigerator until analysis.

2.3.2.2. Biodegradable mulching film MNPs

Approximately 0.05 g of biodegradable mulching film MNPs (<63 µm) was weighed into a pre-burnt glass bottle, and 5 mL of ultrapure water (UPW) was added. The leaching test was performed under the same conditions as for the intact film pieces. After the leaching period, the leachate was divided into two aliquots. The aliquot for LC-MS analysis was filtered through 0.45 µm glass membrane filters (Mini-UniPrep™ G2 syringeless filters), and an internal standard mixture containing d_4-BBP, d_4-DBP, and d_4-DEHP was added to obtain a final concentration of 200 ng mL^{-1}. The aliquot for ICP-MS analysis was filtered through 0.45 µm Minisart cellulose nitrate membrane filters (Sartorius, Goettingen, Germany) and acidified to 2% HNO_3. The aliquots were stored in the refrigerator until analysis.

2.4. Migration testing

Following the same sample selection as in the leaching experiments, migration tests were performed using MNPs produced from biodegradable mulching films (fraction <63 µm) and intact mulching films. The intact film experiments included conventional black, conventional transparent, field-exposed conventional black, and biodegradable mulching films.

2.4.1. Migration testing conditions summary

Migration testing was performed using food simulant B (3% (w/v) acetic acid) to represent contact with acidic foods. The testing conditions were selected to simulate potential contact between strawberries and agricultural mulching films during cultivation.

Migration tests were conducted under the following conditions:

- Food simulant: 3% (w/v) acetic acid
- Contact time: 10 days
- Temperature: 40 °C
- Exposure type: Total immersion

These parameters correspond to conditions equivalent to overall migration condition (OM2) described in Regulation (EU) No 10/2011. The same testing conditions were applied to both intact mulching film samples and biodegradable MNPs samples to ensure comparability.

All migration samples were analysed as soon as possible after the completion of the migration test, as migration solutions should not be stored for longer than 10 days prior to analysis.

2.4.2. Migration testing protocol

2.4.2.1. Intact mulching films

Intact mulching films were initially cut into square samples of 1 dm^2, and their mass was recorded. Field-exposed mulching films were gently rinsed with ultrapure water before cutting to remove any

attached soil particles. Each sample was subsequently divided into four equal pieces and placed into glass migration cells. Glass rods were used to ensure that the film pieces did not come into contact with one another during testing. Glass migration cells suitable for total immersion were selected to allow complete exposure of the material to the simulant.

Each migration cell was filled with 100 mL of preheated 3% (w/v) acetic acid (food simulant B), placed in a preheated oven at 40°C, and kept for 10 days. A calibrated Tinytag data logger was used to continuously monitor and record the temperature throughout the duration of the migration test.

All intact mulching film samples were prepared and tested in triplicate. In addition, three blank samples containing only 100 mL of 3% (w/v) acetic acid were included under the same conditions to serve as controls.

After the migration period, the film pieces were removed from the cells. The migration solution was directly used for ICP-MS analysis, while an aliquot was filtered through 0.45 µm glass membrane filters (Mini-UniPrep™ G2 syringeless filters) for LC-MS analysis, to which an internal standard mixture containing d_4-BBP, d_4-DBP, and d_4-DEHP was added to obtain a final concentration of 200 ng mL^{-1}.

2.4.2.2. Biodegradable mulching film MNPs

To maintain consistency with the intact mulching film experiments, the mass-to-volume ratio used in the migration test was considered. In the intact film study, 1 dm^2 of biodegradable film corresponded to an average mass of 0.416 g and was exposed to 100 mL of food simulant. To preserve a comparable material-to-simulant ratio while reducing material consumption, the experiment with biodegradable MNPs was down scaled. For the down scaled test with biodegradable MNPs, two replicates were prepared with sample masses of 0.0120 g and 0.0115 g. Accordingly, 2.9 mL and 2.8 mL of 3% (w/v) acetic acid were added to the respective samples. In addition, two blank samples containing the 2.9 mL of 3% (w/v) acetic acid without MNPs were included as controls.

After the migration period, the migration solutions were divided into two aliquots. The aliquot for LC-MS analysis was filtered through 0.45 µm glass membrane filters (Mini-UniPrep™ G2 syringeless filters), to which an internal standard mixture containing d_4-BBP, d_4-DBP, and d_4-DEHP was added to obtain a final concentration of 200 ng mL^{-1}. The aliquot for ICP-MS analysis was filtered through 0.45 µm Minisart cellulose nitrate membrane filters (Sartorius, Goettingen, Germany) and analysed directly. The aliquots were stored in the refrigerator until analysis.

3. Sample preparation protocols to analyse plastic additives in strawberries

Different sample preparation protocols were tested to analyze plastic additives in strawberry matrix considering these situations: if mulching films leach into soil and plastic additives taken up by strawberries, or if MNPs are taken up by plant and leach in the strawberry.

Reflux-based extraction for strawberry samples: For this procedure, it is necessary to use freeze-dried strawberry samples because the high water content of fresh fruit would cause phase separation or spraying issues during extraction with toluene. Approximately 0.25 g of the strawberry powder was weighed accurately using an analytical balance. Due to the electrostatic nature of the powder, the sample was weighed by taring the aluminum weighing boat with the sample and calculating the difference after transferring the powder to a pre-burnt glass beaker. To account for volumetric losses

and ensure accurate normalization, a small amount of an internal standard - deuterated benzophenone, was spiked directly into the sample before the solvent was added. For the extraction, 10 mL of toluene was added to the beaker using a glass graduated cylinder. The beaker was then placed on a heating plate at 90°C for 20 minutes. Because a standard condenser was not available, solvent re-condensation was achieved by covering the beaker with an appropriately sized round-bottom flask filled with ice. This procedure was performed in triplicate, alongside reagent blanks, to ensure consistency and check for laboratory contamination.

After the extraction was complete, the supernatant was carefully collected using a Pasteur pipette. If analytes are expected to be below detection limits, the extract could be concentrated under a gentle stream of nitrogen (rotary evaporation should be avoided to prevent the loss of volatile analytes like certain phthalates). Finally, the clear supernatant was carefully collected and filtered through a 0.45µm Mini-UniPrep™ G2 syringeless filters to remove any remaining particulates. Plastic-housed filters were avoided to prevent any extra additives from the filter housing from leaching into the sample. The filtered extracts were then transferred into amber glass vials and stored at ≤-18°C until they were ready for GC-qTOF-MS analysis. A schematic representation of the whole procedure is shown in Figure 4Figure 2.

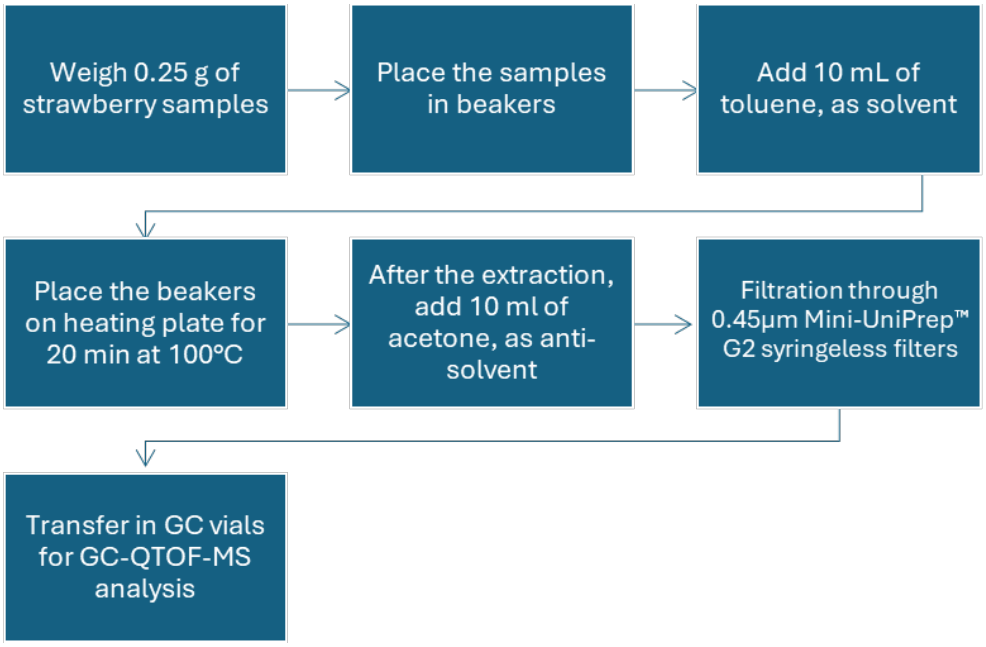

Figure 4. Steps for Reflux Extraction of strawberries

Accelerated Solvent Extraction (ASE): The method for ASE was based on Li et al., 2014. Around, 0.5 g of strawberry was weighed. As shown in Figure 5, an ASE cell was filled partly with Ottawa sand, 0.5g of strawberry powder, spiked internal standard, and refilled with Ottawa sand until the top and closed. The extraction was performed using acetonitrile (ACN), as the solvent, under the following conditions: oven temperature 100°C, pressure 1500 psi, heat time 5 min, static time 5 min (two static cycles), flush volume 50%, and nitrogen purge time 60 s. After the extraction, 10 mL of the extract were collected in a 15 mL glass tube and evaporated to ~0.5 mL under a gentle stream of nitrogen at 45°C. The residue

was filtered through 0.45µm Mini-UniPrep™ G2 syringeless filters and transferred to amber LC vials prior to LC–MS analysis.

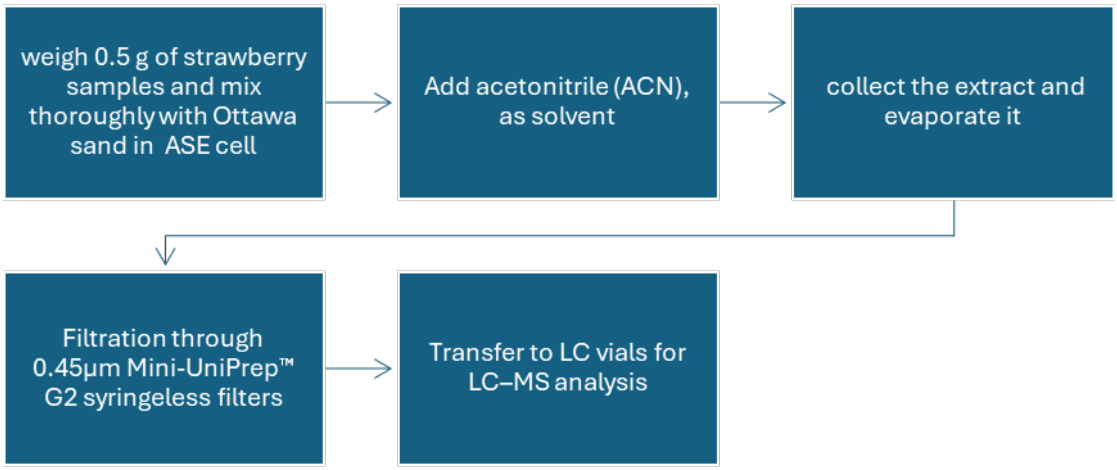

Figure 5. Steps for ASE of strawberries

QuEChERS: Two grams of homogenized sample were weighed into a 40 mL glass centrifuge tube. Freeze-dried samples were reconstituted (1 g with 5 mL water; 1:5, w/v) prior to extraction. An internal standard was added to obtain a final concentration of approximately 0.15 µg mL^{-1} in the extract. Five milliliters of acetonitrile was added, and the samples were shaken for 6 min at 750 rpm (GenoGrinder), ultrasonicated for 15 min, and centrifuged for 10 min at 4°C and 3500 rpm. The supernatant was collected, and the extraction was repeated on the residue; supernatants were combined and stored at −20°C overnight.

The following day, the extracts were centrifuged (5 min, 4°C, 3500 rpm) and subjected to dispersive SPE clean-up by transfer to a tube containing 2.0 g $MgSO_4$, 0.5 g NaCl, 0.1 g C18, and 0.1 g ENVI-Carb. After shaking (2 min) and centrifugation (20 min, 5°C, 4000 rpm), the supernatant was collected and evaporated under N_2 at 40°C to ~0.4 mL. 1 mL of MeOH/H_2O (prepared by adding 3.6 mL water to 100 mL MeOH) was added, and the azeotrope was evaporated to ≤0.5 mL, adjusting with MeOH if necessary. The final extract (~0.5 mL) was filtered through 0.45µm Mini-UniPrep™ G2 syringeless filters and transferred into amber LC vials prior to LC–MS analysis. All steps of the protocol are described in Figure 6.

Figure 6. Steps for QuEChERS of strawberries

Solid phase extraction (SPE): Two grams of homogenized sample were weighed into a 40 mL glass centrifuge tube. For freeze-dried samples, 1 g was first reconstituted with 5 mL of water (1:5, w/v) prior to weighing. The sample was then spiked with an internal standard. Subsequently, 6 mL of n-heptane and 6 mL of methanol/water (75:25, v/v) were added to the tube. The mixture was shaken vigorously for 1 min to ensure efficient extraction and then centrifuged at 3500 rpm for 10 min.

Following centrifugation, 3.6 mL of the methanol/water phase was carefully collected and transferred into a 15 mL glass tube. To adjust the solvent composition for solid-phase extraction (SPE), 2.3 mL of water was added, resulting in a total extract volume of 5.9 mL. The extract was loaded onto a CHROMABOND® C18 ec, 6mL/500mg SPE cartridge previously conditioned with 3 mL of methanol followed by 3 mL of water. After sample loading, the cartridge was rinsed with 5 mL of water to remove matrix interferences. Analytes were eluted with 3 mL of methanol into a 5 mL volumetric flask. Finally,

1 mL of water was added, and the solution was brought to volume with methanol. Prior to analysis, the extract was filtered through 0.45µm Mini-UniPrep™ G2 syringeless filters and transferred to amber LC vials. All steps of the protocol are described in Figure 7.

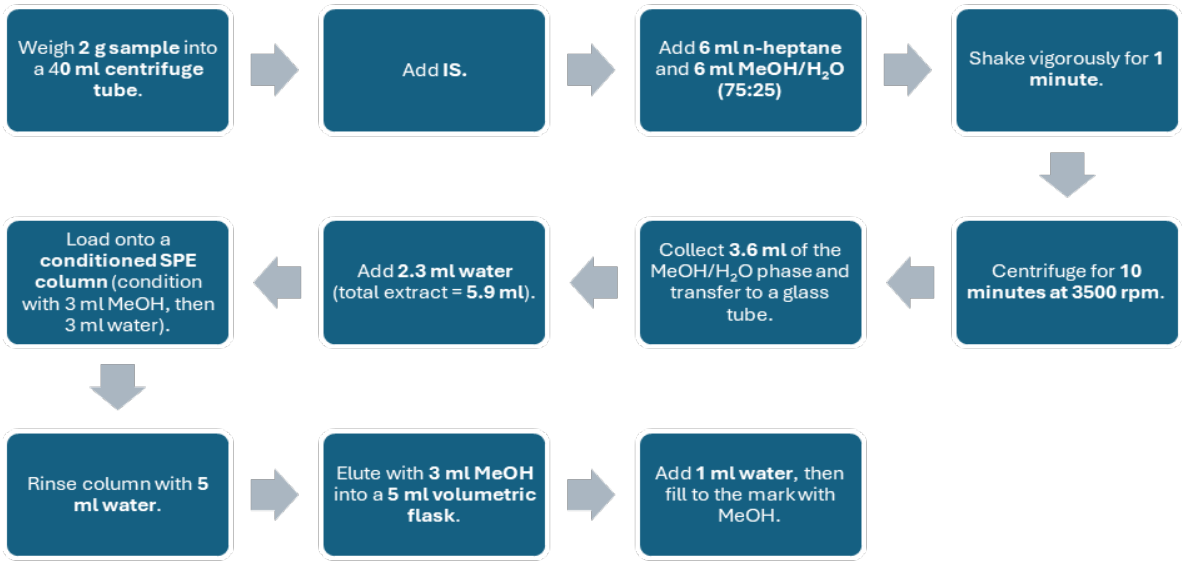

Figure 7. Steps for Solid Phase Extraction of strawberries

4. Instrumental Analysis

4.1. Liquid chromatography-mass spectrometry (LC-MS)

Liquid Chromatography separation was performed on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) equipped with a high-pressure binary pump, degasser, auto sampler and a column heater. The analytes were separated on a Waters ACQUITY UPLC BEH C18 Column with the LC-column held at 35 ◦C. A gradient was used for separating the analytes using 0.1% formic acid in Milli-q grade water (solvent A) and 0.1% formic acid in methanol (solvent B) as mobile phases. The elution gradient was set as follows: 2% of solvent B running at a flow of 0.3 ml/min from 0 to 1 min, a linear gradient to 50% B from 1 to 8 min, another linear gradient from 8 to 10 min to 98% B at a flow of 0.4 ml/min that was held till 12 min. At 12.1 min, the composition of the mobile phase was changed to 2% B and the flow was reduced to 0.3 ml/min and held until 15 min. The total run time was 15 min. The injection volume was set to 5 µL. The Liquid Chromatography system was connected to a Bruker Daltonics, MaXis Q-TOF MS equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2–0.4 min time segment and used for internal calibration of the data files. Hexakisperflouroetoxyphophazene was used as lock mass calibrant to compensate for drift in the mass axis during analysis. The samples were analyzed using Compass Data Analysis software from Bruker.

4.2. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was performed using an Agilent 8890 gas chromatography coupled to an Agilent 7250 Q/TOF-MS detector. A volume of 2 µl sample was injected using programmed temperature vaporization (PTV) inlet in solvent vent mode with temperature starting at 50 °C, held for 0.8 min, ramped to 290 °C and held for 2 min, ramped to 330 °C and held for 10 min. The GC oven temperature used was 70°C held for 3.3min, ramped to 180°C at a rate of 50°C/min held for 0min, ramped to 230°C at a rate of 4°C/min held for 0 min, ramped to 280°C at a rate of 3°C/min held for 10 min, ramped to 310°C at a rate of 14°C/min held for 10 min. The total run time was 56. 81 min. Two chromatographic columns J&W Select PAH capillary column (Agilent Technology, 15 m x 150 µm x 0.1 µm) coupled with a HP5MS ultra inert capillary columns (Agilent Technology, 15 m x 250 µm x 0.25 µm) was used. The two columns were coupled with backflush between the columns to prevent column contamination. The carrier gas used was Helium (1 mL/min). All the analyses were performed in electron ionization positive mode at 70 eV with source temperature 200°C. The MS data were acquired in profile mode that included mass range from 50-500 amu at an acquisition rate of 5 spectra/sec. The samples were analyzed using Agilent Qualitative analysis software (version 10).

4.3. Inductively coupled plasma mass spectrometry (ICP-MS)

The total mass concentrations of Al, As, Ca, Cd, Co, Cr, Cu, Fe, Mn, Na, Pb, Sn, Ti, and Zn in samples following migration and leaching tests were determined by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP TQ ICP-MS instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with an ASX-560 autosampler (Teledyne CETAC Technologies, Omaha, NE, USA).

For migration experiments, food simulant samples obtained after contact with both intact film pieces and MNPs were diluted only by adding an internal standard (IS) mixture containing Rh and Bi to obtain a final concentration of 1 ng mL^{-1}. The resulting dilution factor was taken into account during data evaluation. Calibration standards were prepared in 3% acetic acid to ensure matrix matching, and the same food simulant was used as the rinsing solution. For leaching experiments (intact films and MNPs), calibration standards were prepared in 2% nitric acid. Samples were diluted threefold to a final matrix of 2% nitric acid, and the same IS mixture was added to all samples and calibration standards.

5. Quality assurance and control

For all sample preparation procedures, the same measures were applied to prevent contamination from external plastic additives. Plastic labwares were replaced by glasswares, where feasible. All the glassware used during the sample preparation were burned 450°C for six hours. The scalpel used for cutting the samples was cleaned with ethanol between each sample to avoid cross-contamination. The filters used for filtering samples were based on glass membrane filters (0.45 µm Mini-UniPrep™ G2 syringeless filters). Procedural blanks were included in all sample preparation protocols and treated as the same way as the samples.

To evaluate the extraction efficiency of each developed sample preparation procedure by LC-MS and GC-MS, various plastic additive analytical standards (Table 2 and Table 3) were spiked into test samples prepared in triplicate (or in additional replicates when required).

Method performance was assessed by calculating recovery rates using the following equation:

$$Recovery\ (\%) = \frac{Measured\ concenetration}{Spiked\ concentration} \times 100$$

where measured concentration is the amount of plastic additive measured in the spiked sample, which was calculated as $\frac{Peak\ area\ of\ analytical\ standard}{Peak\ area\ of\ internal\ standard}$

Spiked concentration is the actual amount of plastic additive spiked, which was calculated as $\frac{Peak\ area\ of\ analytical\ standard\ in\ spiked\ matrix}{Peak\ area\ of\ internal\ standard\ in\ spiked\ matrix}$

Method accuracy of ICP-MS was evaluated using the digested certified reference material DORM-5 (fish protein). The CRM was analysed together with the leaching and migration test samples, and certified values were available for all measured elements except Ti. Recoveries ranged from 87.4 to 107.3%, confirming satisfactory analytical performance.

6. Evaluation of extraction efficiency of plastic additives in strawberries

6.1. Evaluation of extraction efficiency of ASE, QuEChERS, SPE

The extraction efficiency of ASE, QuECheRS and SPE were performed using a mix of analytical standards: diphenyl phthalate, dicyclohexyl phthalate, and triphenyl phosphate. The results are presented in Table 2. Each extraction method was evaluated by comparing the measured concentration of each analyte in the final extract to the expected concentration calculated from the known mass spiked into the sample and the final extract volume.

ASE provided the most consistent and robust extraction efficiency, with recoveries ranging from 46–52% for all analytes presented in Table 2. The method showed no extreme suppression or enhancement and handled phthalates, and phosphate ester with similar effectiveness. Overall, ASE demonstrated the most balanced and reliable extraction performance.

QuEChERS exhibited low to moderate recoveries (approximately 5–22%) depending on analyte. Diphenyl phthalate showed poor recovery. Dicyclohexyl phthalate and triphenyl phosphate, hydrophobic compounds, also showed low recovery. This indicates that the QuEChERS cleanup procedure and evaporation steps likely removed a substantial fraction of more hydrophobic analytes. SPE showed the lowest and most variable recoveries. Phthalates were recovered very poorly (3–4%), suggesting breakthrough or insufficient retention on the C18 sorbent. Higher recoveries for hydrophobic analytes (e.g., 47% for triphenyl phosphate) indicate selective behavior rather than broad extraction efficiency. Overall, SPE was the least reliable method under the tested conditions.

Rt (min)	Name	CAS	Mol. formula	Recovery in QuEChERS(%)	Recovery in SPE (%)	Recovery in ASE (%)
5.1	Diphenyl phthalate	84-62-8	$C_{20}H_{14}O_4$	4.7	3.7	52.0
7.4	Dicyclohexyl phthalate	84-61-7	$C_{20}H_{26}O_4$	22.0	2.9	52.2
5.1	Triphenyl phosphate	115-86-6	$C_{18}H_{15}O_4P$	17.1	47.3	46.0

Table 2. List of analytical standards used to evaluate the extraction efficiency of ASE, QuEChERS, and SPE and recovery of analytical standards strawberry matrix

6.2. Evaluation of extraction efficiency using reflux-based extraction

The extraction efficiency of reflux-based extraction were performed using a mix of analytical standards listed in Table 3. The results showed that the recovery of smaller phthalates (DMP, DBP and DEP) were close of acceptable limit (70-110% according to the SANTE guidelines 2017). For the larger plasticizers like DEHA, DEHP, and DNOP, the recoveries were much higher than 100%. This suggests that the sample matrix causes ion enhancement. It has to be noted that DBP and DEHP peaks were observed in blank matrices and in the calculation of recoveries, this peak area was subtracted. BBP behaved differently, with recoveries below 100%, which indicates losses or signal reduction during the analysis. Overall, the results show that matrix effects and background contamination strongly influence the measurements, especially for the heavier plasticizers, and that more matrix-specific calibration or improved cleanup might be needed.

Rt (min)	Name	CAS	Mol formula	Recovery in strawberry (%)
7.2	Dimethyl phthalate (DMP)	131-11-3	$C_{10}H_{10}O_4$	90
8.1	Diethyl Phthalate (DEP)	84-66-2	$C_{12}H_{14}O_4$	84
12.2	Dibutyl phthalate (DBP)	84-74-2	$C_{16}H_{22}O_4$	89
18.0	Hexanedioic acid, bis(2-ethylhexyl) ester (DEHA)	103-23-1	$C_{22}H_{42}O_4$	261
20.2	Benzyl butyl phthalate (BBP)	85-68-7	$C_{19}H_{20}O_4$	54
21.7	Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	$C_{24}H_{38}O_4$	185
25.8	Di-n-octyl phthalate (DNOP)	117-84-0	$C_{24}H_{38}O_4$	398

Table 3. List of analytical standards used to evaluate the extraction efficiency of reflux-based extraction and recovery of analytical standards in strawberry matrix

Summing up, for the LC-MS analysis of plastic additives in strawberries, ASE performed better than other methods. QuEChERS performs moderately but inconsistently, while SPE performs poorly across most analyte classes and is not recommended without major optimization. For the GC-MS analysis of plastic additives in strawberries, reflux based extraction showed good recoveries for most of the phthalates. However, the method needs to be optimized for high molecular weight phthalates.

7. Related virtual trainings

In addition to the hands-on training provided to researchers from JSI and AUA on conducting migration and leaching studies, as well as the analysis of plastic additives using different instrumentation at DTU Food, WP6 activities also included regular virtual trainings and consultations. These online interactions, conducted under Task 6.3 (Virtual consultations and training: Enhancing knowledge exchange on leaching of plastic additives from MNPs), resulted in many fruitful discussions and new collaborations. For example, the collaboration with DTU Sustain on conducting an algae growth inhibition test is one important outcome of these consultations.

This task included several meetings focused on planning activities related to Tasks 6.1 and 6.2, while also providing opportunities to present results obtained within these tasks and initiate new discussions.

The following is a description of the consultations organized within Task 6.3 through Microsoft Teams:

- 14 January 2025 – A meeting was held in preparation for the upcoming staff exchange within Task 6.2. Participants from JSI and DTU Food discussed the theoretical background, objectives, and planned time frame for the activities.
- 23 June 2025 – Following the completion of the first part of the staff exchange within Task 6.2, a virtual consultation was organized where the activities and preliminary results were presented to all InPlasTwin partners.
- 7 January 2026 – DTU Food organized a meeting for JSI and AUA participants who will take part in Task 6.1 activities, as well as the continuation of activities within Task 6.2.
- 20 February 2026 – An online meeting with Dr. Are Sæle Bruvold, a scientist from IMR was held to agree on the details of his involvement in Task 6.1, particularly regarding the data handling process. During the meeting, he presented several open-source platforms used for processing mass spectrometric data.
- 13 and 16 March 2026 - Meetings were held to wrap up the activities conducted within Tasks 6.1 and 6.2. During the meeting, the main outcomes of the activities were reviewed and discussed, and the partners coordinated the preparation and structure of the related deliverables.

8. Conclusions

This deliverable presents the harmonized protocols developed for the leaching and migration testing of plastic additives from agricultural mulching films and corresponding MNPs. The protocols were designed to ensure comparability between experiments performed with intact mulching films and those conducted with biodegradable MNPs, by maintaining consistent material-to-medium ratios and controlled experimental conditions.

Through the knowledge exchange activities conducted within the project, the participating researchers gained practical experience in the designing and implementation of experiments aimed at investigating the release of additives from biodegradable plastics and MNPs under environmentally and food-contact relevant conditions. Importantly, this knowledge transfer has strengthened the analytical capacity of the participating institutions, enabling researchers to independently design and conduct similar experiments at their home institutions. The shared protocols and methodological insights will support future studies on plastic additives and contribute to improving the comparability and reliability of data generated within the project and beyond.

9. References

1. Babalola, B. A., & Adeyi, A. A. (2018). Levels, dietary intake and risk of polybrominated diphenyl ethers (PBDEs) in foods commonly consumed in Nigeria. *Food Chemistry*, *265*, 78–84. https://doi.org/10.1016/J.FOODCHEM.2018.05.073

2. Bhattarai, B., Wedebye, E. B., Nikolov, N. G., Cederberg, T. L., Jensen, L. K., Granby, K., & Pedersen, G. A. (2025). Analytical testing strategy for identification and in silico toxicity assessment of non-intentionally added substances in repeatedly recycled flexible mono-plastic food contact material. Food Packaging and Shelf Life, 48, 101456.

3. Christia, C., Bizani, E., Christophoridis, C., & Fytianos, K. (2015). Pesticide residues in fruit samples: comparison of different QuEChERS methods using liquid chromatography–tandem mass spectrometry. *Environmental Science and Pollution Research 2015 22:17*, *22*(17), 13167–13178. https://doi.org/10.1007/S11356-015-4456-0

4. European Commission Directorate-General for Health and Food Safety (SANTE) (2017). *Guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed* (SANTE/11813/2017). European Commission.

5. EU Regulation. (2011). Commission Regulation (EU) No 10/2011 of 14 January 2011. Official Journal of the European Union, Special ed(L 12), 1–89.

6. Fasano, E., Cirillo, T., Esposito, F., & Lacorte, S. (2015). Migration of monomers and plasticizers from packed foods and heated microwave foods using QuEChERS sample preparation and gas chromatography/mass spectrometry. *LWT - Food Science and Technology*, *64*(2), 1015–1021. https://doi.org/10.1016/J.LWT.2015.06.066

7. Fernandes, V. C., Luts, W., Delerue-Matos, C., & Domingues, V. F. (2020). Improved QuEChERS for Analysis of Polybrominated Diphenyl Ethers and Novel Brominated Flame Retardants in Capsicum Cultivars Using Gas Chromatography. *Journal of Agricultural and Food Chemistry*, *68*(10), 3260–3266. https://doi.org/10.1021/ACS.JAFC.9B07041

8. Genualdi, S., Jeong, N., deJager, L., & Begley, T. (2017). Investigation into perfluoroalkyl substances (PFASs) in a cranberry bog: method development and sampling results.*Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *34*(12), 2181–2189. https://doi.org/10.1080/19440049.2017.1361046

9. Gordan, H., Mahdavi, V., Basij, M., & Mousavi Khaneghah, A. (2025). Optimized QuEChERS-HPLC-MS/MS method for pesticide residue detection in strawberries and associated health risks. *Journal of Agriculture and Food Research*, *21*, 101907. https://doi.org/10.1016/J.JAFR.2025.101907

10. He, M. J., Lu, J. F., Wang, J., Wei, S. Q., & Hageman, K. J. (2020). Phthalate esters in biota, air and water in an agricultural area of western China, with emphasis on bioaccumulation and human exposure. *Science of The Total Environment*, *698*, 134264. https://doi.org/10.1016/J.SCITOTENV.2019.134264

11. Hidalgo-Serrano, M., Borrull, F., Pocurull, E., & Marcé, R. M. (2020). Pressurised Liquid Extraction and Liquid Chromatography–High Resolution Mass Spectrometry for the Simultaneous Determination of Phthalate Diesters and Their Metabolites in Seafood Species. *Food Analytical Methods 2020 13:7*, *13*(7), 1442–1453. https://doi.org/10.1007/S12161-020-01759-7

12. Li, Y., Huang, G., Zhang, L., Gu, H., Lou, C., Zhang, H., & Liu, H. (2020). Phthalate esters (PAEs) in soil and vegetables in solar greenhouses irrigated with reclaimed water. *Environmental*

Science and Pollution Research 2020 27:18, *27*(18), 22658–22669. https://doi.org/10.1007/S11356-020-08882-2

13. Monkley C., Reay M. K., Whelton H. L., Evershed R.P. and Lloyd C.E.M. (2025)'Characterisation of the unknown chemical composition of a commercial biodegradable agricultural plastic mulch film using complementary spectrometric and spectroscopic techniques', Analyst, 150, 3319-3332. Available at: DOI: 10.1039/D5AN00423C (Accessed: 12 March 2026)

14. Reay, M. K., Graf, M., Murphy, M., Li, G., Yan, C., Bhattacharya, M., Osbahr, H., Ma, J., Chengtao, W., Shi, X., Ren, S., Cui, J., Collins, C., Chadwick, D., Jones, D. L., Evershed, R. P., & Lloyd, C. E. M. (2025). Higher potential leaching of inorganic and organic additives from biodegradable compared to conventional agricultural plastic mulch film. Journal of Hazardous Materials, 488, 137147. https://doi.org/10.1016/J.JHAZMAT.2025.137147 (Accessed: 21.3.2025)

15. Scopetani C., Bellabarba A., Selvolini G., Martellini [a d e] T. , Viti [b c] C., Cincinelli [a d e] A. (2025) 'Evaluating additive release from conventional and biodegradable mulch films', 975, Available at: https://doi.org/10.1016/j.scitotenv.2025.179294 (Accessed: 12 March 2026)

16. Scordo, C. V. A., Checchini, L., Renai, L., Orlandini, S., Bruzzoniti, M. C., Fibbi, D., Mandi, L., Ouazzani, N., & Del Bubba, M. (2020). Optimization and validation of a method based on QuEChERS extraction and liquid chromatographic–tandem mass spectrometric analysis for the determination of perfluoroalkyl acids in strawberry and olive fruits, as model crops with different matrix characteristics. *Journal of Chromatography A*, *1621*, 461038. https://doi.org/10.1016/J.CHROMA.2020.461038
17. Shaaban, H., Mostafa, A., Alqarni, A. M., Almohamed, Y., Abualrahi, D., Hussein, D., & Alghamdi, M. (2022). Simultaneous determination of bisphenol A and its analogues in foodstuff using UPLC-MS/MS and assessment of their health risk in adult population. *Journal of Food Composition and Analysis*, *110*, 104549. https://doi.org/10.1016/J.JFCA.2022.104549
18. Yue, Q., Huang, Y. Y., Shen, X. F., Yang, C., & Pang, Y. H. (2020). In situ growth of covalent organic framework on titanium fiber for headspace solid-phase microextraction of 11 phthalate esters in vegetables. *Food Chemistry*, *318*, 126507. https://doi.org/10.1016/J.FOODCHEM.2020.126507