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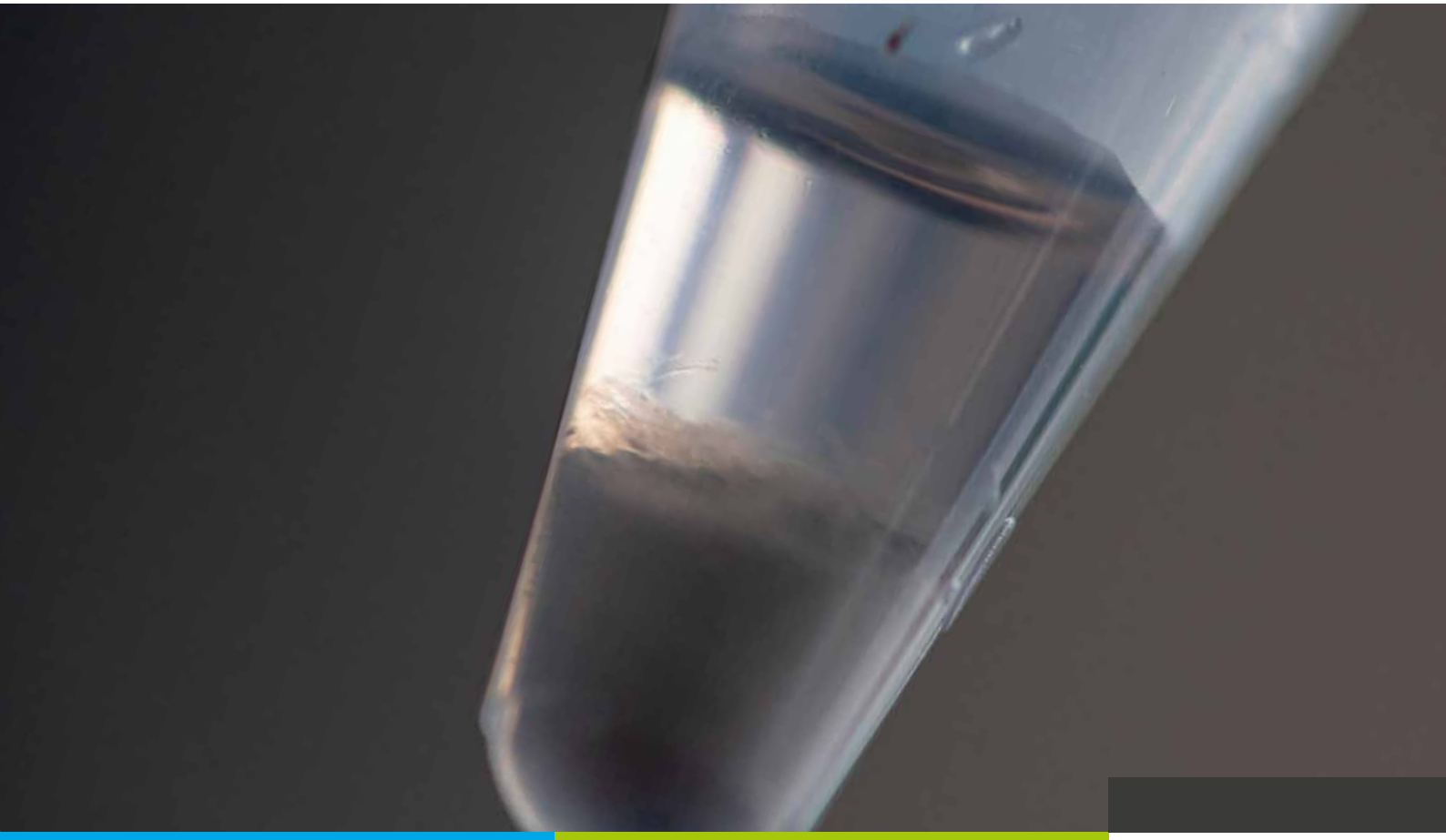

Collection of Sample Preparation Protocols for MALDI-TOF MS Based Identification of Meat, Dairy Products, Fish and Insects

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Collection of Sample Preparation Protocols for MALDI-TOF MS Based Identification of Meat, Dairy Products, Fish and Insects

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The use of matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS) is spreading rapidly in various application areas within food control, veterinary medical diagnostics, clinical microbiology and other fields [1–5]. The typical workflow of a MALDI-TOF MS analysis comprises the six steps in Fig. 1.

1. Definition of the Parameter of Interest	What is to be identified: microorganism, species of animals, fish or insects?
2. Choice of sample material	What will be prepared and measured, e,g. the colony or the pure culture of microorganism, which animal tissues or part?
3. Sample preparation	How can the target molecules be extracted from the sample, transferred on the MALDI-target, measured and analysed?
4. Generation of spectra using MALDI-TOF MS	Which set-up and algorithms should be applied for generation of mass spectra in the relevant mass range?
5. An available database for identification	How to build a MALDI-TOF MS database for your purpose if no commercial one is available?
6. Identification and validation	What are the criteria for identification and validation of the system?

Figure 1: Typical workflow for MALDI-TOP MS based species identification

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One of the most challenging tasks when developing an identification method using MALDI-TOF MS is the extraction of proteins and peptides form the target material. There are several well-known and established sample preparation procedures for microorganisms, such as bacteria and filamentous fungi. These methods are usually suggested by the manufacturers of the instruments (e.g. [6]). For application other than microorganisms the users have to develop an suitable sample preparation procedure.

In order to promote the dissemination of the species identification of microbes, meat, dairy products, sea foods or insects, we initiated 2016 the MALDI User Platform "MALDI-UP" [7]. Up to date, more than 1750 reference spectra are listed there for exchange. Among them there are entries for microbiology and extensive databases for various applications in the food sector. This collection is now being complemented by a selection of practical sample preparation protocols for users from different application areas. These protocols have been written in the form of short protocols to facilitate direct application in the readers' laboratories. Some of the protocols are based on existing procedures described in literature, with modifications or improvements that have proven successful in daily practice.

These protocols are also uploaded on MALDI-UP (https://maldi-up.ua-bw.de) that offers the possibility to every MALDI-TOF MS user of sharing his protocols, experience and spectra with other users.

This collection includes sample preparation protocols for the following matrices:

- **Meat** – Organic Solvent Extraction (OSExtr)
- **Dairy Products** – Organic Solvent Extraction (OSExtr)
- **Fish and Seefoods I** – Trifluoroacetic acid extraction (TFAextr)
- **Fish and Seefoods II** – Magnetic beads extraction (TFAextr C18)
- **Insects** – Ethanol/formic acid extraction modified for insects (EtOH-FA-I)

General Tipps and Recommendation

- HCCA Matrix solution: Either purchase from a provider of MALDI-systems or prepare according to the manufacturer's recommendations, e.g. saturated α-cyano-4-hydroxycinnamic acid, dissolved in OS Solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%).
- Freeze or measure the samples as soon as possible to prevent protein oxidation or degradation that possibly results in signal shifting. Freezing prevents or reduces the aging effects.
- Please always use chemicals of the highest purity available, e.g., HPLC or LC-MS grade.
- All relevant regulation to ensure chemical safety in the workplace must be obeyed.

References

[1] Pavlovic, M., Huber, I. & Busch U. (2020): MALDI-TOF MS profiling based identification of food components. Reference Module in Food Science, pp. 1–5. https://doi.org/10.1016/B978-0-08-100596-5.22767-4

[2] Rahi, P., Vaishampayan, P. (2020): Editorial: MALDI-TOF MS Application in Microbial Ecology Studies; Frontiers in Microbiology 10:2954. doi: 10.3389/fmicb.2019.02954.

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[6] Pranada, A.B., Schwarz, G., & Kostrzewa, M. (2016): Maldi Biotyping for Microorganism Identification in Clinical Microbiology. In R. Cramer (Ed.), Advances in MALDI and Laser-Induced Soft Ionization Mass Spectrometry (pp. 197–222): Springer International Publishing Switzerland; https://doi.org/10.1007/978-3-319-04819-2_11

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Sample Preparation Procedure for

Meat
Organic Solvent Extraction (OSExtr)

General Information

This protocol for meat, provided by CVUA Stuttgart, is based on the short description of *Post & Dikler* [2] and was further developed by P. Stoll within the scope of a student internship for biotechnology at the University of Applied Sciences Esslingen [4]. It was used to create and validate a reference database for muscle meat described before [1, 3].

A comprehensive collection of reference spectra (msp) and single spectra generated using this protocol is available for exchange on the MALDI-UP homepage (https://maldi-up@ua-bw.de).

Field of Application

Muscle meat of mammals, birds and reptiles in natural form, raw, frozen or heated (cooked, grilled). Also applicable for liver and kidney.

Chemicals and Material

- 1.5 ml reaction tubes and tips
- Silica beads (0.1 mm diameter)
- Micropestle, fitted for the tubes used
- Benchtop centrifuge
- HCCA Matrix solution (please see »Tipps and Recommendation«, page 2)
- OS Solvent (acetonitrile 50 %, water 47.5 % and trifluoroacetic acid 2.5 %)

References

[1] Hiller, E., Männig, A., Rau, J. (2017): Tierartendifferenzierung bei Fleisch – Mit MALDI-TOF-MS von der Datenbank zur Validierung. Deutsche Lebensmittelrundschau 113: 12–16.

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Meat:

Organic Solvent Extraction (OSExtr)

Extraction Procedure

- Add silica beads (about 5 mm high) into a 1.5 ml reaction tube
- Add 100–200 µl OS
- Add about 3 mm^3 muscle tissue
  For the best results please use material from the inner part of the tissue to avoid possible oxidation and contamination on the surface
- Homogenize/grind the sample using a micropestle
- Mix thoroughly by vortexing for 10–20 seconds
- Centrifuge at 12,000–14,000 rcf in a benchtop centrifuge for 2 minutes
- Pipet 1 µl supernatant onto a target sample spot (We recommend spotting the supernatant in duplicate or triplicate)
- As soon as the sample spot has dried, overlay the sample with 1 µl HCCA matrix solution (to prevent oxidation reactions leading to peak shifting)
- Allow the sample spot to air dry before analysis ➔ MALDI measurement

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Sample Preparation Procedure for

Diary Products

Organic Solvent Extraction (OSExtr) – (incl. Variant with Protein Precipitation)

General Information

This protocol for diary products was provided by CVUA Stuttgart and used to create and validate a reference database for feta and mozzarella cheese [1, 2].

A comprehensive collection of reference spectra (msp) and single spectra generated using this protocol is available for exchange on the MALDI-UP homepage (https://maldi-up@ua-bw.de).

Field of Application

Dairy products, e.g. milk, yogurt or cheese.
For pure milk it is necessary to perform protein precipitation (steps 1 – 4).
For yoghurt or (fresh-)cheese (already "precipitated" casein) please start with step 5.

Chemicals and Material

- 2.5 ml reaction tubes and tips.
- Spatula
- Centrifuge with centrifuge tubes and temperature control
- Small beaker
- Heating block
- Benchtop centrifuge
- Dilute acetic acid (50 %)
- Aqua dest/bidest
- HCCA Matrix solution (please see »Tipps and Recommendation«, page 2)
- OS Solvent (acetonitrile 50 %, water 47.5 % and trifluoroacetic acid 2.5 %)

References

[1] Männig, A., Hiller, E., Rau, J. (2017): MALDI-TOF MS zur Tierartenbestimmung bei Milch und Käse. 46; Poster at the "Deutscher Lebensmittelchemikertag" 25. – 27.09.2017, Würzburg. https://maldi-up.ua-bw.de/docs/CVUAS_Maennig_et_al_Tierarten_MALDITOF_2017.pdf

[2] Rau, J., Korte, N., Dyk, M., Wenninger, O., Schreiter, P., Hiller, E. (2020a): Rapid Animal Species Identification of feta and mozzarella cheese using MALDI-TOF mass-spectrometry. Food Control, 107349. https://doi.org/10.1016/j.foodcont.2020.107349

Diary Products:
Organic Solvent Extraction (OSExtr) – incl. Variant with Protein Precipitation

Protein / Peptides Precipitation

1. Separation of Fat/Skimming

For milk with especially low fat content, e.g. mare milk, this step can be skipped.

- Add approx. 30 ml milk to a centrifuge tube
- centrifuge at 3,345 rpm and 8 °C for 12 minutes
- To remove fat, please carefully decant the liquid into a small beaker; the fat should cling to the wall of the centrifuge tube and is to be discarded.

2. Precipitation of Protein / Peptides (mostly casein)

- Add a few drops of dilute acetic acid to the skimmed milk
- Heat the liquid to 40 – 45 °C (e.g. in a heating block) for at least 15 minutes until sufficient casein is precipitated.

3. Separation of Casein and Whey

- Transfer the liquid with the precipitate to 1.5 ml reaction tubes
  For mare milk you will need to pool the content of about 6 Eppendorf tubes, since mare milk does not seem to contain as much casein as other milk types. For other milk types less tubes are required
- Centrifuge at 12,000 – 14,000 rcf in a benchtop centrifuge for 2 minutes
- Discard the supernatant (= whey) and merge the pellets (= casein)

4. Washing

- Add 1 ml aqua dest. and stir with a spatula
- Centrifuge at 12,000 – 14,000 rcf in a benchtop centrifuge for 2 minutes
- Discard the supernatant

Extraction Procedure

5. Organic Solvent Extraction Procedure

- Transfer a small amount (about 3 mm^3) of the sample or protein precipitate into a 1.5 ml tube
- Add 200 µl OS
- Homogenize by stirring with a spatula
- Mix thoroughly by vortexing for 10 – 20 seconds
- Centrifuge at 12,000 – 14,000 rcf in a benchtop centrifuge for 2 minutes
- Pipet 1 µL supernatant onto a target sample spot (We recommend spotting the supernatant in duplicate or triplicate)
- As soon as the sample spot has dried, overlay the sample with 1 µL HCCA matrix solution (to prevent oxidation reactions leading to peak shifting)
- Allow the sample spot to air dry before analysis ➔ MALDI measurement

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Sample Preparation Procedure for

Fish and Seafood

Trifluoroacetic Acid Extraction (TFAextr)

General Information

This protocol for fish and seafood, provided by CVUA Karlsruhe, is based on the description of Mazzeo et al. [1] with modification, e.g. sample weight. It contains no purification step with magnetic beads. This method was used to create and validate a reference database for muscle meat of fish and seafood, which will be continuously expanded.

A selection of reference spectra (msp) and single spectra using this protocol is available for exchange on the MALDI-UP homepage (https://maldi-up@ua-bw.de).

Field of Application

Muscle meat of fish, crustaceans and bivalve molluscs in natural form, raw, cooked or frozen.

Chemicals and Material

- 1.5 ml reaction tubes
- Pipettes and tips for volumes from 1–500 µl
- Silica beads (0.1 mm resp. 0.5 mm diameter)
- Micropestle, compatible with the tubes used
- Bead mill (optional)
- Benchtop centrifuge
- HCCA matrix solution (please see »Tipps and Recommendation«, page 2)
- Trifluoroacetic acid (TFA) 0.1 %

References

[1] Mazzeo, M. F., Giulio, B. D., Guerriero, G., Ciarcia, G., Malorni, A., Russo, G. L. & Siciliano R. A. (2008): Fish Authentication by MALDI-TOF Mass Spectrometry. J Agric Food Chem 56(23): 11071–11076.

[2] Stephan, R., Johler, S., Oesterle, N., Näumann, G., Vogel, G. & Pflüger, V. (2014): Rapid and reliable species identification of scallops by MALDI-TOF mass spectrometry. Food Control 46: 6–9.

Fish and Seafood:

Trifluoroacetic Acid Extraction (TFAextr)

Extraction Procedure

- Add approx. 5 mg muscle without skin from the depth of the tissue into a 1.5 ml reaction tube
- Extract proteins either manually:
  - » Add 100 µl TFA (0.1 %) and approx. 100 mg silica beads into the tube
  - » Homogenize/grind the sample using a micropestle
  - » Add 400 µl TFA (0.1 %) and mixing thoroughly by vortexing for 10 – 20 sec.

  or mechanically:
  - » Add 500 µl TFA (0.1 %) into the tube
  - » Disrupt the cells using a bead mill with a frequency of 40 Hz for 3 minutes
- Leave for about 10 minutes (to solve proteins)
- Centrifuge at 15,900 – 18,400 rcf in a benchtop centrifuge for 10 minutes
- Pipet 1 µl supernatant onto a target sample spot (we recommend spotting the supernatant in duplicate or triplicate)
- As soon as the sample spot has dried, overlay the sample with 1 µl HCCA matrix solution (to prevent oxidation reactions which might cause peak shifts)
- Allow the sample spot to air dry before analysis ➔ MALDI measurement

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Sample Preparation Procedure for

Fish and Seafood

Magnetic Beads Extraction (TFAextr C18)

General Information

This protocol for fish, provided by CVUA Karlsruhe, is based on the description of Mazzeo et al. [1] with modification for e.g. sample weight and the purification step with magnetic beads. This method was used to create and validate a reference database for muscle meat of fish and seafood, which will be continuously expanded.

A selection of reference spectra (msp) and single spectra using this protocol is available for exchange on the MALDI-UP homepage (https://maldi-up@ua-bw.de).

Field of Application

Muscle meat of fish, crustaceans, bivalve molluscs and fish eggs in natural form, raw, cooked or frozen.

Chemicals and Material

- 1.5 ml reaction tubes
- Pipettes and tips for volumes from 1-500 µl
- Silica beads (0.1 mm resp. 0.5 mm diameter)
- Magnetic stand
- Micropestle, compatible with the tubes used
- Bead mill (optional)
- Benchtop centrifuge
- HCCA matrix solution (please see »Tipps and Recommendation«, page 2)
- Trifluoroacetic acid (TFA) 0.1 %
- Desorption solution (50 % acetonitrile in 0.1 % TFA)
- Magnetic beads (surface modified with C18 alkyl groups)

References

[1] Mazzeo, M. F., Giulio, B. D., Guerriero, G., Ciarcia, G., Malorni, A., Russo, G. L. & Siciliano R. A. (2008): Fish Authentication by MALDI-TOF Mass Spectrometry. J Agric Food Chem 56(23): 11071 – 11076.

[2] Stephan, R., Johler, S., Oesterle, N., Näumann, G., Vogel, G. & Pflüger, V. (2014): Rapid and reliable species identification of scallops by MALDI-TOF mass spectrometry. Food Control 46: 6 – 9.

[3] MoBiTec 2012, M-Beads-Proteomics S-C4, S-C8, S-C18

Fish and Seafood:

Magnetic Beads Extraction (TFAextr C18)

Protein Extraction

1. Protein Extration

- Add approx. 5 mg muscle without skin from the depth of the tissue into a 1.5 ml reaction tube
- Extract proteins either manually:
  - » Add 100 µl TFA (0.1 %) and approx. 100 mg silica beads into the tube
  - » Homogenize / grind the sample using a micropestle
  - » Add 400 µl TFA (0.1 %) and mixing thoroughly by vortexing for 10 – 20 sec.

  or mechanically:
  - » Add 500 µl TFA (0.1 %) into the tube
  - » Disrupt the cells using a bead mill with a frequency of 40 Hz for 3 minutes
- Leave for about 10 minutes (to solve proteins)
- Centrifuge at 15,900 – 18,400 rcf in a benchtop centrifuge for 10 minutes

Prurification of Protein Extract with Magnetic Beads

2. Conditioning / Resuspension Magnetic Beads

- Pipette 20 µl of the magnetic beads solution into a 1.5 ml reaction tube, place the tube on the magnet, remove the supernatant with a pipette and discard it
- Remove the reaction tube from the magnet, add 100 µl TFA (0.1 %) and resuspend the magnetic beads
- Place the tube on the magnet, remove the supernatant with a pipette and discard it, repeat this washing step a second and a third time
- Add 10 µl TFA 0.1 % and resuspend the magnetic beads

3. Protein/Peptide adsorption

- Add 25 µl sample solution from step 1 into the tube containing magnetic beads and leave for 10 minutes at ambient temperature
- Place the tube on the magnet, remove the supernatant with a pipette and discard it
- Remove the reaction tube from the magnet, add 50 µl TFA (0.1 %) and re-suspend the magnetic beads
- Place the tube on the magnet, remove the supernatant with a pipette and discard it, repeat this washing step a second and a third time

4. Protein/Peptide desorption

- Add 10 µl desorption solution to the sample-coated magnetic beads from step 3, resuspend and incubate for 8 minutes at ambient temperature
- Pipet 1 µl supernatant onto a target sample spot (we recommend spotting the supernatant in duplicate or triplicate)
- As soon as the sample spot has dried, overlay the sample with 1 µl HCCA matrix solution (to prevent oxidation reactions which might cause peak shifts)
- Allow the sample spot to air dry before analysis ➔ MALDI measurement

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Sample Preparation Procedure for

Insects

Ethanol / Formic Acid Extraction Modified for Insects (EtOH-FA-I)

General Information

This protocol for insects, provided by CVUA Freibrug, is based on the sample preparation procedure using ethanol/formic acid by Bruker Daltonik GmbH [1] with modification for sample quantity, homogenization and purification. It was used to create and validate a reference database for insects, which will be continuously expanded.

A selection of reference spectra (msp) and single spectra using this protocol is available for exchange on the MALDI-UP homepage (https://maldi-up@ua-bw.de).

Field of Application

Insects: whole, ground, processed, in natural form, raw, cooked, frozen or freeze-dried

Chemicals and Material

- 1.5 ml reaction tubes
- Pipettes and tips for volumes from 1 – 1000 µl
- Silica beads (0.1 mm resp. 0.5 mm diameter)
- Micropestle, compatible with the tubes used
- Bead mill (optional)
- Benchtop centrifuge
- HCCA matrix solution (please see »Tipps and Recommendation«, page 2)
- Ethanol absolute
- 70 % Formic acid
- Ultrapure water
- Acetonitrile (ACN)

References

[1] Bruker Daltonik GmbH. Ethanol/formic acid extraction sample preparation procedure.

Insects:

Ethanol / Formic Acid Extraction, Modified for Insects (EtOH-FA-I)

Extraction Procedure

- Transfer up to 5 mg of sample material into a clean reaction tube
- Pipet 100 µl water into the tube and homogenize / grind the sample using a micropestle
- Add 200 µl water and mix thoroughly (if the suspension is jellylike, start again by using less sample material)
- Add 900 µl ethanol into the tube and mix thoroughly
- Centrifuge at 18,400 rcf for 2 minutes and decant the supernatant
- Wash the pellet by adding 300 µl water to the tube and mix thoroughly
- Centrifuge at 18,400 rcf for 2 minutes, decant the supernatant or remove it from the pellet by using a pipet
- Dry the pellet for some minutes to increase the extraction efficiency
- Add between 50 – 200 µl 70 % formic acid to the pellet and mix thoroughly by pipetting
- Add silica beads and mix thoroughly for about 1 minute (alternatively use a bead mill to disrupt the cells, e.g. at frequency of 50 Hz for 2 minutes)
- Add acetonitrile in the same amount as formic acid and mix for about 1 minute by pipetting (if the suspension is jellylike, add 70% formic acid and acetonitrile in equal volumes until the suspension is liquid)
- Centrifuge at 18,400 rcf for 2 minutes
- Pipet 1 µl supernatant onto a target sample spot (we recommend spotting the supernatant in duplicate or triplicate)
- As soon as the sample spot has dried, overlay the sample with 1 µl HCCA matrix solution (to prevent oxidation reactions which might cause peak shifts)
- Allow the sample spot to air dry before analysis ➔ MALDI measurement

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