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Animal Species Identification of Meat using MALDI-TOF Mass Spectrometry

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Abstract

One of the main aspects of food control regarding meat, seafood and milk products is the inspection and verification of the declared animal species. The potential of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for this purpose has already been demonstrated in principle. In fact, this tool has become an integral part of our official food analysis. In our study we confirm that MALDI-TOF MS is an easy, fast and reliable tool for the identification of animal species when analyzing the meat from pigs, cattle, goats, sheep, horses, turkeys, and chickens. Using a simplified extraction procedure and the Bruker MALDI-Biotyper system, we generated a MALDI-TOF MS database containing more than 550 reference spectra of muscle meat from over 260 confirmed different animal species. In order to accelerate the expansion of this database, we listed the spectra generated in this study on the MALDI-User Platform "MALDI-UP" (https://maldi-up.ua-bw.de) for exchange with other laboratories.

1. Introduction

The 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 agitates consumers and forces the competent authorities to perform more focused monitoring activities (Everstine et al., 2013; Rahmati et al., 2016; European Commission, 2015). One particular example of food fraud is the substitution of high-quality ingredients with cheaper alternatives without declaration. In the case of high-priced food of animal origin, fraudulent intentions are most frequently commited by manufacturers, suppliers or restaurant owners (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 their food expenditure on meat and meat products, with an annual market value of over 20 billion euros (Statistisches Bundesamt, 2016). Legislation in the EU provides clear rules for the declaration of animal species processed in food products (Regulation (EC) No 1169/2011). A spate of recent scandals concerning horse and game meat, among others, has caused enormous uncertainty among consumers, accompanied by a loss of trust in authorities and industrial food businesses (Everstine et al., 2013; Bayrischer Landtag, 2008). The fraudulent supplementation or substitution of the declared meat has presented a recurring 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 often occurs further down in the food supply chain, particularly in unpacked products sold directly to consumers or in ingredients used in gastronomy. In order to effectively counteract such widespread activities, food control laboratories require rapid, reliable, easy to use, and cheap tools with high throughput capabilities for the authentication of foodstuffs.

A wide range of analytical methods is available for animal species identification in food. These are mainly comprised of DNA-based techniques and 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). These approaches commonly focus on the detection of

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specific marker molecules, which enables qualitative species identification (Marbaix et al., 2016; von Bargen et al., 2014; Waiblinger et al., 2017; Skouridou et al., 2019). Other techniques, such as the sequencing of marker genes or, recently, complex "metabarcoding", which combines 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 previous years, methods based on mass spectrometry were developed to identify animal species in meat-based products by analyzing their proteins (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 food analysis laboratories for the identification of microorganisms (Pavlovic et al., 2013; Quintela-Baluja et al., 2014). Furthermore, MALDI-TOF MS has been demonstrated to be a suitable tool for the identification of scallops, shrimps, fish, and edible insects, as well as animal products such as cheese, gelatin, and 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 the 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 generated 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 to human nutrition. The suitability of this rapid method for routine food control as well as for 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 facilitate the exchange of database entries among interested users, additional information on each reference spectra is listed on the MALDI-User Platform "MALDI-UP" (https://maldi-up.ua-bw.de) (Rau et al., 2016a).

2. Materials and Methods

2.1. Sample collection

A collection of 1,088 raw animal flesh samples were received mainly from veterinary pathology units and official food control laboratories of several institutes in Germany: Chemisches und Vetärinäruntersuchungsämte (Chemical and Veterinary Investigations Offices (CVUAs)) in 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. At the time of study the collection included material from 132 mammalian species, 115 bird species and 18 reptilian species. A selection of spectra from 527 independent muscle samples, comprising 320 from mammals, 187 from birds and 20 from reptiles, was consolidated into the MALDI-meat reference database (Table 1). Overall, 1,088 samples were integrated in

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

Class	Number of		
Order	Species	Samples	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 orders)	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 orders)	18	29	23
Reptilia	**18**	**21**	**20**
Crocodilia	4	5	5
Squamata	8	8	8
Testudines	7	8	7
Sum	**265**	**1,088**	**527**

the validation part of the study (Supplement 1). Immediately after performing the gross pathology, or in the case of food samples, immediately after the initial organoleptic analysis has been completed, meat samples were then frozen at -18 °C (+/- 2 °C) until preparation for MALDI-TOF MS.

2.2. Organic solvent sample preparation (OSextr)

Proteins were extracted from meat in accordance with 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 while still fresh and again after long-term freezing. Fresh meat (pork, beef, chicken, turkey) was prepared directly after purchase using 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 months, mass spectra of these samples were taken and compared with the initial mass spectra.

To investigate the feasibility of MALDI-TOF MS for identifying the animal species of a meat sample after exposure to high temperatures during food preparation such as 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 each meat sample was boiled in water for 15 min. The second slice was roasted in a pan for 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 were cut off and stored at -18 °C until analysis. Sample preparation, measurement and identification were performed as described in Sections 2.4 – 2.6. This experiment was repeated three times.

2.4. MALDI-TOF MS measurement and analysis

The MALDI-TOF mass spectra were 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). The default parameter settings were: positive linear mode, laser frequency 60 Hz, ion source 1 = 120 kV, ion source 2 = 18 kV; Bruker's MBT_FC and MBT_AutoX methods; mass range: 2,000 – 20,000 Da. The Bruker IVD bacterial test standard (BTS) was used for mass-calibration in accordance with the manufacturer's instructions (c.f. Rau et al., 2020).

2.5. Generation of the MALDI-TOF MS meat database

Reference entries were generated and evaluated in accordance with the basic manufacturer's instructions (Pranada et al., 2016). In brief, the protein extract from a meat sample was detected on eight spots and measured in triplicate to create at least 24 raw spectra for one sample. Control and processing of raw spectra was performed with FlexAnalysis software (version 3.4), and reference main spectra (MSP) were calculated by the "Biotyper MSP Creation Standard Method" using the MBT software package as previously described (Rau et al., 2020). These reference entries for meat were presented in the project folder of the MBT database module (Biotyper OC 3.1). Detailed information about the generated reference entries are listed in Supplement 1, and on MALDI-UP (https://www.maldi-up.ua-bw.de).

The most prominent and common *m/z* signals in terms of intensity are collected in peak lists and form the signal fingerprint for the respective meat type. Several average *m/z* signals for meat of major farm animals (pig, cattle, sheep, goat, horses, chicken, and turkey) are shown in Supplement 2.

2.6. Identification criteria

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

2.7. Validation study

The validation of the animal species identification by MALDI-TOF MS follows a parameter-based concept used for microorganisms and cheese (cf. Rau et al., 2016b, Rau et al., 2020). In the first step, the identification rate of the respective parameter, that means the ratio of identified samples to all probed samples, is calculated. To assess the significance of an identification result related to the parameter, a simple validation procedure based on the true 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 a 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 a 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 negatives (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. Accordingly, 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, a minimum of 20 independent validly assigned sample materials for a parameter were used to test the complete system, consisting of a 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 peptide fingerprints of a sample with those in a suitable database. As proteins are the main component of 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 the feasibility of MALDI-TOF MS for identifying animal species from muscle meat in routine food control. Indispensable to 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. So far, there has been 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 for more than 260 animal species. Typical single mass-spectra of skeletal muscle from pigs, cattle, sheep, goats, horses, chicken and turkey are shown in Figure 1 (p. 5). Even though the mass-accuracy of the MALDI-TOF MS system used is limited, it is sufficient for defining tolerance ranges 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 patterns of reference MSPs.

In order to cover the diversity within a species, several MSPs from different individuals within each species, if available, were generated (Supplement 1). Considering the requirements for food control, we focused on sampling the skeletal muscles commonly used in food production. As shown in Figure 1g) and 1h) for turkey leg and breast, respectively, mass spectra varied slightly, even if the samples had been 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 different muscle parts and stages of aging for 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); some from animal species of minor relevance for European eating habits (deer, hare and rabbit, ducks and geese); as well as 'exotic' animals (ostrich, kangaroos, camels, zebras, antelopes, crocodiles, and guinea-pigs). More than 150 other species were analyzed for comparison, some of which are 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, p. 6). It demonstrated three clearly separate 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*), as well as the family of *Anatidae*, including domestic (mallard-)duck (*Anas platyrhynchos*), Muscovy duck (*Cairina moschata*) and domestic goose (*Anser anser*) (Figure 2b, and 2c, p. 6).

3.2. Validation of the database

To verify the reliability of the identifications using the database, a validation procedure was conducted following 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 by official food control laboratories. In total, 1,088 meat samples were prepared as described in Section 2.2. 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). Similarly, 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.

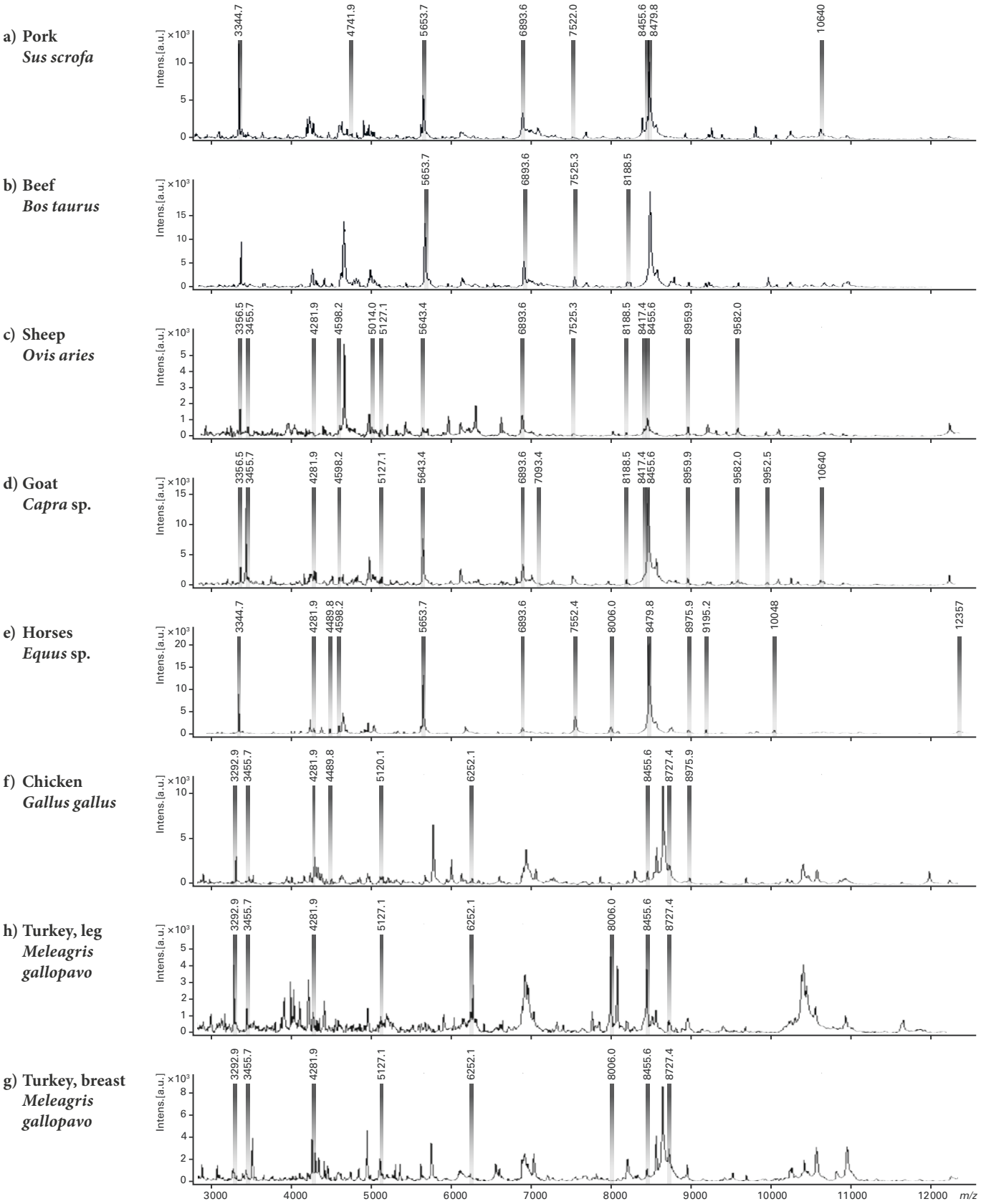
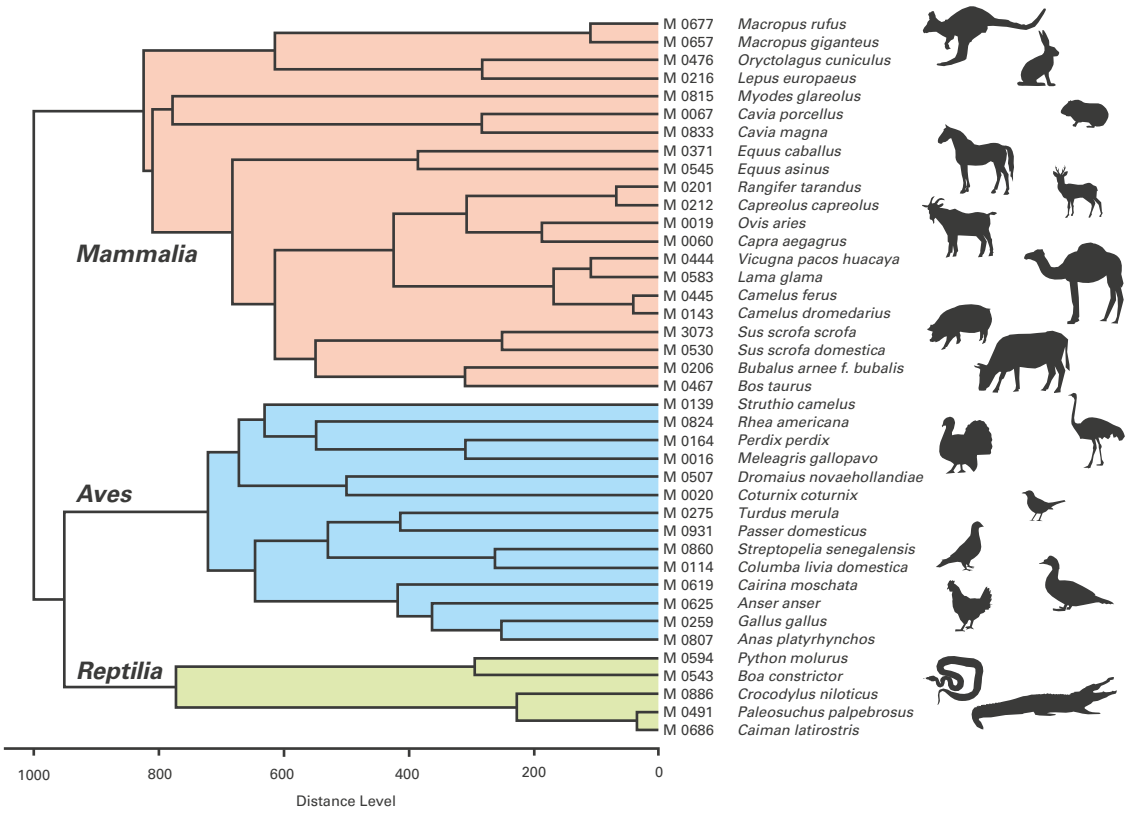
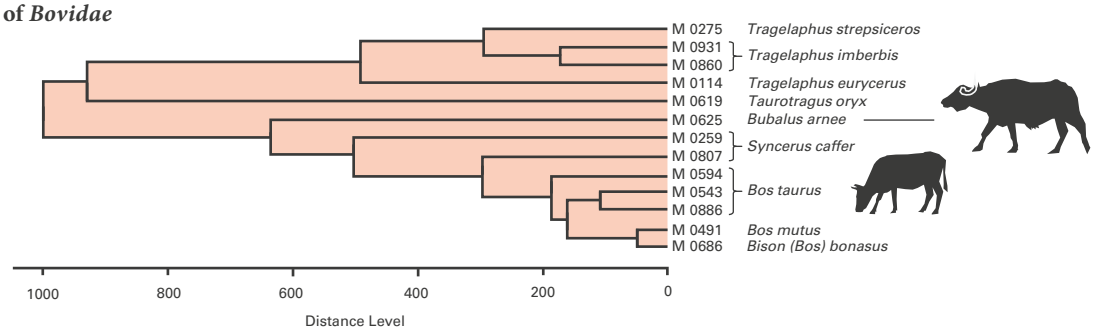


Figure 1. Typical MALDI-TOF mass spectra for muscle meat in the mass range from 2,800 to 12,500 *m/z*. a) pork (*Sus scrofa*); b) beef (*Bos taurus*); c) sheep (*Ovis aries*); d) goat (*Capra* sp.); e) horses (*Equus* sp.); f) chicken (*Gallus gallus*); g) turkey (*Meleagris gallopavo*) breast, and h)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

a) Overview



b) Subfamily *Bovinae* of *Bovidae*



c) Family *Anatidae*

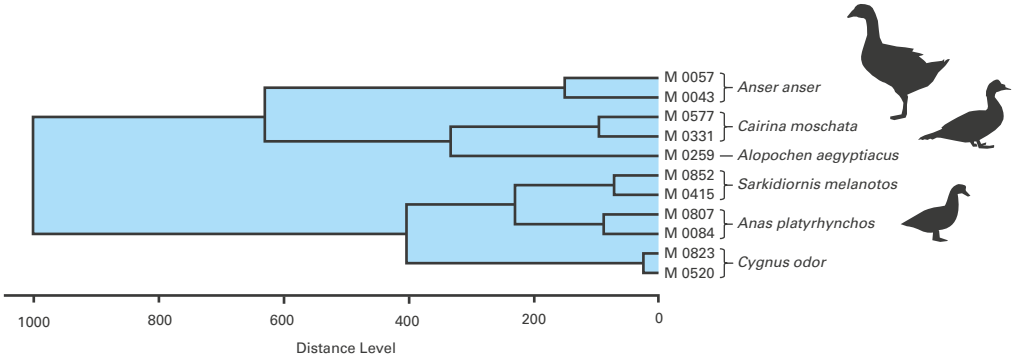


Figure 2. Cluster analysis of reference main spectra (MSP) obtained by MALDI-TOF MS from a collection of species, including animals relevant to the human diet. a) Overview; b) subfamily *Bovinae* of *Bovidae*; c) family *Anatidae*. Details of the samples are listed in Supplement 2 and on https://maldi-up.ua-bw.de.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

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	Number of samples	Mean of scores	SD of scores	Number of samples identified	Identification rate (%)	True positive	False negative	True positive rate of identified samples (%)	False negative rate of identified samples (%)	Number of samples in the control group	Number of Samples identified	Identification rate (%)	True negative	False positive	True negative rate of identified samples (%)	False positive rate of identified samples (%)
Mammals																	
Pig	*Sus scrofa*	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

Due to the limited availability of samples, several rare animal species were combined and validated as a family-level parameter. The following identification rates were achieved for these taxonomic families: 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). No false identification was obtained for these families. The control groups revealed an identification rate of higher than 95 % for all species and family groups, and no false positive identification was obtained (Table 2). The score values achieved for meat lay between 2.001 and 2.806 and were thereby comparable with those obtained in the identification of microorganisms and cheese, using 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 spectra scores for 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. This indicates that freezing and storing at -18 °C is an appropriate method for preserving 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 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 from four different animal species. After roasting or cooking, the species of all meat samples was identified cor-

rectly 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 of concern for consumers and keeps consumer protection authorities and food inspection laboratories all over the world occupied (European Commission, 2015; Rahmati et al., 2016; Everstine et al., 2013). The price-determining components, such as meat and dairy protein, are most frequently affected (Wisniewski & Buschulte, 2019). The most prominent, economically motivated food fraud case in the meat sector was the horse meat in lasagna in 2013. Other incidents that gained national attention such as the game meat scandal in Germany have also contributed to consumer confusion (Bayrischer Landtag, 2008; On, 2016).

DNA-based and immunological methods are the prevalent techniques for identifying the animal species of meat containing food (Waiblinger, 2017; Li et al., 2020; Rahmati et al., 2016). However, the commercial kits used for these analyses are both time consuming and associated with high costs. Protein or peptide analysis using mass spectrometry gives a different approach for the inspection of protein-rich food (Ortea et al., 2016). Over the past years, use of 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 gelatin 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. So far, an easy-to-use and comprehensive database for meat identification has not yet been commercially available.

One of the official food control activities is the monitoring of labels. For this purpose, we focused on 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. We were thus 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 performance time from sample preparation to reliable identification result to 20 minutes.

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 preparation procedures, such as freezing, cooking and roasting.

4.1. Reference database

The most important key to species identification using MALDI-TOF MS or other fingerprinting technologies is the database used, which must contain appropriate mass profiles (mass lists or MSPs), in order to compare the resulting sample spectra. Using MALDI-TOF MS, meat from the major livestock animals can be clearly distinguished by several species-specific *m/z*-signals (Supplement 2, Figure 1, p. 5). Using the OSextr protocol, a simplified procedure without tryptic digestion, we have obtained species-specific mass meat profiles of more than 260 animal species (Supplement 1). Furthermore, the results of the identification made via the Biotyper algorithm and the MSP-dendrogram highlights the specificity of the MALDI method for the analyzed meat samples (Figure 2, p. 6). Consequently, all reference spectra of skeletal muscle meat were compiled in the same database. This collection compensates for 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 species identification by MALDI-TOF MS is negligible. This also provides an easy and suitable way to preserve 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 meat spectra at different maturation stages to mirror proteolytic changes during ripening (Lametsch et al., 2002, Supplement 2).

Turkey meat from the breast and leg are examples of the similarity in protein mass-spectra of different skeletal muscles (Figure 1, g and h, p. 5). 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 combining their own databases and methods with applications of MALDI-TOF MS for the differentiation of animal meat on a small scale and/or addressing specific issues (Flaudrops et al., 2015; On, 2016). The focus of the current work was on the validation of the whole system, using the Bruker MALDI-Biotyper combined with our 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, in every case, the control group comprised 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 results occurred for 979 single spectra from other animals (Table 2). High identification rates (> 85 %) were also achieved for beef (*Bos taurus*), goats (*Capra genus*), horses (*Equus genus*), chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*), and no misinterpretation of results were determined, neither from the parameter itself nor from the extensive control group. In the case of sheep, the rate of identified samples sunk to 72 %, due to the similarity of spectra to other members of the Tribus Caprini. Nevertheless, successful identifications were in any case correct (Supplement 2). Due to insufficient amounts of individual material available for validation, the horses, goats, hares (family *Leporidae*), deer (*Cervidae*), kangaroos (*Macropodidae*), and the family of ducks and geese (*Anatidae*) were evaluated as groups (Table 2). The identification results obtained were also reliable. We concluded, therefore, that the in-house database reached sufficient identification rates for all meat categories investigated. We also demonstrated that the species of major meat categories relevant to the market could be reliably identified by MALDI-TOF MS. So far, no false identifications have occurred for any parameters validated (Table 2).

If reference spectra for meat of other animal species can be added and if the number of reliable sample spectra for validation can be increased, the following issues are expected to be better resolved using MALDI in the future:

Differentiation between wild boar meat (*Sus scrofa scrofa*) and pork (*Sus scrofa domestica*) has not yet been successful using the simple evaluation techniques (Supplement 2).

Within the subfamily Bovinae, meat spectra for the representatives of the tribe Tragelaphini (Spiral-horned antelopes) were distinguished from the spectra derived from the tribe Bovini (Bovinans) (Figure 2b). Within 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. Differentiation by MALDI failed for the two genera from the subtribe Bovina, *Bos* and *Bison*, (Supplement 2). Hassanin & Ropiquet (2004) questioned 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 the major livestock species *Anatidae*, domestic goose, mallard, and Muscovy duck showed significant differences in the spectra that resulted in separate branches in the MSP-cluster diagram (Figure 2c). However, the number of independent samples and the MSPs derived from them are still too small to identify the animal at a 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 temperatures could interfere with the animal species identification, MALDI-TOF MS profiles of meat samples from four animal species were acquired after roasting or cooking 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 decreased 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 animal identification by MALDI-TOF MS, as long as the heat is not extreme.

As shown in our study, raw and heated meat can be assigned to the same animal species when using the current procedure. 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 also be recorded, especially if corresponding reference spectra are included in the compilation of the database. Further factors such as quality defects (PSE and DFD) of meat, the slaughtering process and, of course, aging (either controlled, such as dry aging, or uncontrolled, such as spoilage), are not completely covered by the current method. So far, offal has not been 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

Given that sample preparation for MALDI-TOF MS takes only minutes, the reagents are inexpensive, and only 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 for the disadvantage of the expensive equipment. As shown in this study, the meat method has been validated for all animal species relevant to the human diet. An important limitation of this direct and rapid MALDI-TOF procedure, however, is that only the animal species of the major meat component of a mixed sample is identified. Other mass peak evaluation methods or elaborate mass-spectrometry techniques were more promising for detecting small amounts of meat admixtures in meat products such as minced meat (von Bargen et al., 2014; Montowska & Spychaj, 2018; Prandi et al., 2017).

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 minimally 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 for uncovering food fraud, MALDI-TOF MS has turned out to be a suitable, rapid, high throughput technology for identifying animal species even beyond the use as food, e.g. for issues of illegal trade of wildlife or farmed animal species. In particular, the monitoring of protected species for the enforcement of 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 grounds for increasing emphasis on the rapid identification of muscle meat. For these applications, the databases as well as the collection of material and 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 process can be accelerated by increasing opportunities for exchange among interested MALDI users

5. Conclusion

Using direct protein extraction and MALDI-TOF MS in combination with a comprehensive database, we demonstrated a rapid, easy and robust method for identifying the animal species of meat, in both its raw state and 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 specialized 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 are available for this paper at https://doi.org/10.48414/aspects2021/14.

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