Theses for a doctoral dissertation (PhD)

Examination of genetic homogeneity of different plant seeds using mass spectroscopy technology

by:
Csilla Bojté
PhD candidate

Supervisor:
Dr. Szilárd Tóth

Associate supervisor:
Dr. Adrienn Micsinai

UNIVERSITY OF DEBRECEN

Kerpely Kálmán Doctoral School



Debrecen, 2022

1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL THESIS

With the growth of humanity and our accelerated world, the challenge of satisfying needs is enormous. Fulfilling the challenges and tasks within the agricultural sector is a key factor for quality food and feed in our society. The production and use of high quality and genetically homogeneous seeds is a fundamental prerequisite for a secure and successful yield.

The MALDI-TOF mass spectrometer is a device for the rapid identification of microbiological samples on a protein basis. J. J. Thomson, who is also considered the father of mass spectroscopy, concluded in his studies that positively charged gas ions accelerated in an electric field after ionisation follow different trajectories in the magnetic field and then hit the detector at different locations. This opens up the possibility of separating the ions from the sample on the basis of molecular weight/charge (m/z) (HTTP4). In the 1980s, newer ionisation techniques were developed: fast atom bombardment, FAB; plasma desorption, PD; laser desorption, LDI ionisation. The invention of laser desorption ionization was the most important technique for the study of biopolymers. In 1988, HILLENKAMP and KARAS created the MALDI system, the newly developed MALDI source was coupled to a time-of-flight (TOF) analyser (KARAS and HILLENKAMP, 1988).

This instrument was already capable of analysing samples with a molecular weight of 10 kDa. KOICHI TANAKA was the first to report (1987) on the application of laser desorption ionisation, for which he was awarded the Nobel Prize in 2002 (ZIMMERMANN, 2015). Mass spectrometry is an efficient method for structural analysis in which the molecules to be analysed are introduced into a gas phase, ionised and accelerated by a high-voltage electric field. Ions are separated and measured by magnetic, electrostatic or radiofrequency fields based on their mass and charge. MALDI-TOF technology is one of the most important analytical methods for the analysis of biologically active molecules (proteins, enzymes). Thanks to new engineering developments, the instruments are extremely fast, accurate and easy to use.

My Ph.D. thesis was carried out at WESSLING Hungary Ltd. in Budapest, where I had the opportunity to develop methods and prepare and measure samples on the company's MALDI-TOF MS instrument. A member of the internationally renowned Hungarian company group had already started the development of a maize seed testing system, which provided a successful and secure basis for the start and successful completion of my further work.

With their tremendous professional and human help, I have been able to test and develop a method for seeds of 4 economically important industrial crops. The results of the maize trials provided the basis for further trials on sunflower, soybean and wheat seeds with the MALDI-TOF MS instrument and software background.

The main objectives of my research were:

1. To determine which extractants most accurately reflect the analysed parameters in the selected crops (maize, sunflower, soybean, wheat):
    - varietal identity/homogeneity
2. Testing of extractants and development of sample preparation procedures:
    - NaCl (neutral and acidic) and 60% ethanol buffer process
    - 1- or 2-propanol buffer process
    - Significance of SA matrix solution and HCCA matrix solutions; differences and deviations
    - Hexane pre-treatment for sunflower and soya
3. Optimisation of MALDI-TOF MS instrument parameters:
    - Automatic fast measurement by optimizing measurement parameters (AutoXrun)
    - The results obtained are used to design a measurement method, i.e. evaluation files.

2. MATERIAL AND METHOD

2.1. Calibration Standard

In the scope of the analyses, the measurement methods were calibrated with the Bruker protein mix standard. The calibration material, which covered the expected range of measurement weights, contained the following standards, Insulin, Ubiquitin, Cytochrome C, Myoglobin (Table 1, Figure 1)

TABLE 1: PROTEIN MIX I CALIBRATION STANDARD

Protein Mix I	(M+H)avg
Insulin	5734.51 Da
Ubiquitin	8565.76 Da
cytochrome C	1236.97 Da
Myoglobin	16952.30 Da

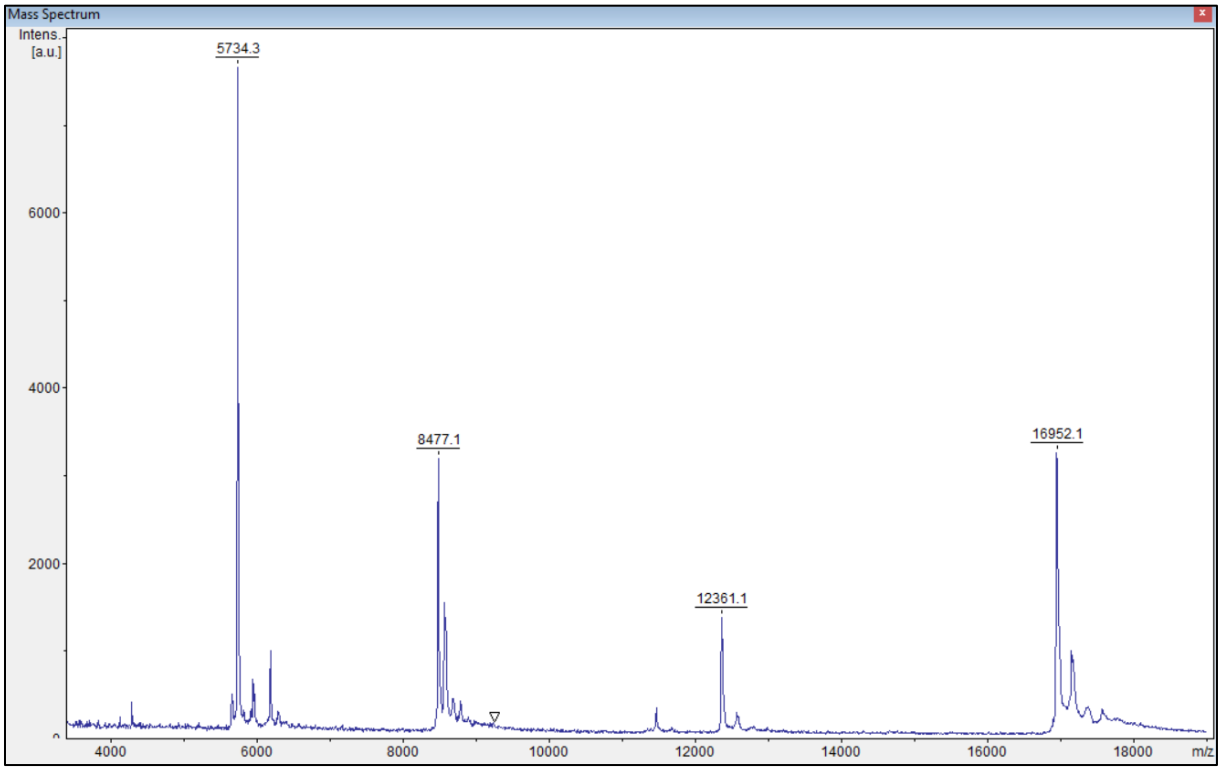


FIGURE 1: CALIBRATION STANDARD SPECTRUM OF PROTEIN MIX

2.2. Optimisation of the extraction method

To obtain the most informative mass spectra, we need to optimise the extraction conditions. The reserve proteins are divided into two main groups in terms of solubility, the "water-soluble" fraction and the "alcohol-soluble" fraction, and therefore different extraction mixtures were used for the extraction. The extraction is influenced by several parameters (pH, ionic strength, mixing mode, extraction time, extraction temperature, concentration of reducing agent), the effect of which must also be considered when optimising the method. For this purpose, extraction experiments are carried out on average samples of seeds with known protein composition, in order to apply the most appropriate parameters to the real samples. The application of extracts of water-soluble fractions to the MALDI-TOF target is usually not problematic (distinct droplets can be applied), although the literature suggests that these extracts should also be diluted to a given composition of acetonitrile - TFA (30 % ACN - 0,6 % TFA). The solution thus prepared gives a more homogeneous spot and also improves the shape and resolution of the mass spectrometric peaks.

2.3. Characteristics of alcohol soluble extractants

a) *the material quality of the extracting agent:*
   - Analytical purity or equivalent on all nights

b) *pH of the extracting agent:*
   - neutral pH (without buffer);
   - acid pH (pH adjustment with acetic acid (1% v/v);
   - slightly basic pH (pH adjustment with TRIS buffer)

c) *Ionic strength of the extractant:*
   - Concentration of sodium chloride (0.2 - 0.4 - 0.6 - 0.8 - 1.5 - 5.0 % m/v)

2.4. Amount of reducing agent

Several concentrations can be used in the tests, the most typical of which are:

- Dithiothreitol (DTT): 0.0 - 0.1 - 0.2 - 0.4 - 0.8 - 1.0 % m/v

2.5. The extraction methods

- ❖ Ultrasound-assisted extraction;
- ❖ Extraction with a rotary mixer;
- ❖ Extraction on a shaking machine

2.6. *Extraction time and temperature*

a) *Extraction time:* 40 minutes;

b) *Extraction temperature:* room temperature (20-24 °C);

2.7.*Extraction work solutions*

- **NaCl extraction buffer:** 100 ml of deionized water was measured into a 150 ml screw-top flask, 0.58 g NaCl and 0.31 g Dithiothreitol (DL-DTT) were measured and mixed thoroughly.
- **NaCL acid extraction buffer:** 100 ml of deionized water was measured into a 150 ml screw-top flask, 0.58 g NaCl and 0.31 g Dithiothreitol (DL-DTT) and 1 ml acetic acid were added and mixed thoroughly.
- **Propanol extraction buffer:** 50 ml of 1-propanol was measured into a 150 ml screw-top bottle, 47 ml of deionized water, 1 ml of 100% acetic acid and 2 g of dithiothreitol (DL-DTT) were added.
- **2-propanol extraction buffer:** 50 ml of 2-propanol was measured into a 150 ml screw-top jar , 47 ml of deionized water, 1 ml of 100% acetic acid and 2 g of dithiothreitol (DL-DTT) were added.
- **60% EtOH:** 60 ml of analytical grade absolute ethanol is weighed in a 200 ml graduated cylinder and 40 ml of deionized water is added. This solution is poured into a 150 ml screw-top bottle and mixed thoroughly.

Due to the large sample size, these solutions were used in 4 studies, but can be stored for up to 30 days at 4 °C.

2.8. Eluting solution

TA25 solution: 25 ml of acetonitrile (ACN), 75 ml of deionized water and 100 µl of trifluoroacetic acid were added to a 150 ml screw-top flask and mixed thoroughly.

2.9. Matrix solutions

- **Matrix solution HCCA:** 450 µl of the deionized water was pipetted into a 1.5 ml Eppendorf tube, 500 µl of acetonitrile and 25 µl of 100% trifluoroacetic acid were added.

- **Matrix solution SA:** 450 µl of the deionised water was pipetted into a 1,5 ml Eppendorf tube, 450 µl of acetonitrile and 35 µl of 100 % trifluoroacetic acid were added.

2.10. Sample preparation

2.10.1. Sample holder plates

2.10.1.1. Preparation of sample plates

This cleaning process was developed by Csaba Lovász, a chemical engineer at WESSLING Hungary Ltd. The first step is to prepare the sample plates as follows:

1. After completion of the MALDI-TOF test, the contaminated sample plates are soaked in 70 % ethanol for 5-10 minutes.
2. After soaking, the residue of the matrix or extracts was washed off with warm running water and then
3. rubbed the plates with a fine aqueous suspension of aluminium oxide, then
4. they were dried with a paper towel.
5. The plates were then soaked in methanol for 5 - 10 minutes and
6. the residual methanol was allowed to evaporate in a laminar booth.
7. The plates were rubbed with a lint-free wipe dipped in 2-propanol.
8. After drying, 50 ml of 80 % trifluoroacetic acid solution was pipetted onto the centre of the plates in a laminar booth and the plates were thoroughly rubbed with an ear cleaning stick, then
9. the residue of trifluoroacetic acid was washed off with deionised water.

10. The dried plates were rubbed with a little graphite powder, and the rest of the graphite powder was removed with a fine brush.
11. The prepared sample plates were stored in Petri dishes until the tests were started.

In multiple laboratories using MALDI-TOF or MALDI-TOF-TOF instruments, the simple detergent and distilled, i.e. deionised water procedure is used:

1. First soak in distilled water for 10 minutes and then
2. dishwashing liquid is applied to the plate, then
3. wiped with a general dishwashing sponge
4. again soaked in distilled water and wiped dry after 5 minutes.

2.11. Calibrating Standard Solution

2.11.1. Standard solution

In the procedure, the protocol published by Bruker was used. 1 ml of the calibration standard solution was dropped onto a position in row A of the prepared sample holder plate and allowed to evaporate. If possible, 1 ml of matrix solution (SA or HCCA) was dropped onto the calibration standard immediately after drying and the solvent was allowed to evaporate in a laminar box. A new pipette tip was used to apply each sample, and after the sample had dried on the plate, the matrix solution was then measured on the samples within a maximum of 10 min. The sample plates were allowed to dry at room temperature and were ready for MALDI-TOF and MALDI-TOF-TOF analyses after drying.

2.11.2. Preparation of the SA matrix

The SA matrix solution (25 mg/ml concentration) was prepared by mixing a 1:1 solution of deionized water containing 0.6 % TFA and ACN with crystalline synaptic acid to obtain the desired 25 mg/ml concentration, according to a study published by DIECKMANN et al. Dissolution was promoted by the use of vortex. The matrix solution should be stored at 4ºC, protected from light, and should be prepared fresh on the day of use, as it can be used for up to 1 day.

2.11.3. Preparation of the HCCA matrix

The α-HCCA matrix solution (20 mg/ml concentration) was prepared by mixing a 7:3 solution of deionized water and ACN containing 5 % formic acid and crystalline α-HCCA to obtain the desired 20 mg/ml concentration, according to a study published by SIGNOR and ERBA (2013). Dissolution was promoted by the use of vortex followed by an ultrasonic water bath. The matrix solution should be stored protected from light at 4ºC, should be prepared fresh on the day of use, and should be used for up to 1-2 days.

2.12. Physical preparation of samples

Due to the novelty of the method, we had to make a complete batch of seeds for each seed lot and plant species. From the seed lots obtained, 20 seeds were ground, 1 mg was extracted into a 2 ml eppendorf tube and, depending on the plant species, 500 µl of the appropriate extractant was added. For soybean and sunflower, the hexane procedure was essential. This was necessary to map out which biomarkers we should obtain for the grain-by-grain assays.

For the grain-by-grain tests, i.e. homogeneity tests, the samples are peeled in the sunflower stubble. For each variety, they were ground one by one using an ultracentrifugal grinder (Retsch ZM200). The ground grain was placed in 2 ml sterile eppendorf.

2.13. Extraction procedures

2.13.1. Steps for application of hexane buffer extracts

For sunflower seeds and soybean seeds, the crushed and ground grains were soaked in 1 ml of analytical grade hexane for 30 min prior to the extraction procedure. This special procedure was necessary because our preliminary experiments showed that the high oil and fat content influences the final result.

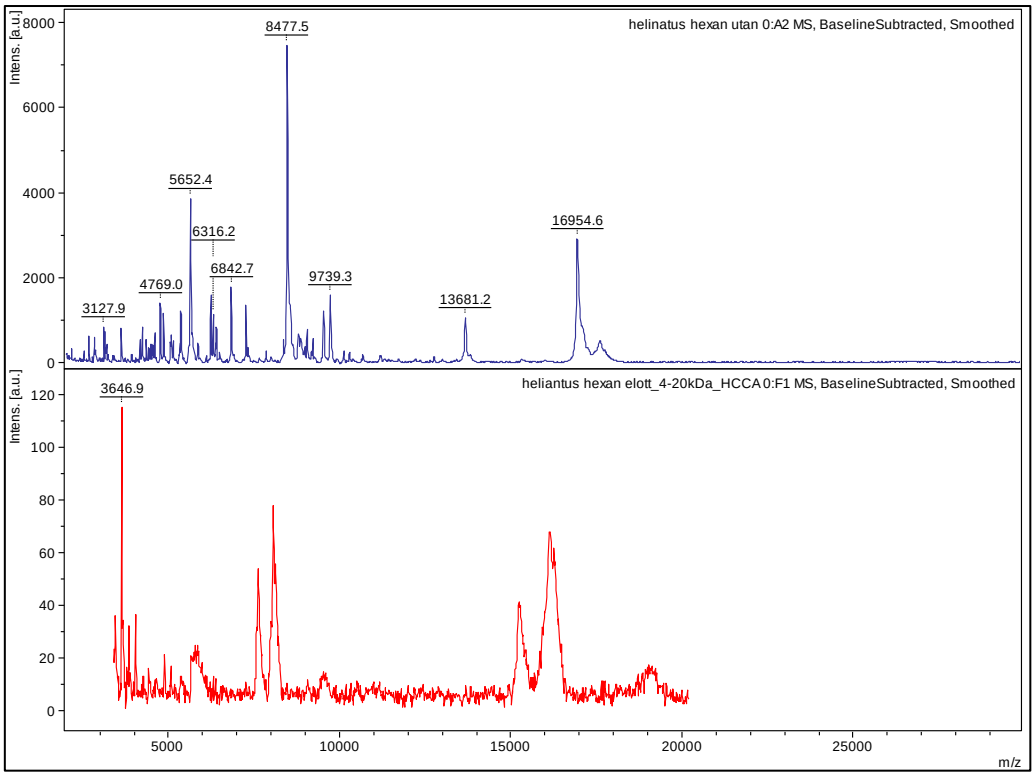


FIGURE 2: HEXANE TREATMENT OF SUNFLOWER HULLING - BLUE SPECTRUM BEFORE AND RED SPECTRUM AFTER

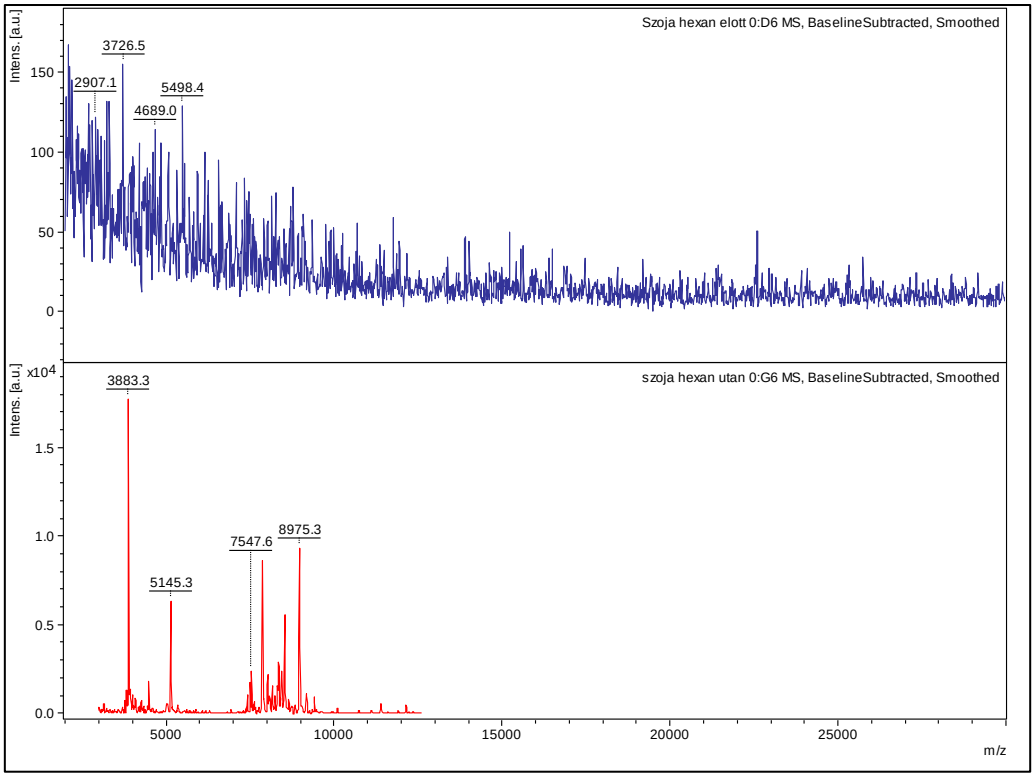


FIGURE 3: HEXANE TREATMENT FOR SOYBEAN - BLUE SPECTRUM BEFORE AND RED SPECTRUM AFTER

2.13.2 Extraction with aqueous (neutral) NaCl buffer

- Samples were broken into pellets and 10 mg were weighed into a 2.0 ml Eppendorf centrifuge tube.
- 300 µl of NaCl extractant was pipetted onto the ground samples and vortexed. They were then placed in an ultrasonic bath for 30 minutes. Samples in NaCl were placed in a rotary vertical shaker and shaken for a further 30 minutes. After the extraction was completed, the samples were centrifuged (3 min, 14100 rpm) and 50 µl of the supernatant was pipetted into a clean 1.5 ml new Eppendorf tube. Before applying the samples, the extract was vortexed again and centrifuged again (3 min, 14100 rpm). 1-1 µl was then dropped in two parallel drops onto the prepared sample plate as samples. After drying, 1 µl of HCCA or SA matrix solution was dropped onto the applied samples and allowed to evaporate in a laminar box and dry as a sample.

2.13.3. Extraction with acid NaCl buffer

10 mg of the samples were weighed into a 2.0 ml Eppendorf centrifuge tube. Then 300 µl of NaCl extractant was pipetted into the centrifuge tubes. They were vortexed and placed in an ultrasonic bath for 30 minutes. The samples were then placed in a rotary vertical shaker and extraction continued for another 30 min. After completion of this procedure, the samples were centrifuged (3 min, 14100 rpm) and 50 µl of the supernatant was pipetted into a clean 1.5 ml Eppendorf tube. Before applying the samples, the extract was thoroughly vortexed again and centrifuged once more (3 min, 14100 rpm). 1-1 µl was then dropped onto the prepared sample plate in two parallel runs as samples. After drying, 1 µl of HCCA or SA matrix solution was dropped on each sample one by one and the solvent was allowed to evaporate in a laminar box and the sample dried.

2.13.4. Extraction with 2-propanol buffer

10 mg of the samples were weighed into a 2.0 ml Eppendorf centrifuge tube. 300 µl of 1-propanol extraction solvent was pipetted onto the minced samples. It was mixed with a vortex and placed in an ultrasonic bath for 30 minutes. The samples were then placed in a rotary vertical shaker and continued for 30 min, as soon as this process was complete the samples were centrifuged (3 min, 14100 rpm) and 50 µl of the supernatant was pipetted into a clean 1,5 ml Eppendorf tube. After pipetting off the supernatant, 50 µl of TA25 solution was added to the samples. This step is important for the application of the sample extract.

The pure 2-propanol extract will run off on the plate, but when supplemented with TA25 solution, application is possible. Our experiments have shown that this substance does not affect the further results. Prior to sample application, the extract was again thoroughly vortexed and centrifuged (3 min, 14100 rpm). 1 µl was then dropped onto the prepared sample plate in two parallel drops as a sample. After drying, 1 µl of matrix solution was dropped onto the samples and the solvent was allowed to evaporate in a laminar buffer and the sample dried.

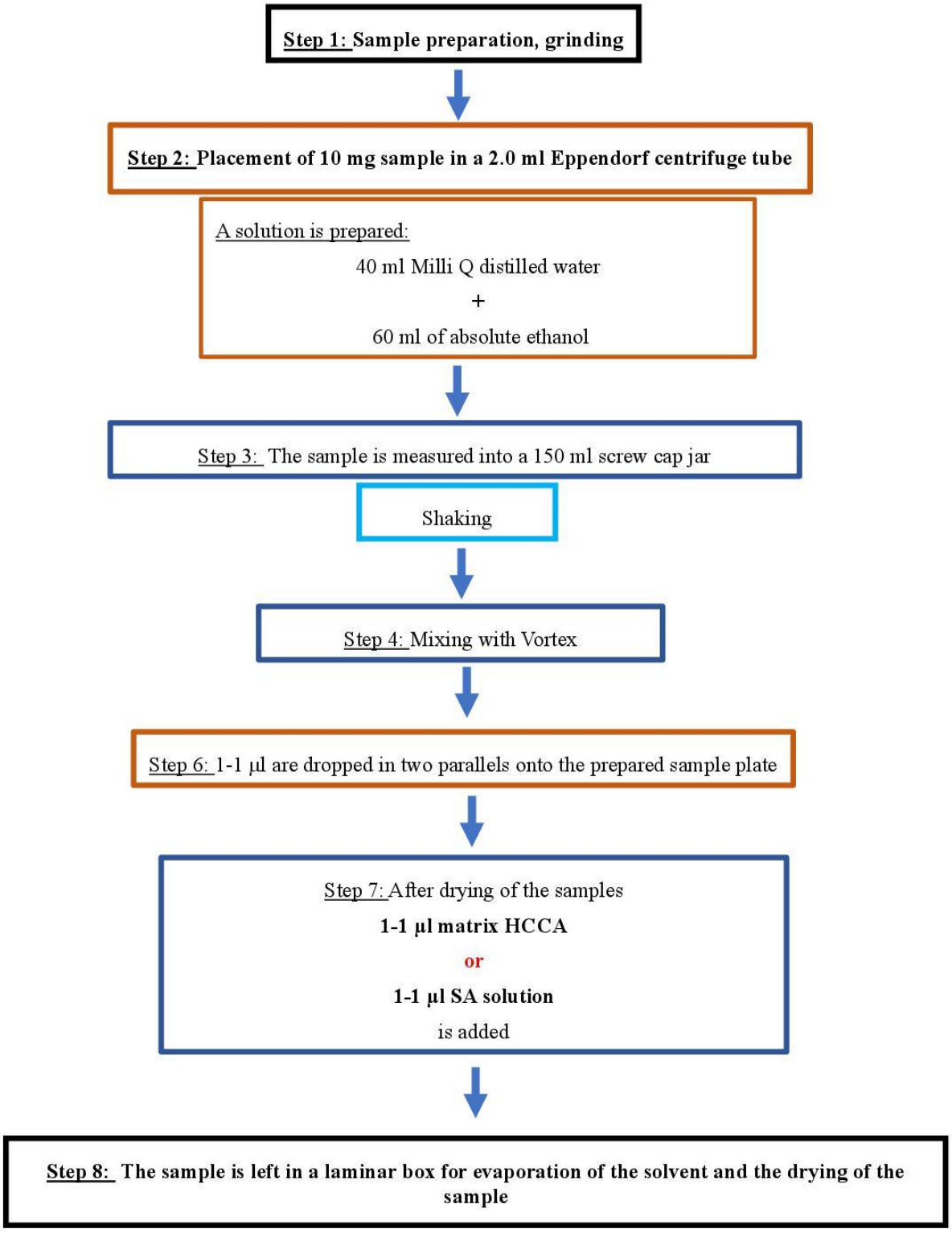
2.13.5. Extraction with 1-propanol buffer

10 mg of each sample was measured separately into a 2.0 ml Eppendorf centrifuge tube. Then 300 µl of 1-propanol extractant was pipetted into the centrifuge tubes. They were thoroughly vortexed and placed in an ultrasonic bath for 30 minutes. The samples were then placed in a rotary vertical shaker and the extraction continued for another 30 minutes. After the extraction was completed, the samples were centrifuged (3 min, 14100 rpm) and 50 µl of the supernatant was pipetted into a clean 1.5 ml Eppendorf tube. After pipetting off the supernatant, 50 µl of TA25 solution was added to the samples. This step is important for the application of the sample extract. The pure 1-propanol extract will run off on the plate, but with the addition of TA25 solution, uptake is possible. Our experiments have shown that this material does not affect the further results. Prior to sample application, the extract was again thoroughly vortexed and centrifuged (3 min, 14100 rpm). 1 µl was then dropped onto the prepared sample plate in two parallel drops as a sample. After drying, 1 µl of matrix solution was dropped onto the samples and the solvent was allowed to evaporate in a laminar buffer and the sample dried.

2.13.6 Application of 60% ethanol buffer and extracts

The ground grains were placed in a 2.0 ml Eppendorf centrifuge tube. The solution was prepared by weighing and mixing 40 ml Milli Q distilled water and 60 ml absolute ethanol in a 150 ml screw-cap flask. The extract was again thoroughly vortexed and centrifuged (3 min, 14100 rpm) before sample application. 1 µl was then dropped onto the prepared sample plate in four parallel drops as a sample. After drying, 1-1 µl of matrix HCCA or 1-1 µl of SA solution was dropped onto the samples and the solvent was allowed to evaporate in a laminar box and the sample dried.

Preparation of wheat samples for 60% EtOh test



2.14. MEASUREMENT

For the measurement a *Bruker microflex LT* MALDI-TOF mass spectrometer was used at Wessling Hungary Ltd. In 2000, the Department of Applied Chemistry of the University of Debrecen installed a state-of-the-art MALDI-TOF-TOF mass spectrometer, also manufactured by Bruker; its type is Autoflex Speed.

The prepared sample holder plate was introduced into the ion source through a sluice system. The movement required to scan the samples is performed by a precise mechanism that can move the sample holder plate under the UV laser source with great accuracy. A low resolution digital camera can also be used to monitor the sample in the current position. This both helps to check that the scan is being performed from the correct sample position and aids manual spectral acquisition in case of possible sample inhomogeneity.

A general instrument control software (*flexControl - Bruker Daltonik*) can be used to set the parameters required for mass spectra acquisition, such as the laser energy, the high voltage required to accelerate the ions, the movement pattern of the sample holder plate and the range of mass numbers to be tested. This software can also be used to calibrate the mass number scale of the instrument, to define the function for the conversion of time-of-flight to mass number. The principle is simple, a protein mix of known protein composition is used as a calibration standard for this purpose.

After checking the vacuum, the laser was set to the calibration position (A3) and in automatic spectrum recording mode, the average mass spectrum of the calibration standard was acquired in the mass number range 2000 – 25,000 amu *(atomic mass unit)*, preferably from the average of 200-500 spectra. After noise filtering (smoothing) and baseline correction, the calibration was performed (the instrument searched for the reference mass numbers and fitted the appropriate function to the mass numbers). The deviation of the reference mass numbers was checked and the calibration was accepted only if the deviation was less than 500 ppm for all mass numbers.

2.14.1. Own method files for automated measurement

To test the genetic homogeneity of maize seeds, we used the method file /csilla/MBT_FC_Kukorica_14526.*par*. The instrument automatically traversed the sample positions and the software performed the mass spectra recording.
To test the genetic homogeneity of sunflower seeds, the method file /csilla/MBT_FC_napraforgo_14476.*par* was used. The instrument automatically ran through the sample positions and the software performed the mass spectra acquisition .

To test the genetic homogeneity of the soybean seeds, the method file /csilla/MBT_FC_szoja_*14852.par* was used. The instrument automatically traversed through the sample positions and the software performed the mass spectra acquisition in the process.

To test the genetic homogeneity of wheat seeds, the method file /csilla/MBT_FC_buza *_14999.par* was used. The instrument automatically traversed the sample positions and the software performed the mass spectra acquisition.

2.14.2. Statistical evaluation methodology

For the evaluations, the data were summarized and plotted using Microsoft Excel spreadsheet, while statistical analyses were performed using SPSS v13.0.

The statistical analysis was performed in the statistical environment R 4.1.3 (R CORE TEAM, 2022) with the graphical interface RStudio (HTTP7) using the packages "agricolae". Graphs were generated using Microsoft Excel 2019. The statistical analysis was performed with a first-order error of 5% (alpha = 0.05). To investigate the effect of the extractant on M/Z values, a repeated measure analysis of variance model was constructed following the example of HUZSVAI and BALOGH (2015). The syntax of the repeated measures model in the statistical environment R:

model<- aov(M/Z~Extractor+Error(sample_identifier/extractor),
data=source_database)
summary(model)

The mean comparison of the measured M/Z data was performed using the least significant difference (LSD) method, in which we determined the least significant difference in an R statistical setting for each repeated measurement model specified, which was performed using the following syntax for each model separately for post hoc tests of degrees of freedom (df) and mean squared errors (MSE) individually (HUZSVAI and BALOGH, 2015):

```
df=df.residual(model$"error:error")
mse=deviance(model$"error:error")/df
LSD <- with(database, LSD.test(M/Z, model_significant_effect, df, mse, console = T))
```

3. RESULTS

3.1. Evaluation of the results of the MALDI-TOF tests

With FelxAnalysis software, we were able to view mass spectra of samples of seeds from different plant species one by one. Once the spectra were scanned, we performed noise filtering and baseline correction using a method file we developed. Although the instrument produced and averaged 640 mass spectra from a single sample, the intensity of the spectra obtained was completely different. This difference is due to the fact that the sample portions used for extraction did not contain the same amount of the different reserve proteins that were analysed.

For genetic homogeneity studies, it is always necessary to map the characteristic proteins, so extracts are prepared from average samples from several grains and these are transferred to the sample plate and then measured to see what spectral curves are characteristic of the particular batches. This allowed us to find the so-called marker proteins that are typically detected in the different batches. In general, these appeared as peak pairs with close mass numbers in the mass spectra of the lots. After selection of the marker proteins, mass spectra of the extracts prepared from the hybrids were analysed and the presence of marker proteins was sought. If the selected marker peaks were present in the spectra, they were declared genetically homogeneous in the hybrids.

For quality control purposes, two parallel tests were performed on each extract. Extracts from "defective" hybrid grains, which are not genetically pure, were repeated several times and only then were the final results accepted.

In addition, the extracts of the samples were stored in a refrigerator at 4 °C, because our experience shows that the extracts retain their quality for at least 1 month. *Tables 2* until *Tables 5* clearly show that in the case of corn (Zea mays), soybean [Glycine max (L.) Merr.] and wheat (Triticum aestivum) it did not drop below 90%, while for sunflower (Heliantus annus) it did not exceed a 4% decrease after 1 month.

TABLE 2: STORABILITY TEST RESULTS FOR MAIZE (*ZEA MAYS*) SAMPLES

Zea mays 1-206	100%
Zea mays 2-89	100%
Zea mays 3-236	100%
Zea mays 4-167	95%
Zea mays 5-137	93%
Zea mays 6-99	100%
Zea mays 7-125-15	100%
Zea mays 8-103-15.4	100%
Zea mays 9-169-15.25	100%
Zea mays 10-165-15	99%

TABLE 3: STORABILITY TEST RESULTS FOR SOYBEAN (*GLYCINE MAX (L.) MERR.*) SAMPLES

Glycine max (L.) Merr. 1- 1	100%
Glycine max (L.) Merr. 2-2	100%
Glycine max (L.) Merr. 3-78-16	100%
Glycine max (L.) Merr. 4-4	100%
Glycine max (L.) Merr. 5-5	98%
Glycine max (L.) Merr. 6-169	98%
Glycine max (L.) Merr. 7-58.59	100%
Glycine max (L.) Merr. 8- mentor	91%
Glycine max (L.) Merr. 9- Elio5-16	100%
Glycine max (L.) Merr. 10-blend	100%

TABLE 4: STORABILITY TEST RESULTS FOR SUNFLOWER (*HELIANTUS ANNUS*) SAMPLES

Heliantus annus 1-Sun 1	96%
Heliantus dose 2-33tetel sun	98%
Heliantus dose 3-8tetel	100%
Heliantus dose 105-15	100%
Heliantus dose 5-sun.2018.10.11	97%
Heliantus dose 6-122-15	100%
Heliantus annus 7-F1	99%
Heliantus annus 8- 7 new	100%
Heliantus annus 9-9new	99%
Heliantus dose 10-10_SA	99%

TABLE 5: STORABILITY TEST RESULTS FOR WHEAT (*TRITUCUM AESTIVUM*) SAMPLES

Triticum aestivum1-h7	100%
Triticum aestivum 2-F2	100%
Triticum aestivum 3-20tetel Buza	100%
Triticum aestivum 4-4tetel Buza	98%
Triticum aestivum 5-21	100%
Triticum aestivum 6- 10telel Buza	97%
Triticum aestivum 7-balibuza	100%
Triticum aestivum 8-h2	100%
Triticum aestivum - 9 9tetel Buza	100%
Triticum aestivum 10-h22	97%

3.2. Selection of extraction agents based on spectra for the analysed plant species

For the wheat samples, we found that the highest M/Z value (20409.2) was obtained with the 60% EtOH and the lowest with the NaCl isolant (1893.2), but there were also significant differences in the M/Z values obtained with the other three isolants, which when tested on $LSD_{5\%-}$ gave significantly ($p<0.05$) poorer spectral results compared to the 60% EtOH extractant (Figure 4).

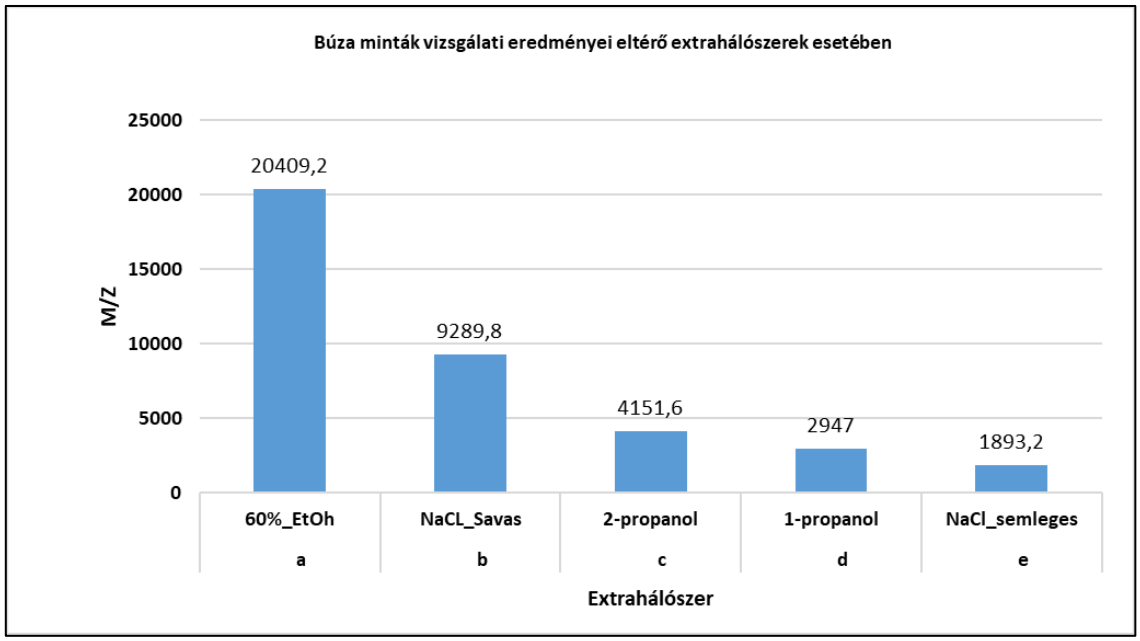


FIGURE 4: TEST RESULTS OF WHEAT SAMPLES USING DIFFERENT EXTRACTANTS

A similar trend was observed for the maize samples, but the NaCl acid and neutral isolates gave the lowest (3987.26 and 2765.48), almost identical M/Z values. When tested on $LSD_{5\%-}$, it was found that 1-propanol gave significantly ($p<0.05$) the best M/Z (17857.84) for maize compared to the spectra of 60% EtOH and NaCl acidic and NaCl neutral extractants, respectively (Figure 5).

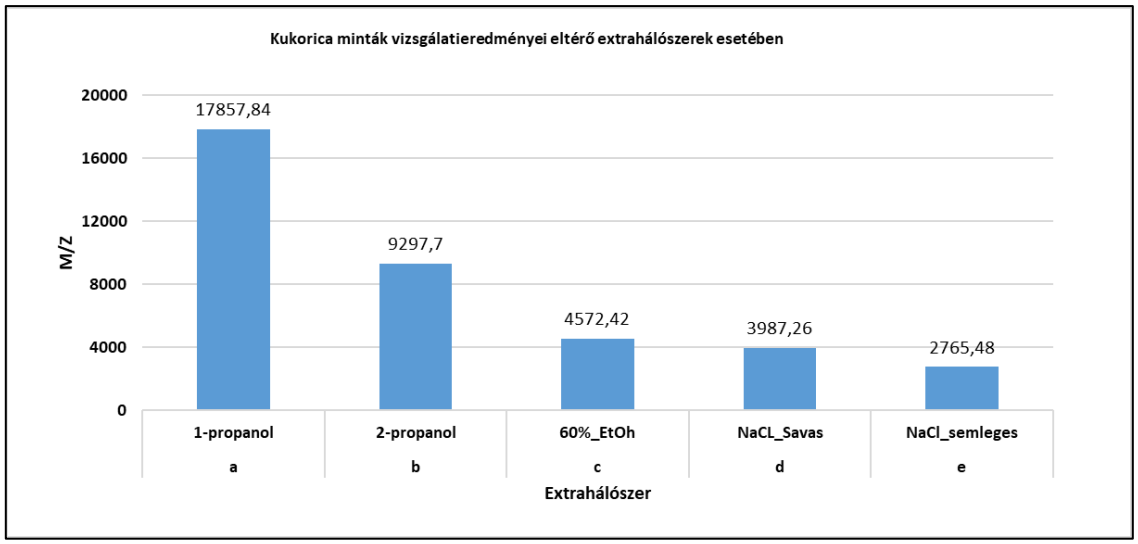


FIGURE 5: TEST RESULTS OF MAIZE SAMPLES WITH DIFFERENT EXTRACTANTS

For the sunflower achene samples, the 2-propanol isolate gave the best M/Z value (22056.78) among the five extractants, which was also significantly different ($p<0.05$) when tested on $LSD_{5\%-}$ compared to the 60% EtOH, 1-propanol, NaCl neutral and NaCl acidic isolates (Figure 6). This shows that not all extractants are suitable for the analysis of a given plant species.

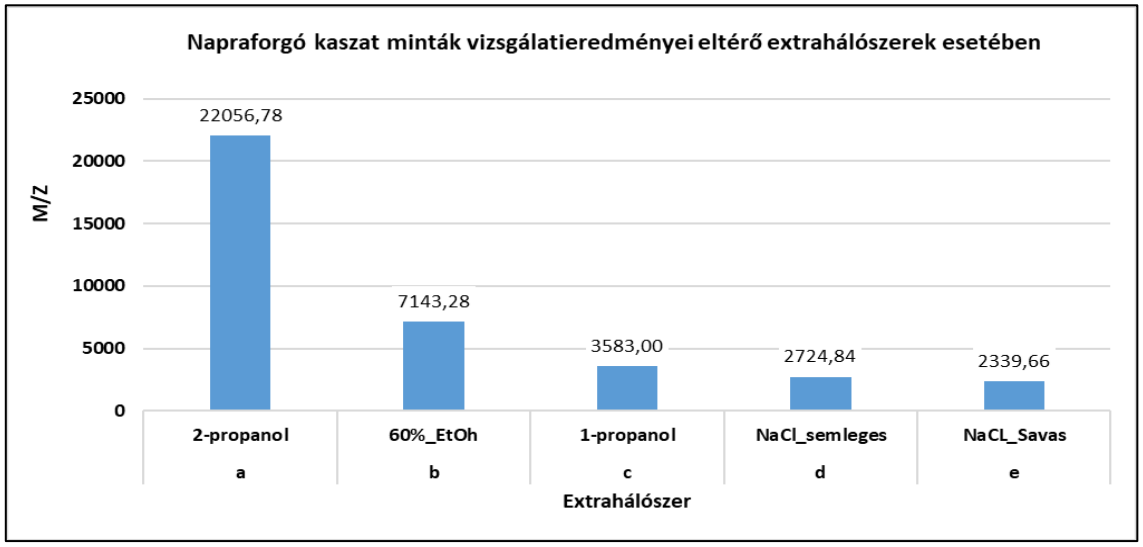


FIGURE 6: TEST RESULTS OF SUNFLOWER ACHENE SAMPLES WITH DIFFERENT EXTRACTANTS

For soybean samples, both NaCl acid and NaCl neutral isolates gave high M/Z values (18028 and 17902), while 1-propanol and 2-propanol and 60% EtOH gave significantly the lowest ($p<0.05$) M/Z values compared to the two NaCl extractants (tested on $LSD_{5\%}$) (Figure 7)

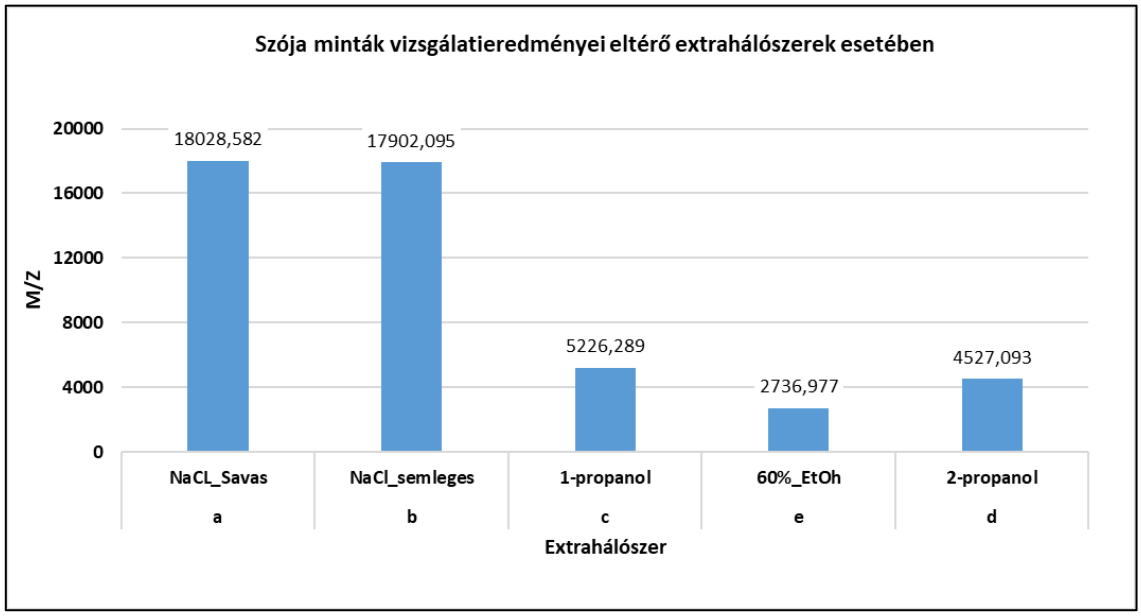


FIGURE 7: TEST RESULTS OF SOYBEAN SAMPLES WITH DIFFERENT EXTRACTANTS

3.3. Correlation between the extractant and the M/Z value examined via Pearson analysis

Further analysing the results obtained, we sought to determine the suitability of a given isolate for the determination of protein markers of the field crop under study. The correlation was tested using Pearson's analysis and the results showed that for all the crop samples tested, a strong relationship between a particular extractant and M/Z value was observed, which may contribute to the refinement of the new method (Table 6-9). The analysis confirmed that the M/Z value of wheat is best determined with 60 EtOH, maize with 1-propanol, sunflower crops with 2-propanol, while for soybean with the two NaCl extractants, which expresses the time of flight mass of proteins, showing a strong relationship for all four plant species (Wheat 0.661**, Maize 0.600**, Sunflower 0.634**, Soybean 0.656**).

TABLE 6: PEARSON'S ANALYSIS OF THE CORRELATION BETWEEN THE EXTRACTANT USED FOR THE WHEAT SAMPLE AND THE M/Z VALUE

Extractants used for wheat sample	60% EtOH	NaCl acidic	2-propanol	1-propanol	NaCl neutral
M/Z	**0.661****	0.197	0.152	0.129	0.106

** Correlation significant at 1% level; * Correlation significant at 5% level

TABLE 7: PEARSON'S ANALYSIS OF THE CORRELATION BETWEEN THE EXTRACTANT USED FOR THE MAIZE SAMPLE AND THE M/Z VALUE

Extractants used for maize samples	1-propanol	2-propanol	60% EtOH	NaCl in acidic	NaCl neutral
M/Z	**0.600****	0.193	0.157	0.134	0.112

** Correlation significant at 1% level; * Correlation significant at 5% level

TABLE 8: PEARSON'S ANALYSIS OF THE CORRELATION BETWEEN THE EXTRACTANT USED FOR THE SUNFLOWER SAMPLE AND THE M/Z VALUE

Extractants applied to sunflower samples	2-propanol	60% EtOH	1-propanol	NaCl neutral	NaCl acidic
M/Z	**0.634****	0.197	0.163	0.127	0.122

** Correlation significant at 1% level; * Correlation significant at 5% level

TABLE 9: PEARSON'S ANALYSIS OF THE CORRELATION BETWEEN THE EXTRACTANT USED FOR THE SOYBEAN SAMPLE AND THE M/Z VALUE

Extractants used for soybean samples	NaCl acidic	NaCl neutral	1-propanol	60% EtOH	2-propanol
M/Z	**0.656****	**0.623****	0.177	0.125	0.169

** Correlation significant at 1% level; * Correlation significant at 5% level

4. NEW SCIENTIFIC RESULTS

1. Maldi-TOF MS Reserve Protein Create a mass spectrum-based database for genetic purity of 10-10 wheat, corn, sunflower and soy seeds, from which the genetic homogeneity of the items tested can be retrieved at any time and can be compared for years.
2. The method is cost -effective and environmentally gentle than the methods used to date. Maldi-TOF MS technology allows you to measure 102 or 362 samples in a short period of time, depending on the type of device.
3. During our work, not only hybrids but also parent lines were examined. This is of great importance in the fact that when producing hybrid seeds, parental lines are also homozygot and genetically clean, as this is one of the important conditions for the genetic homogeneity of the hybrid type.
4. During the analysis of the Maldi-TOF MS MS MS, it has been proven that they can be evaluated in the 2000-2500 m/z range when examining reserve proteins with a molecular weight of 2-30 kD.
5. Our research results prove that the Maldi-TOF MS spare protein mass spectra are capable of examining genetic homogeneity of various self-fertilized plant species in the range of 1-25000 m/z.

5. PRACTICAL RESULTS

1. In the case of corn, wheat, sunflower, and soybean seeds, the method we developed can be used in seed certification, depending on the method of sample preparation. We recommend grinding samples of different seed types of the examined plant species before the analyses.
2. In the case of plant species with a high oil/fat content, the results of the homogeneity test are affected by the oil and/or fat content, therefore, after grinding, the sunflower and soybean seeds must be degreased with a 250 µl ml hexane solution. In the absence of this, the obtained results do not reflect reality.
3. Based on the analyses, the SA matrix (at a concentration of 3mg/250µl) or the HCCA matrix (at a concentration of 2,5mg/250µl) are the most suitable for testing the reserve proteins of the mentioned plant species.

BIBLIOGRAPHY

1. *DIECKMANN, R. - HELMUTH, R. - ERHARD, M. - MALORNY B.* (2008): Rapid Classification and Identification of Salmonellae at the Species and Subspecies Levels by Whole-Cell Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. Applied and Environmental Microbiology. 74 (24): 7767-7778.
2. *HTTP4*: https://pubsapp.acs.org/subscribe/archive/tcaw/12/i09/pdf/903chemchron.pdf (letöltve: 2019.11.15)
3. *HTTP7*: R CORE TEAM. (2022) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/ (letöltve: 2020.09.10)
4. HUZSVAI L. – BALOGH P.: 2015. Lineáris modellek az R-ben. Seneca Books, Debrecen. 28-34; 109–124. http://seneca-books.hu/doc/Linearis_modellek.pdf (letöltve: 2020. 09.10)
5. *KARAS, M. – HILLENKAMP, F* (1988): Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons, Anal. Chem., 60 (20): 2299-2301.
6. *SIGNOR, L. - ERBA, E. B.* (2013): Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometric Analysis of Intact Proteins Larger than 100 kDa. Journal of Visualized Experiments. 79: 1-7.
7. *ZIMMERMANN, S.* (2015): Modern Techniques for pathogen detection. Markono Print Media Pte Ltd. Singapure. 221-222.

PUBLICATIONS OF THE CANDIDATE IN RELATION TO THE SUBJECT OF THE THESIS

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK//2022.PL
Subject: PhD Publication List

Candidate: Csilla Bojté
Doctoral School: Kálmán Kerpely Doctoral School
MTMT ID: 10056782

List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (3)

1. **Bojté, C.**, Helenkár, A., Nagy, N. E., Tóth, B., Hajósné Novák, M., Micsinai, A., Parádi, I., Szemán-Nagy, G., Tálas, L., Bojtor, C., Nagy, J.: Búza (Triticum aestivum L.) vetőmagok minősítése MALDI-TOF MS módszerrel.
   Növénytermelés. 70 (1), 45-57, 2021. ISSN: 0546-8191.
2. Kaszab, E., Háhn, J., **Bojté, C.**, Jiang, D., Kriszt, B., Szoboszlay, S.: Rhodococcus típustörzsek atrazine biodegradációjára utaló biomarkerinek azonosítása MALDI-TOF MS módszerrel.
   Ökotoxikol. 2 (2), 15-16, 2020. EISSN: 2732-2556.
3. **Bojté, C.**, Lovász, C., Lajkó, L., Tímár, E., Miccsinai, A., Tóth, S. Z.: MALDI-TOF módszer alkalmazhatóságának vizsgálata napraforgó genetikai azonosság vizsgálatokban.
   Agrártud. Közl. 72, 33-38, 2017. ISSN: 1587-1282.

Foreign language scientific articles in Hungarian journals (1)

4. **Bojté, C.**, Lovász, C., Tímár, E., Lajkó, L., Nagy, J., Nagy, N. E., Bodnár, K. B., Czerődiné Kempf, L., Horváth, B., Micsinai, A., Tóth, S. Z.: Development of seed analyses by means of various matrix solutions and the MALDI-TOF MS technique.
   Agrártud. Közl. 2019 (1), 53-57, 2019. ISSN: 1587-1282.
   DOI: http://dx.doi.org/10.34101/actaagrar/1/2370

Hungarian conference proceedings (1)

5. Hajósné Novák, M., Daood, H. G., **Bojté, C.**: A klasszikus mutációs szójanemesítés (Glycine max. L Merrill) eredményei a SZIE genetikai és biotechnológia intézetében az 1990-es és 2010-es évek között.
   In: Növénynemesítés a 21. század elején: kihívások és válaszok : XXV. Növénynemesítési Tudományos Nap 2019. Szerk.: Karsai Ildikó, MTA Agrártudományok Osztályának Növénynemesítési Tudományos Bizottsága, Budapest, 313-317, 2019. ISBN: 9789638351456

Egyetemi és Nemzeti Könyvtár

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Hungarian abstracts (4)

6. Hajdú, E., Hajósné Novák, M., Szűcsné Varga, G., Vinogradov, S., **Bojté, C.**: A C. 50x Cserszegi fűszeres hibridpopuláció morfológiai és termesztési értékeinek értékelése.
In: XXVI. Növénynemesítési Tudományos Napok : összefoglaló kötet. Szerk.: Bóna Lajos, Karsai Ildikó, Matuz János, Pauk János Polgár Zsolt, Veisz Ottó, Gabonakutató Nonprofit Közhasznú Kft., Szeged, 85, 2020. ISBN: 9789635089307

7. **Bojté, C.**, Bojtor, C., Hajósné Novák, M., Illés, Á., Horváth, B., Lovász, C., Nagy, J., Czerődiné Kempf, L., Nagy, N. E., Tímár, E., Micsinai, A.: A szója (Glycine max L.) vetőmagok genetikai homogenitás vizsgálata Maldi-TOF Készülékkel.
In: Hungalimentaria 2019 : "Ésszel a kosárba" : konferencia és kiállítás, Wessling Hungary Kft., Budapest, 24-25, 2019.

8. **Bojté, C.**, Lovász, C., Tímár, E., Lajkó, L., Lukács, J., Micsinai, A., Tóth, S. Z.: Minta-előkészítési eljárások fejlesztése napraforgó vetőmagok MALDI-TOF MS vizsgálatára.
In: Tavaszi szél konferencia 2018 Nemzetközi multidiszciplináris konferencia : Absztraktkötet. Szerk.: Keresztes Gábor, Doktoranduszok Országos Szövetsége, Budapest, 65, 2018. ISBN: 9786155586262

9. **Bojté, C.**, Lovász, C., Tímár, E., Lajkó, L., Lukács, J., Micsinai, A., Tóth, S. Z.: Napraforgó magvak vizsgálata MALDI-TOF MS módszerrel.
In: XXIV. Növénynemesítési Tudományos Nap : Összefoglalók. Szerk.: Karsai Ildikó, Polgár Zsolt, Keszthelyi Burgonyáért Egyesület, Keszthely, 68, 2018. ISBN: 9786150014692

Foreign language abstracts (2)

10. Kaszab, E., Dongze, J., **Bojté, C.**, Micsinai, A., Kriszt, B., Szoboszlay, S.: The application of maldi-tof ms in the characterization of opportunistic pathogen pseudomonas aeruginosa.
In: A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium : Absztraktfüzet, Magyar Mikrobiológiai Társaság, Keszthely, 16, 2008.

11. **Bojté, C.**, Lovász, C., Tímár, E., Lajkó, L., Lukács, J., Micsinai, A., Tóth, S. Z.: Homogenity testing of sunflower (Hellianthus annuus) seeds by MALDI-TOF MS technique.
In: Fiatal Biotechnológusok Országos Konferenciája. Szerk.: Tamás László, Zelenyánszki Helga, JATEPress, Szeged, 112, 2018. ISBN: 9789633153703



UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

Hungarian scientific articles in Hungarian journals (1)

12. Nagy, N. E., **Bojté, C.**, Tatárvári, K.: Eltérő szójatermesztési technológiák összehasonlító elemzése az igen korai éréscsoportban.
Gradus. 6 (2), 47-52, 2019. ISSN: 2064-8014.

Foreign language scientific articles in Hungarian journals (1)

13. Huzsvai, L., Fejér, P., Illés, Á., Bojtor, C., **Bojté, C.**, Horváth, É., Demeter, C.: Analysis of sweet corn nutritional values using multivariate statistical methods.
Agrártud. Közl. 1, 103-108, 2021. ISSN: 1587-1282.
DOI: http://dx.doi.org/10.34101/ACTAAGRAR/1/8587

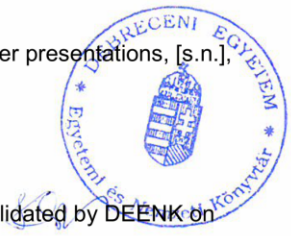
Hungarian conference proceedings (2)

14. Nagy, N. E., Popovics, T., **Bojté, C.**, Tatárvári, K.: Napjainkban elfogadott helyes agrotechnológia hatása a közép-magyarországi régióban termesztett gmo mentes szójabab termésátlagára, fehérje- és olajtartalmára.
In: XXXVII. Óvári Tudományos Napok, 2018. november 9-10. : Fenntartható agrárium és környezet, az Óvári Akadémia 200 éve - múlt, jelen, jövő. Szerk.: Szalka Éva, VEAB Agrártudományi Szakbizottság, Széchenyi István Egyetem Mezőgazdaság- és Élelmiszertudományi Kar, Mosonmagyaróvár, 302-308, 2018. ISBN: 9786155837159

15. Nagy, N. E., Pepó, P., Tatárvári, K., **Bojté, C.**: Szójatermesztésben alkalmazható csávázó- és oltóanyagok hatása a termésátlagra és beltartalomra kisparcellás körülmények között.
In: XXXVII. Óvári Tudományos Napok, 2018. november 9-10. : Fenntartható agrárium és környezet, az Óvári Akadémia 200 éve - múlt, jelen, jövő. Szerk.: Szalka Éva, VEAB Agrártudományi Szakbizottság, Széchenyi István Egyetem Mezőgazdaság- és Élelmiszertudományi Kar, Mosonmagyaróvár, 158-165, 2018. ISBN: 9786155837159

Foreign language abstracts (1)

16. Illés, Á., Bojtor, C., **Bojté, C.**, Nagy, J.: Effects of biostimulant foliar fertilization on maize (Zea mays L.) hybrid zinc content and yield parameter.
In: 4th Biostimulants World Congress : Abstract book for Oraland poster presentations, [s.n.], [Barcelona], 203, 2019.



The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

25 May, 2022