European Union Reference Laboratory

for Plant Parasitic Nematodes

Diagnostic protocols and analytical methods

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Extraction methods for *Meloidogyne chitwoodi* and *M. fallax*

Foreword

These methods were recommended by:

ANSES - Plant Health Laboratory - Nematology Unit

Address: Domaine de la Motte au Vicomte - BP 35327 - 35653 Le Rheu Cédex - France

ILVO - Plant Unit-Nematology

Address: Burg. Van Gansberghelaan 96 - 9820 Merelbeke - Belgium

The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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1 Introduction

The root-knot nematodes (RKN), *Meloidogyne chitwoodi* and *Meloidogyne fallax* are plant-parasitic nematodes which cause significant damage to susceptible agronomic important crops. Both of these root-knot nematode species are classified as quarantine organisms in the European Union (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2019/2072) and are subject to mandatory control measures to avoid their introduction into and spread within the EU territory in geographical areas where these pests are not present.

The purpose of these EURL recommended protocols[1] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostics analyses, by providing them with details on analytical operating procedures for the detection and identification of *M. chitwoodi* and *M. fallax*.

These protocols are suitable for official testing, in particular in the framework of plant health controls for these pests under the EU territory, including import and export surveillance.

2 Method validation

These EURL recommended protocols are based on EPPO standards. Some of these diagnostic protocols, including operational procedures, were chosen as the basis, and with the scientific experience and technical expertise of the EURL team, some of these protocols have been modified, optimised and further validated by the EURL laboratory.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

[1] The use of chemical brands or equipment in these recommended protocols does not imply the exclusion of others, which may also be appropriate.

3 Terms, abbreviations and definitions

- AZC: Automated Zonal Centrifuge
- Container: bowl, beaker, vial, tube, pot, jar, device, etc
- DGO: Dorsal Gland Opening
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- L/h: Litres per hour
- Ml/min: Millilitres per minute
- $MgSO_4$: Magnesium sulfate
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes
- PVC: PolyVinyl Chloride (plastic polymer)
- RKN: Root-Knot Nematodes
- Swollen stages: sedentary immobile stages inside the plant, third-stage juveniles (J3) and fourth-stage juveniles (J4) and females.
- Vermiform: second-stage juveniles (J2) and males

4 Nematode extraction methods

4.1 Purpose and scope

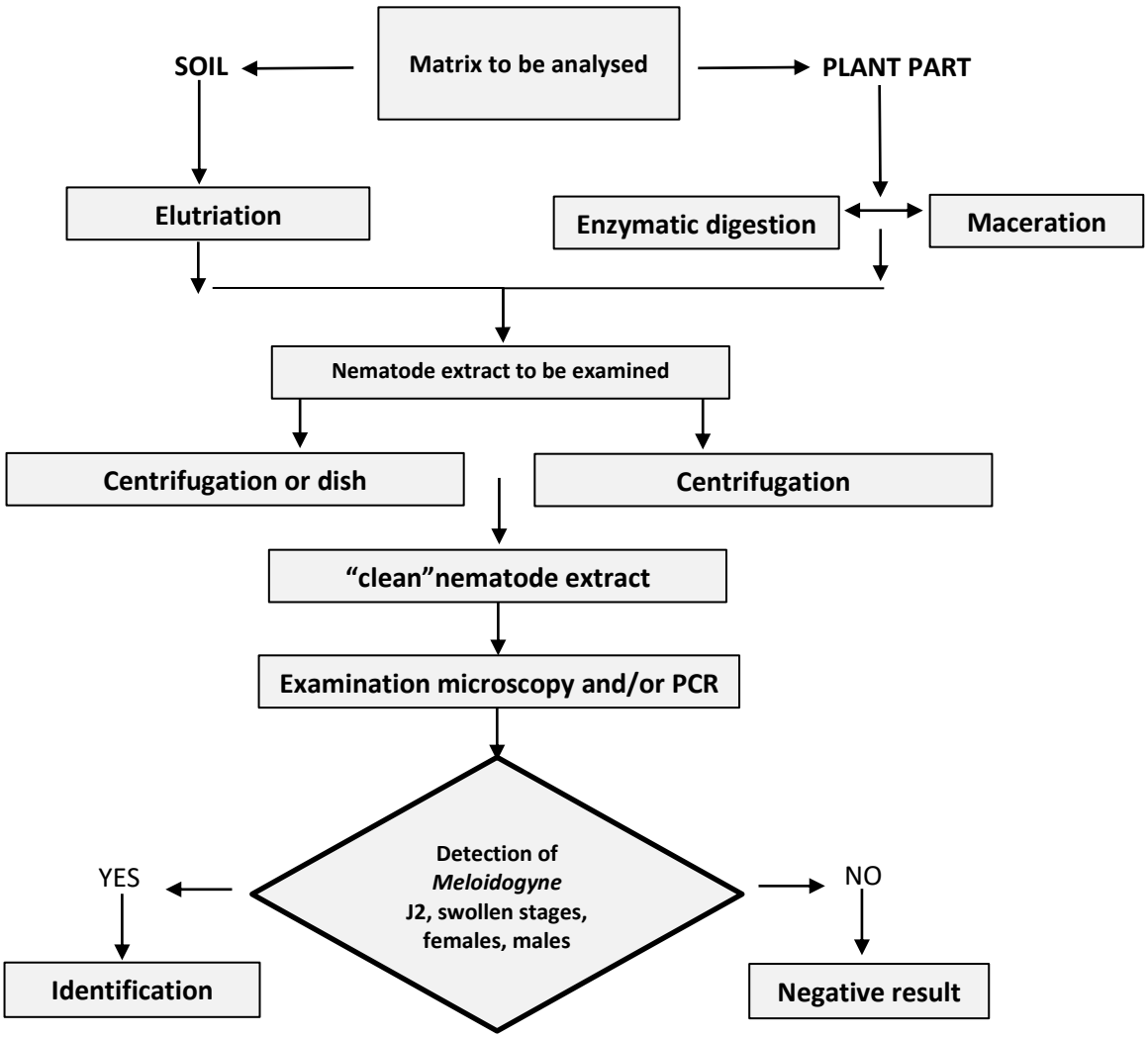
The purpose of this procedure is to provide guidance on the different analytical methods to extract root-knot nematodes, especially *Meloidogyne chitwoodi* and *M. fallax* from soil, plant roots and tubers. These methods also extract other Plant-Parasitic Nematodes (PPNs), except cysts.

These recommended protocols are suitable for the extraction of *Meloidogyne* sp. in their vermiform or swollen stages. However, the choice of the method depends, among other variables, on the purpose of the extraction, equipment available, and preferences of the person performing the extraction.

These extraction techniques are applied to:

- Soil, soil waste containing or not crop substrates;
- Subterranean plant organs, such as roots, tubers and other plant tissues.

Schematic procedure for nematode detection from soil and plant material



4.2 Homogenising sample

1) The items that are reused when preparing the samples, such as blender beaker, blender beaker lid, knife, spoon, mixing container, sieves, etc., are thoroughly rinsed before starting sample extraction and between samples.
2) Homogenising: the sample must be thoroughly mixed before a sub-sample is taken. Sample homogenisation can be done either in a plastic bag or in a mixing container.
3) Plastic bag or mixing container: Make sure that the volume is at least 4 times larger than that of the sample.
4) The sieves used in sample preparation are cleaned according to the following way:
    - Rinse thoroughly so that soil and/or organic material is mostly removed. A clean sieve is used for each new sample. You can clean the sieves by using a dishwasher or an Ultrasonic bath for 10 minutes to remove sand grains and nematodes, avoiding cross-contamination between samples.
    - **Important:** If using the Ultrasonic bath, it is advised to change the bathwater between samples. Clean grinder or blender between samples.

4.3 Controls and their purpose

These controls are highly recommended or advised. These are added to evaluate if the nematode extraction process was correctly performed and serves as a control for further morphometric and molecular identification analysis.

Extraction Control	Purpose		Control application	Expected Results
Negative process control*	When analysing a series of samples**, water is considered a negative control**. The water undergoes the same operations as a sample.		Highly recommended	Negative no nematodes
Positive process control**	With each series of samples to be analysed, **one sample from the same matrix and infested with *Meloidogyne* sp.**, is also analysed.	When analysing potato tubers for *Meloidogyne* sp. one infected tuber is peeled and further treated like the rest of the samples. ✓ If no positive tubers are present, a suspension of juveniles from a culture may be added to a beaker with mixed potato peels from a negative sample. This is to simulate a positive tuber.	Optional	Positive Presence of *Meloidogyne*
		Use positive infected soil samples. ✓ In the absence of a positive soil sample, a suspension of juveniles from culture may be added to a negative soil sample, in order to simulate a positive soil sample.	Optional	Positive Presence of nematodes
		Use positive infected root samples. ✓ In the absence of a positive root sample, a suspension of juveniles from culture may be added to a negative root sample in order to simulate a positive root sample.	Optional	Positive Presence of nematodes

*During nematode extraction, process the samples in the following order: samples, positive control, followed by the negative control.

**The *Meloidogyne* sp. is detected on the positive control before starting the morphometric analysis of the remaining samples. In this way, one is sure that the nematodes were extracted from the samples. The presence of other nematodes in the samples is an additional guarantee for the extraction.

4.4 Protocol for extraction from soil samples

4.4.1 Principle

This protocol allows the extraction of vermiform motile nematodes of the genus *Meloidogyne* from the soil. The techniques[2] are based on principles of size and shape of nematodes (sieving) and on the density of them (elutriation, centrifugation).

The techniques described in this protocol for soil samples, such as elutriation and further cleaning by centrifugal flotation and Oostenbrink dish including their application are based on EPPO standard PM 7/119 (1)[3], and the EURL experience and operational procedures. However, soil samples can be treated directly by centrifugal flotation without previously having passed through the elutriator (described in EPPO PM 7/119 (1)). The EURL (ILVO Plant Unit laboratory, Belgium) uses the Automated zonal centrifugation[4] (AZC) (Illustration at EPPO PM 7/119 (1)) as an automatic variant of centrifugal flotation. However, as most NRLs are not equipped with this equipment, the AZC utilisation for nematode extraction from a matrix (soil or plant tissues) is not mentioned in this protocol.

The detailed protocols for nematode extraction by direct treatment of soil samples by AZC centrifugal flotation and Oostenbrink dish can be found in the above cited EPPO standard.

[2] The techniques make it possible to extract other vermiform nematodes from soil (all stages of ectoparasites and mobile stages of endoparasitic nematodes).

[3] EPPO (2013). PM 7/119 (1): Nematode extraction. Bulletin EPPO 43 (3), 471-495.

[4] Automated zonal centrifuge: The machine was built by a small company; Wim de Koning, Industrieweg 7, 4301 RS Zierikzee, Nederland; wldekoning@zeelandnet.nl.

4.4.2 Materials and consumables

4.4.2.1 Equipment and small materials

Elutriation

- Oostenbrink elutriators
- Sieve with openings of 4 mm and 40 µm to 45 µm (1 to 4 sieves)

Centrifugation

- Centrifuge
- Revolving Mixer or Shaker
- Balance
- Centrifuge containers adapted to your equipment
- Sieve mesh size 1 mm
- Sieve mesh size of 20 µm
- Wash Bottle
- Beakers
- Stick, Spoon, Spatula, Whisk

4.4.2.2 Consumables

Centrifugation

- Water
- Kaolin (quantity to be adjusted, according to the centrifuge container diameter (e.g. approximately 10 g for a container of ~ 8.6 cm of diameter)
- $MgSO_4$ Solution (for preparation **see Appendix §5.2**)

4.4.3 Sample preparation

1) For optimum results, root fragments should be first separated from soil by hand or using a sieve. Both fractions (organic and mineral) can then be analysed. For sample homogenisation (see **§4.2**).
2) Manually remove plant debris and stones (use a 4 mm sieve if necessary).
3) Take a subsample of approximately 100 to 300 ml (preferentially 200 ml) of soil from several different areas of the sample.
4) Pour the subsample into a blender or a revolving shaker and cover with water (to be adjusted according to the amount of soil taken)
5) Homogenise the suspension (soil sampling + water) until a fluid mud is obtained.
6) Clay soil and heavy loam soil are kneaded in a quantity of water so that the sample passes more smoothly through the 1 mm sieve into the elutriator. This kneading is less intensive when the sample was soaked in water for 24 hours.

4.4.4 Elutriation

1) Before starting to use the Oostenbrink elutriator, close the side-outlet and the opening at the bottom.
2) Empty the soil suspension into a sieve with apertures of 1 mm at the top of the Oostenbrink elutriator[5] and rinse with a water jet. The soil is entrained into the elutriator. After almost all soil suspension passes through the sieve into the elutriator, add more water to the soil suspension using a sprayer, but take care not to exceed the level A (Fig. 1A). Proceed with this step as soon as possible to avoid settling of the soil in the lower area.
3) Fill the elutriator funnel from below by opening the lower water tap, creating an up-flow water current. Initially, set an intense water current of maximal flow rate to re-suspend the soil suspension up to the level B (Fig. 1A), and then adjust to approximately 35-40 L/h (i.e. 625 ml/min) to separate the nematodes.
4) The weaker current (35-40 L/h) prevents the nematodes from settling below the funnel base (Fig. 1A). Soil particles are layered according to their density. Nematodes are separated from heavier soil particles by their specific gravity in an up-flow water current.
5) Once the level C is reached (Fig. 1A), the recovery side-outlet is opened, and the suspension is filtered through a series of moistened sieves (3 to 4 sieves) with an aperture of 40 to 45 µm, stacked upon each other. The up-flow water current (adjusted to 35-40 L/h) is maintained during recovery.
6) The contents of the collection sieves (40 to 45 µm aperture) are immediately collected in a container and further cleaned by centrifugation (**see §4.4.5)** or by passing on an Oostenbrink dish (Fig. 2 and **Appendix §5.1**).

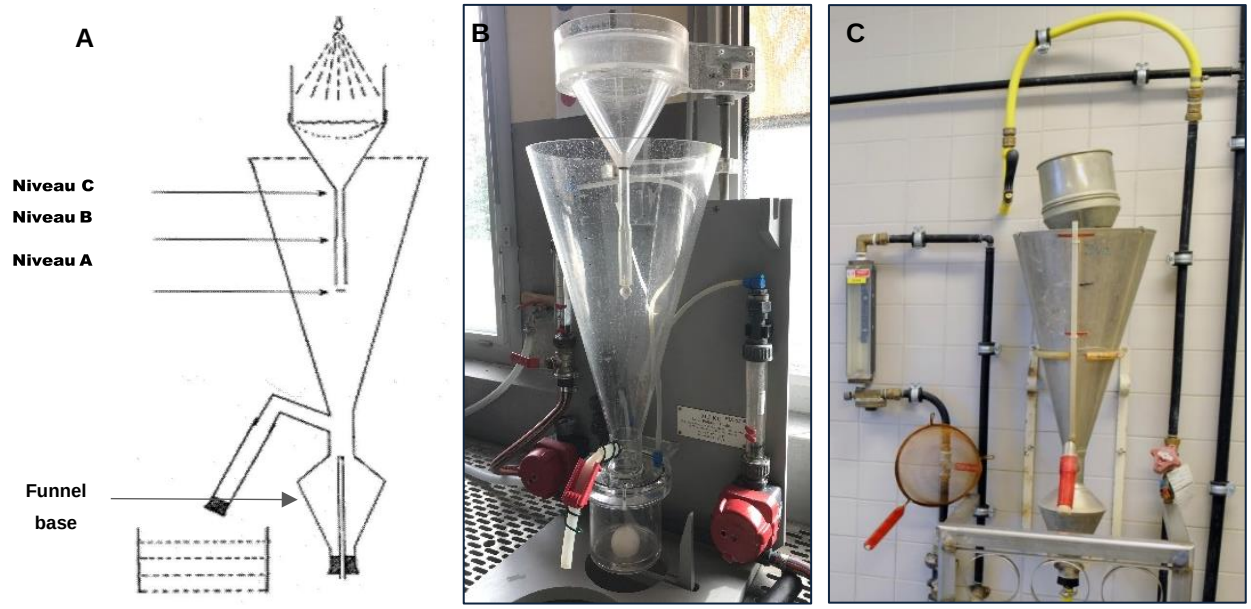


Figure 1. Oostenbrink elutriators for extracting nematodes from soil. (A) Schematic diagram of the Oostenbrink elutriator. Oostenbrink elutriator made of (B) acryl glass (www.meku-pollaehne.de) or (C) stainless steel. (Picture: ANSES-LSV, France and ILVO, Plant Unit, Belgium).

[5] The protocol presented here is based on the acrylic Oostenbrink elutriator of the brand MEKU. However, the protocol can be adapted for other elutriators.

4.4.5 Cleaning of the nematode suspension by centrifugation

4.4.5.1 Homogenisation of the suspension and centrifugation with kaolin

1) After elutriation, the contents of the collection sieves (40 to 45 µm aperture) are immediately transferred to a centrifuge container.
2) Put a spoonful of kaolin in the centrifuge container (quantity to be adjusted, see **§4.4.2.2**).
3) Fill the container to about three-quarters of its volume with water.
4) Homogenise the suspension with a spatula or a whisk. Use a clean utensil for each sample.
5) Centrifuge the suspension for 4 min at 1800g
    - ***Note:*** *Centrifugation[6] speeds and times are approximate*
6) Remove the supernatant by gently emptying the container, so as not to lose the pellet into a sink or similar.
7) Add magnesium sulfate solution ($MgSO_4$) (density 1.18) and mix it with the pellet.
    - ***Important****: Make sure that the entire pellet is suspended, tilting the container to see the bottom.*
8) Centrifuge the suspension for 2 min at 900 g
9) Empty the supernatant through a pre-moistened 20 µm sieve. Rinse immediately and abundantly to remove any remaining $MgSO_4$ and collect the contents of the 20 µm sieve into a collecting container using a wash bottle with water.
10) The obtained suspension is kept at room temperature if nematode detection is carried out the same day, otherwise stored at 4°C.
11) The nematode suspension can be further used for nematode detection by morphometric, and/or by molecular analysis.

[6] EPPO (2013). PM 7/119 (1) describes that centrifugation speed and time are not critical and can vary from **2 to 5 min** and from **700 to 2900 g.**

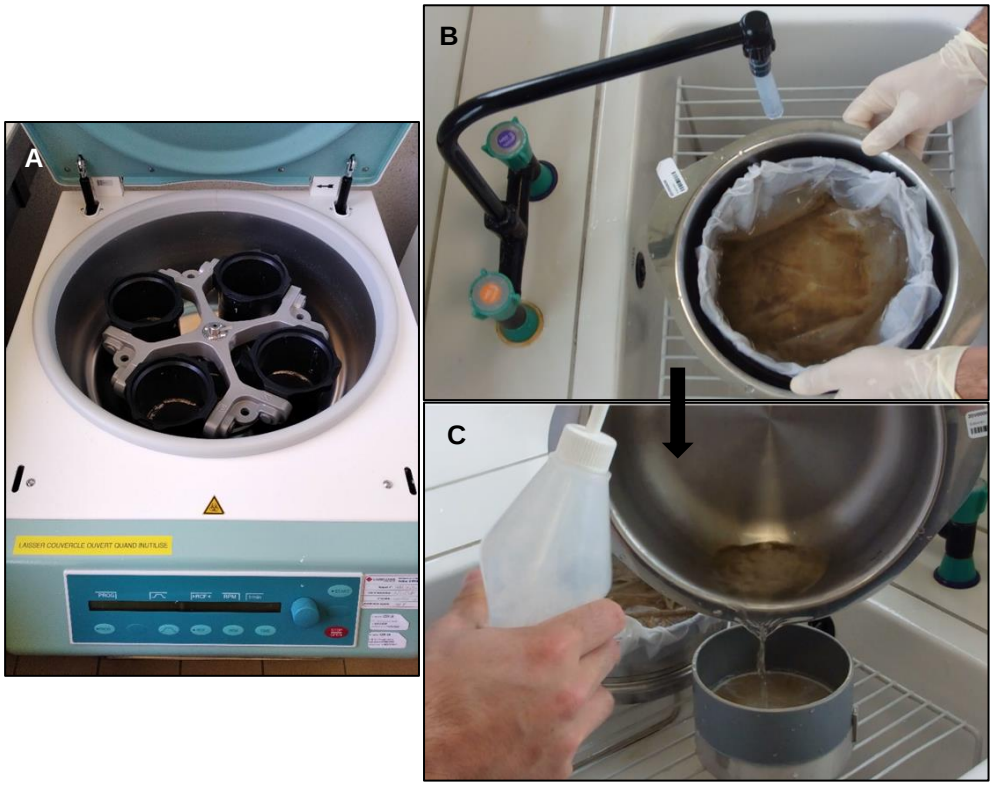
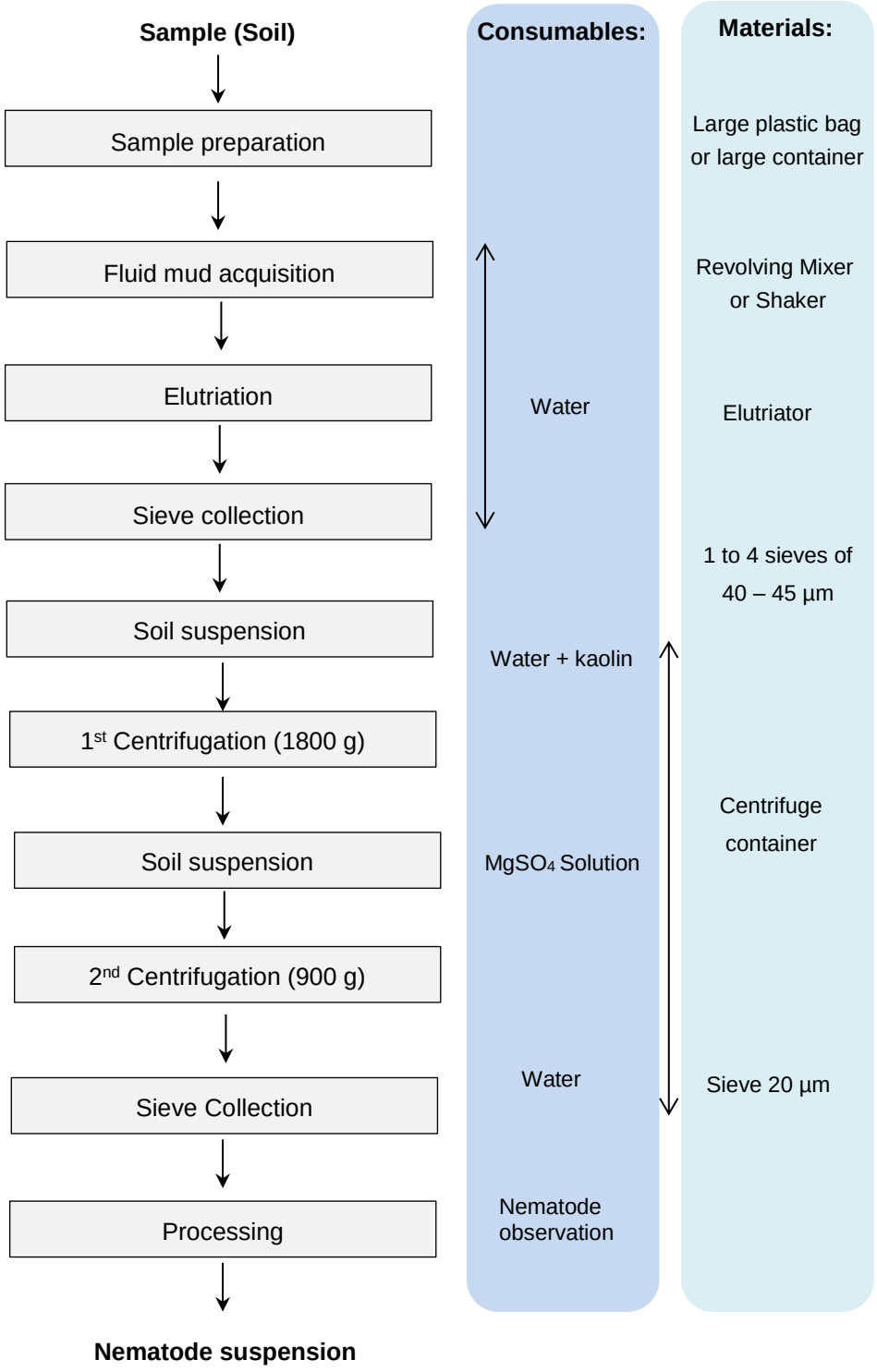


Figure 2. (A) Centrifuge used at the EURL to extract nematodes from plant material or soil or clean up nematode suspensions. (B) Oostenbrink dish set-up consisting of a steel dish supporting sieve of 160 µm water-covered for cleaning nematode suspension after elutriation of a soil sample. (C) Empty the nematode suspension from the steel dish into the 20 µm sieve to collect the nematodes (Picture ANSES - Nematology Unit, France).

Schematic procedure for nematode extraction using elutriation followed by centrifugation



4.5 Protocol for nematode extraction from plant samples

4.5.1 Enzymatic digestion of potato tubers and roots

4.5.1.1 Principle

The presented technique describes the extraction of different stages (swollen or vermiform) of RKN, including *Meloidogyne chitwoodi* and *M. fallax* using cellulolytic and pectinolytic enzymes. This technique avoids the need to grind the sample. The protocol can also be applied to both motile and immotile stages of migratory and sedentary endoparasitic nematodes from plant tissues. This extraction method is applied on samples of bulbs/tubers/rhizomes as well as on roots of various non-woody or slightly woody plants.

4.5.1.2 Material and consumables

4.5.1.2.1 Equipment and small materials

- Centrifuge
- Balance
- Shaker
- Blender
- Sieves (160 µm (for female) or 40 µm (for J2), 600 µm and 20 µm sieves)
- Centrifuge containers
- beakers
- Chopping knife or peeler
- Scissors
- Spatula
- Labelled containers (e.g. a piece of aluminium foil or a plastic dish)

4.5.1.2.2 Consumables

- Water
- Kaolin
- $MgSO_4$ Solution (for preparation **see Appendix §5.2**)
- Enzyme mixture (for preparation **see Appendix §5.3**)

4.5.1.3 Sample preparation

The *Meloidogyne* spp. (females) and egg masses, if already produced, are located just below the potato tuber skin. They can be located up to the vascular ring, but around 90% are present in the first 3 - 4 mm depth of the potato tuber peel (Fig. 3). External symptoms are visible sometimes (Fig. 4), but usually not. Potato tuber sampling[7] can be conducted by visual inspection, and by further enzymatic extraction of females and other nematode stages.

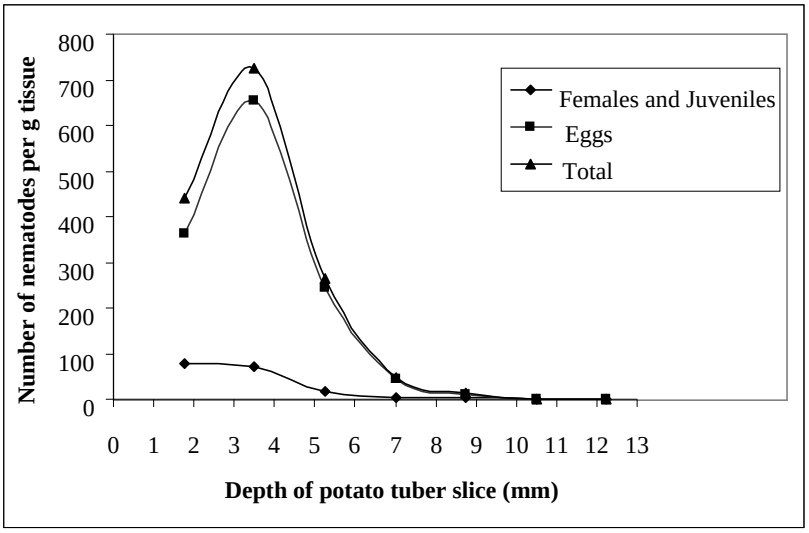


Figure 3. Distribution of nematodes (*M. chitwoodi*) in consecutive slices of a potato tuber (based on 5 tubers) (Viaene et al., 2007).

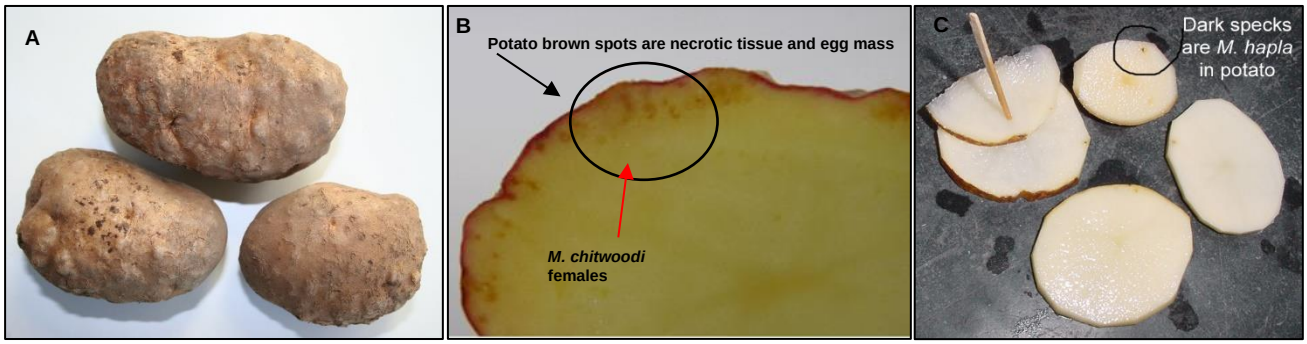


Figure 4. *Meloidogyne* spp. symptoms on potato tubers. (A) Potatoes with clear external symptoms of *Meloidogyne* spp. infection; (B) Females of *Meloidogyne chitwoodi* are visible as white globules (red arrow) in the cortex layer, and brown spots due to necrotic tissue and egg masses (black circle), and (C) Photo showing similar symptoms of *M. hapla* infection in potato tubers, females of *M. hapla* can be seen as black dots. (A, B: Pictures ILVO, Belgium, C: https://nematode.unl.edu/melhapsy.htm).

[7] The sampling of potato tuber from lots is well described in the EPPO (2019). PM 3/69 (2): *Meloidogyne chitwoodi* and *M. fallax*: sampling potato tubers for detection. Bulletin EPPO 49 (3), 486-487.

1) Remove most of the soil (if present) by shaking or washing the sample.
2) Cut at least 1 peel, about 3 mm thick (± 1 mm) and about 2 cm^2 (2 cm x 1 cm), from each potato tuber in the sample and put it in the labelled container. If analysing root samples, cut the roots into 1 to 2 cm sections.
3) Weigh all potato peels collected from the sample (e.g. in case of 200 potato tubers, a minimum of 105 g to a maximum of 150 g). If the sampling is from plant roots, collect a representative sampling of around 30 – 50 g.
    - ***Note:*** *If analysing 200 potato tubers (standard size of a sample), it is important that 200 tubers are sampled, and that each tuber is analysed. If typical symptoms are visible on one or a few tubers, include their symptomatic peels in the sample. If enough material is available, analyse these peels separately to enhance the chance for detection.*
    - ***Note:*** *If extraction is not possible on the same day as taking the peels, the collected and weighted potato peels are stored at 4°C.*

4.5.1.4 Enzymatic digestion

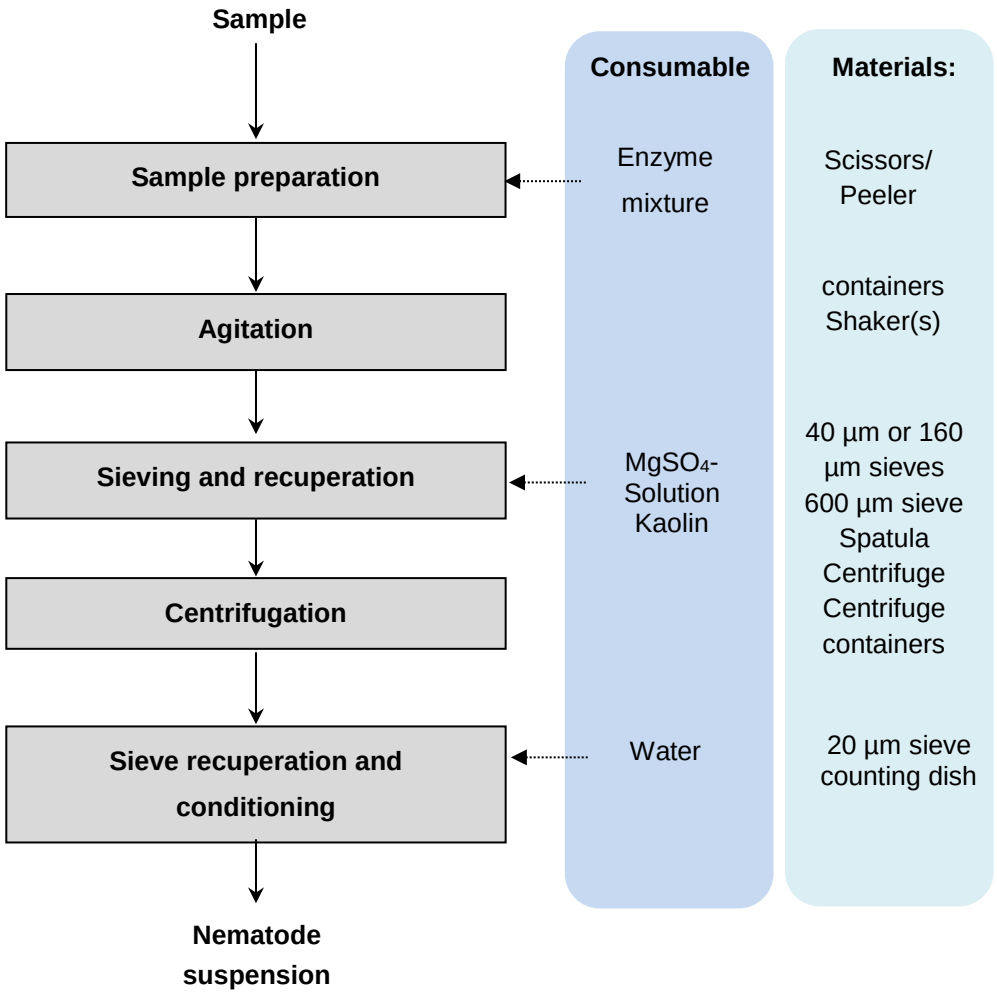
4) Place the sample in a container for shaking. Add the enzyme mixture to cover the plant material (potato peels or plant roots).
5) Place the preparation on a shaker and let stir for a **minimum of 8 hours for potato peels** and **4 hours for plant roots**.
6) Pour the digestion product onto a 600 µm sieve placed on the top of a 160 µm sieve for extraction of swollen nematodes, or in a 40 µm sieve for extraction of filiform nematodes.
7) Wash the contents of the 600 µm sieve with a stream of water and break up the retained debris to facilitate the passage of the digested juice.
8) Discard the contents of the 600 µm sieve.
    - ***Note:*** *If the extract obtained is sufficiently clear after sieving, centrifugation is not essential;* nematodes can be observed for detection and identification (morphology/morphometric and/or molecular analysis).
9) Gather the contents from the 160 or 40 µm sieve with a jet of $MgSO_4$. Transfer the suspension to a centrifuge container, using a **wash-bottle of $MgSO_4$**.
10) Put a spoonful of kaolin in the centrifuge container (quantity to be adjusted, according to the amount of product collected).
    - ***Note:*** *If necessary, rinse the sides of the container with a wash-bottle of $MgSO_4$.*
11) Homogenise the suspension with a spatula or similar material.
    - ***Note:*** *Make sure to use a clean utensil for each sample.*
12) Centrifuge the suspension for 2 min at 900 g
13) To maximise nematode and egg recovery, and if any egg masses are present after enzyme treatment, empty the contents of the container (supernatant) onto a 20 µm sieve previously moistened.

14) Collect the particles retained by the 20 µm sieve with a wash-bottle of water.

15) Transfer the contents of the 20 µm sieve to a beaker using a wash-bottle of water.

- ***Note:*** *The suspension obtained is kept at room temperature if nematode detection is carried out on the day of extraction, otherwise, store at 4°C.*

Schematic procedure for nematode extraction via enzymatic method



4.5.2 Maceration and centrifugal flotation of root plants

4.5.2.1 Principle

This method extracts RKN, including *Meloidogyne chitwoodi* and *M. fallax,* as well as other various stages of nematodes present in plant tissues using mechanical maceration (blending) followed by centrifugation. The blending releases the nematodes from the plant tissues into the water, and the centrifugal flotation separates the nematodes from the macerated plant tissues and other particles, e.g. soil. The extraction method can be applied to potato peels, whole plants, root systems, or root fragments.

However, the protocol described below it's written for plant roots.

4.5.2.2 Material and consumables

4.5.2.2.1 Equipment and small materials

- Centrifuge
- Balance
- Grinder or blender
- Sieves (600 to 850 µm and 20µm mesh)
- Centrifuge containers
- counting dish
- Scissors, Knives or peeler, Spoon, Stainless Steel Spatula
- Labelled beakers or containers and plates

4.5.2.2.2 Consumables

- Water
- Paper towels
- Kaolin
- $MgSO_4$ Solution (**Appendix §5.2**)
- Antifoam (aqueous silicone oil emulsion) (The brands generally used in the EURL laboratory: Grosseron 426R and Neodisher Entschaumer S)

4.5.2.3 Sample preparation

1) Put the root sample into a container, and carefully rinse the root system with water, removing as much soil debris and dirt.
   - ***Note:*** *To increase detection chances, do not rinse the sample with a hard jet of water, but rather allow the root system to move gently back and forth in water so that any egg masses of Meloidogyne spp., containing eggs and juveniles, are not washed away.*
2) Analyse the whole sample if less than 30 g of root material, otherwise cut a fraction of roots from each plant received. Homogenise the whole sample and take a sampling of approximately 30g in several takes distributed over the whole product to be analysed.

- ***Note:*** *In the case of salsify, chicory root or carrot, if there is a shortage of rootlets, the outer layer of the taproot can be peeled (with peeler or knife). These sample peels are randomly added to the rootlets to obtain the requested amount of roots.*

3) Blot the roots dry with a paper towel to remove most of the water and weigh.
4) Cut the roots into fragments (**from 0.5 to 1.5 cm long**).

Figure 5. Rinsing of blender beaker after mixed content was poured on an 850 µm sieve on top of a 1 L beaker.

4.5.2.4 Maceration and centrifugal flotation

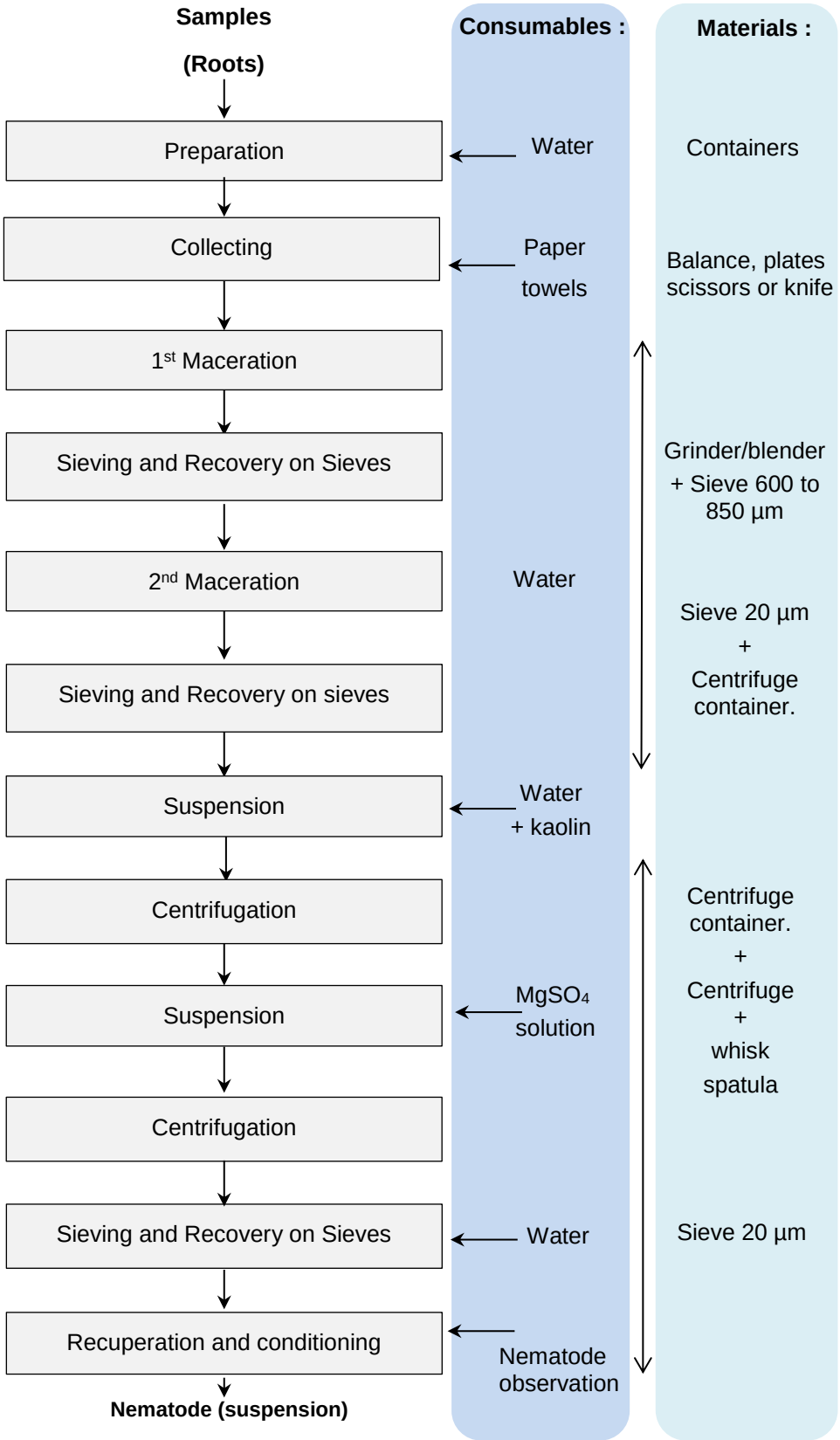
5) Put the root fragments to be analysed into the grinder container or blender and add water to cover root fragments and blades.
6) Add a few drops of antifoam if the roots are likely to foam profusely (lily of the valley roots, for example).
7) Mix the sample for 1 minute at high speed.
8) Pour the contents of the container onto a 600 µm to 850 µm sieve placed over a beaker. Rinse the material retained in the sieve thoroughly. Rinse the container and pour over the sieve as well (Fig. 5).
9) Remix the root contents to extract the maximum of nematodes; Pour the root contents of the 850 µm or 600 µm sieve into the grinding bowl or blender and add the necessary volume of water to cover the material and the blades.
10) Mix the sample for around 3 minutes at high speed.
11) Empty the contents of the container onto the 850 µm or 600 µm sieve placed above the beaker. Rinse the container and empty it onto the sieve. Rinse thoroughly and then lightly tap the residue retained by the sieve to extract the residue.
12) Pass the suspension collected in the beaker over the 20 µm sieve to reduce the water volume.
13) Empty the contents of the 20 µm sieve into a centrifuge container, using a wash-bottle of water if preferable.

- ***Note:***
  - *i. Carry out a final mix (about 3 minutes at high speed) if necessary.*
  - *ii. The centrifuge container should not be filled more than 4 cm from the rim. If necessary, reduce the suspension after decanting by passing part of it over the 20 µm sieve.*
  - *iii. The suspension prepared in the centrifuge container is to be kept cold overnight if centrifugations cannot be started immediately.*

14) Add a spoonful of kaolin to the container (quantity to be adjusted, according to the amount of product collected)
15) Fill the container to about three-quarters of its volume with water.
16) Homogenise the suspension with a spatula or similar material.
    - ***Note:*** *Make sure to use a clean utensil for each sample.*
17) Centrifuge the suspension for 4 min at 1800 g.
18) Remove the supernatant by gently emptying the container over the sink so as not to lose the pellet.
19) Add the $MgSO_4$ solution.
20) Blend the pellet with a utensil making sure it is clean for each sample.
    - ***Note****: Make sure that the entire pellet is in solution.*
21) Centrifuge the suspension for 2 min at 900 g.
22) Pour the supernatant through a 20 µm sieve, previously moistened.
23) Rinse rapidly to remove the $MgSO_4$ solution and collect the contents of the 20 µm sieve using the Wash-Bottle.
24) Transfer the contents of the 20 µm sieve to a pot (Figure 6) so that nematodes can be observed for detection and identification (morphology/morphometric and/or molecular analysis).
    - ***Note:*** *The suspension obtained is kept at room temperature if the analysis is carried out on the day of extraction, otherwise, store at 4°C.*

Figure 6. Transferring the contents of the 20 µm sieve containing the extracted nematodes to a plastic beaker. Suspension can be stored and observed through the beaker in the stereomicroscope (Picture ANSES - Nematology Unit, France).

Schematic procedure for nematode extraction via maceration and centrifugal flotation



5 Appendices

5.1 Cleaning the nematode suspension via Oostenbrink dish

1) At the end of the elutriation, the contents of the collection sieves (40 to 45 µm aperture) are immediately collected and further cleaned by passing on an Oostenbrink dish.
2) Place the soil extract on wiping paper or milk filters (e.g. cotton-wool milk filter) which is held by support (e.g. sieve with a mesh size greater than 40 µm); maximum soil layer: 2-3 mm.
3) Place the sieve in a dish and fill the dish with water so that the contents of the sieve is in permanent contact with the water (add more if absorbed). Avoid the formation of air bubbles under the sieve (if bubbles are present, remove them by tilting the sieve).
4) Nematodes migrate through the paper/filter and sink to the bottom of the dish.
5) Allow nematodes to migrate for at least 24 hours.
6) Empty the nematode suspension contained in the dish onto a 20 µm sieve previously moistened to reduce the volume of water.
7) Recuperate nematodes in a beaker:
    - **If the suspension filtered through the sieve is clear**: the extraction is finished, and nematodes can be observed for detection and identification (morphology/morphometric and/or molecular analysis).
    - **If the suspension filtered through the sieve is dirty**: clean via centrifugation (**see § 4.4.5**)
8) The obtained suspension is kept at room temperature if nematode detection is carried out on the same day of extraction, otherwise at 4°C.

5.1.1 Materials and consumables

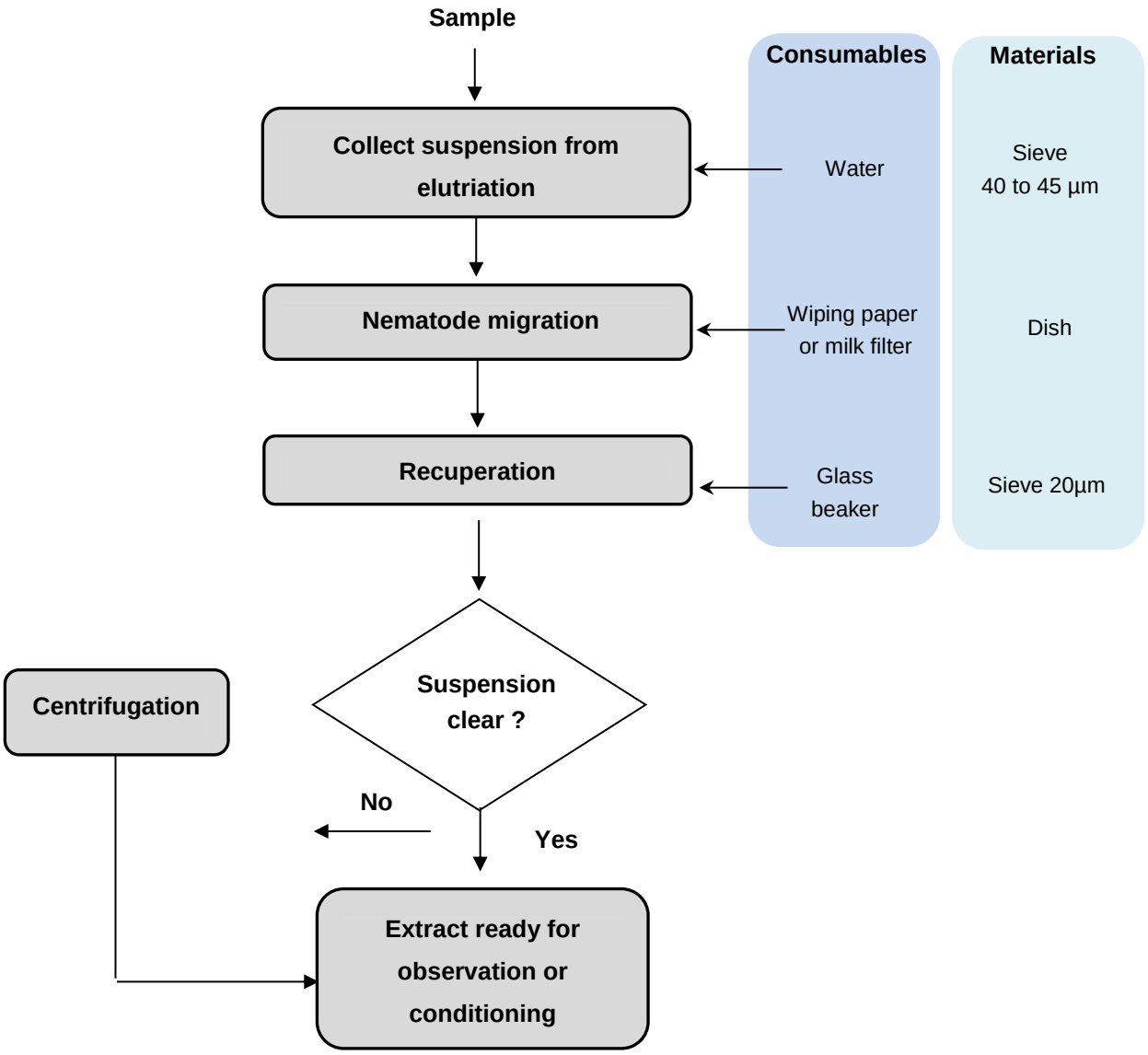
5.1.1.1 Equipment and small materials

- Sieve with apertures greater than or equal to 40 µm for migration
- Sieve with apertures of 20 µm for suspension recuperation
- Dishes, container or basins in plastic or stainless steel

5.1.1.2 Consumables

- Milk Filters and/or Wiping paper
- Beaker

Schematic procedure for RKN extraction using Oostenbrink elutriator and dish



5.2 Preparation of a solution of $MgSO_4$ with a 1.18 density

1) Salt Dilution

   For example, to prepare about 20 L of a solution with a density close to 1.18:

   - Weigh about 7.2 kg of $MgSO_4$.
   - Add 12L of hot water,
   - Mix until $MgSO_4$ is fully dissolved,

2) Cooling: Allow to cool to room temperature (e.g. overnight).

3) Adjustment of Density, if needed.

   - Measure the density of the solution with the density meter,
   - Adjust the density to 1.18 by adding water (at room temperature) or $MgSO_4$.

4) Filtration, Conditioning and Storage

   - Filter through a sieve of maximum 40 µm and store the solution in a plastic tank at room temperature.
   - Keep the solution away from light to prevent algae growth.

   - ***Note:*** *Adjust the quantities if other products than* $MgSO_4$ are used. *The quantities of product to be added, depending on the desired density, are shown in the following table:*

Required quantities in grams of each product to be diluted in water adjusting the quantity to obtain a final solution of 1 litre at the indicated densities (based on SOUTHEY, 1986)

Desired Density (20°C)	**1,15**	**1,18**	**1,22**
Sugar	401	484	588
$MgSO_4$ (pure)	166	200	245
$MgSO_4$ $7H_2O$	339	409	503
$ZnSO_4$ (pure)	156	187	229
$ZnSO_4$ $7H_2O$	279	335	410

Check the density with a densimeter before use of the final product.

5.3 Preparation of an enzyme mixture for the nematode extraction

Volume Measurement:

Components	Composition	For 1 L of Solution	For 5 L of solution
Pectinex	15%	150 mL	750 mL
Celluclast	30%	300 mL	1500 mL
Water	55%	550 mL	2750 mL

1) Mixing of the Product: Close the bottle and turn it over several times until a homogeneous mixture is obtained.
2) Storage:
   - Keep the mixture in a cool place in the refrigerator.
   - If mould appears in the bottle, pass the mixture through a paper filter before use.
     - ***Note:*** *Store enzymes in the cold room, before and after opening the canister.*

Warning: The risks associated with the use of enzymes require the operator to wear a mask, goggles and gloves, to put lids on the jars when stirring and to put the room air extraction system into operation.

Further information

If you have any question about the extraction methods for Meloidogyne chitwoodi and M. fallax, please, send an e-mail to eurl.nematodes@anses.fr

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