Chemical Pretreatment of Cells for Enhanced MALDI-TOF-MS Discrimination of Clinical Staphylococci Including MRSA

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Abstract

BACKGROUND: Limited success has been reported for matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) differentiation of staphylococci, including methicillin resistant *Staphylococcus aureus* (MRSA) strains. Chemical pretreatment of cells prior to MALDI-TOF-MS analysis has not been systematically investigated for enhanced discrimination of *S.aureus* strains.

OBJECTIVES: To evaluate various chemical pretreatment of cells for MALDI-TOF-MS discrimination of clinical staphylococcal isolates, with a focus on differentiation of MRSA from methicillin sensitive *S. aureus* (MSSA) strains and from other staphylococcal species.

METHOD: MALDI-TOF-MS of a well-characterised *S. aureus* strain(s) was optimised with respect to matrix chemical(s), matrix solvent and target plating method. Various chemical pretreatments (solvents, reductants, detergents) and pretreatment application methods were then evaluated for enhancement of spectral richness. The three most promising pretreatments were applied to MALDI-TOF-MS discrimination of three set of clinical isolates comprising non-*S.aureus* staphylococci (77 isolates), MSSA (36) and MRSA (43), with analysis by total or set specific resolved peaks.

RESULTS: The optimized MALDI-TOF-MS protocol involved α-cyano-4-hydroxycinnamic acid (CHCA) as matrix chemical (in 1:2 acetonitrile:H_2O and 2% trifluoroacetic acid), with application as an overlay onto smeared cells (on-probe). On-probe application of chemical pretreatment was most effective at enhancing MALDI-TOF-MS spectral richness. Use of reductants and detergents as pretreatments were ineffective. The three most effective solvents/acid pretreatments - ethanol:formate, ethanol:acetate and formate:isopropanol - each generated reproducible and distinct spectra over the 2,000 -10,000 *m/z* range. For the combined sets of clinical isolates (114), all three of these pretreatments increased the total number of resolved peaks in comparison with no pretreatment controls. The ethanol:formate pretreatment gave 100% clustering of non-*S. aureus* staphylococci, based on total resolved peaks. The formate:isopropanol pretreatment generated the

largest increase in number of MRSA set specific peaks (from 18 to 32; 78% increase) and clustered the majority (77%) of the MRSA strains together, although compete discrimination of the MSSA and MRSA was not achieved.

CONCLUSION: MALDI-TOF-MS discrimination of clinical isolates of staphylococci is enhanced through chemical pretreatment of cells. Three chemical pretreatments, not previously applied to staphylococci, are highlighted for enhancing spectral richness and offering new opportunities for improved discrimination of staphylococci, including MRSA and MSSA strains.

Keywords

MALDI-TOF-MS; *Staphylococcus; S. aureus*; MRSA; MSSA;

1. Introduction

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) for rapid identification of microorganisms is well established, particularly for bacteria. In 1996, Claydon *et al*. [9] and Holland *et al*. [14] showed that bacterial cells could be analysed directly by MALDI-TOF-MS, using a simple technique known as intact cell MALDI-TOF-MS that requires minimal sample preparation, and identifies bacteria according to differences in cell wall composition. The merits and limitations of MALDI-TOF-MS biomedical mass spectrometry for identification of clinical etiological agents have recently been reviewed and evaluated, including assessment of the impact on time to identification and cost-effectiveness [3 ,27]. Despite considerable recent advances in the application of intact cell MALDI-TOF-MS for identification of bacteria, the differentiation of bacteria at the species level remains problematic. Commercially available MALDI-TOF-MS systems, such as the BrukerBiotyper, identify bacteria based on analysis of unique spectral profiles derived from whole cells or cell extracts, by comparison against databases of reference spectra (see [1] and references therein). Christensen *et al*. [8] applied the Bruker Daltonics microflex LT system to 90 well-characterised gram-positive cocci and found that more than half of the collection of strains obtained low score (identification) values due to taxa not being included in the database. Alatoom *et al*. [1] evaluated the BrukerBiotyper system to identification of 305 clinical isolates of staphylococci, streptococci and related genera. After exclusion of isolates not present in the Biotyper library, 69 and 20% of isolates were identified to species-level using direct colony testing and preparatory extraction respectively; the latter involving analysis of supernatant samples following cell lysis with 100% ethanol. These studies highlight a limitation for identification of gram-positive cocci through database comparison (i.e. the suitability/completeness of the database), as well as chemical extraction of cells as a means of enhancing individual spectral profiles leading to improved identification. Off-probe pretreatment of cells using cell-wall digesting enzymes has also been reported to enhance species specific MALDI-TOF-MS spectra from gram-positive bacteria [25] and

from yeasts [24]. Cassagne *et al*. [7] reported that complete extraction methods are better suited for MALDI-TOF-MS-based identification of yeasts in the clinical laboratory although they are more labour intensive. Knot *et al*. [19] compared direct smear (on-probe) and extraction methods on a taxonomically diverse collection of bacterial isolates and found the latter method yielded higher identification scores for the majority of isolates.

Preparation of supernatant samples or off-probe pretreatment of cells prior to MALDI-TOF-MS analysis is time consuming compared to pretreatment of direct smears. Other studies have adopted various on-probe chemical pretreatments of cells as a means of enhancing MALDI-TOF-MS spectra. Madonna *et al*. [19] applied 40% ethanol as a bacterial pretreatment in MALDI-TOF-MS detection of proteins above 15 KDa. Discrimination of *Escherichia coli* strains by MALDI-TOF-MS using an extraction solvents comprising formate:isopropanol:water has also been reported [6,10]. Meetani et al. [22] reported new MALDI-TOF-MS peaks in the mass range 2-80 kDa following on-probe pretreatment of whole cell gram-positive and gram-negative bacteria with surfactants. Qian *et al*. [23] reported that simple on-probe pretreatment of yeast cells with 50% methanol significantly improved the mass signature quality. We have recently reported [18] on a range of on-probe chemical pretreatments of cells – including various solvents, reductants, detergents - for enhanced discrimination of clinical yeasts by MALDI-TOF-MS. Furthermore, Zhang and Li [29] showed that MALDI-TOF-MS analysis with a two-layer matrix/sample preparation method can be used for direct analysis of protein digests with no or minimal sample cleanup after proteins are digested in a solution containing the surfactants, including sodium dodecyl sulfate (SDS). The feasibility of MALDI-TOF-MS analysis for proteomic samples following treatment with the reducing agent dithrothreitol (DTT) [5] and β-mercaptoethanol [16] has also been demonstrated. These reports highlight a wide scope of potentially beneficial pretreatments of intact bacterial cell prior to their attempted discrimination by MALDI-TOF-MS analysis.

MALDI-TOF-MS discrimination of bacterial strains within species generally presents a greater challenge than inter-species discrimination. The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) strains in hospital environments is recognised as a world-wide healthcare problem [26]. Early studies on optimisation of a MALDI-TOF-MS procedure for identification and discrimination of MRSA, has met with limited success. In a series of papers [13,17,28], Edwards-Jones and co-workers investigated a range of growth, plating, instrument and data process parameters for MALDI-TOF-MS discrimination of MRSA from other *S. aureus* strains and *Staphylococcus* species. From an organism set comprised of 26 staphylococcal isolates, they demonstrated the potential for discrimination between staphylococcal species and showed discrimination between methicillin sensitive *S. aureus* (MSSA) and two major MRSA strains [28]. Direct deposition of cells onto the MALDI target plate, without careful regulation of absolute numbers of bacterial cells, was shown to provide the best and most reliable spectra [17]. This study, involving a selection of 10 clinical isolates, also demonstrated the potential for differentiation of two epidemic-MRSA strains. Du *et* al. [12] analysed 76 strains of *S. aureus* and reported that seven isolates lacking the *mec* A gene were incorrectly identified as MRSA by MALDI-TOF-MS. Bernardo *et al*. [4] compared the MALDI-TOF-MS spectra from to a well-characterised MSSA and a MRSA strain to those from clinical isolates of *S. aureus*. They reported the bacterial 'fingerprints' obtained proved to be specific for any given strain, but a uniform signature profile for MRSA could not be identified.

Chemical pretreatment of cells prior to MALDI-TOF-MS analysis has not been systematically investigated for enhanced discrimination of *S.aureus* strains. We report here on optimisation of an MALDI-TOF-MS protocol for discrimination of clinical staphylococcal isolates, with a focus on discrimination of *S. aureus* from other species and on discrimination of MRSA from MSSA strains. MALDI-TOF-MS of a well-characterised *S. aureus* strains was optimised with respect to matrix

chemical(s), matrix solvent and target plating method. Various chemical pretreatments (solvents, reductants, detergents) and pretreatment application methods were then evaluated for enhancement of spectral richness. The three most promising pretreatments were applied to MALDI-TOF-MS discrimination of three set of clinical isolates comprising: non-*S.aureus* staphylococci (77 isolates), MSSA (36) and MRSA (43).

2. Materials and Methods

2.1 Organisms and culture conditions

Clinical isolates (114) from individual patients - obtained from the clinical microbiology laboratory of the Leicester Royal Infirmary-National Health Service (LRI-NHS) Trust, Leicester, UK - were included in the study. These isolates were previously identified as to the species level by standard laboratory procedures (including Gram staining and slide and tube coagulase) and by biochemical profiling using API® Staph (bioMérieux, Inc ., Durham, UK). *S. aureus* strains were further distinguished as methicillin sensitive *S. aureus* (MSSA) or MRSA according to their sensitivity/resistance to oxacillin and cefoxitin, with testing according to the British Society Antimicrobial Chemotherapy (BSAC) guidelines. Confirmation of MRSA strains was by detection of the penicillin binding protein 2' using MASTALEX™- MRSA (MAST Group Ltd., Merseyside, UK). Confirmation of the identification and the presence of the *mec* A in these strains was by testing with GenoType® MRSA (Hain Lifesciences UK Ltd., Byfleet, UK). The isolates were characterised as (number of isolates in parentheses): *Staphylococcus auricularis* (2); *Staphylococcus capitis* (3); *Staphylococcus chromogenes* (4); *Staphylococcus cohnii* (3); *Staphylococcus epidermidis* (2); *Staphylococcus haemolyticus* (3); *Staphylococcus hominis* (2); *Staphylococcus intermedius* (5); *Staphylococcus lugdunensis* (4); *Staphylococcus saprophyticus* (3); *Staphylococcus schleiferi* (3); *Staphylococcus sciuri* (3); *Staphylococcus simulans* (3); *Staphylococcus warneri* (3); *Staphylococcus xylosus* (5); MSSA (36); and

MRSA (30). *S. aureus* NCTC 10702 and 9315 (Public Health England, Salisbury, UK) were used as test strains for initial evaluation of MALDI-TOF-MS protocols.

The bacteria were cultivated on Muller Hinton agar (CM0337B, Oxoid) in the absence or presence (MRSA strains) of oxacillin at 4mg mL^{-1}. The agar was sterilised by autoclaving at 121°C for 20 min; filter sterilised oxacillin was added after cooling of the medium. Growth incubation was at 37°C for 48h.

2.2 MALDI-TOF-MS analysis

2.2.1 Matrix system and plating method optimisation

Three matrix chemicals - α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and ferulic acid (FA) - were tested to select the most suitable matrix chemical for detection of bacterial sample peaks over the *m/z* 2,000 – 20,000 range. For each matrix chemical (10mg/mL of CHCA, SA or FA), four matrix solutions of 1:1 acetonitrile:water (ACN:H_2O) with 0.3, 1, 2, 3 or 4 % trifluoroacetic acid (TFA) were prepared. *S. aureus* NCTC 10702 was used as the test strain. Each of the 15 matrix variants were applied to bacterial samples by three methods: direct colony transfer methods A and B, and a sandwich method. Direct colony transfer Method A involved applying one colony onto the target plate and allowing to dry in ambient air. Matrix solution (1µL) was applied to the plated sample, and dried in ambient air for approximately 20min (based on [12]); the target plate was then incubated at 60°C overnight to inactivate cells. Direct colony transfer Method B was as Method A but with the incubation at 60°C carried out *prior* to applying the matrix chemical. In the sandwich method**,** cells were mixed with wet matrix and, following drying, the cells were overlaid with a further 0.5µL of matrix solution and again dried (based on [11]); the target plate was then incubated at 60°C

overnight to inactivate cells. The influence of varying the ACN:H_2O ratio from 1:1 to 3:2 and 2:1, with CHCA as matrix chemical, was also investigated.

The quality of spectra and peak detection obtained through use of a single matrix chemical (CHCA, SA or FA) was compared to use of two or three matrix chemicals (combinations of CHCA, SA and/or FA). Single matrix chemical (10mg ml^{-1} matrix in 2:1 ACN:H_2O with 2% TFA) solutions were prepared and combined in 1:1 or 1:1:1 ratios by volume (i.e. CHCA:SA, CHCA:FA, SA:FA or CHCA:SA:FA) in eppendorf tubes and applied to cells on the target plates by the direct colony transfer method. In other experiments, single matrix solutions were applied to target plates as separate layers in a bi- or tri-matrix combination. The following sequence of layers within the bi- or tri-matrix combinations were investigated: CHCA-FA; CHCA-SA; FA-CHCA; FA-SA; SA-CHCA; SA-FA; CHCA-FA-SA; CHCA-SA-FA; FA-CHCA-FA; FA-CHCA-SA; SA-CHCA-FA; SA-FA-CHCA.

2.2.2 Pretreatment chemicals and their application

Pretreatment chemicals (Table 1) were applied to bacterial cells by three different methods (i to iii), with target plating using a modification of the direct colony transfer Method B using CHCA in 2:1 ACN:H_2O with 2% TFA. Pretreatment methods: (i) on-probe pretreatment: overlay of pretreatment chemical (0.5 µl) onto plated cells immediately prior to application of matrix; (ii) in-solution pretreatment: cells from one colony were washed in 500µL HPLC grade water, the cell pellet was resuspended in pretreatment solution (100µl), vortexed and incubated at room temperature for 5min and cells again pelleted; (iii) in-solution pretreatment with cell washing: as (ii) but with washing off of pretreatment chemical(s) from cells using HPLC grade water. Matrix only and pretreatment only controls were also prepared.

2.2.3 MALDI-TOF-MS spectra acquisition and data processing

All bacterial strains were analysed on Kratos PCAxima CFRplus MALDI-TOX-MS instrument (Kratos Analytical, Manchester, UK) with data acquisition and analysis via Launchpad 2.8.4 software

(Shimadzu Biotech, Manchester, UK). Spectra were acquired over the *m/z* 2,000 – 20,000 range by combining 150 profiles (five laser shots per profile), obtained by manual firing in linear positive mode. Replicate spotting and analysis (typically *n* = 3-5) was carried out to confirm that each plating/pretreatment method produced consistent spectra (also see Fig. 1). Sample chamber vacuum was 10^{-6} to 10^{-7} mbar. A solution of peptides and proteins (MS-CAL1, SigmaAldrich) with a molecular weight range of 757 to 16,952 Da was used to calibrate the mass spectrometer.

The Launchpad software selected the most intense peaks within spectra and processing was optimised to obtain representative spectra for samples by adjusting the following peak processing settings (optimised value in parentheses): average method smoothing (30); baseline subtract (80); threshold (0.3mV); and pairwise cutoff (0.6). Peak lists were aligned using SPECLUST software (web-based application; Alm *et al.* [2]) prior to analysis using SPSS 19 software. Hierarchical clustering for between group linkages was by the Jaccard method; a method particularly suited to handling asymmetric binary attributes by considering only those present in either one or both spectra.

3. Results

3.1 Matrix and plating method optimisation using S. aureus NCTC 10702

Visual comparison of spectra acquired for *S. aureus* NCTC 10702 with CHCA as matrix in 2:1 ACN:H_2O and 2% TFA showed the greatest number of resolved peaks by the direct colony transfer Method B, in comparison with the direct colony transfer Method A and sandwich methods. Changes to the TFA concentration reduced the number of resolved peaks within spectra over the *m/z* 2,000-10,000 range. Spectra acquired with SA or FA as matrix chemical over the range of TFA concentrations were poor in comparison with use of CHCA, with fewer resolved peaks and higher background interference observed for all three plating methods; the order for matrix chemical with regards to spectral richness was CHCA>SA>FA. Changing the ACN:H_2O, over the range given in Section 2.2.1, had little influence on the overall quality of spectra obtained using the three plating methods.

For bi- and tri-matrices combinations (in 2:1 ACN:H_2O with 2% TFA), the quality of the spectra obtained from *S. aureus* NCTC 10702 was lower than that obtained for CHCA or SA as sole matrix chemical, regardless of whether applied to cells on the target plate as pre-mixed matrix combination or as separate layers.

Based on the above, CHCA in 2:1 ACN:H_2O and 2% TFA with target plating by direct colony transfer Method B was selected for studies on the influence of pretreatment chemicals in MALDI-TOF-MS discrimination of clinical isolates.

3.2 Pretreatment chemicals

Of the three pretreatment chemical application methods (i to iii, Section 2.2.2), the on-probe method (method i) was the most consistent at generating peak rich spectra from *S. aureus* NCTC 10702. This method was used to compare the effectiveness of pretreatment chemicals for enhancing spectral richness obtained from two NCTC strains of *S. aureus* and from a clinical MRSA isolate. Evaluation of improved spectral richness was based on number of resolved peaks detected using the Launchpad software and overall visual inspection of spectra, both in comparison with no pretreatment control. Only three of the pretreatment regimes shown in Table 1 enhance spectral richness for all three organisms according to these criteria. All detected peaks were within the *m/z* 2,000 to 10,000 range, with none detected at higher *m/z* values. Application of ethanol:formate was the most promising of the three chemical pretreatment, producing the highest percentage increase in number of resolved peaks within spectra for each of the three organisms tested (Table 2). For all three chemical pretreatments, MALDI-TOF-MS spectra derived from replicate treatments were highly reproducible; spectra from three replicate analyses on ethanol:formate treated cells are shown in Fig.1. As illustrated in Fig. 2, each of the three chemical pretreatments generated spectra

that were clearly distinct, comprising common peaks, but also unique peaks that were not present in other pretreatment regimes.

The three chemical pretreatments shown in Table 2 were further evaluated by analysing three sets of clinical isolates: non-*S. aureus* staphylococci (48 isolates); MSSA (36 isolates); MRSA (30 isolates). For the combined sets (114 isolates), all three pretreatments increased the total number of resolved peaks in comparison with no pretreatment controls, with the highest increase (18-19%) shown for the ethanol:formate and formate:isopropanol pretreatments. As illustrated in Fig. 3, these two pretreatments also produced the highest increase in number of set specific resolved peaks, at 15 and 9% for ethanol:formate and formate:isopropanol respectively; the proportion of set specific peaks to total peaks for the three pretreatments was in the range 14 to 22%. "Set specific peaks" are those peaks found only in some or all spectra from a particular set of isolates. Strain clustering based on all resolved peaks within spectra showed that each of the three pretreatments enhanced clustering of organisms within one or more of the sets (Table 3). Ethanol:formate pretreatment gave 100% clustering on non-*S. aureus* staphylococci, and also marginally enhanced clustering of the MSSA set. Improved clustering of strains within the non-*S.aureus* staphylococci and MRSA sets was evident for the ethanol:acetate pretreatment, while the formate:isopropanol pretreatment marginally improved clustering of the MRSA strains. Based on set specific resolved peaks, all three pretreatments greatly enhanced clustering of the non-*S. aureus* staphylococcal strains (Table 3); clustering of the MRSA and non-*S.aureus* sets of clinical isolates is illustrated in Fig. 4. However, this was not evident for the MSSA and MRSA sets (Table 3). Based on total resolved peaks within the MSSA and MRSA sets only, ethanol:formate and formate:isopropanol pretreatments showed slight improvement in clustering of the MRSA set (Table 3). Based on set specific resolved peaks for the MSSA and MRSA sets, it was only possible to generate SPSS dendrograms for the formate:isopropanol pretreatment derived spectra. This reflects the large increase in number of

MRSA set specific peaks (from 18 to 32; 78% increase) produced by this pretreatment; there was little or no increase in MRSA specific peaks generated by the other two pretreatments (Table 3). This evident enhancement of MRSA set specific peaks through formate:isopropanol pretreatment led to clustering of the majority (77%) of the MRSA strains, although compete discrimination of the MSSA and MRSA was not achieved.

4. Discussion

We have previously shown that pretreatment of yeasts with a cell wall digesting enzyme [24] or with formate:isopropanol enhances discrimination of yeasts by MALDI-TOF-MS [18]. Other researchers have applied various solvent, reductant or detergent chemicals in MALDI-TOF-MS analysis of intact microorganisms or proteins/peptides (Table 1). In the present work, following optimisation of a MALDI-TOF-MS protocol (with respect to matrix solution and plating method, pretreatment application method), 27 novel chemical pretreatments - as well as eight others reported on previously – were compared for their efficacy at enhancing spectral richness and for discrimination of the non-*S. aureus* staphylococci, MRSA and MSSA sets of clinical isolate. An on-probe method of pretreatment was found to be most effective, which has the advantage of being less time consuming than off-probe preparation of cell supernatants. Of the various categories of chemical pretreatments, only three solvents/acid mixtures enhanced the richness of MALDI-TOF-MS spectra derived from *S. aureus*, with each capable of generating reproducible and distinct spectra from a MRSA isolate. An ethanol:formate pretreatment generated spectra that separated non-*S. aureus* staphylococci from MRSA and MSSA isolates, based on clustering involving all resolved peaks. A formate:isopropanol pretreatment generated the large increase in number of MRSA set specific peaks (from 18 to 32; 78% increase), with clustering of the majority (77%) of the MRSA strains together, although compete discrimination of the MSSA and MRSA was not achieved. This is consistent with Camera *et al*. [8] who reported on the efficacy of formate:isopropanol pretreatment for MALDI-TOF-MS discrimination of *E. coli* strains.

MALDI-TOF-MS analysis in the linear mode preserves covalent hydrogen bonds and it is therefore unlikely that large biopolymers within the bacterial cell wall are cleaved to give fragments within the 2,000-10,000 *m/z* range. Protein and peptide molecules associated with the bacterial cell wall are likely to contribute to diagnostic signals (peaks) within the spectra. However, the richness of spectra obtained suggests that other molecules are also ionised and contribute to spectral richness. Gluckmann *et al*. [15] reported that, based on different preparation protocols, distinct differences in the desorption/ionisation process for carbohydrates in contrast to peptides/proteins can be elucidated by MALDI-TOF-MS. These authors confirmed laser desorption and gas-phase cationisation as the principal mechanism in ionisation for neutral oligosaccharides, which can be initiated even for particulate analyte material or deposits onto a matrix surface. Interaction of pretreatment solvents with the bacterial cell wall leading to unfolding of cell surface molecules and exposure of smaller molecules to the matrix may account for the increased richness of spectral signals obtained. Solvent pretreatment may also promote release of cell membrane or cytosolic/ribosomal materials to the bacterial cell surface [23], giving rise to exposure of an abundance of medium-sized molecules to the matrix chemical. Majcherczyk *et al*. [21] compared MALDI-TOF-MS spectra from isogenic strains of MRSA differing in their expression of resistance to methicillin with changes in pulsed-field electrophoresis profiles or peptidoglycan muropeptide digest patterns of these strains. They proposed that MALDI-TOF-MS might be useful to detect strain-specific differences in ionisable components released from bacterial surfaces and not from their peptidoglycan network.

5. Conclusion

We conclude that MALDI-TOF-MS discrimination of clinical isolates of staphylococci is enhanced through chemical pretreatment of cells. Three chemical pretreatments - ethanol:formate, ethanol:acetate and formate:isopropanol - not previously applied to MALDI-TOF-MS analysis of

staphylococci, are highlighted for enhancing spectral richness and offering new opportunities for improved discrimination of such isolates, including MRSA and MSSA strains.

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Table 1. Chemical pretreatments for staphylococcal cells

Pretreatment chemical	Reference/origin
Solvent	
H_2O	Control pretreatment
Acetonitrile (50%)	novel
Methanol (50%)	Qian *et al*. [23] - yeasts and filamentous fungi
Methanol:formate:H_2O (5:1:4)	novel
Methanol:acetate:H_2O (5:1:4)	novel
Methanol:TFA:H_2O (25:3:22)	novel
Ethanol (40%)	Madonna *et al*. [20] - bacteria
Ethanol:formate:H_2O (4:1:5)	novel
Ethanol:acetate:H_2O (4:1:5)	novel
Ethanol:TFA:H_2O (20:3:27)	novel
Formic acid:isopropanol:H_2O (17:33:50)	Camara *et al*. [6]; Domin *et al*. [10]–bacteria
Isopropoanol (50%)	novel
Isopropanol:formate:H_2O (5:1:4)	novel
Isopropanol:acetate:H_2O (5:1:4)	novel
Isopropanol:TFA:H_2O (25:3:22)	novel
Acetone (50%)	novel
Acetone: formate:H_2O (5:1:4)	novel
Acetone:acetate:H_2O (5:1:4)	novel
Acetone:TFA:H_2O (25:3:22)	novel
Reductant	
DTT (20mmol L^{-1})	Bodzon-Kulakowsha*et al*. [5]– proteins
DTT (20mmol L^{-1}):formate:H_2O (5:1:4)	novel
DTT (20mmol L^{-1}):acetate:H_2O (5:1:4)	novel

DTT (20mmol L^{-1}):TFA:H_2O (25:3:22)	novel
β-ME (2% v/v)	Jaafar, Moukadiri and Zueco [16]- proteins/peptides
β-ME (2%):formate:H_2O (5:1:4)	novel
β-ME (2%):acetate:H_2O (5:1:4)	novel
β-ME (2%):acetate:H_2O (5:1:4)	novel
Detergent	
CHAPS (0.1%)	Zhang and Li [29] - protein digests
SDS (0.1%)	*ibid*
SDS (0.1%):formate:H_2O (5:1:4)	novel
SDS (0.1%):acetate:H_2O (5:1:4)	novel
SDS (0.1%):TFA:H_2O (25:3:22)	novel
[a]BugBuster x1 or x10 solution	Jenkins *et al*.[18] – yeasts
[a]Bugbuster x10:formate:H_2O (1:1:8)	novel
[a]Bugbuster x10:acetae:H_2O (1:1:8)	novel
[a]Bugbuster x10:TFA:H_2O (1:6:84)	novel

[a]Novagen®; DTT, dithiothreitol; β-ME,β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid.

Table 2. Chemical pretreatments for *S. aureus* affording greatest enhancement of spectral richness over the *m/z* 2,000 – 10,000 range

	Organism and number of resolved peaks		
Pretreatment	***S. aureus* NCTC 10702**	***S. aureus* NCTC 9315**	**MRSA (clinical)**
Ethanol:formate	57	53	41
Ethanol:acetate	27	50	39
Formate:isopropanol	30	48	40

Ethanol:formate= ethanol:formate:H_2O (5:1:4); Ethanol:acetate= ethanol:acetate:H_2O (5:1:4); Formate:isopropanol = formate:isopropanol:H_2O (17:33:50). Detection of peaks within spectra was as described in Section 2.2.3. Overall visual assessment of spectra also identified these three pretreatments as those that most improved the spectral richness for the test organisms.

Table 3. Comparison of effectiveness of three chemical pretreatments for discrimination of three sets of clinical staphylococci isolates by MALDI-TOF-MS

Pretreatment and set comparison (number of peaks in SPSS analysis)	Number of isolates in each sets clustered together (as percentage)		
	non-SA	**MSSA**	**MRSA**
All sets: total resolved peaks			
No pretreatment (1437)	37 (77)	13 (36)	13 (43)
Ethanol:formate (1706)	*48 (100)	*16 (44)	11 (37)
Ethanol:acetate (1517)	*41 (85)	13 (36)	*14 (47)
Formate:isopropanol(1694)	36 (75)	11 (31)	*16 (53)
All sets: set specific resolved peaks			
No pretreatment (203)	28 (58)	22 (61)	21 (70)
Ethanol:formate(237)	*46 (96)	19 (53)	13 (43)
Ethanol:acetate (210)	*47 (98)	13 (36)	11 (37)
Formate:isopropanol(221)	*43 (90)	16 (44)	14 (47)
MSSA *vs*. MRSA sets: total resolved peaks			
No pretreatment (619)	-	14 (39)	11 (37)
Ethanol:formate(733)	-	14 (39)	*12 (40)
Ethanol:acetate (706)	-	13 (36)	10 (33)
Formate:isopropanol (701)	-	13 (36)	*13 (43)
MSSA *vs*. MRSA sets: set specific resolved peaks			
No pretreatment (8 MSSA, 18 MRSA)	-	dnf	dnf
Ethanol:formate(6 MSSA, 23 MRSA)	-	dnf	dnf
Ethanol:acetate (11 MSSA, 18 MRSA)	-	dnf	dnf
Formate:isopropanol (10 MSSA, 32 MRSA)	-	*22 (61)	*23 (77)

non-SA, non-*S. aureus* staphylococci; dnf, dendrogram not formed by SPSS analysis; *, enhanced clustering relative to 'no pretreatment' control.

Figure legends

Figure 1. Replicate MALDI-TOF-MS spectra generated from a clinical MRSA isolate following on-probe pretreatment of cells with ethanol:formate:H_2O (5:1:4). The matrix chemical was CHCA in 2:1 ACN:H_2O and 2% TFA, with target plating by the direct colony transfer B method.

Figure 2. Distinct MALDI-TOF- MS spectra generated from *S. aureus* NCTC 9315 following different on-probe chemical pretreatments of cells. The matrix chemical was CHCA in 2:1 ACN:H_2O and 2% TFA, with target plating by the direct colony transfer B method.

Figure 3. Comparative increase in spectral richness (over the m/z 2,000 – 10,000 range) for three strains of *S. aureus* following chemical pretreatment of cells. The percentage increases in number of resolved peaks was relative to no-pretreatment controls. Pretreatments: (A) ethanol:formate:H2O (5:1:4); (B) ethanol:acetate:H2O (5:1:4); (C) formate:isopropanol:H2O (17:33:50). Detection of peaks within spectra was as described in Section 2.2.3.

Figure 4. Hierarchical clustering (Jaccard) showing separation of MRSA and non-*S.aureus* sets of clinical isolates, based on set specific peaks. On-probe chemical pretreatment of cells was with ethanol:acetate:H_2O (5:1:4); matrix chemical was CHCA in 2:1 ACN:H_2O and 2% TFA, with target plating by the direct colony transfer B method.