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Comparison of sample preparation methods for the identification of *Staphylococcus Aureus* by MALDI-FOF MS

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SUMMARY

Coagulase-positive staphylococci include 3 species, Staphylococcus aureus, S. hyicus *and* S. intermedius*. Of these three species,* S. aureus *is the most well-known human pathogen.* S. aureus *is part of the human and animal normal microbiota, however, it is capable of producing several staphylococcal enterotoxins (SEs) that cause intoxication symptoms of varying intensity in humans after consuming contaminated food. Selective media which are used for the determination of coagulase-positive staphylococci from foods are not able to identify isolates at a species. With the MALDI-TOF MS technique, we can identify* S. aureus *cheaper and faster than by using molecular methods. This paper describes the results of the study of the presence of coagulase-positive staphylococci and* S. aureus *in many food products, and the application of three sample preparation methods: direct sample preparation, formic acid suspension and ethanol extraction.*

Keywords: *Coagulase-positive staphylococci, MALDI-TOF MS, sample preparation, foodborne pathogen,* S. aureus

INTRODUCTION

Staphylococcus is the main genus of the Staphylococcaceae family in the order of Bacillales, Bacilli class and Firmicutes phylum. Staphylococci are non-spore forming, non-motile, Gram-positive cocci, 0.5 to 1 micrometer in diameter, catalase and coagulase positive.

Coagulase-positive staphylococci include 3 species, *S. aureus, S. hyicus* and *S. intermedius* (ANSES, 2011; EURL CPS, 2014). The most virulent pathogen species of the genus *Staphylococcus* is *S. aureus* (Foster, 1996) (Jain and Daum, 1999).

Staphylococcus aureus is facultatively anaerobic, and can be found on the skin and mucous membranes of 20-30% of people and warm-blood animals. These microbes have also been isolated in the natural environment, hospital environment and foodstuff (Manukumar and Umesha, 2017). *Staphylococcus aureus* forms golden-yellow colonies on blood agar (Taylor and Unakal, 2017) and produces several toxins, including staphylococcal enterotoxins responsible for staphylococcal food-poisoning outbreaks (Ercoli et al., 2017).

Many *S. aureus* biotypes have been isolated from various hosts (human, poultry, cattle and sheep/goat) that show a close adaptation of the microorganism to the host cell (Hennekinne et al., 2012). The most common cause of subclinical mastitis in cattle are coagulase-positive staphylococci, but other animals (pigs, poultry, horses) can also be infected. Therefore, these microorganisms may often be present in foods (Rajic-Savic et al., 2015).

Staphylococcal food poisoning is one of the most common food-borne illnesses (Jain and Daum, 1999) (Kadariya et al., 2014). Enterotoxigenic strains of coagulase-positive staphylococci produce enterotoxins, mainly *Staphylococcus aureus* and very rarely other *Staphylococcus* species, such as *Staphylococcus intermedius* (Loir et al., 2003). Staphylococcal food poisoning symptoms develop rapidly (2–8 hours), including nausea, vomiting, abdominal cramps, with or without diarrhea. The disease usually does not require treatment and typically resolves after 24 to 48 hours of recovery (Kadariya et al., 2014). Occasionally, the infection may prove to be serious enough to require hospital treatment, especially when infants or elderly are affected (Argudín et al., 2010).

In order to ensure the microbiological safety of food, both EU level and national regulation exists. Regulation (EC) No. 2073/2005 sets criteria – among others – for coagulase-positive staphylococci in several food product categories of animal origins as process hygiene parameters to be investigated by the set of EN ISO 6888 norms. National Hungarian regulation (Ministry of Health regulation No. 4/1998) also sets microbiological limits. Of these, *Staphylococcus aureus* is prescribed as a technological limit criterion with regard to several foodstuffs with constituents of animal origin.

Selective media which are used for the determination of coagulase-positive staphylococci from foods are not able to identify isolates at a species. Thus, it is important to develop such new methods for identification that allow reduction of time-to-result for the food and feed industry. The aim was to look into the possibility of applying MALDI-TOF MS technique for the identification of coagulase-positive staphylococci.

The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique provide information on the protein and macromolecule profiles of the sample. Obtained mass

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spectra serve as base for the routine identification of the microbes, compared to a validated database (Singhal et al., 2015) to uncover markers or marker sets which can reliably identify *Staphylococcus* species (Pavlovic et al., 2013). Furthermore, this technique provides information about the microorganism variety of sources: e.g. isolates from clinical, livestock, food, feed or environmental sources (Sandrine et al., 2012).

MATERIALS AND METHODS

Bacterial strains, isolates and culture conditions

In the present study, 20 coagulase-positive staphylococci isolates were collected from different foodstuffs (*Table 1*). Based on the MSZ EN ISO 6888-1:2008 standard, the isolates were isolated by culturing on selective and non-selective growth media. On Baird-Parker selective medium (Biokar, Fr), staphylococci formed typical colonies and all strains showed positive coagulase reaction. For the coagulase test (Microgen Bio Product, UK), colonies were grown at 37 °C for 24±1 h on Columbia Blood agar (Neogen, UK). The strains and their sources are indicated in *Table 1*.

Table 1

The sources of the different *Staphylococcus* spp. strains isolated from food matrices exhibiting typical features on Baird-Parker medium and testing positive in coagulase latex test

Category of food		ID number	Type of food sample
Milk products		SA-1	Cheese
		SA-10	Cheese
		SA-18	Milk
Dried pasta		SA-2	Dried pasta
		SA-7	Dried pasta
Meat	Poultry	SA-12	Chicken wings
		SA-13	Duck liver
		SA-16	Chicken breast
		SA-17	Goose liver
		SA-20	Duck meat
	Pork	SA-4	Bacon
		SA-5	Pork sausage
		SA-6	Pork chops
		SA-9	Pork shoulder
		SA-14	Pork sausage
		SA-15	Pork greaves
		SA-19	Pork chops
	Beef	SA-3	Beef
		SA-8	Beef
		SA-11	Beef

Sample preparation

For identification with the MALDI-TOF MS technique, the isolates were grown on Columbia Blood agar. Three different types of sample preparation for MALDI-TOF MS analysis were used. The direct sample preparation was based on the standard Bruker Daltonic Inc. protocol. In this case, samples were taken from colonies with sterile sampling loops, then transferred directly onto the target plate and 1–1 µl 70 v/v% formic acid was added to them. After drying, 1 µl α-HCCA (10 mg/ml) matrix solution was added to the samples and the spots were crystallized by air drying.

In the second sample preparation protocol, formic acid suspending was used: a single colony was picked up with an inoculation loop and it was suspended in 40 µl of formic acid in an Eppendorf tube for 30 seconds. As a next step, 40 µl of acetonitrile was added to the suspension and mixed thoroughly. Finally, 1 µl of the suspension was transferred onto the target plate and when dried, it was overlaid with 1 µl α-HCCA (10 mg/ml) matrix solution and left to dry again.

In the third sample preparation protocol, ethanol extraction was used: a single colony was suspended in 300 µl of distilled water in an Eppendorf tube. Next, 900 µl absolute ethanol was added to the suspension, vortexed for 30 seconds and centrifuged at 14,500 rpm (18188 x g) for 3 minutes. After the supernatant was removed, the pellet was resuspended in 40 µl of 70 v/v% formic acid. Following this, 40 µl acetonitrile was added to the suspension, mixed and centrifuged thoroughly. Finally, 1 µl of the suspension was transferred onto the target plate, and after the sample dried, 1 µl of α-HCCA (10 mg/ml) was added.

MALDI-TOF MS measurement parameters

Mass spectra were obtained by the application of Bruker Microflex LT MALDI-TOF mass spectrometer operating in positive linear mode, in the molecular

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mass range of 2.0–25 kDa. We used the MALDI BioTyper 3.1 software to identify *Staphylococcus* spp. More than 200 shots gave adequate spectra with appropriate signal-to-noise ratio. Before the measurement, the calibration was carried out with *Bruker Bacterial Test Standard*. For the aim of analysis, 640 shots were performed mass spectra of *Staphylococcus* spp. Following the measurement, the mass data files were transferred to flexAnalysis 3.4 software (Bruker Daltonics). All spectra were processed by baseline correction, Gaussian smoothing, and peak finding. The obtained mass spectra were analysed individually for characteristic peaks.

The 20 isolates were analysed in parallel, the obtained mass spectra were compared to MALDI Bruker's Biotyper-specific database of mass spectra. The obtained results are reported as numeric score values based on similarity with the reference spectra. Scores below 1.699 reported as non-reliable genus identification, scores of 1.700–1.999 were classified as probable genus identification, scores of 2.000–2.299 were secure genus identification and scores of 2.300–3.000 designated as highly probable species identification.

RESULTS AND DISCUSSION

Identification *Staphylococcus* spp.

By using the direct sample preparation method, it was possible to obtain identification scores higher than 1.700 in the case of all (100%) isolates by comparison with the Bruker MALDI-Biotyper database. Seven *Staphylococcus* spp. isolates gave score values ≥2.300, therefore, the species *Staphylococcus aureus* was safely identified. Eleven isolates had score values between 2.000 and 2.300, i.e., the isolates' genus level identification was secure. Two strains had a probable genus level identification with a score in the 1.700–1.999 range.

The summary of the identification results of the direct sample preparation protocol including the best and second best matching organism names and scores are shown in *Table 2*. According to Manukumar et al. (2017), similar results were obtained when foodstuffs were investigated: 34 out of 36 coagulase positive *Staphylococcus* isolates were identified with a >2.000 score value, and two isolates had score values between 1.700–1999 when direct sample preparation was used.

Table 2

Score values of identification for the direct sample preparation method

Analyte name	Organism (best match)	Score value	Organism (second best match)	Score value
SA-1	*Staphylococcus aureus*	2.185	*Staphylococcus aureus*	2.156
SA-2	*Staphylococcus aureus*	2.214	*Staphylococcus aureus*	2.115
SA-3	*Staphylococcus aureus*	2.219	*Staphylococcus aureus*	2.047
SA-4	*Staphylococcus aureus*	2.278	*Staphylococcus aureus*	2.124
SA-5	*Staphylococcus aureus*	2.158	*Staphylococcus aureus*	2.023
SA-6	*Staphylococcus aureus*	2.391	*Staphylococcus aureus*	2.264
SA-7	*Staphylococcus aureus*	2.372	*Staphylococcus aureus*	2.195
SA-8	*Staphylococcus aureus*	2.136	*Staphylococcus aureus*	2.102
SA-9	*Staphylococcus aureus*	2.271	*Staphylococcus aureus*	2.224
SA-10	*Staphylococcus aureus*	2.233	*Staphylococcus aureus*	2.172
SA-11	*Staphylococcus aureus*	2.416	*Staphylococcus aureus*	2.370
SA-12	*Staphylococcus aureus*	2.441	*Staphylococcus aureus*	2.326
SA-13	*Staphylococcus aureus*	2.374	*Staphylococcus aureus*	2.211
SA-14	*Staphylococcus aureus*	2.413	*Staphylococcus aureus*	2.409
SA-15	*Staphylococcus aureus*	1.838	*Staphylococcus aureus*	1.795
SA-16	*Staphylococcus aureus*	2.158	*Staphylococcus aureus*	2.141
SA-17	*Staphylococcus aureus*	2.213	*Staphylococcus aureus*	2.182
SA-18	*Staphylococcus aureus*	1.813	*Staphylococcus aureus*	1.804
SA-19	*Staphylococcus aureus*	2.252	*Staphylococcus aureus*	2.167
SA-20	*Staphylococcus aureus*	2.356	*Staphylococcus aureus*	2.249

The results of the formic acid suspension sample preparation did not differ from the direct suspension method. Seven coagulase-positive staphylococci isolates gave score values ≥2.300, eleven isolates had score values between 2.000 and 2.300, and two strains had scores in the 1.700–1.999 range (*Table 3.*). The SA-15 and SA-18 isolates had the lowest score values for both of the two sample preparation methods.

The results of the ethanol extraction sample preparation method did not differ fundamentally from the direct and formic acid suspension methods. In this case, eight coagulase-positive staphylococci isolates had score values ≥2.300, nine isolates had score values between 2.000 and 2.300, and three strain gave scores in the 1.700–1.999 range. The best and second best matching organism names and scores are shown in *Table 4.*

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Table 3

Score values of identification for the formic acid suspending sample preparation method

Analyte name	Organism (best match)	Score value	Organism (second best match)	Score value
SA-1	*Staphylococcus aureus*	2.238	*Staphylococcus aureus*	2.163
SA-2	*Staphylococcus aureus*	2.252	*Staphylococcus aureus*	2.118
SA-3	*Staphylococcus aureus*	2.217	*Staphylococcus aureus*	2.209
SA-4	*Staphylococcus aureus*	2.246	*Staphylococcus aureus*	2.231
SA-5	*Staphylococcus aureus*	2.188	*Staphylococcus aureus*	2.162
SA-6	*Staphylococcus aureus*	2.429	*Staphylococcus aureus*	2.344
SA-7	*Staphylococcus aureus*	2.365	*Staphylococcus aureus*	2.307
SA-8	*Staphylococcus aureus*	2.216	*Staphylococcus aureus*	2.081
SA-9	*Staphylococcus aureus*	2.273	*Staphylococcus aureus*	2.268
SA-10	*Staphylococcus aureus*	2.172	*Staphylococcus aureus*	2.132
SA-11	*Staphylococcus aureus*	2.405	*Staphylococcus aureus*	2.386
SA-12	*Staphylococcus aureus*	2.312	*Staphylococcus aureus*	2.309
SA-13	*Staphylococcus aureus*	2.333	*Staphylococcus aureus*	2.215
SA-14	*Staphylococcus aureus*	2.461	*Staphylococcus aureus*	2.447
SA-15	*Staphylococcus aureus*	1.887	*Staphylococcus aureus*	1.829
SA-16	*Staphylococcus aureus*	2.215	*Staphylococcus aureus*	2.081
SA-17	*Staphylococcus aureus*	2.211	*Staphylococcus aureus*	2.094
SA-18	*Staphylococcus aureus*	1.866	*Staphylococcus aureus*	1.837
SA-19	*Staphylococcus aureus*	2.252	*Staphylococcus aureus*	2.164
SA-20	*Staphylococcus aureus*	2.373	*Staphylococcus aureus*	2.271

Table 4

Score values of identification for the ethanol extraction sample preparation method

Analyte name	Organism (best match)	Score value	Organism (second best match)	Score value
SA-1	*Staphylococcus aureus*	2.281	*Staphylococcus aureus*	2.194
SA-2	*Staphylococcus aureus*	2.301	*Staphylococcus aureus*	2.297
SA-3	*Staphylococcus aureus*	2.124	*Staphylococcus aureus*	2.119
SA-4	*Staphylococcus aureus*	1.797	*Staphylococcus aureus*	1.786
SA-5	*Staphylococcus aureus*	2.195	*Staphylococcus aureus*	2.073
SA-6	*Staphylococcus aureus*	2.396	*Staphylococcus aureus*	2.308
SA-7	*Staphylococcus aureus*	2.331	*Staphylococcus aureus*	2.310
SA-8	*Staphylococcus aureus*	2.127	*Staphylococcus aureus*	2.065
SA-9	*Staphylococcus aureus*	2.162	*Staphylococcus aureus*	2.115
SA-10	*Staphylococcus aureus*	2.164	*Staphylococcus aureus*	2.034
SA-11	*Staphylococcus aureus*	2.376	*Staphylococcus aureus*	2.084
SA-12	*Staphylococcus aureus*	2.336	*Staphylococcus aureus*	2.311
SA-13	*Staphylococcus aureus*	2.412	*Staphylococcus aureus*	2.319
SA-14	*Staphylococcus aureus*	2.462	*Staphylococcus aureus*	2.343
SA-15	*Staphylococcus aureus*	1.793	*Staphylococcus aureus*	1.728
SA-16	*Staphylococcus aureus*	2.253	*Staphylococcus aureus*	2.192
SA-17	*Staphylococcus aureus*	2.187	*Staphylococcus aureus*	2.037
SA-18	*Staphylococcus aureus*	1.913	*Staphylococcus aureus*	1.879
SA-19	*Staphylococcus aureus*	2.210	*Staphylococcus aureus*	2.201
SA-20	*Staphylococcus aureus*	2.382	*Staphylococcus aureus*	2.259

Table 5 summarizes the results of the different sample preparation methods. The direct sample preparation and the formic acid suspension yielded basically the same identification results. Seven strains gave score values ≥ 2.300, eleven isolates had score values between 2.000 and 2.300, and two isolates had the lowest score values for each of the two sample preparation methods. The results of the ethanol method differed slightly from the results of the other two methods. In that case eight isolates had score values ≥ 2.300, nine strains gave score values between 2.000–2.300, and three isolates gave scores in the 1.700–1.999 range.

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Table 5

Summary table of result of identification

	Sample preparation		
Score value	Direct sample preparation	Formic acid suspending	Ethanol extraction
2.300 ≤	7	7	8
2.000–2.300	11	11	9
1.700–1.999	2	2	3

Table 6 summarizes the best score value of mean and deviation of different sample preparation. The best score value of mean and deviation were defined using Microsoft Excel 2007. The largest deviation was obtained in SA-4 sample (0.269), the smallest standard deviation was found at the SA-20 sample (0.013). The largest mean of score values was determined in the SA-14 sample (2.445), the lowest mean of score value was determined in the SA-15 sample.

Table 6

Summary table of mean and deviation of best score value

Analyte name	Best score of direct sample preparation	Best score of formic acid suspension	Best score of ethanol extraction	Mean of score value	Standard deviation of score value
SA-1	2.185	2.238	2.281	2.235	0.048
SA-2	2.214	2.252	2.301	2.256	0.044
SA-3	2.219	2.217	2.124	2.187	0.054
SA-4	2.278	2.246	1.797	2.107	0.269
SA-5	2.158	2.188	2.195	2.180	0.020
SA-6	2.391	2.429	2.396	2.405	0.021
SA-7	2.372	2.365	2.331	2.356	0.022
SA-8	2.136	2.216	2.127	2.160	0.049
SA-9	2.271	2.273	2.162	2.235	0.064
SA-10	2.233	2.172	2.164	2.190	0.038
SA-11	2.416	2.405	2.376	2.399	0.021
SA-12	2.441	2.312	2.336	2.363	0.069
SA-13	2.374	2.333	2.412	2.373	0.040
SA-14	2.413	2.461	2.462	2.445	0.028
SA-15	1.838	1.887	1.793	1.839	0.047
SA-16	2.158	2.215	2.253	2.209	0.048
SA-17	2.213	2.211	2.187	2.204	0.014
SA-18	1.813	1.866	1.913	1.864	0.050
SA-19	2.252	2.252	2.210	2.238	0.024
SA-20	2.356	2.373	2.382	2.370	0.013

The difference in the summarised results of the ethanol extraction method to the other two methods with regard to the number of strains showed two strains switching identification levels. Strain SA-2 was securely identified at a species level (score value 2.301) with the ethanol extraction method contrary to the other two method which offered only secure genus level identification. However, the standard deviation of score values for the SA-2 strain between the three methods is smaller than the average standard deviation of the 20 strains (0.049). Consequently, this change in identification might not be significant. The other strain that had its identification levels changed when using the ethanol extraction method is SA-4. This strain's score was 2.278 and 2.246 with the direct sample preparation and the formic acid suspension testing, respectively, whereas the ethanol extraction gave only a 1.797 score. The reason for this difference is not well understood, maybe a protein rich fraction's behaviour is behind the phenomenon.

CONCLUSIONS

The MALDI-TOF MS technique can be used to assess the taxonomic position of coagulase-positive staphylococci from several sources, including foodstuffs. Based on result of direct sample preparation and the formic acid suspension sample preparation 19 out of 20 coagulase-positive *Stapylococcus* isolates were securely identified at genus level and seven isolates were identified at species level with a high probability. During ethanol extraction sample preparation, eight isolates were identified at species level with a high probability, but three strains had a score in the 1.700–1.999 range, therefore, in these cases, the identification of genus failed. Based on a first

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and second best score value, all isolates were evaluated as *Staphylococcus aureus.*

Two sample preparation methods (direct and formic acid techniques) yielded the same identification evaluation results. The result of the third sample preparation method (ethanol suspension technique) is slightly different from the other two processes, therefore, this procedure needs to be investigated for the causes of the discrepancy, since the quality of the obtained spectrum of isolates can affect the score value of identification.

The MALDI-TOF MS technique is a simple, quick and exact tool for a more reliable and even faster identification and confirmation of the taxonomic position of coagulase-positive staphylococci and, more specifically, of *Staphylococcus aureus.* This method can be implemented into routine diagnostic microbiology due to its high throughput and fast time-to-result capabilities. With the use of the MALDI-TOF technique, streaking of typical or suspect colonies on nutrient agar and the classical biochemical confirmations could be replaced, thus the analysis time of *Staphylococcus* spp. could be reduced by at least 24 hours.

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