Animal Species Identification of Meat using MALDI-TOF Mass Spectrometry

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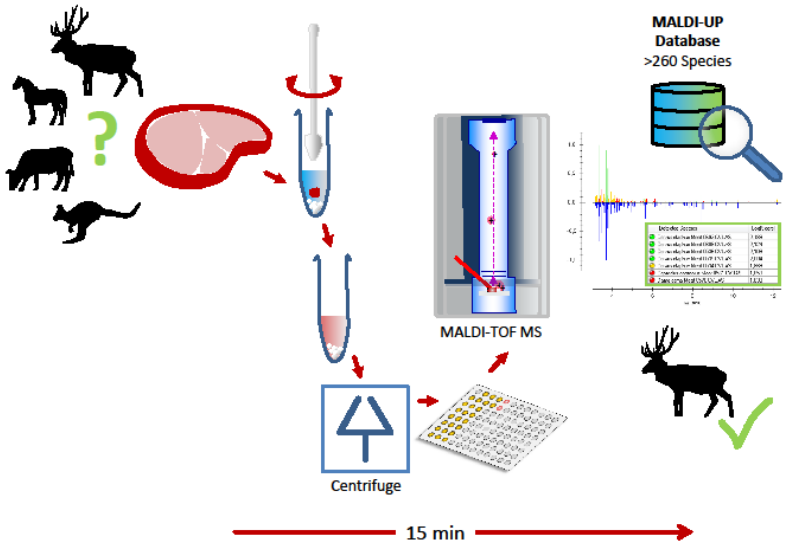
Highlights

- MALDI-TOF MS enables rapid and reliable animal species identification of muscle meat
- The most important animal species for human consumption are covered by the method
- Database with more than 260 confirmed different animal species
- The validated method is easily transferable in laboratories with existing equipment
- Spectra exchange is facilitated by the MALDI-user platform https://maldi-up.ua-bw.de



Abstract

One of the main topics of food control for meat, seafood or milk products is the detection of undeclared substitution with regard to the animal species. For this purpose, the potential of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has already been demonstrated in principle. In our study, for the meat from pig, cattle, goat, sheep, horse, turkey, and chicken, we validated the animal species identification by MALDI-TOF MS as an easy, fast and reliable tool, which is now an integral part of our official food analysis. Using a simplified extraction and the Bruker MALDI-Biotyper system, we generate a MALDI-TOF MS database, which combines more than 550 reference spectra of muscle meat from more than 260 confirmed different animal species. In order to speed up database expansion, we offer the spectra generated via the MALDI user platform MALDI-UP for exchange with other laboratories (https://maldi-up.ua-bw.de).

Keywords: Meat authentication; MALDI-TOF MS; food fraud; consumer protection; animal species; database; validation; MALDI-UP

1. Introduction

Incorrect declaration and adulteration of food is a relevant issue of consumer protection at every level of the food chain (Wisniewski & Buschulte, 2019; European Commission, 2019). Food fraud unsettles consumers and enforces focused control activities of the competent authorities (Everstine et al., 2013; Rahmati et al., 2016; European Commission, 2015). In particular, high-value ingredients were substituted by cheaper alternatives without declaration. Therefore, high-priced food of animal origin is affected by fraudulent intentions of manufacturers, suppliers or restaurant owners most frequently (Everstine et al., 2013; European Commission, 2019; Wisniewski & Buschulte, 2019). Meat and meat products from mammals represent one of the most valuable food categories. In 2013, German consumers spent on average 16.6% of food expenditure on meat and meat products with annual market value over 20 billion Euros (Statistisches Bundesamt, 2016). Legislation in the EU provides clear rules for the declaration of the animal species processed in food products (Regulation (EC) No 1169/2011). Recent scandals concerning horsemeat, game meat, or other cases show the enormous uncertainty for consumers, accompanied by a loss of trust in authorities and industrial food business (Everstine et al., 2013; Bayrischer Landtag, 2008). Hence, fraudulent supplementation or substitution of the declared meat has been a recurrent challenge for many years, arousing wide media attention when longer supply chains are affected (Everstine et al., 2013; Rahmati et al., 2016). Furthermore, fraudulent declaration occurs often in the later food supply chain, in particular in unpacked products sold directly or in ingredients used in gastronomy. In order to counteract such widespread activities effectively, food control laboratories require rapid, reliable, easy to use, and cheap tools for the authentication of foodstuffs with high throughput possibilities.

A wide range of analytical methods is available for animal species identification in food. These are mainly comprised of DNA-based techniques, immunological and chromatographic methods with different detectors, including mass-spectrometry (Li et al., 2020; Waiblinger et al., 2017; Iammarino et al., 2016; Rahmati et al., 2016; von Bargen et al., 2014). Commonly, the focus of these approaches is on the detection of specific marker molecules, which enables qualitative species identifications (Marbaix et al., 2016; von Bargen et al., 2014; Waiblinger et al., 2017; Skouridou et al., 2019). Other techniques, such as sequencing of marker genes or recently complex "metabarcoding" by combination of information from several discriminative genes, are time consuming (>8 h) and require trained personnel and/or expensive materials (Kumar et al., 2015; Staats et al., 2016).

In the last years, methods based on mass spectrometry were developed to identify animal species in meat-based products by analyzing their proteins (Verma & Ambatipudi, 2016; Ortea et al., 2016; von Bargen et al., 2014). Generally, these methods combine a chromatographic separation of trypsin digested protein extracts with the detection of specific target peptides using MS (Marbaix et al., 2016; von Bargen et al., 2014). Alternatively, the protein/peptide mass fingerprints are analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technique has been widely established in laboratories for food analysis to identify microorganisms (Pavlovic et al., 2013; Quintela-Baluja et al., 2014). Furthermore, MALDI-TOF MS has been demonstrated to be a suitable tool for the detection of animal species of scallops, shrimps, fish, cheese, edible insects, gelatine, and also meat (Stephan et al., 2014; Stahl & Schröder, 2017; Rau et al., 2020; Ulrich et al., 2017; Flaudrops et al., 2015; On, 2016; Pavlovic et al., 2020).

According to our postulation, MALDI-TOF MS can be used as an easy and robust technology for rapid and reliable animal species identification of skeletal muscle meat in a food control laboratory. Starting from previous feasibility studies (Stoll & Rau, 2015; Hiller et al., 2017) we have extensively expanded our in-house meat database in terms of the number of animal species and the number of reference materials used for validation. By skipping any additional digestion step for sample preparation and using device settings common for microorganisms, a comprehensive reference spectra database for muscle meat in a wide range of species was created for the Bruker MALDI-Biotyper. Using the concept described by Rau et al. (2016b) this meat database was extensively validated for the identification of several animal species of relevance in human nutrition. The suitability of this rapid method for the routine food control as well as the influence of commonly used food-processing technologies, such as heating and freezing, were shown. The workflow from sample preparation to result can be easily adapted and established in a laboratory with basic experience in MALDI-TOF MS. In order to allow the exchange of database entries among interested users, additional information to each reference spectra is listed on the MALDI-UP page (https://maldi-up.ua-bw.de) (Rau et al., 2016a).

2. Materials and Methods

2.1 Sample collection

A collection of 1088 raw animal flesh samples were received in majority from veterinary pathology units and governmental food control laboratories of several institutes in Germany: the Chemical and Veterinary Analysis Agencies (CVUA) Stuttgart, Karlsruhe, Krefeld and Freiburg, the Bavarian Health and Food Safety Authority (LGL), Erlangen, and the Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin. In addition to domestic animals, these institutes receive samples from different zoos or other owners of exotic animals. The

collection included material from 132 mammalian species, 115 bird species and 18 reptilian species at the time of study. A selection of spectra from 527 independent muscle samples, comprising 320 from mammals, 187 from birds and 20 from reptiles, was consolidated to the MALDI-meat reference database (Table 1). Overall, 1088 samples were integrated in the validation part of the study (Supplement 1). Immediately after gross pathology, or in the case of food samples, immediately after the initial organoleptic analysis has been completed, meat samples were frozen at -18°C (+/- 2°C) until preparation for MALDI-TOF MS.

2.2 Organic solvent sample preparation (OSextr)

Proteins were extracted from meat according to Post & Dikler, 2010, with a modified organic solvent protocol described previously (Stoll & Rau, 2015; Rau et al., 2020). Each sample was prepared at least in duplicate, unless otherwise noted. A short protocol of this sample preparation is available on the MALDI-UP homepage (Dyk et al., 2020). The MALDI-TOF MS measurement of the spot yielding the higher score value for the identification was considered for further evaluation.

2.3 Effect of Freezing and Heat-treatment on MALDI-TOF MS spectra

To analyze potential effects of freezing on spectra, a set of samples were analyzed both fresh and after long-term freezing. Fresh meat (pork, beef, chicken, turkey) was prepared directly after purchasing with OSextr as described above and the MALDI-TOF mass spectra were acquired. A portion (about 20 g) of each sample was frozen at -18°C. After storage for 54 month, mass spectra of these samples were taken and compared with the initial mass spectra.

To investigate the feasibility of MALDI-TOF MS to identify the animal species of the meat sample after exposure to high temperatures during food preparation like cooking and roasting, spectrum analysis was carried out with samples from the same four animal species as in the

freezing test. The meat pieces were cut into two portions (approx. 100 g each). One 1 cm thick slice of every meat was boiled in water for 15 min. The second slice was roasted in a pan, three minutes per side, using a small amount of canola oil. After cooling down to room temperature ca. 20 g of heat treated samples comprising the surface as well as core meat were cut off and stored at -18°C until analysis. Sample preparation, measurement and identification were performed as described in the corresponding sections. This experiment was repeated three times.

2.4 MALDI-TOF MS measurement and analysis

The MALDI-TOF mass spectra was acquired by a microflex LT mass spectrometer (Bruker) using the manufacturer's software FlexControl (version 3.4) and the MALDI-Biotyper software (MBT, version 3.1) with the default parameter settings: positive linear mode, laser frequency 60 Hz, ion source 1: 20 kV, ion source 2: 18 kV; Bruker's MBT_FC and MBT_AutoX methods; mass range: 2,000 – 20,000 Da. According to the manufacturer's instructions, the Bruker IVD bacterial test standard (BTS) was used for mass-calibration (c.f. Rau et al., 2020).

2.5 Generation of the MALDI-TOF MS meat database

Reference entries were created and evaluated in accordance with the basic manufacturer's instructions (Pranada et al., 2016). Briefly, the protein extract from a meat sample was spotted on eight spots and measured in triplicate to create at least 24 raw spectra for one sample. Control and processing of raw spectra was done in the FlexAnalysis software (version 3.4), and reference main spectra (MSP) were calculated by the "Biotyper MSP Creation Standard Method" in the MBT software package as described previously (Rau et al., 2020). Thes reference entries for meat were organized in the project folder of the MBT database module

158 (Biotyper OC 3.1). Detailed information about the generated reference entries are listed in
159 Supplement 1, and on MALDI-UP (https://www.maldi-up.ua-bw.de).
160
161 The most prominent and common *m/z* signals in terms of intensity are collected in peak lists
162 and form the signal fingerprint for the respective meat type. Several average *m/z* signals for
163 meat of major farm animals (pig, cattle, sheep, goat, horses, chicken, and turkey) are shown in
164 Supplement 2.

165 *2.6 Identification criteria*

166 The same procedure used for the identification of microorganisms was performed for the
167 identification of meat by MALDI-TOF MS (Pranada et al., 2016). Briefly, using a pattern
168 matching approach, including signal position and intensity, MBT software compares sample
169 mass spectra with the MSPs present in the database. A hit list is generated with the best
170 matching MSPs in descending order, expressed in terms of a log-score value (Pranada et al.,
171 2016). For identification of meat, only the first two hits are taken into account: A sample is
172 regarded as identified if the first hit has a score value $\geq$2.0 and the species of the second hit
173 (score $\geq$2.0) agrees with that of the first one. If these criteria are not met, the sample is not
174 considered as identified.

175 *2.7 Validation study*

176 The validation of the animal species identification by MALDI-TOF MS follows a parameter-
177 based concept used for microorganisms and cheese (cf. Rau et al., 2016b, Rau et al., 2020). In
178 the first step, the identification rate of the respective parameter, that means the ratio of
179 identified samples to all probed samples, is calculated. To assess the significance of an
180 identification result related to the parameter, a simple validation procedure based on the true
181 positive rate (TPR) and the true negative rate (TNR) is applied. If the identification result of a

meat sample is in accordance with the expected animal species, the result is considered to be true positive (TP). To test a parameter of interest (e.g. chicken - *Gallus gallus*), a control group was defined, comprising all meat samples other than the respective parameter (meat, but not chicken). A result is regarded as false positive (FP) if the spectra of a sample within this control group is identified as the parameter of interest. All other identified samples of the control group were considered as true negative (TN). The TPR is calculated as the ratio of the number of TP to the number of all samples of the parameter with an identification result. Analogue, the TNR is calculated as the ratio of the number of TN to all samples of the control group with an identification result. Depending on sample availability, the minimum of 20 independent valid assigned sample materials for a parameter were used to test the complete system, consisting of mass spectrometer and database.

3. Results

3.1 MALDI-TOF MS meat reference database

MALDI-TOF MS systems are commonly used to identify microorganisms. The identification is based on the mass spectral comparison of protein and peptides fingerprints of a sample with those in a suitable database. As proteins are the main component in muscle tissue, the method has been shown to be applicable for species identification of meat and protein from several animal orders (Ulrich et al., 2017; Stephan et. al., 2014; Stahl & Schröder, 2017; Flaudrops et al., 2015). The aim of this study was to test feasibility of MALDI-TOF MS to identify animal species of muscle meat in routine food control. Indispensable for species identification using MALDI-TOF MS is the existence of a database containing appropriate mass profiles (mass lists or MSPs) for the species of interest. Until now, there is no commercial or public meat database available. Therefore, the in-house meat database generated for the previous studies was expanded (Stoll & Rau, 2015; Hiller et al., 2017). Using the OSextr protocol without

tryptic digestion we have obtained species-specific mass profiles of meat of more than 260 animal species. Typical single mass-spectra of skeletal muscle from pig, cattle, sheep, goat, horses, chicken and turkey are shown in Figure 1. Even though the mass-accuracy of the MALDI-TOF MS system used is limited, it is sufficient to define tolerant windows for relevant *m/z* signals. Therefore, both *m/z* signals common to more than one animal species as well as species-specific signals were detected (Supplement 2). Such *m/z* signals are the backbone of signal pattern of reference MSPs.

In order to cover the diversity within a species, for each species several MSPs from different individuals, if available, were created (Supplement 1). Considering the demands of a food control laboratory, we focused on sampling of the skeletal muscles commonly used in food production. As shown in Figure 1g) and 1h) for turkey leg and breast, mass spectra vary slightly even if the samples were taken from the same animal, but from different skeletal muscle parts. To facilitate the correct identification, MSPs of different meat types were integrated in the database.

Currently (as of May 2020), this database contains more than 520 reference entries of meat from 265 different animal species (Table 1). This collection includes MSPs of the major livestock animals (cattle, n=21; pig; n=23; horses, n=19; sheep, n=8; goats, n=8; chicken, n=12; turkey, n=9) from different muscle parts and aging stages, some other MSPs of meat from animal species of minor relevance to the European nutrition (deer, hare and rabbit, ducks and geese), as well as ‘exotic’ animals (ostrich, kangaroos, ‘camels’, zebras, ‘antelopes’, ‘crocodiles’, guinea-pig) and more than 150 other species for comparison, among them some consumed in several regions of the world (Supplement 1). For an overview a selection of MSPs of meat from 40 animal species covering a wide taxonomic range were compiled in a dendrogram (Figure 2a). It demonstrated three clearly separated main branches for mammals,

birds and reptiles. Additionally, two detailed dendrograms were created for the taxonomic groups of the subfamily *Bovidae,* including cattle (*Bos taurus*) and Asian water buffalo (*Bubalis bubalis*), and the family of *Anatidae,* including domestic (mallard-)duck (*Anas platyrhynchos*), Muscovy duck (*Cairina moschata*) and domestic goose (*Anser anser*) (Figures 2b, and 2c).

3.2 Validation of the database

To verify the reliability of the identifications using the database, a validation procedure was conducted according to the concept of Rau et al., 2020. The collection of raw flesh samples with reliably confirmed species names were provided by our project partners from veterinary pathology as well as official food control laboratories. So, 1088 meat samples were prepared as described in "methods". The MALDI-TOF mass spectra were generated and identified using the MBT in combination with the meat database.

The validation study focused on the major relevant livestock animals: for pork samples 96.3% (n=109) were correctly identified with a score value >2.0 (Supplement 1; Table 2). Analogously, 88.0% of beef (n=92), 100% of horses (n=35; including nine species), 86.4% of chicken (n=81), and 86.7% of turkey meat (n=45) were identified correctly without false identifications. Meat from sheep and goats have similar *m/z* patterns (Supplement 2), therefore, the rate of samples which fulfill the criteria for identification was reduced to 72.0% for sheep (n=75), and 93.3% for goats (n=30; including three species). Since all identification-results showed the expected species, these results are also reliable, regardless of the proportion of technically successful identifications.

Due to the limited availability of samples, several rare animal species were combined and validated as family-level parameter. For these taxonomic families the following identification

rates were achieved: deer (*Cervidae*, including nine species) 97.6% (n=85), hares (family of *Leporidae*, including three species) 100% (n=31), kangaroos (*Macropodidae*) 100% (n=21), and ducks and geese (*Anatidae*) 97.9% (n=47) (Table 2). For these families no false identification was obtained. For all species and family groups the control groups reveal identification rate higher than 95%, and no false positive identification was obtained (Table 2). The score values achieved for meat between 2.001 and 2.806 were comparable with those in the identification of microorganism or for cheese with the Bruker MBT-system (Rau et al., 2016b; Rau et al., 2020).

3.3 Storage conditions

The effects of frozen storage on the spectrum were evaluated. Comparison of the matching scores of spectra of pork, beef, chicken and turkey generated before storage (2.261, 2.318, 2.419 and 2.257, respectively) with those from the same material after storage at -18°C for 54 month (2.296, 2.443, 2.509, 2.371, respectively) revealed no significant changes in the protein profile. That indicates that freezing and frozen storage at -18°C is an appropriate method to preserve meat material for MALDI-TOF MS analysis.

3.4 Identification of animal species of meat after heat-treatment

Food samples originating from gastronomy represent a significant part of official food inspection. Meat samples arrive in food control laboratories in different conditions: raw or prepared for ready-to-eat, with or without preservation, such as cooking, roasting, curing or salting. Reference spectra are mainly based on raw material, therefore, the effect of cooking and roasting on identification performance was investigated using meat of four different animal species. After roasting or cooking the species of all meat samples was identified correctly with moderately reduced score values for the first hit compared with the raw control sample (Supplement 3). Only burnt meat could not be assigned.

4. Discussion

Economically motivated food fraud is worrying consumers and occupying consumer protection authorities and food inspection laboratories all over the world (European Commission, 2015; Rahmati et al., 2016; Everstine et al., 2013). The price-determining components, such as meat and dairy protein, were most frequently affected (Wisniewski & Buschulte, 2019). The most prominent, economically motivated food fraud case in the meat sector was the horsemeat in lasagna in 2013. In addition, various incidents with national attention such as the game meat scandal in Germany contributed to consumer confusion (Bayrischer Landtag, 2008; On, 2016).

To detect the animal species of meat containing food DNA-based and immunological methods are the prevalent techniques (Waiblinger, 2017; Li et al., 2020; Rahmati et al., 2016). However, they are either time consuming or associated with high costs caused by commercial kits. Protein or peptide analysis using mass spectrometry gives a different approach for inspection of protein-rich food (Ortea et al., 2016). Over the past years, MALDI-TOF MS has been established in many food-microbiology laboratories for the routine identification of microorganisms (Quintela-Baluja et al., 2014; Pavlovic et al., 2013; Ulrich et al., 2016). This technique has been applied to species differentiation of seafood, fish and fungi (Stephan et al., 2014; Stahl & Schröder, 2017; Pavlovic et al., 2020), as well as gelatine and meat (Flaudrops et al., 2015; Hiller et al., 2017; On, 2016). Flaudrops and co-workers demonstrated the differentiation of a small number of meat samples from different animal species using the MALDI-TOF MS Biotyper platform. In that study the score based identification could not be established, therefore, a cluster-based approach was applied to a basic animal species differentiation of meat. Until now, an easy-to-use and comprehensive database for meat identification has not yet been commercially available.

One of the official food control activities is labelling control. For this purpose, we focused on the examination of the animal species of meat from livestock animals with strong market presence. In order to establish a simple and rapid protocol for protein profiling of meat we facilitated sample preparation procedures used in other studies by skipping the tryptic digestion and were able to generate species-specific mass signal patterns using Bruker MALDI-Biotyper system for all investigated animals. This direct extraction protocol reduces the analytical costs and the total performing time to 20 minutes from sample preparation to reliable identification result.

The first part of the study covers the generation of a representative reference spectra collection (MSP-database) using a standardized protein extraction method. Subsequently this in-house database is validated using the concept introduced by Rau et al. (2016b). In the last step we verify the applicability of the method for meat samples after common food preparing procedures, such as freezing, cooking and roasting.

4.1 Reference database

The most important key to the species identification using MALDI-TOF MS or other fingerprinting technologies is the database used, containing appropriate mass profiles (mass lists or MSPs), to compare the resulting sample spectra. By means of MALDI-TOF MS, meat from the major livestock animals can be clearly distinguished by several species-specific *m/z*-signals (Supplement 2, Figure 1 a-h). Using the OSextr protocol, a simplified procedure without tryptic digestion, we have obtained species-specific mass profiles of meat of more than 260 animal species (Supplement 1). Furthermore, the results of the identification via the Biotyper algorithm and the MSP-dendrogram highlights the specificity of the MALDI method for the analyzed meat samples (Figure 2 a-c). Consequently, all reference spectra of skeletal

muscle meat were compiled in the same database. This collection overcomes the current lack of a commercial or public meat database for routine analysis.

On (2016) observed an acceptable change in the MALDI-TOF MS spectra of three animal species after storage at -20°C for 2 months. Our investigation proved that the influence of freezing and long-term frozen storage on the species identification by MALDI-TOF MS is negligible. This also provides an easy and suitable way to conserve reference material with respect to its quality. Consequently, the majority of the samples used in this study were stored frozen and catalogued in the MALDI-UP list for further scientific exchange.

For the major livestock species, e.g. pork (*Sus scrofa)* or cattle *(Bos taurus*), a number of reference spectra from independent individuals exist. If the information on the variances of races and age of the animals is available, the respective variability is covered. A further point to round off this database is the integration of spectra of meat at different maturation stages to mirror proteolytic changes during ripening (Lametsch et al., 2002) (Supplement 2).

Turkey meat from breast and leg are examples of the similarity in protein mass-spectra of different skeletal muscle (Fig. 1g and 1h). Despite variations, the *m/z* profiles of these samples are clearly assigned to the animal species. The differences in the spectra for leg and breast meat could be used to distinguish between these qualities (On, 2016), if both the corresponding reference spectra and a targeted validation based on reliable materials are available.

4.2 Validation

A few previous studies have described the applications of MALDI-TOF MS combined with their own databases and methods to meat animal differentiation on a small scale and/or

regarding specific issues (Flaudrops et al., 2015; On, 2016). The focus of the current work was on the validation of the whole system, consisting of the Bruker MALDI-Biotyper combined with the own meat-database, for routine use in an official food control laboratory. The validation followed the concept introduced by Rau et al. (2016b). Consequently, every parameter was evaluated separately, and the control group comprised in every case more than 900 spectra from a wide range of species (Table 2).

Using MALDI-TOF protein mass profiles, pork (*Sus scrofa*) can be clearly distinguished from meat from other animal species (Figure 1). More than 95% of all pork samples were identified correctly, no false positive result occurred for 979 single spectra from other animals (Table 2). High identification rates (>85%) were achieved also for beef (*Bos taurus*), meat from goats (*Capra* genus), horses (*Equus* genus), chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*), and in all cases no misinterpretation of a result was obtained, neither from the parameter itself nor from the extensive control group. In the case of meat from sheep, the rate of identified samples reduced to 72% due to the similarity of spectra with other members of the Tribus Caprini, nevertheless, successful identifications are in any case correct. (Supplement 2). Due to insufficient numbers of individual material available for validation the horses and the goats, the hares (family *Leporida*e), the deer (*Cervidae*), the kangaroos (*Macropodidae*), and the family of ducks and geese (*Anatidae*) were evaluated as groups (Table 2). The identification results obtained are also reliable. We thus concluded that for all meat categories investigated the in-house database reached sufficient identification rates. We also demonstrated that the species of the major meat categories relevant to the market could be reliably identified by MALDI-TOF MS. So far no false identifications occurred for all parameter validated (Table 2).

If reference spectra of meat from further animal species may be added and if the number of reliable sample spectra for validation can be increased, the following species and groups are expected to be better resolved per MALDI in future:

The differentiation of wild boar (*Sus scrofa scrofa*) from pork (*Sus scrofa domestica*) has not yet been successful using the simple evaluation techniques (Supplement 2).

Inside the subfamily Bovinae, meat spectra for the representatives of tribe Tragelaphini (Spiral-horned antelopes) were detached from the spectra derived from tribe Bovini (Bovinans) (Fig. 2b). Inside the Bovini, *Bubalus bubalis*, and *Syncercus sp.* were separated from the *Bison/Bos* group, which is in concordance with the affiliation to the genetically separated subtribe Bubalina. For the two genera from subtribe Bovina, *Bos* and *Bison*, a differentiation by MALDI failed (Supplement 2). Hassanin & Ropiquet (2004) interrogated the taxonomic classification of the subtribe Bovina using genetic sequence data and suggested that *Bos* and *Bison* should be regarded as a synonym of *Bos*. The close relationship and the derived taxonomic consequences are still under discussion (Zeyland et al., 2012).

Meat materials from major livestock species *Anatidae*, domestic goose, mallard, and Muscovy duck show significant differences in the spectra that resulted in separate branches in the MSP-cluster diagram (Fig 2c). However, the number of independent samples and the MSPs derived from them is still too small to identify the meat animal at species level.

4.3 Effect of common food preparing procedures

Meat is seldom eaten raw. To evaluate whether coagulation and chemical transformation of the proteins at high temperature could interrupt the animal species identification, MALDI-TOF MS profiles of meat samples from four animal species after roasting or cooking were acquired and their match scores were determined. All four meat species were successfully identified. Compared with the raw control sample the score values for the first hit of the cooked or roasted samples reduced moderately (Supplement 3). Only spectra derived from

burnt surface parts of roasted meat could not be identified for two of the four kinds of meat. This means that the heating process during food preparation does not significantly affect the animal identification of a heated meat sample by MALDI-TOF MS as long as it is not extreme.

As shown in our study, using the current procedure raw and heated meat can be assigned to the same animal species. Besides heating, there are other factors influencing the proteins in meat and the resulting spectra (On 2016; Flaudrops et al., 2015). Different skeletal muscle types (e.g. leg, breast) can be also recorded, especially if corresponding reference spectra are included in the compilation of the database. Further factors like quality defects (PSE and DFD) of meat, the influence of the slaughtering process and of course aging, either controlled such as dry aging or uncontrolled such as spoilage, is not covered by the current method completely. So far, offal was not considered, although the first database entries for heart, liver or kidney have already been created. Important food processing procedures, like salting and curing, also have to be evaluated.

4.4 Application

As sample preparation for MALDI-TOF MS takes only minutes, low-price reagents and small sample amounts are necessary, it is easy for a laboratory to handle large numbers of samples in a short timeframe at low cost. That is of special importance in times of crisis. Dual-use of the MALDI-TOF MS system with other applications, e.g., identification of microorganisms, cheese or fish (Rau et al., 2020; Stahl & Schröder, 2017), compensates the disadvantage of the expensive equipment. As shown in this study, the meat method has been validated for all animal species relevant to diet. An important limitation of this direct and rapid MALDI-TOF procedure is that only the animal species of the major meat component of a mixed sample is identified. Other mass peak evaluation methods or other elaborate mass-spectrometry

techniques were more promising to detect small amounts of meat admixtures in meat products like minced meat (von Bargen et al., 2014; Montowska & Spychaj, 2018; Prandi et al., 2016).

Easy sample preparation, analogue to known procedures, standardized settings of the system, widely used algorithms for the decision, and a transparent presentation of results and validation greatly facilitate the acceptance of MALDI-TOF MS in routine use. The method developed in this study has already been successfully implemented in routine food control for the identification of low processed meat (Gmeiner & Rau, 2020). The results give good reason to believe that further kinds of meat (exotic meat, game, etc.) can be identified with regard to the animal species by means of MALDI-TOF MS combined with a continuously expanding database. One of the key advantages of Bruker MBT is the easy exchange of mass spectra with other users of the same technique and device. In order to facilitate this beneficial exchange with other laboratories, a selection of our database reference entries and single spectra with additional information is listed on the MALDI-UP website https://maldi-up.ua-bw.de/ (Rau et al., 2016a).

4.5 Outlook

In addition to the verification of meat-declaration to uncover food fraud MALDI-TOF MS appears to be a suitable rapid high throughput technology to identify animal species even beyond the use as food, e.g. for issues of illegal trade with wildlife or farmed animal species. In particular, the monitoring of protected species to enforce the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) or the growing interest in monitoring wildlife consumption in various countries in the wake of the COVID-19 virus pandemic could be an increasing emphasis on rapid identification of muscle meat. For these applications the material collection, the databases, as well as the collection of reliable single spectra for validation have to be expanded extensively, e.g. for muscle meat from common

game and globally traded exotic meat (ostrich, crocodile). This can be accelerated by increased exchange among interested MALDI-users.

5. Conclusion

Based on direct protein extraction and using MALDI-TOF MS combined with a comprehensive database, we demonstrated a rapid, easy and robust method to identify the animal species of meat, raw or even after some heat treatment. The validation of the method has already covered the most important meat-producing livestock species. This method can be easily implemented for routine analysis in laboratories with existing MALDI-TOF MS equipment without additional costs or specific knowledge. The exchange of reference spectra to accelerate the expansion of the database entries is facilitated by the MALDI-user platform (https://maldi-up.ua-bw.de).

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Statements

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Supplementary material

Supplementary data to this article can be found at the end of this manuscript.

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641 ***Table 1***

642 Number of animal species, individual meat samples used, and reference spectra (MSPs)
643 created for the MALDI-TOF MS meat database (for details see Supplement 1).
644

Class Order	**n of species**	**n of samples**	**n of MSPs**
Mammalia	**132**	**719**	**320**
Artiodactyla	47	466	158
Carnivora	34	104	64
Perissodactyla	9	36	20
Primates	15	24	24
Lagomorpha	3	31	10
Diprotodontia	5	21	12
Rodentia	12	27	23
Other (from 5 order)	7	10	9
Aves	**115**	**348**	**187**
Accipitriformes	10	17	13
Anseriformes	12	47	25
Ciconiiformes	4	8	6
Columbiformes	5	14	9
Falconiformes	3	5	4
Galliformes	14	142	36
Passeriformes	18	22	21
Pelecaniformes	6	10	8
Psittaciformes	21	36	31
Strigiformes	3	7	5
Struthioniformes	1	11	6
other (from 9 order)	18	29	23
Reptilia	**18**	**21**	**20**
Crocodilia	4	5	5
Squamata	8	8	8
Testudines	7	8	7
Sum	**265**	**1088**	**527**

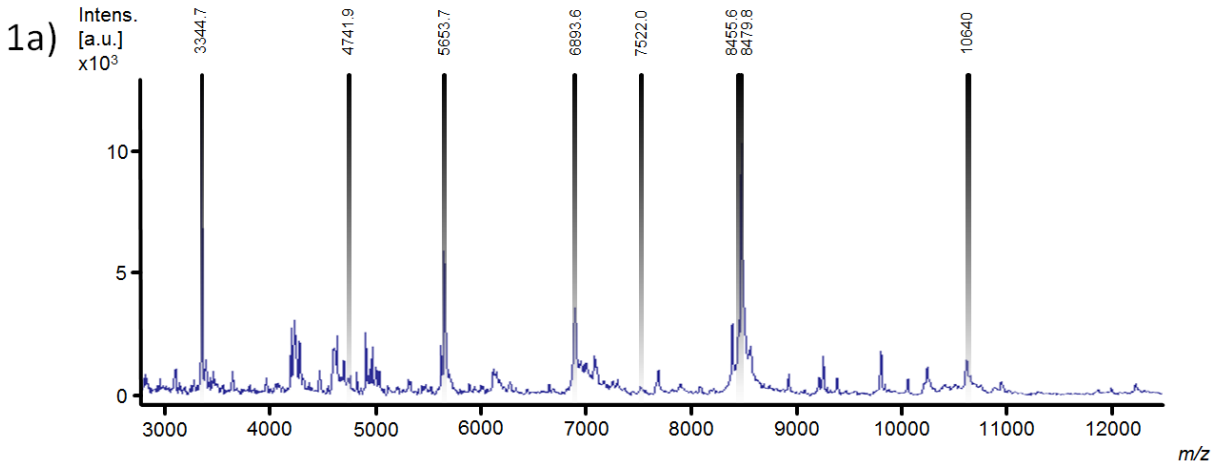
Table 2

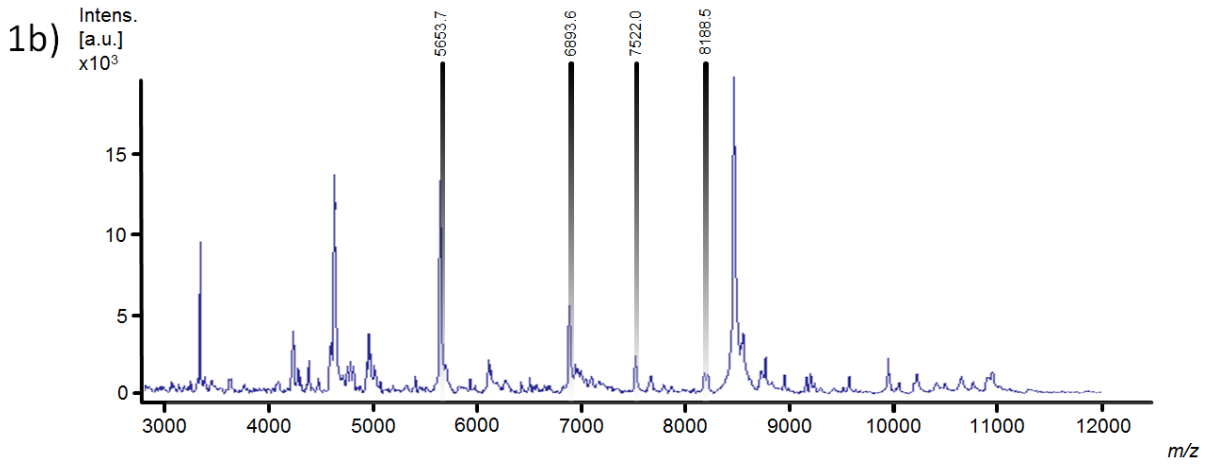
Results of animal species identification of meat samples by MALDI-TOF MS. True/False: the animal species was correctly/not correctly identified. All samples within the control group did not belong to the parameter (= species / genus / family) of interest. Individual results for any sample were given in Supplement 1.

Group animal species	**Parameter of interest**	**n of samples**	***Score mean***	***Score STD***	**Samples identified**	***Identification rate (%)***	**True positive**	**False negative**	***True positive rate (%) of identified samples***	***False negative rate (%) of identified samples***	**n of samples of the control group**	**Samples identified**	***Identification rate (%)***	**True negative**	**False positive**	***True negative rate (%) of identified samples***	***False positive rate (%) of identified samples***
Mammals																	
Pig	*Sus scofa*	109	*2.296*	*0.171*	105	*96.3*	105	0	*100*	*0*	979	940	*96.0*	940	0	*100*	*0*
Cattle	*Bos taurus*	92	*2.224*	*0.187*	81	*88.0*	81	0	*100*	*0*	996	961	*96.5*	961	0	*100*	*0*
Sheep	*Ovis aries*	75	*2.186*	*0.144*	54	*72.0*	54	0	*100*	*0*	1013	976	*96.4*	976	0	*100*	*0*
Goats	*Capra* (genus)	30	*2.308*	*0.190*	28	*93.3*	28	0	*100*	*0*	1058	999	*94.4*	999	0	*100*	*0*
Deer	*Cervidae* (family)	85	*2.373*	*0.174*	83	*97.6*	83	0	*100*	*0*	1003	961	*95.8*	961	0	*100*	*0*
Horses	*Equus* (genus)	35	*2.397*	*0.170*	35	*100*	35	0	*100*	*0*	1053	1010	*95.9*	1010	0	*100*	*0*
Hares	*Leporidae* (family)	31	*2.328*	*0.165*	31	*100*	31	0	*100*	*0*	1057	1014	*95.9*	1014	0	*100*	*0*
kangaroos	*Macropodidae* (family)	21	*2.526*	*0.157*	21	*100*	21	0	*100*	*0*	1067	1024	*96.0*	1024	0	*100*	*0*
Birds																	
Chicken	*Gallus gallus*	81	*2.276*	*0.235*	70	*86.4*	70	0	*100*	*0*	1007	974	*96.7*	974	0	*100*	*0*
Turkey	*Meleagris gallopavo*	45	*2.201*	*0.189*	39	*86.7*	39	0	*100*	*0*	1043	1006	*96.5*	1006	0	*100*	*0*
ducks and geese	*Anatidae* (family)	47	*2.388*	*0.202*	46	*97.9*	46	0	*100*	*0*	1041	999	*96.0*	999	0	*100*	*0*

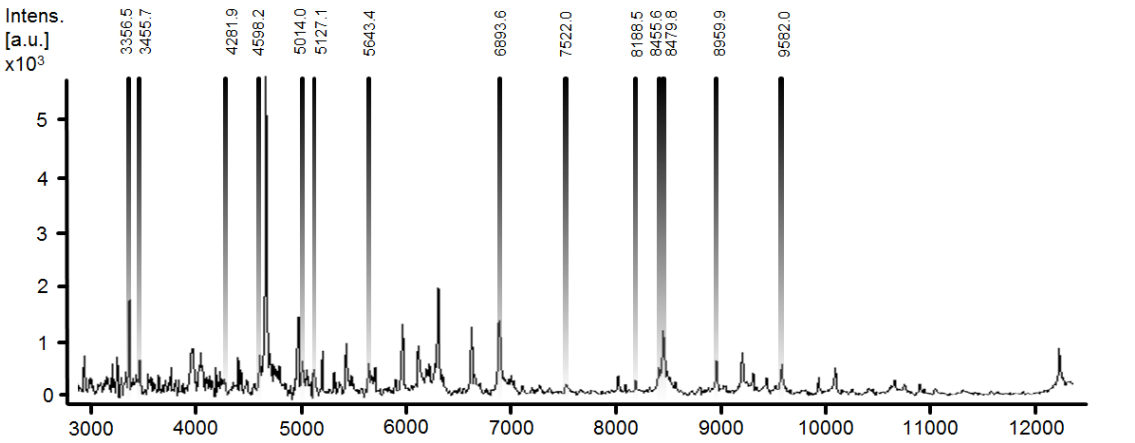
Figure 1

Typical MALDI-TOF mass spectra for muscle meat in the mass range from 2,800 to 12,500 *m/z*: **Fig. 1a)** beef (*Bos taurus*), **Fig. 1b)** pork (*Sus scrofa*), **Fig. 1c)** sheep (*Ovis aries*), **Fig. 1d)** goat (*Capra* sp.), **Fig. 1e)** horses (*Equus* sp.), **Fig. 1f)** chicken (*Gallus gallus*), **Fig. 1g)** turkey breast (*Meleagris gallopavo*), and **Fig. 1h**) turkey leg. The colored bars indicate the selected *m/z* values according to Supplement 2.



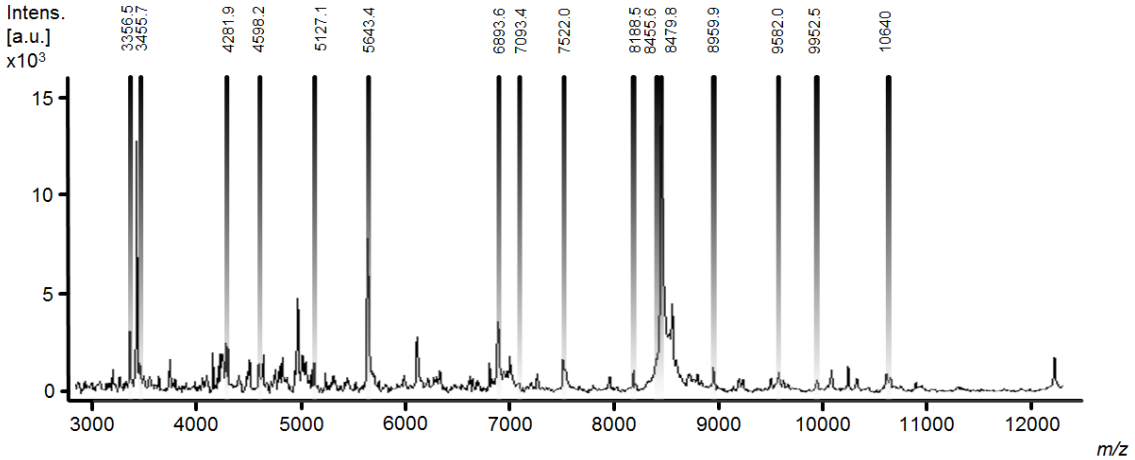


1c)



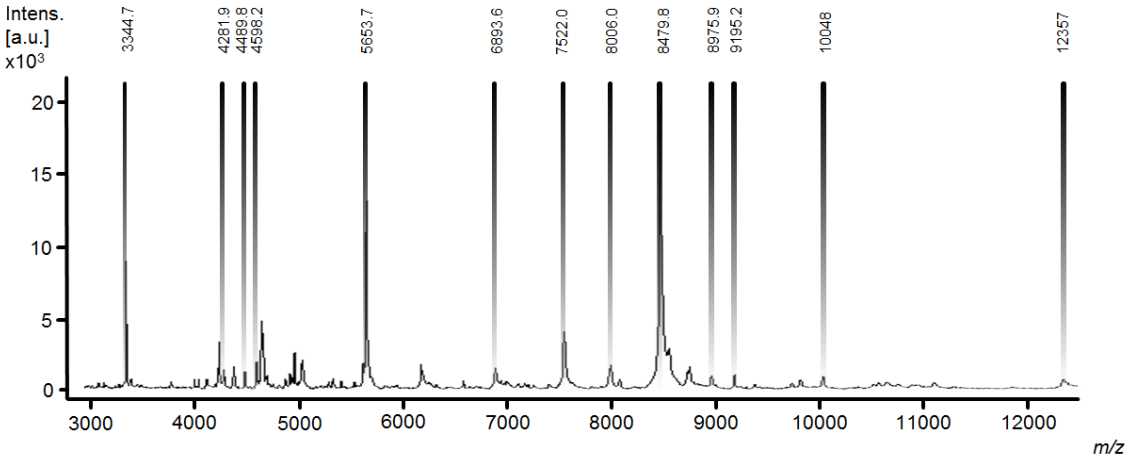
657

1d)



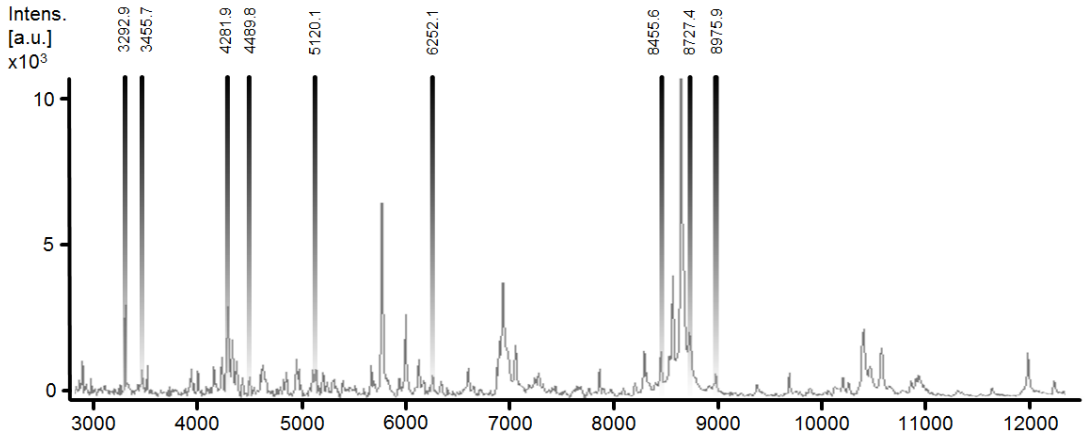
658

1e)



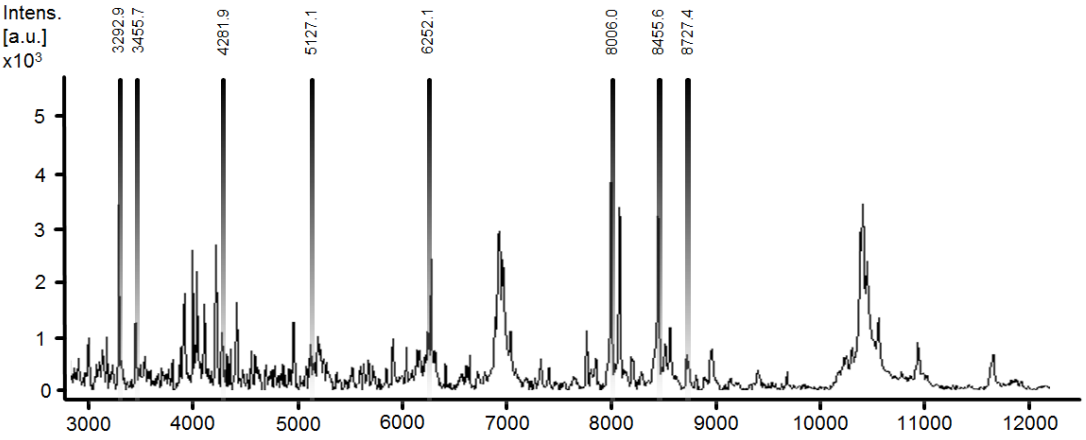
659

1f)



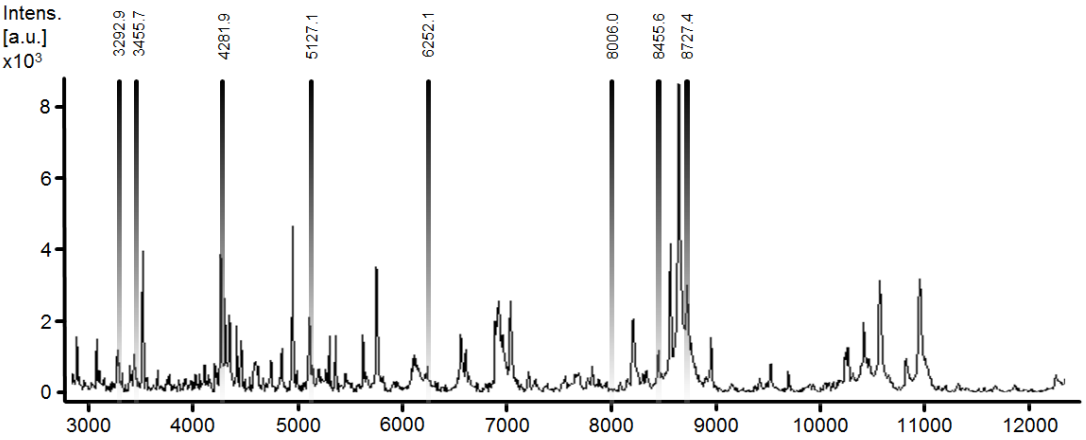
660

1g)



661

1h)



662

663

Figure 2

Cluster analysis of reference main spectra (MSP) obtained by MALDI-TOF MS from a collection of species, including animals relevant to human diet (**Fig. 2a**, Overview, **Fig. 2b**, subfamily *Bovinae* of *Bovidae*, **Fig. 2c,** family *Anatidae*). Details of the samples are listed in Supplement 2 and on https://maldi-up.ua-bw.de. Cluster analysis was done by the Biotyper OC software with setting correlation for distance measure to build a score-oriented dendrogram in average linkage mode.

Fig. 2a

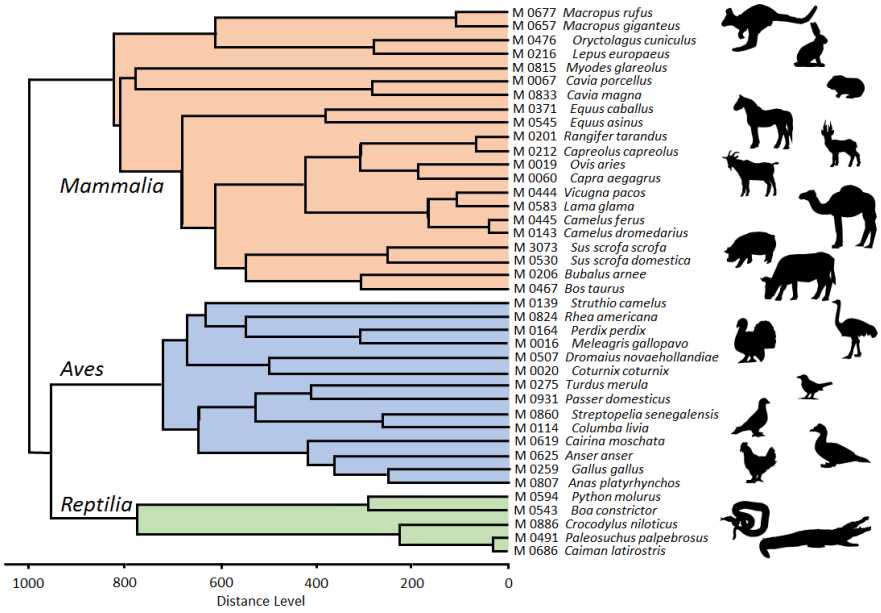


Fig. 2b

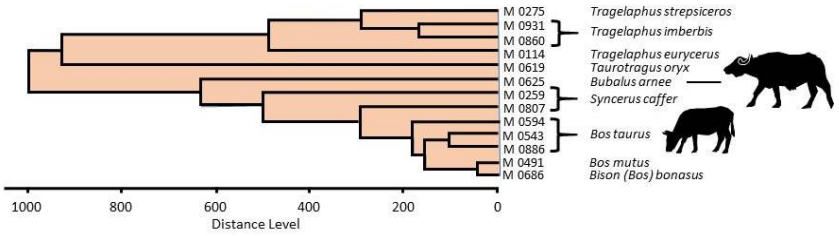
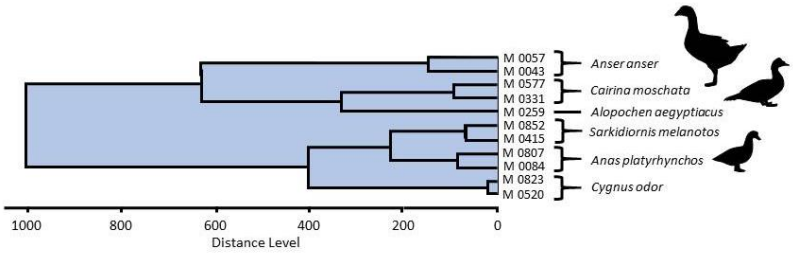


Fig. 2c



Supplementary Material

Animal Species Identification of Meat using MALDI-TOF Mass spectrometry

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Supplement 1

Provided in a separate file: *Supplement 1 Database and Validation Spectra.xlsx*

Meat material, MALDI-TOF MS meat reference database and identification results of validation spectra (an excerpt from the MALDI-User Platform, MALDI-UP https://maldi-up.ua-bw.de).

Excel file including three worksheets:

Worksheet “Database”: MSPs included in the MALDI-TOF MS meat reference database.

Worksheet “Validation Spectra and Results”: Muscle meat samples used and their identification results obtained in the validation study.

Workheet “Taxonomic Summary”: Summary of MSPs and single spectra used in taxonomic rank “order”

Supplement 2

Selection of specific MALDI-TOF MS *m/z* signals obtained from meat of pig (n=109), cattle (n=92), goats (genus *Capra*; n=30), sheep (n=75), horses (genus *Equus*; n=35), chicken (n=81) and turkey (n=45). The intensity of *m/z* signals in relation to the highest peak is given as the mean-value of all spectra received from the type of meat considered. # = no signal >5% intensity for >80% of all spectra in a +/- 800 ppm *m/z*-window. [1] Signal-frequency lower than 80%.

	Skeletal muscle / Meat						
	Pig	**Cattle**	**Sheep**	**Goats**	**Horses**	**Chicken**	**Turkey**
	Sus scrofa	*Bos taurus*	*Ovis aries*	*Capra* (genus)	*Equus* (genus)	*Gallus gallus*	*Meleagris gallopavo*
***m/z* +/- 800 ppm**	**relative intensities (%)**						
3292.9	#	#	#	#	#	54.1	43.2
3344.7	62.3	#	#	#	18.8	#	#
3356.5	#	#	31.1	30.2	#	#	#
3455.7	#	#	12.6	8.4	#	23.6	21.5
4281.9	#	#	20.9	23.0	8.8	31.7	36.7
4489.8	#	#	#	#	7.3	21.7	#
4598.2	#	#	26.2	18.9	14.9	#	#
4741.9	14.2	#	#	#	#	#	#
5014.0	#	#	16,6	#	#	#	#
5120.1	#	#	#	#	#	46.9	#
5127.1	#	#	11.4	7.4	#	#	20.8
5643.4	#	#	37	43.5	#	#	#
5653.7	38.8	43.1	#	#	50.1	#	#
6252.1	#	#	#	#	#	18.1	16.1
6893.6	35.8	38.5	47.8	44.3	16.4	#	#
7093.4	#	#	#	8.7	#	#	#
7525.3	40.4	28.0	23.5	23,3	#	#	#
7552,4	#	#	#	#	14.1	#	#
8006.0	#	#	#	#	10.3	#	71.5
8188.5	#	9.3 [1]	13.7	10.0	#	#	#
8417.4	#	#	10.5	9.6	#	#	#
8455.6	40.4	#	76.1	81.0	#	23.7	25.9
8479.8	66.0	#	#	#	92.8	#	#
8727.4	#	#	#	#	#	13.6	19.3
8959.9	#	#	11.4	12.5	#	#	#
8975.9	#	#	#	#	6.9	9.2	#
9195.2	#	#	#	#	8.5	#	#
9582.0	#	#	13.4	14.0	#	#	#
9952.5	#	#	#	6.9	#	#	#
10048	#	#	#	#	7.6	#	#
10640	12.2	#	#	12.3	#	#	#
12357	#	#	#	#	6.6	#	#

Supplement 3

Identification of meat from different species after cooking or roasting. Three independent samples of each preparation were analysed by MALDI-TOF in triplicate. Only the highest scored value of each location/preparation is listed.

Species of meat sample	Food preparation	Sampling location	Score value first hit	Score value second hit	Result: meat identified as
pig	raw	surface	2.31	2.31	*Sus scrofa*
	cooking	inside	2.08	1.83	*Sus scrofa*
		surface	2.14	1.83	*Sus scrofa*
	roasting	inside	2.18	2.04	*Sus scrofa*
		surface	0	0	not identified
cattle	raw	surface	2.09	2.08	*Bos taurus*
	cooking	inside	2.29	1.94	*Bos taurus*
		surface	2.30	1.84	*Bos taurus*
	roasting	inside	2.20	2.02	*Bos taurus*
		surface	2.18	2.09	*Bos taurus*
chicken	raw	surface	2.15	1.93	*Gallus (G.) gallus*
	cooking	inside	2.06	1.95	*G. gallus*
		surface	2.11	2.01	*G. gallus*
	roasting	inside	2.06	1.98	*G. gallus*
		surface	0	0	not identified
turkey	raw	surface	2.37	2.00	*Meleagris (M.) gallopavo*
	cooking	inside	2.18	2.08	*M. gallopavo*
		surface	2.09	1.77	*M. gallopavo*
	roasting	inside	2.06	1.65	*M. gallopavo*
		surface	2.01	1.53	*M. gallopavo*