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Optimization of Sample Preparation Method, and Evaluation of Formic Acid Protein Extracts for MALDI-typing of *Staphylococcus aureus*

By

Hussein Hassan El Hage

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To my loving mother, I dedicate this work.

Optimization of Sample Preparation Method, and Evaluation of Formic Acid Protein Extracts for MALDI-typing of *Staphylococcus aureus*

Hussein El Hage

Abstract

Matrix Assisted Laser Desorption Ionization mass spectrometry (MALDI-MS) has been recently introduced in to the field of microbiology. The use of this technique overcomes some of the limitations of current phenotypic and genotypic methods. MALDI-typing of microorganisms is an approach based on the differentiation of MALDI-acquired protein fingerprints. Employed in clinical settings, it allows rapid identification of microorganisms down to the strain level. Leading to high morbidity and mortality rates, antibiotic resistant strains of *Staphylococcus aureus* have become a worldwide concern. Herein, we carried out a comparative study of 20 variations of an acid/alcohol bacterial protein extraction method using a clinical isolate of *S. aureus*. Protein fingerprints of these extracts acquired in linear mode (800-20,000 Da) were used for assessment of information content (number of peaks) and identity (size of proteins/peptides, m/z). Two methods were shown to be most efficient for sample preparation, i.e. formic acid/methanol and trifluoroacetic acid/ethanol, yielding 28 peaks each. Proteins obtained by the classic formic acid/ethanol extraction were separated using 2-dimensional electrophoresis (2-DE) yielding more than 50 protein spots. Nine proteins were successfully identified using peptide mass fingerprinting (PMF), two of which are virulence related proteins, SpoVG and endonuclease IV. These proteins are considered prospects for MALDI-typing.

Keywords: *Staphylococcus aureus,* Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS), Protein Fingerprint, MALDI-Typing, 2-Dimensional Electrophoresis (2-DE), Peptide Mass Fingerprinting (PMF).

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GLOSSARY

2-DE: Two-dimensional elctrophoresis

MALDI- MS: Matrix Assisted Laser Desorption Ionization Mass Spectrometry

MLST: Multi-locus sequence typing

MRSA: methicillin-resistant *Staphylococcu aureus*

PFGE: Pulsed-field gel electrophoresis

PMF: Peptide mass fingerprinting

PVL: Panton-Valentine leukocidin

S. aureus: *Staphylococcus aureus*

SCC: staphylococcal chromosomal cassette

***spa* CC:** *spa* clonal complex

ST: Sequence type

TSA: Tryptone Soy Agar

Chapter One
Introduction

1.1 MALDI-MS and Microbiology

A variety of phenotypic and genotypic methods are typically applied for the identification of highly pathogenic bacteria (De Bruyne *et al.*, 2011). Matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS) analysis of bacterial proteins, provides an alternative approach for bacterial identification. This technique has been recently introduced into the field of microbiology. Using MALDI-MS, rapid, reliable, easy to perform, and high-throughput bacterial identification techniques have been developed (De Bruyne *et al.*, 2011; Drevinek *et al.*, 2012). Several studies demonstrate the ability of MALDI-MS to generate specific protein fingerprints, spectra, for different microorganisms. These protein fingerprints can be used for strain specific differentiation and characterization of bacteria (Wahl *et al.*, 2002; Drevinek et al, 2012). In 2008, Barbuddhe *et al.* differentiated *L. monocytogenes* clonal lineages using MALDI-MS analysis. In 2011, Carbonelle *et al.* achieved species level identification of *Listeria* and *Pneumococci*.

Software such as Biotyper and ClinProTools (Bruker), implement different algorithms for the classification and differentiation of bacterial species. These tools are available for commercial use. Identification is accomplished by comparing a selection of detected masses to a reference database (Carbonelle *et al.*, 2012).

The use of MALDI-MS analysis in clinical microbiology laboratories was first reported by Seng *et al.* (2009). Novel studies demonstrate the ability of MALDI-MS to identify bacteria directly form positive blood cultures. Chen *et al.* (2013), Leli *et al.* (2013), and Schubert *et al.* (2011) report high accuracy, cost effective, and rapid identification of micro-organisms directly from cultures using MALDI-MS coupled with Bruker's Biotyper software. Proteins that are highly abundant in all microorganisms, are measured by the MALDI Biotyper. The software then matches the

protein fingerprint acquired from one microorganism to an open database, thus achieving accurate species level identification.

MALDI-MS analysis has been proven superior to phenotypic techniques in the identification of micro-organisms down to the genus and species level (El-Bouri *et al.*, 2012). Nevertheless, the capabilities of MALDI typing of microorganisms, to the strain level, remain a question of interest.

Two recent studies on the micro-organism used in this project, i.e. *Staphylococcus aureus* (*S. aureus*), identified peptide biomarkers for the classification and discrimination of methicillin-resistant strains by MALDI-MS (Wolters et al., 2011; Lu et al., 2012).

1.2 *Staphylococcus aureus*

Staphylococci are facultative anaerobe, Gram-positive, round shaped bacteria that grow in clusters. *S. aureus* is a member of the Micrococcaceae family (Stapleton and Taylor, 2002). It was first discovered by Sir Alexander Ogston in the 1880s as a main cause of wound infections (Archer, 1998), and was first isolated in a pure culture by Rosenbach. This human pathogen was annotated as *S. aureus* (golden, in Latin) for its characteristic surface pigmentation (Liu *et al.*, 2005).

S. aureus is a catalase and coagulase positive organism (Engemann *et al.*, 2003). It is part of the normal flora of the skin, oral cavity, and the gut (Todar, 2009). *S. aureus* is responsible for a wide array of human diseases, such as endocarditis, food poisoning, septicemia, skin boils, and soft tissue infections (Kim, 2009).

The emergence of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* has become a major concern (Brady *et al.*, 2006). According to reports from the National Nosocomial Infections Surveillance System *S. aureus* is the most common cause of nosocomial infections; however, during the past decade, it has emerged in community based settings (Saïd-Salim *et al.*, 2003; Huang *et al.*, 2006).

1.2.1 Virulence

S. aureus is a highly versatile organism capable of evading the immune system through several mechanisms. These include, capsular polysaccharides, protein A (spa), toxin production (gamma-hemolysin and Panton-Valentine leukocidin), and biofilm formation (Brady *et al.*, 2006).

Protein A, a 42 kDa virulence factor is encoded by the *spa* gene (Palmqvist *et al.*, 2002). Protein A has an antiphagocytic role due to its ability to bind immunoglobulin G (IgG) through the Fc portion thus rendering IgG dysfunctional (Shakeri *et al.*, 2010).

Panton-Valentine leukocidin (PVL) is a cytotoxin that highly contributes to the pathogenicity of the organism (McGrath *et al.*, 2008; Bittar *et al.*, 2009). PVL targets polymorphonuclear cells leading to necrotizing pneumonia as well as necrotizing skin and soft tissue infections (Bittar *et al.*, 2009).

Hospital acquired infections caused by *S. aureus* are typically associated with biofilm formation, making it difficult to eradicate. Cells in a biofilm are attached to each other forming a community, and are entrenched in a milieu of extracellular polymeric substance. The main advantages accompanied with this mode of growth are: increased antibiotic resistance, evading the immune system, and dissemination by detaching into the blood stream (Brady *et al.*, 2006).

Worldwide emergence of methicillin resistant *S. aureus* MRSA strains have caused increased morbidity and mortality rates in hospitals (Wolters *et al.*, 2011). Clinical isolates of MRSA are often resistant to a broad range of antibiotics.

MRSA is a result of the presence of the staphylococcal chromosomal cassette (SCC) *mec,* a mobile genetic element containing the *mec* A gene (Saïd-Salim *et al.*, 2003; Wang and Archer, 2010).

1.3 Phenotypic Identification and Molecular Typing

Rapid identification of different *S. aureus* strains is essential for the development of effective infection control measures (Sabet *et al.*, 2012).

In clinical settings, conventional approaches for identification of *S. aureus* are mainly based upon phenotypic methods (Morot-Bizot *et al.*, 2004), such as microscopic appearance, colonial morphology, staining reactions, and biochemical assays that check for key enzyme activities (Song *et al.*, 2003). However, phenotypic techniques are unable to accurately differentiate MRSA strains (Palavecino, 2007).

To determine the genetic relatedness of different *S. aureus* strains, a number of genotypic techniques have been developed; i.e. pulse field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and *spa* typing. Genotypic techniques have several limitations; they are technically demanding, potentially biased, and expensive (Conway *et al.*, 2001; Deurenberg *et al.*, 2007).

1.4 MALDI-typing of Microorganisms

Bacterial strain differentiation based on MALDI-MS acquired protein fingerprints is known as MALDI-typing. This is achieved by the identification of discriminatory protein peaks for different strains within one species (Rettinger *et al.*, 2012).

Protein fingerprints of crude bacterial extracts in the range of 2-20 kDa, mainly represent highly conserved species specific ribosomal proteins. These proteins are the key component in the process of species differentiation (Kornienko *et al.*, 2013). In 2012, Rettinger *et al.* differentiated strains of *Leptospira* species using MALDI-MS. The group deployed a simple and cheap method, using ethanol and formic acid for

protein extraction. The significance of Rettinger *et al.*'s results was comparable to sequencing of the16S rRNA gene and multi locus sequence typing (MLST).

Kornienko *et al.* (2013), attempted to differentiate *S. aurues* strains using MALDI-MS, based on the presence or absence of α and β-hemolysins. The two virulence proteins were picked randomly as prospects for MALDI-typing of *S. aureus*. Hemolysins, have molecular weights greater than the MALDI-typing range (800-12,000 Da); however, sample handling might lead to protein degradation or fragmentation. The authors could not achieve accurate strain differentiation, nor did they identify peaks indicative of the absence or presence of hemolysins. Yet, they suggest that further data analysis might lead to successful typing.

Identifying the protein content of a sample is one step towards MALDI-typing of microorganisms. Protein identification can be achieved via peptide mass fingerprinting (PMF). The procedure of PMF includes, the cleavage of proteins by a site specific enzyme (e.g. trypsin), MALDI-MS analysis, database search (Clauser *et al.*, 1999).

In this study, we evaluated 20 variations of an acid/alcohol bacterial protein extraction method by MALDI-MS analysis. Acquired data were analyzed using stringent criteria. Moreover, we assessed the suitability of formic acid/ethanol *S. aureus* protein extracts for MALDI-typing. Protein extracts were run on 2-DE gels, gel spots excised, and peptide mass fingerprinting was performed (PMF), in an attempt to identify proteins within or outside the MALDI-typing range of 800-12,000 Da. The suitability of typing was then addressed based on the identity, size, and function of the extracted proteins.

Chapter Two

MATERIALS AND METHODS

2.1 Clinical Isolate

A *S. aureus* sample was obtained from Dr. Sima Tokajian as a clinical isolate annotated "SA-16". This isolate has a staphylococcal cassette chromosome mec (SCCmec) of type IVc, and is Panton-Valentine Leukocidin (PVL) positive. The multilocal sequence typing (MLST) is of ST-30, grouped into the clonal complex of CC30. The allelic profile of SA-16 is 2-2-2-2-6-3-2 and classified at t318 Spa type, under the spa clonal complex spa-CC021. The E-test susceptibility was observed with Norfloxacin (NX) and Tetracyclin (TC) antibiotics.

2.2 *S. aureus* Protein Extraction

Protein extractions were carried out using nine different acids, i.e. acetic, adipic, boric, citric, formic, malonic, oxalic, succinic, and trifluoroacetic acid, as well as HPLC-grade water in combination with two alcohols, i.e. ethanol, and methanol (20 method variations). In all cases, 70% (w/v) or 70% (v/v) solutions were prepared depending on the physical nature of the acid (solid vs. liquid). For each acid three pH readings were recorded and averaged. The pH was determined using a Milwaukee Instruments MI151 pH/ORP/Temp Bench Meter.

A modified protocol by Wolters *et al.* (2011) was used for extraction of *S. aureus* proteins. In the original protocol, Columbia blood agar was used for culture of *S. aureus*, and vortexing steps were not timed.

SA-16 was cultured on Tryptone Soy Agar (TSA) plates for 22-24 hrs at 37 °C. Under sterile conditions, a full loop of colonies was suspended in 300 µL of HPLC-grade water. Alcohol (900 µL) was then added and the suspension vortexed vigorously. The mixture was then centrifuged at 12,000×g for 2 min at room

temperature. The supernatant was carefully removed, and centrifugation repeated to remove any residual alcohol. The pellet was resuspended in 50 µL of acid, and vortexed vigorously for 15 min (to solubilize the whole pellet). Acetonitrile (50 µL) was then added, and suspension vigorously vortexed for 15 min. Centrifugation was then applied at 20,000×g for 2 min, and 80 µL of the supernatant were collected (protein extract). Samples were spotted directly or stored at −20 ºC until use.

2.3 MALDI-MS

In preparation for MALDI-MS analysis, 1 µL aliquots of the *S. aureus* protein extracts were spotted onto a stainless steel target plate (Opti-TOF TM 384 Well Insert, 123×81 mm RevA, Applied Biosystems, USA) and air dried for 15 min. The sample spot was then overlaid with 1 µL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid, CHCA, in 50% acetonitrile with 0.1% trifluoroacetic acid) and again air dried for 15 min. MALDI-MS analysis was performed using a 4800 MALDI TOF/TOF Analyzer instrument operated by the 4000 Series Explorer software version 3.5.3 (Applied Biosystems, USA). Spectra were acquired in linear positive mode at a laser intensity of 5000 within a mass range from 800 to 20,000 Da. Acceleration voltage was 20 kV, IS2 voltage was maintained at 19.3 kV, the extraction delay time was 160 ns, and laser frequency was set to 500 MHz. Peak detection criterion was set to a minimum signal to noise ratio S/N= 10. For each sample spot, an average spectrum was accumulated from 1000 measurements. Protein extraction was performed in three biological replicates. Each extract was analyzed in multiplets (11 replicates), resulting in 33 mass spectra for each extraction method, and a total of 726 mass spectra for the whole study.

Peak lists with centroid masses, masses at peak centers, were collected form the 4000 Series Explorer Software version 3.5.3 (Applied Biosystems, USA) without further processing.

2.4 Calibration and Error Analysis

Calibration was performed using 4700 Proteomics Analyzer Calibration mixture (4700 Cal Mix) (Applied Biosystems). The average theoretical masses of the five standards were over a mass range of 904.46 to 3,678.93 Da. The standards used are listed in Table 2.1 and 2.2. Error was calculated in parts per million (ppm) after comparing measured values of 4700 Cal Mix standards (Linear mode) to their theoretical ones.

Table 2.1. 4700 Cal Mix standards used for instrument calibration for ethanol sample runs.

Standards	Theoretical Mass (Da)	Measured Mass (Da)	Error (ppm)
des-Arg-Bradykinin	904.4681	904.4139	60
Angiotensin I	1296.6853	1297.0666	294
Glu-Fibrinopeptide B	1570.6774	1570.6819	3
ACTH(clip1-17)	2093.0867	2092.5941	235
ACTH(clip18-39)	2465.1989	2466.1605	390
ACTH(clip 7-38)	3657.9294	3660.4324	684
Average Error (ppm)			278

Table 2.2. 4700 Cal Mix standards used for instrument calibration for methanol sample runs.

Standards	Theoretical Mass (Da)	Measured Mass (Da)	Error (ppm)
des-Arg-Bradykinin	904.4681	903.9250	600
Angiotensin I	1296.6853	1296.0868	462
Glu-Fibrinopeptide B	1570.6774	1570.2392	279
ACTH(clip1-17)	2093.0867	2092.1745	436
ACTH(clip18-39)	2465.1989	2465.7118	208
ACTH(clip 7-38)	3657.9294	3656.5774	370
Average Error (ppm)			393

2.5 Data Analysis

Peak lists of the acquired mass spectra were analyzed using mMass version 5.4.1, an open source software developed by Martin Strohalm (Strolham et al., 2008). During preliminary analysis, one peak list was created for every extraction method by a two-step process. Primarily, 11 peak lists corresponding to the MALDI replicates acquired for every biological replicate were used as input data. At this point, a table containing all the measured masses, and showing the incidence of each was generated. Peak binning, an averaging process of centroid masses, which fall within the determined experimental error interval (278 ppm for ethanol treated samples, and 393 ppm for methanol treated samples), was then performed in house. A peak was considered present (reproducible) if found on ≥60% of MALDI replicates and in ≥2/3 of biological replicates. At this point, one peak list for every extraction method was obtained. Peak lists of acid/ethanol methods and acid/methanol methods were then compared, and for every alcohol a list of unique and common peaks (peak present in two or more different extraction methods) amongst the acids was created. Moreover, the observed spectral profiles for an acid used with either methanol or ethanol treatment were compared.

2.6 Protein Quantification

Proteins were dried using Speed-vac and resolubilized in HPLC-grade water prior to quantification. Protein concentrations were determined using the Pierce BCA (bicinchonic acid) Protein Assay Kit (p/n: 23337, ThermoScientific), and bovine serum albumin for calibration, according to manufacturer's instructions. The measurements were carried out on a 96-well plate using absorbance wavelength 595 nm on a Multiscan micro-plate reader (ThermoScientific), three readings were acquired and averaged for each of the three biological replicates.

2.7 One-Dimensional SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Formic acid extracts of SA-16 were used for 1D and 2D gel electrophoresis. Polyacrylamide gels with a short stacking gel consisting of 5% acrylamide followed by a homogenous 15% acrylamide resolving gel were prepared. Three biological replicates of protein extracts were dried by Speed-vac, and reconstituted in 80 µL of Laemmli buffer (62.5 mM Tris-HCL, 25% glycerol, 2% SDS, 0.01% bromophenol blue). Proteins were then denatured by boiling at 95 °C for 5 min before loading on the gel. Boiled protein extracts (30 µL) were loaded on the gel. A 2.5-26.5 kDa peptide ladder (Bio-Rad) was also loaded into three wells, 15 µL/well. Electrophoresis was performed on a PROTEAN® II XL Cell (Bio-Rad). Gel size was 18.3 x 20 cm (W x L). Run parameters were as follows: 150 V for 90 min, 200 V for 3 hrs, and 250 V for 2 hrs. Gels were washed with distilled water for 30 min, then fixed for 30 min. using a 40% methanol, 10% acetic acid solution. Staining was performed using Bio-safe coomassie (Bio-Rad) for 1 hr. Gels were destained for 1 hr by washing with distilled water. Bands were then visualized, and images acquired using a GS-800 calibrated densitometer (Bio-Rad).

2.8 Two-Dimensional Electrophoresis (2-DE)

Prior to 2D gel electrophoresis run, the three biological protein extraction replicates were cleaned using the ReadyPrep 2D cleanup kit (Bio-Rad). Cleaned samples (400 µg protein) were then resolubilized in 300 µL 2D sample buffer (Bio-Rad).

Samples were applied on 17 cm IPG strips (pH range 3–10) for 12-16 hrs. Isoelectric focusing (IEF) was performed in linear gradual mode on a PROTEAN® i12™ IEF System (Bio-Rad). After focusing, the IPG strips were equilibrated twice for 10 min in equilibration buffer (6 M Urea, 0.375 M Tris-HCl (pH 8.8), 2% w/v SDS, 20% v/v glycerol). In the first equilibration, 2% w/v DTT (dithiotreitol) was

added to the equilibration buffer, and the second, 2.5% w/v iodoacetamide. The equilibrated strips were gently rinsed with 1X Tris-Glycine-SDS buffer (TGS), blotted to remove excess buffer, and then applied on 15% polyacrylamide gels in a PROTEAN® II XL Cell electrophoresis system (Bio-Rad). Electrophoresis was performed in two steps: 16 mA/gel for 30 min, 24 mA/gel for 5 hrs. After SDS-PAGE, the 2-DE gels were washed 3 times for 10 min each with fresh distilled water, and afterwards stained with BioSafe Coomassie (Bio-Rad) for 60 min. Gels were destained by washing twice for 30 min with distilled water, and left overnight in the fridge for better results. The three Coomassie-stained gels were scanned using Bio-Rad's GS-800 calibrated imaging densitometer and the Quantity One Software version 1.0.8 (Bio-Rad).

2.9 In-Gel Protein Digestion

In-gel digestion procedure was performed according to a modified protocol used at the University of California, San Francisco mass spectrometric facility.

Protein spots were manually excised from the gels, and cut into 1-mm pieces. The gel pieces were washed three times with 100 µL (or enough to cover) of 25 mM NH_4HCO_3/50% ACN and vortexed for 10 min, then dried in a Speed-vac. A volume of 25 µL (or enough to cover) of 10 mM DTT in 25 mM NH_4HCO_3 (1.5 mg/mL) was added to each of the gel pieces, proteins were reduced for 1 hr at 56 ºC. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 25 mM NH_4HCO_3 (10 mg/mL). Alkylation reaction was left to proceed for 45 min at ambient temperature in the dark. Gel pieces were then washed with 100 µL of 25 mM NH_4HCO_3, and dehydrated with 100 µL of 25 mM NH_4HCO_3/50% ACN. The liquid phase was removed, and the gel pieces were completely dried with a Speed-vac. The pieces were swollen in a digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/µL TCPK treated trypsin (Sigma-Aldrich) at 4 ºC for 10 min. An adequate volume of 25 mM NH_4HCO_3 was added to cover the gel pieces. Enzymatic cleavage was accomplished by incubating the samples for 3 hrs at 37

ºC. The digest solutions were transferred to clean 1.5 mL siliconized Eppendorf tubes. Peptides were extracted by three changes of 30 µL (enough to cover) 50% ACN/5% formic acid. Each change was carried out by a vortex step of 20-30 min and a spin step. The extracted digests were vortexed, and the volume reduced to 30 µL using Speed-vac. C18 ZipTips (Millipore) were used for sample cleanup according to manufacturer's instructions.

2.10 Protein Identification

MALDI-MS

In preparation for MALDI-MS analysis,1 µL aliquots of the in-gel digested *S. aureus* protein extracts were spotted onto a stainless steel target plate (Opti-TOF TM 384 Well Insert, 123×81 mm RevA, Applied Biosystems, USA) and air dried for 15 min. Sample spots were then overlaid with 1 µL of CHCA matrix solution and again air dried for 15 min. MALDI-MS analysis was performed using a 4800 MALDI TOF/TOF Analyzer instrument operated by the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA). Instrument was externally calibrated using 4700 Cal Mix standards. Spectra were acquired in reflector positive mode at a laser intensity of 4600 within a mass range from 500 to 4,000 Da. Acceleration voltage was 20 kV, IS2 voltage was maintained at 16.1 kV, the extraction delay time was 300 ns, and laser frequency was set to 500 MHz. Peak detection criterion was set to a minimum signal to noise ratio S/N= 20. For each sample spot, an average spectrum was accumulated from 800 measurements. Mass lists were collected from the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA). Mass list processing was performed using Peak Erazor version 2.01 (Lighthouse data). An exclusion list (Table A-3 in the appendix) covering matrix, keratin peptides, and trypsin autolysis masses was created. Peak masses matching the exclusion list were removed prior to database search.

Instrument calibration was performed using 4700 Proteomics Analyzer Calibration mixture (4700 Cal Mix) (Applied Biosystems). The theoretical masses of the five standards cover a mass range of 904.46 to 3,678.93 Da. The standards used are listed in Table 2.3. Error was calculated in parts per million (ppm) after comparing measured values of 4700 Cal Mix standards (Reflector mode) to their theoretical ones.

Table 2.3. 4700 Cal Mix standards used for instrument calibration for reflector mode run of in-gel digested proteins.

Standards	Theoretical Mass (Da)	Measured Mass (Da)	Error (ppm)
des-Arg-Bradykinin	904.4681	904.4043	70
Angiotensin I	1296.6853	1296.5694	89
Glu-Fibrinopeptide B	1570.6774	1570.6102	43
ACTH(clip1-17)	2093.0867	2093.0440	20
ACTH(clip18-39)	2465.1989	2465.1422	23
ACTH(clip 7-38)	3657.9294	3657.6193	85
Average Error (ppm)			55

Peptide Mass Fingerprinting

Protein identification was accomplished by the input of the processed mass lists into the Mascot 2.2 Server (Matrix Science, UK), and performing searches against the NCBInr (NCBI= National Center for Biotechnology Information non-redundant) database (version 10130210) and Swiss-Prot (Swiss Protein Sequence Database version 2013_03). The following search parameters were used: taxonomy, firmicutes; enzyme, trypsin; variable modification, oxidation (M), constant modifications, cabamidomethyl (C). Up to one missed cleavage was allowed, and peptide mass tolerance was set to ±110 ppm. Protein scores greater than 61 and 78 were considered significant in Swiss-Prot and NCBInr respectively with $p < 0.05$.

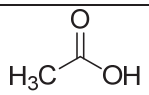
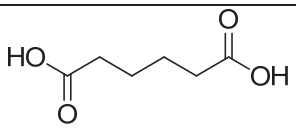
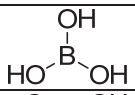
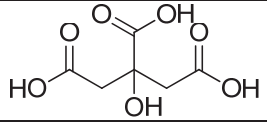
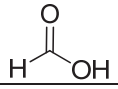
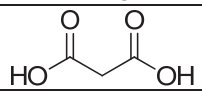
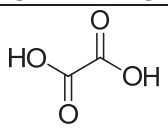
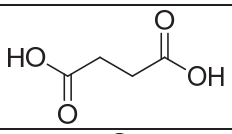
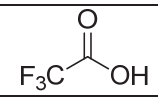
Chapter Three

Results

3.1 Acids pH

The measured pH values of the prepared 70% acid solutions ranged from pH= -0.67 to 3.37, with TFA being the most acidic and boric acid being the least acidic (Table 3.1)

Table 3.1. Name, abbreviation, molecular weight, structure and pH of acid solutions employed in this study.

Acid	Abbreviation	Molecular Weight	Structure	pH of 70% aqueous solution*
Acetic Acid	AcA	60.05		1.64 ± 0.04
Adipic Acid	AdA	146.14		2.8 ± 0.1
Boric Acid	BA	61.83		3.37 ± 0.06
Citric Acid	CA	192.12		2.65 ± 0.05
Formic Acid	FA	46.03		1.23 ± 0.03
Malonic Acid	MA	104.06		2.13 ± 0.03
Oxalic Acid	OA	90.03		1.86 ± 0.05
Succinic Acid	SA	118.09		3.00 ± 0.02
Trifluoroacetic Acid	TFA	114.02		-0.67 ± 0.01

*pH of HPLC-grade water used for preparation of acidic solutions is 9.20± 0.02.

3.2 Protein Quantification

Nine different acids (acetic acid, adipic acid, boric acid, citric acid, formic acid, malonic acid, oxalic acid, succinic acid, TFA) and HPLC-grade water were used for protein solubilization during extraction procedure (Table 3.1). Bovine Serum Albumin (BSA) was used to draw a standard curve, and protein concentrations were calculated from measured absorbance (Table 3.2 and Figure 3.1).

Table 3.2. Absorbance for standard BSA solutions of known concentration at a wavelength of λ = 595 nm.

BSA standard	Conc. (µg/mL)	Abs. (λ=595)	Error (%)
A	1000	0.688	1.05
B	500	0.362	1.68
C	250	0.190	2.13
D	125	0.103	1.94
E	25	0.021	2.79
F	0	0.000	0.00

Values represent the average of three readings. Experimental error was calculated and is expressed in percent.

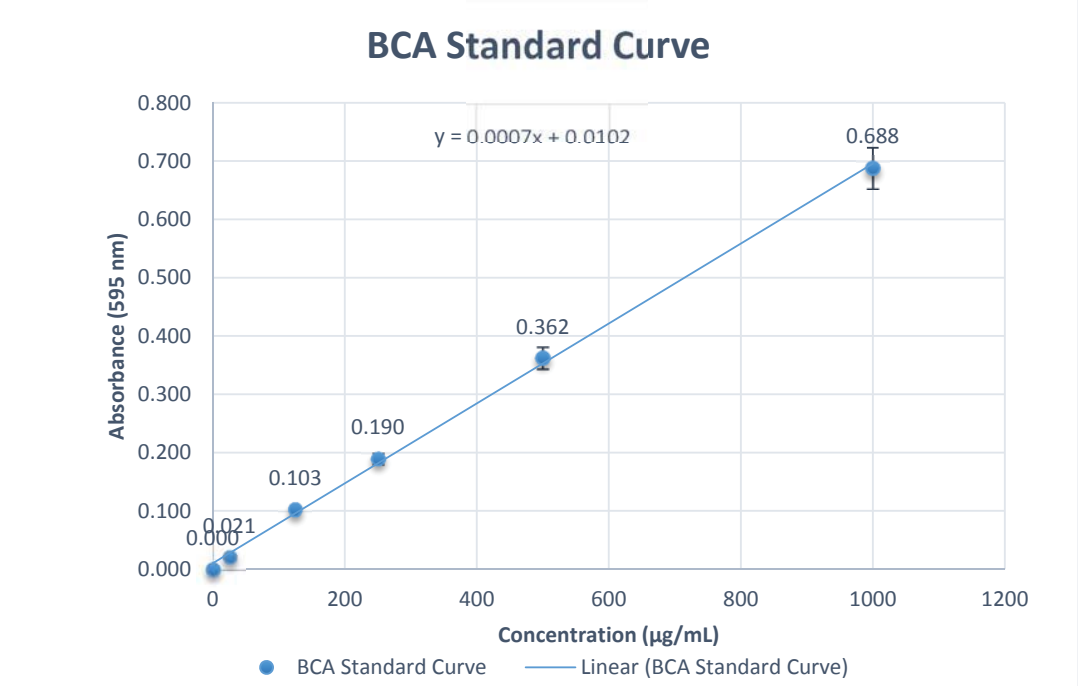


Figure 3.1. BSA standard curve used for protein quantification with linear fit (with R^2 of 0.9988).

Table 3.3 shows the averaged protein concentrations calculated based on the BSA standard curve equation. Experimental error was below five percent for all extractions.

Table 3.3. Calculated protein concentration obtained for each of the acid/alcohol extraction methods.

	Ethanol		Methanol	
	Concentration (µg/mL)	Error (%)	Concentration (µg/mL)	Error (%)
Acetic	375	2.1	407	4.4
Adipic	322	4.9	316	2.3
Boric	576	3.2	540	3.3
Citric	372	2.5	353	1.9
Formic	630	3.6	687	2.9
Malonic	314	2.7	326	2.7
Oxalic	407	2.3	324	4.1
Succinic	495	1.9	468	2.7
TFA	821	2.1	1131	4
Water	522	3	482	1.8

Three readings (technical replicates) were recorded per biological replicate. Concentration was averaged over three biological replicates in µg/mL for the different acid-alcohol extraction protocols.

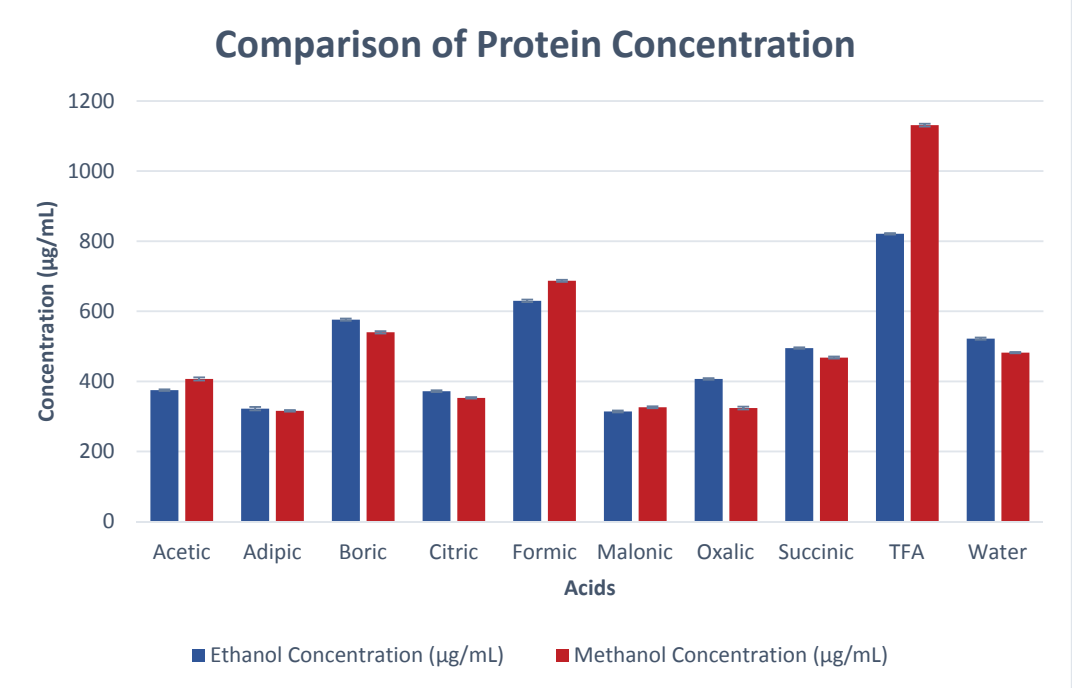


Figure 3.2. Comparison of protein concentration for each acid and HPLC-grade water in ethanol and methanol treated extracts.

Protein concentration ranged from 314 to 1,131 µg/mL across all extraction protocols (Table 3.3). The average values of measured protein concentrations for each extraction protocol indicated that the use of TFA with either ethanol or methanol treatment yielded the highest protein concentration, 821 µg/mL and 1,131 µg/mL respectively (Figure 3.2). Moreover, the lowest protein concentrations were observed when adipic, malonic, and oxalic acid were used. The use of HPLC-grade water for protein extraction yielded an intermediate amount of proteins, higher than six of the acids (acetic, adipic, citric, malonic, oxalic, and succinic) when either alcohol was used for treatment (Table 3.3). No correlation between the pH of the solution and the concentration of extracted protein was observed.

3.3 MALDI-MS Analysis of Protein Extracts

Spectral Acquisition

Figures 3.3 to 3.7 represent MALDI-MS analysis spectra of the protein extracts. The majority of the detected peaks were in a m/z range of 800-11,000. Only TFA-based protein extracts revealed peaks with a m/z ratio up to 16,000. However, these peaks were only reproducible when TFA/ethanol method was used compared to the TFA/methanol method, Figure 3.3.

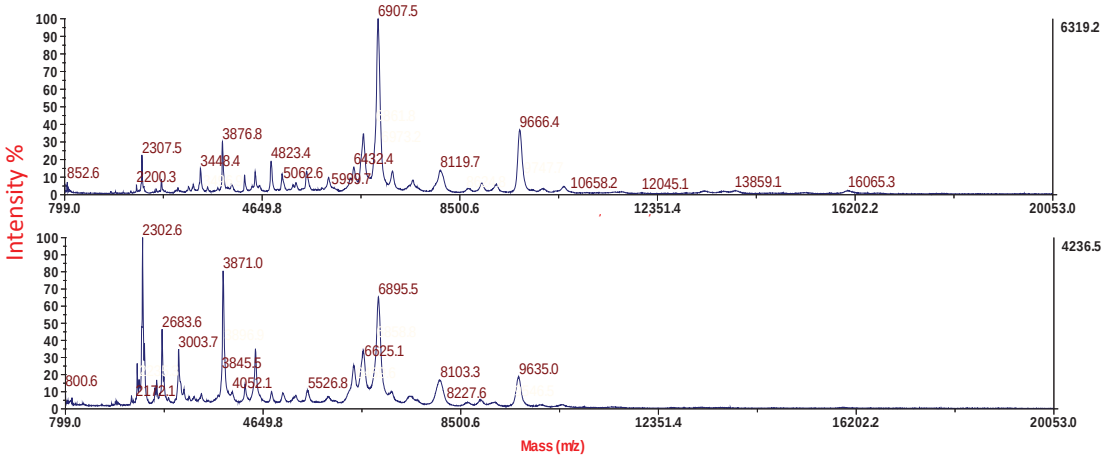


Figure 3.3. MALDI-MS spectra for *S. aureus* protein extracts obtained using TFA in combination with ethanol (top) and methanol (bottom) treatment. Spectra were acquired in linear mode with a mass range of 800-20000 Da.

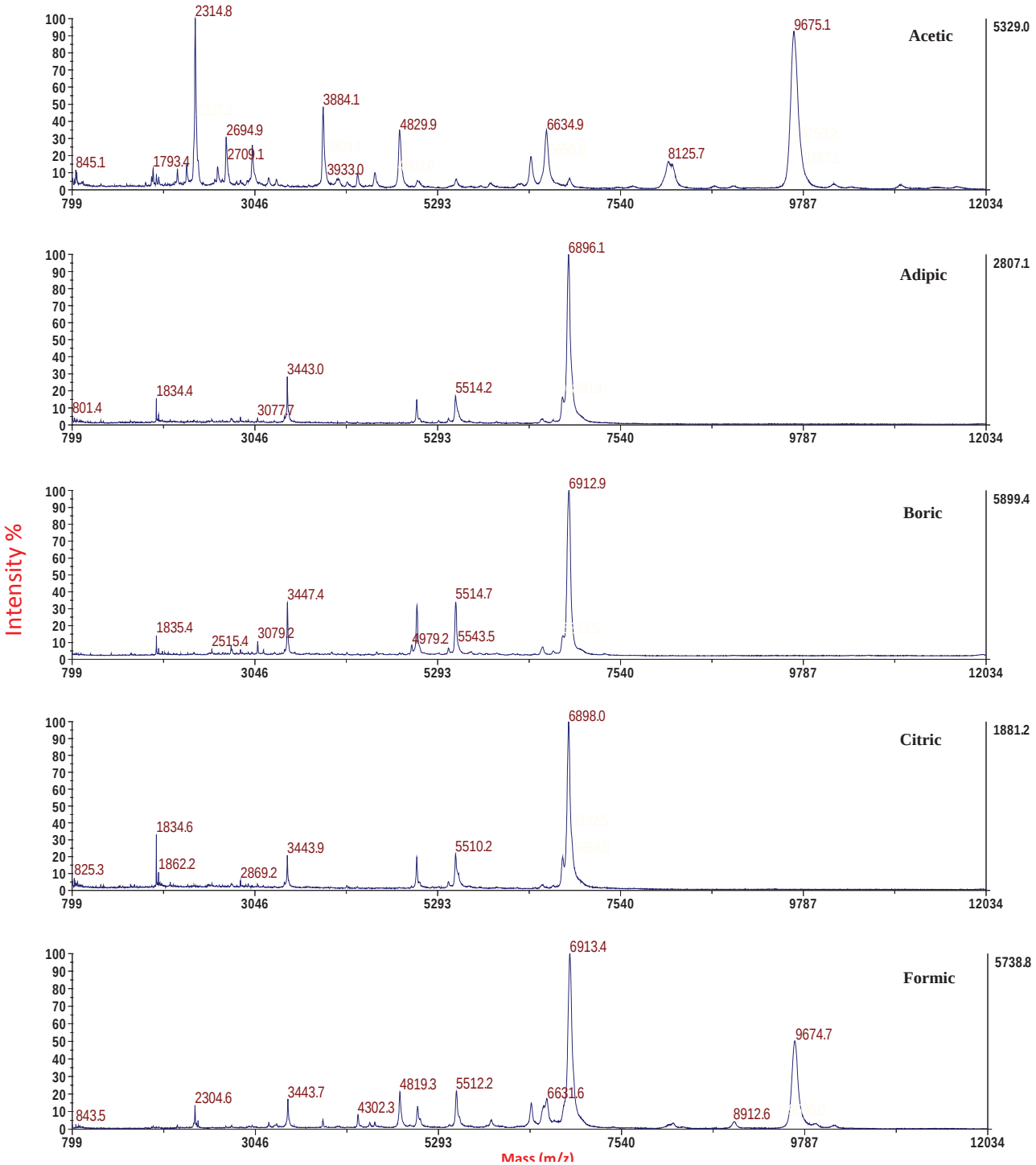


Figure 3.4. MALDI-MS spectra for *S. aureus* protein extracts obtained using acetic, adipic, boric, citric, and formic acid (top to bottom) in combination with ethanol treatment. Spectra were acquired in linear mode with a mass range of 800-12000 Da.

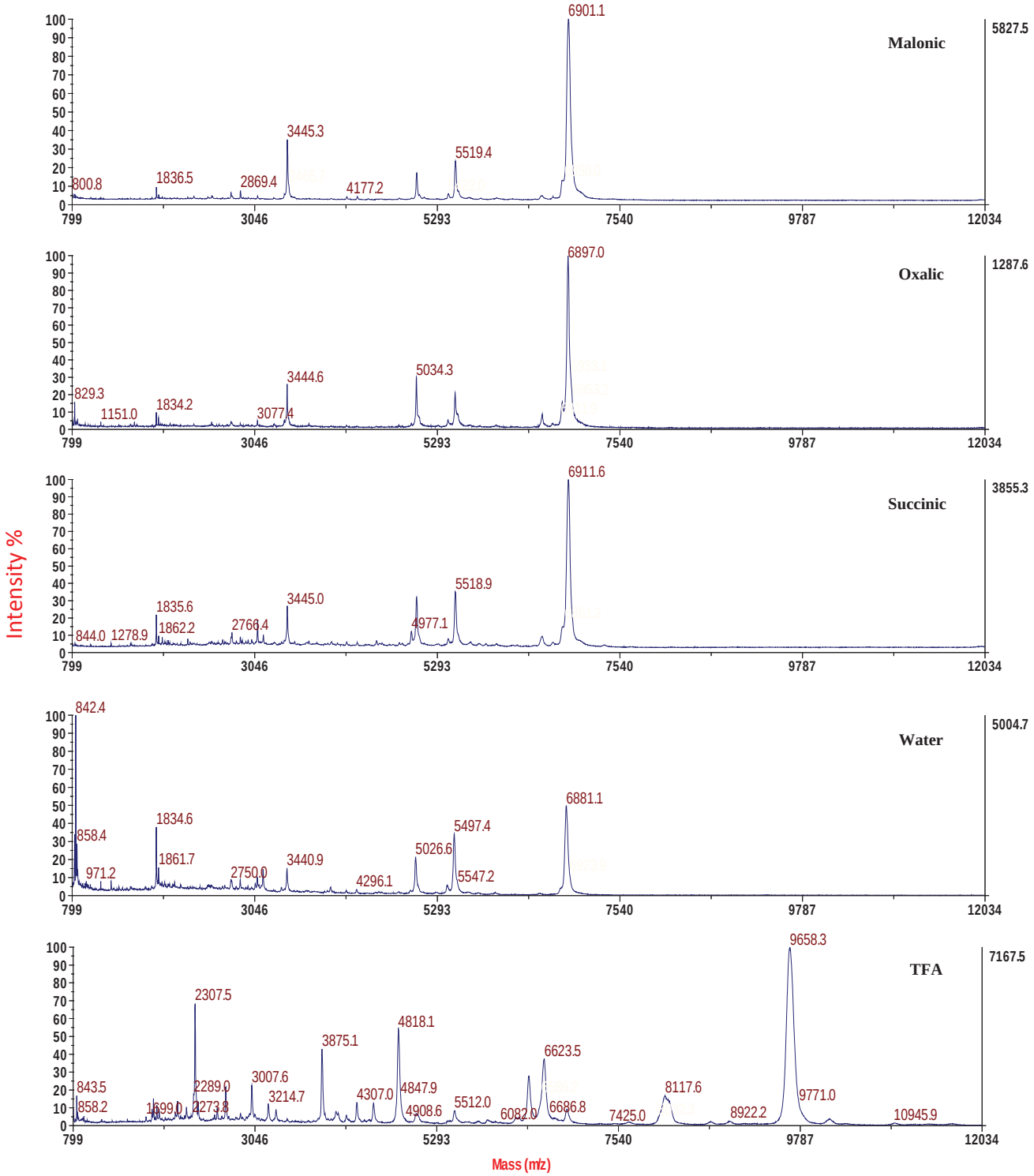


Figure 3.5. MALDI-MS spectra for *S. aureus* protein extracts obtained using malonic, oxalic, succinic, HPLC-grade water, and trifluoroacetic acid (top to bottom) in combination with ethanol treatment. Spectra were acquired in linear mode with a mass range of 800-12000 Da.

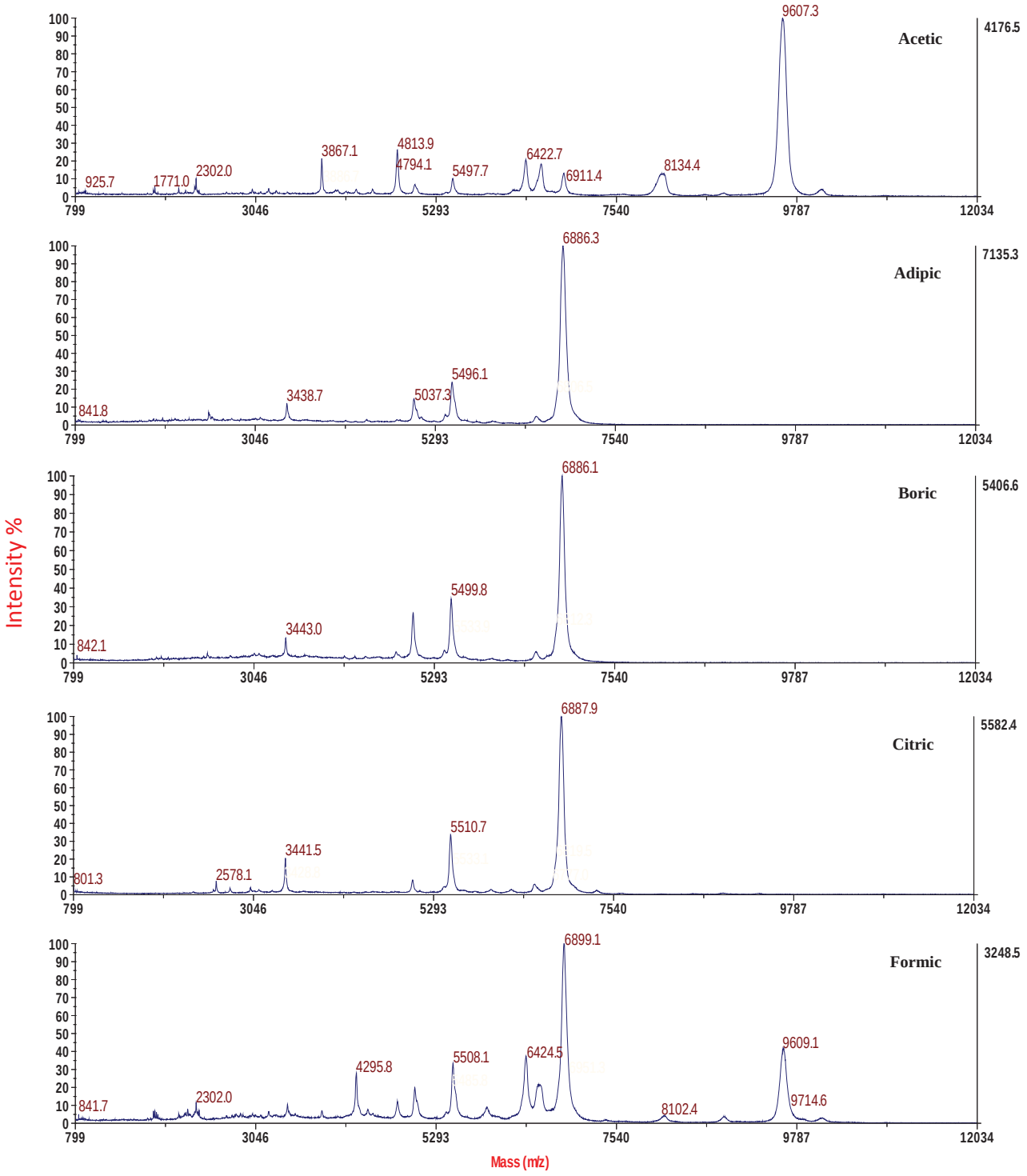


Figure 3.6. MALDI-MS spectra for *S. aureus* protein extracts obtained using acetic, adipic, boric, citric, and formic acid (top to bottom) in combination with methanol treatment. Spectra were acquired in linear mode with a mass range of 800-12000 Da.

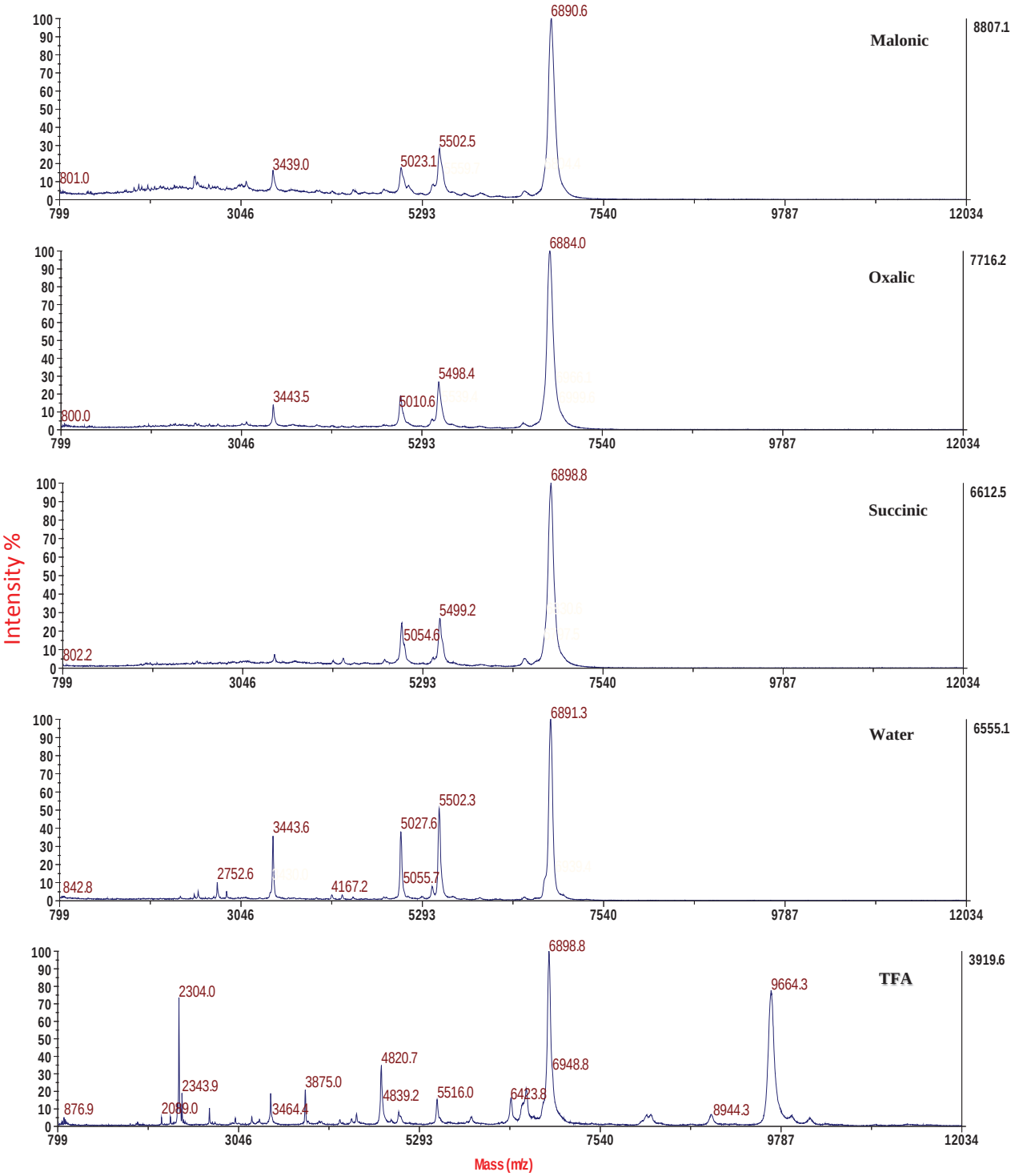


Figure 3.7. MALDI-MS spectra for *S. aureus* protein extracts obtained using malonic, oxalic, succinic, HPLC-grade water, and trifluoroacetic acid (top to bottom) in combination with methanol treatment. Spectra were acquired in linear mode with a mass range of 800-12000 Da.

3.4 Peak List Analysis

For peak count assessment, 33 spectra were collected and analyzed for every acid-alcohol combination. Figure 3.8 represents the number of reproducible peaks obtained from every variation after peak list analysis and binning.

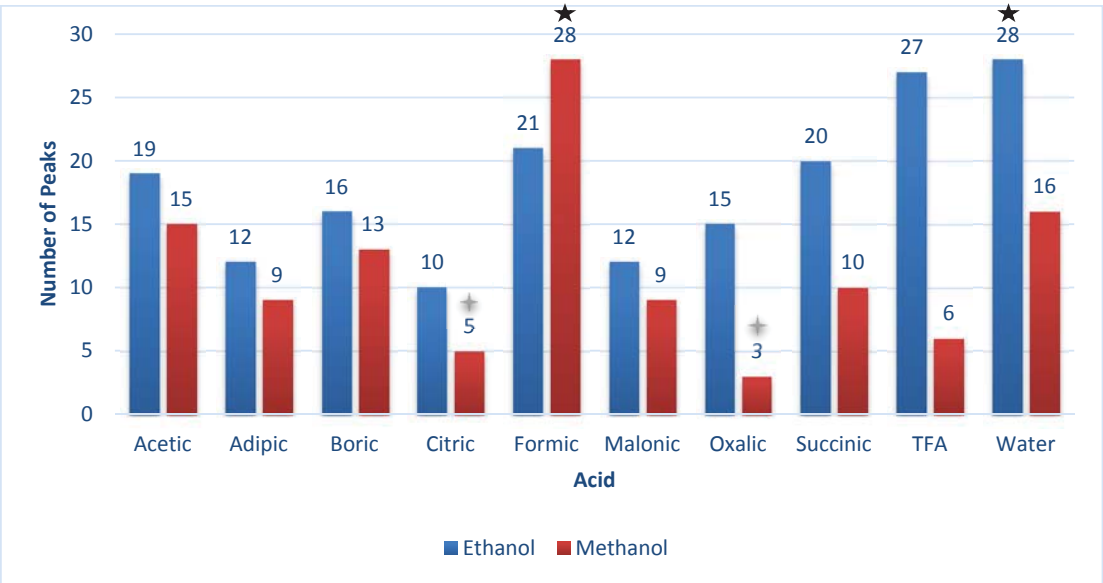


Figure 3.8. Comparison of the number of peaks considered to be present when different acid-alcohol combinations are used. Only peaks consistently present in ≥2/3 of extractions and ≥60% of MALDI replicates were considered for analysis. Data is presented in the appendix Tables A-1 and A-2.

✦ Lowest ★ Highest

The highest number of peaks, 28, was observed when formic acid/methanol or HPLC-grade water/ethanol extraction methods were used. The use of oxalic acid/methanol extraction protocol, produced the lowest number of peaks, 3. Formic acid and TFA extractions also resulted in a high number of peaks, 21 and 27 respectively upon treatment of samples with ethanol prior to protein solubilization. Methanol treatment resulted overall in a lower number of peaks than ethanol treatment, except when used with formic acid. The highest peak observed was at m/z ratio of 10,094 when acetic acid was used for extraction.

Upon ethanol treatment, HPLC-grade water produced the highest number of peaks; however, no peak having a m/z ratio greater than 7,000 was observed. Extraction using TFA yield 27 peaks with a m/z ratio up to 16,000, Figures 3.3 and 3.8.

Table 3.4. Table of peaks found to be consistently present for two or more extraction protocols when samples are treated with ethanol. The number of common peaks produced by every acid is also presented.

Peak (m\z)	Solvent										
	AcA	AdA	BA	CA	FA	MA	OA	SA	TFA	Water	Abundance across Acids
829							x			x	2
843					x				x	x	3
867		x		x							2
1835	x	x	x	x		x	x	X		x	8
1863		x	x	x			x	X		x	6
2089	x				x						2
2306	x				x				x		3
2344	x				x						2
2517			x					x			2
2754			x			x		x			3
2765			x					x			2
2869		x	x	x		x	x	x		x	6
3080			x				x	x			3
3152			x					x			2
3212	x				x						2
3307	x				x				x		3
3446		x	x	x	x	x	x	x			7
3877	x				x				x		3
4307	x				x				x		3
4514	x				x				x		3
4824	x				x				x		3
4974			x					x			2
5038		x	x	x	x	x	x	x	x		8
5071		x		x							2
5428		x	x			x	x	x			5
5515	x		x		x	x	x	x			6
5945					x				x		2
6435	x				x				x		3
6582			x				x	x			3
6834			x			x	x	x			4
6906		x	x		x	x	x	x			6
6912		x		x	x				x		4
8926					x				x		2
9670	x				x				x		3
Number of common peak	13	10	16	8	19	9	12	16	13	5	
Solvent	**AcA**	**AdA**	**BA**	**CA**	**FA**	**MA**	**OA**	**SA**	**TFA**	**Water**	

*Acids: AcA = acetic acid, AdA = adipic acid, BA = boric acid, CA = citric acid, FA = fromic acid, MA = malonic acid, OA = oxalic acid, SA = succinic acid, TFA = Trifluoroacetic acid.

Abundance across acids = the number of times every peak appears across all the acids.

Common peaks = Peak present in extracts of two or more different acids.

Analyzing data acquired from ethanol treated samples, 34 peaks with a m/z ratio between 830 and 9,671 were found to be consistently present in two or more acids, Table 3.4. Results showed that the two peaks at m/z ratio of 1,835 and 5,039 were present in extracts for eight out of the ten acids. The peak at m/z ratio of 5,039 was not present in both acetic acid and HPLC-grade water, and the peak at 1,835 was not present in both formic acid and TFA extracts. Boric and succinic acid have 16 peaks in common which represent the whole set of common peaks each produced. Formic acid and TFA share 13 peaks, which represent all peaks for TFA. Although, acetic and formic acid have only 12 peaks in common they share the highest m/z peak with TFA at m/z ratio of 9,671.

Table 3.4 also depicts the number of peaks produced by every acid. The use of formic acid resulted in the highest number of common peaks, 19. The lowest number of common peaks, 5, was observed when HPLC-grade water was used.

Table 3.5. Table of peaks found to be consistently present for two or more extraction protocols when samples are treated with methanol. The number of common peaks produced by every acid is also presented.

Peak (m/z)	Acid										
	AcA	AdA	BA	CA	FA	MA	OA	SA	TFA	Water	Abundance across acids
841		x					x				2
842			x							x	2
2286	x				x						2
2304	x				x						2
2467		x	x			x				x	4
2478		x				x		x			3
3006	x				x						2
3110		x				x					2
3443		x	x	x		x	x	x		x	7
4174			x					x		x	3
4301								x	x	x	3
4817			x		x			x		x	4
5032			x	x		x		x			4
5423		x	x	x		x	x	x			6
5503	x									x	2
5508		x	x			x		x			4
6558		x								x	2
6566			x					x			2
6889		x	x					x			3
Number of common Peaks	4	9	10	3	4	7	3	10	1	8	
	AcA	**AdA**	**BA**	**CA**	**FA**	**MA**	**OA**	**SA**	**TFA**	**Water**	

*Acids: AcA = acetic acid, AdA = adipic acid, BA = boric acid, CA = citric acid, FA = fromic acid, MA = malonic acid, OA = oxalic acid, SA = succinic acid, TFA = Trifluoroacetic acid.

Abundance across acids = the number of times every peak appears across all the acids.

Common peaks = Peak present in extracts of two or more different acids.

Analyzing data acquired from methanol treated samples, 19 peaks with a m/z ratio between 842 and 6,889 were found to be consistently present in two or more acids, Table 3.5. Being the most common, the peak at m/z ratio of 3,444 appeared in seven out of the ten acids. Moreover, the peak at m/z of 5,424 was present in six acids.

Comparison of the common peaks, produced by the extraction methods when methanol was used for treatment, Table 3.5, showed that boric and succinic acid have the highest number of common peaks, 10. Furthermore, when looking at the common peaks each produces, eight out of the ten peaks are common for both, making them the most similar for the whole set. On the other hand, TFA/methanol extracts produced one common peak only at m/z 4,302.

Table 3.6. Peak list comparison between malonic acid-ethanol and malonic acid-methanol treatment showing highly unique profiles.

Peaks (m/z)	Malonic acid-Ethanol	Malonic acid-Methanol
1835.856	*	
2221.776		*
2467.967		*
2478.136		*
2647.859		*
2754.809	*	
2870.461	*	
3110.774		*
3412.677	*	
3444.796		*
3446.984	*	
3464.395	*	
5032.416		*
5039.06	*	
5425.364		*
5429.298	*	
5508.448		*
5516.22	*	
5552.171	*	
6834.271	*	
6906.954	*	

The comparison of peaks obtained from one acid when either ethanol or methanol treatment was used demonstrated high uniqueness for all the acids. Acetic, adipic, boric, citric, formic, malonic, oxalic, and succinic acid produced a whole set of unique peaks with different alcohol treatments, Table 3.6. For TFA, one common peak at m/z of

5,523 was present, Table A-20. However, ten peaks were found to be common between the two extraction protocols using HPLC-grade water, Table A-21.

3.5 1-DE and 2-DE Gels of Formic Acid/Ethanol *S. aureus* Protein Extracts

Twenty protein bands were observed on the one dimensional gel ranging from approximately 3 to 150 kDa (Figure 3.9). Bands were cut, digested, and peptide mass finger printing was attempted using MALDI-MS; however, no statistically significant identification could be completed.

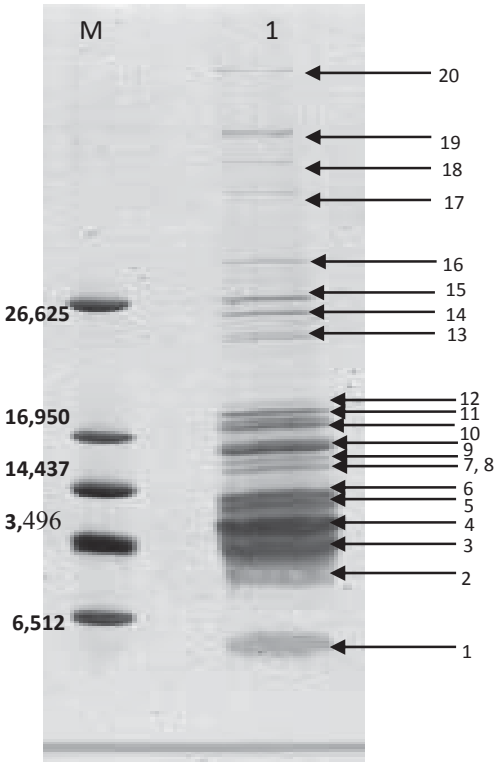


Figure 3.9. Proteins were separated by SDS-PAGE on a 15% polyacrylamide gel and stained with Bio-Safe Coomassie stain. MW standards are in Da. Lane M contains 15 µL of a polypeptide standard. Lane 1 was loaded with 30 µL of boiled *S. aureus* protein extract.

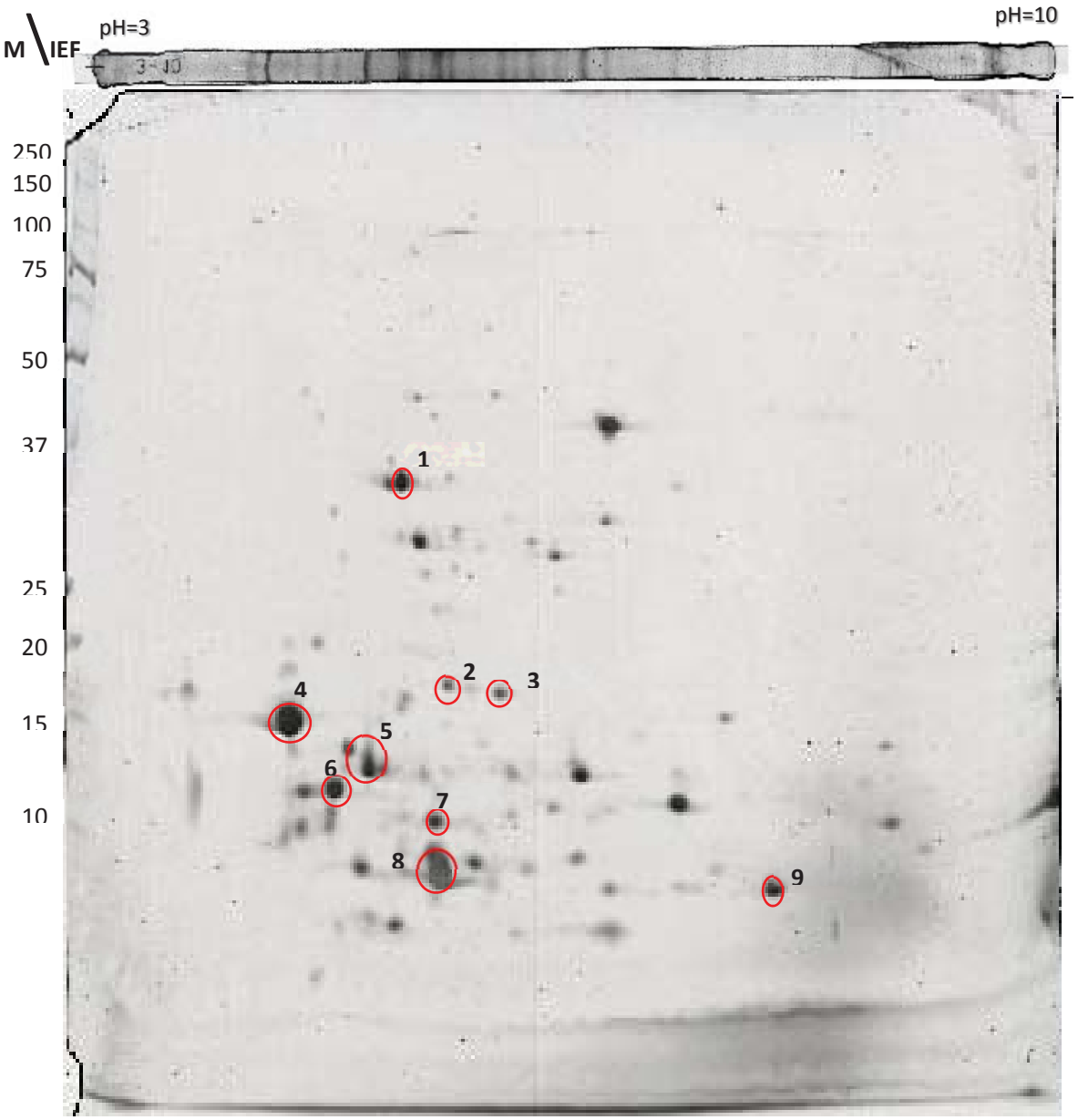


Figure 3.10. A two dimensional electrophoresis (2-DE) representative gel of *S. aureus strain* SA-16 proteins extracted by formic acid/ethanol protocol. A volume of 325 µL containing 400 µg of protein was loaded on isoelectric focusing (IEF) strip with pH range 3-10. More than 50 protein spots are visible. The gel is 15% acrylamide, and the spots in circles are proteins identified by PMF. A 10-250 kDa standard plug ladder was used.

The protein profile of *S. aureus* SA-16 extracts was observed on 2-DE gels (Figure 3.10). IPG strips of pH 3-10 were used; well resolved protein spots were observed and accurate excision was possible. Protein spots were observed throughout the region of pI 3-10. Protein molecular weights were up to 50 kDa. A total of 22 spots were excised from the gel, and nine proteins were successfully identified by peptide mass

fingerprinting (Figure 3.12 and Table 3.7). Peak lists for the identified proteins are represented in the Appendix, Tables A-4 to A-12.

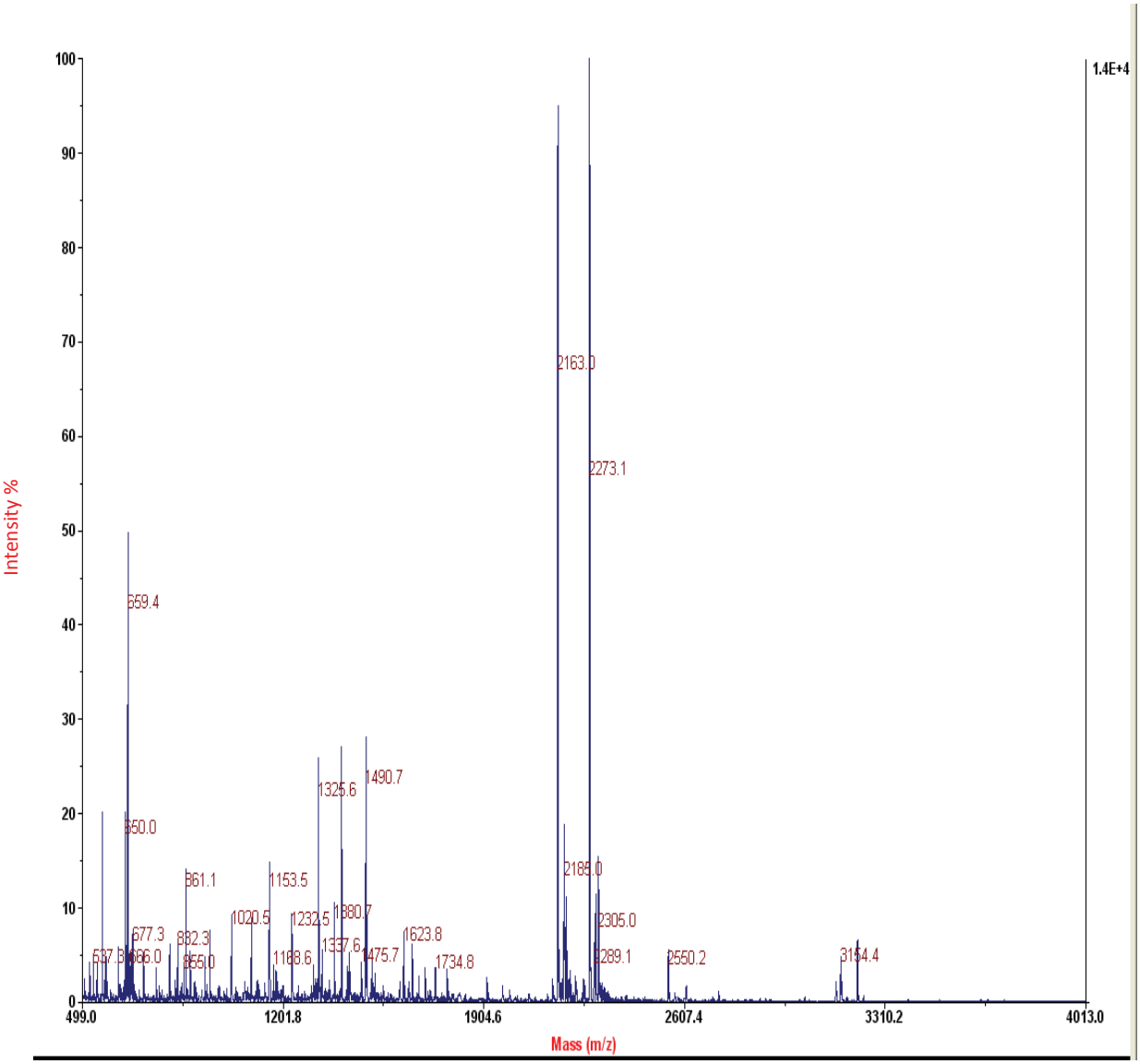
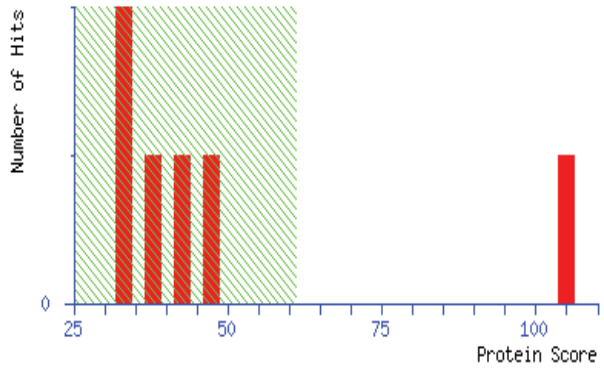


Figure 3.11. MALDI-MS spectrum acquired from in-gel digestion of spot number 6 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 61 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Preferred taxonomy All entries

Re-Search All | Search Unmatched

1. ESXA_STAAC Mass: 11029 Score: 105 Expect: 2.2e-06 Matches: 5
Virulence factor EsxA OS=Staphylococcus aureus (strain COL) GN=esxA PE=3 SV=1

Figure 3.12. Mascot score histogram showing the result of peptide mass fingerprinting using Swissprot database for protein identification. A statistically significant score at p<0.05 is above 61.

Proteins identified by PMF were in the size range of 9,300 to about 33,200 Da, Table 10. Eight out of the nine proteins were identified for *S. aureus*. Protein spot 1 showed two significant identifications as endonuclease 4 of *S. haemolyticus* species and putative endonuclease 4 of *S. warneri*.

Table 3.7. *S. aureus* proteins identified by MALDI-MS-based peptide mass finger printing and database search.

Spot No.	***Database MW (Da)***	***ID No.***	***Protein***	***Species***	***Database***	***MOWSE Score***	***Number of matched peptides***	***% Sequence coverage***
1	33131	gi|239637631	putative endonuclease 4	*Staphylococcus warneri*	NCBInr	84	10	33
	33207	gi|70726360	endonuclease IV	*Staphylococcus haemolyticus*	NCBInr	82	10	30
2	17300	gi|417893437	DivIVA domain protein	*Staphylococcus aureus*	NCBInr	126	10	63
3	16564	NDK_STAA1	Nucleoside diphosphate kinase	*Staphylococcus aureus*	Swissprot	82	5	40
4	13301	Y1663_STAAN	UPF0342 protein SA1663	*Staphylococcus aureus*	Swissprot	130	11	96
5	13143	GPSB_STAA1	Cell cycle protein GpsB	*Staphylococcus aureus*	Swissprot	92	6	55
6	11029	ESXA_STAAC	Virulence factor EsxA	*Staphylococcus aureus*	Swissprot	105	5	48
7	10000	Y197A_STAAN	UPF0457 protein SA1975.1	*Staphylococcus aureus*	Swissprot	74	5	63
8	9717	RL31B_STAA1	50S ribosomal protein L31 type B	*Staphylococcus aureus*	Swissprot	77	7	73
9	9386	gi|418896879	putative septation protein spoVG	*Staphylococcus aureus*	NCBInr	113	7	79
	11283	gi|49482725	regulatory protein SpoVG	*Staphylococcus aureus*	NCBInr	100	7	67

Chapter Four
Discussion

To the current date, no universal approach for MALDI-typing of microorganisms has been developed (Drevinek *et al.*, 2012). MALDI-typing is based on the differentiation of protein fingerprints, acquired from different strains of one microorganism. This can be achieved by the identification of discriminatory protein peaks for different strains within one species (Rettinger *et al.*, 2012). According to De Bruyne *et al.* (2011), the quality and reproducibility of MALDI-MS generated fingerprints is affected by several factors; including: bacterial culture media, sample preparation, protein extraction method, and matrix used.

Four bacterial protein extraction methods were assessed by Drevinek *et al.* (2012). Two of the methods employed acids for extraction, TFA extraction method and fromic acid/ethanol method, were performed according to protocols by Lasch *et al.* (2008) and Marklein *et al.* (2009), respectively. The two other methods were chloroform extraction (Liu *et al.*, 2007), and ACN extraction (Hernychova, *et al.*, 2008). The formic acid/ethanol extraction method yield the highest protein concentration and best spectral quality. Thus it was determined as the sample preparation method of choice for MALDI-MS analysis of bacteria.

Based on the work of Drevinek *et al.* (2012), we evaluated 18 organic acid/alcohol sample preparation methods, as well as protein extraction using HPLC-grade water. Moreover, peptide mass fingerprinting was performed on formic acid/ethanol *S. aureus* protein extracts in an attempt to identify proteins within or outside the MALDI-typing range of 800-12,000 Da (Kornienko *et al.*, 2013).

4.1. Assessment of Methods for Sample Preparation

MALDI-MS analysis of protein extracts obtained from one microorganism frequently yield fingerprints that differ in identification power due to differences in sample preparation (Drevinek *et al.*, 2012). Thus, there is a need to develop a standard

method for bacterial typing using MALDI-MS. Moreover, databases of fingerprints need to be vested with genus, species, and strain specific information. Standardized data acquisition, processing, and analysis methods ought to be established. In our course of work, signal to noise ratio was set to 10, thus any peak with intensity lower than 10 was considered not present. Moreover, any peak present in less than 60% of the MALDI replicates and in <2/3 of the biological replicates was considered not reproducible.

Spectral quality is considered as the crucial determinant of successful analysis. The relation of protein concentration to the number of peaks and the m/z range detected by MALDI-MS was assessed in our study. The effectiveness of each extraction method was first evaluated by measuring the protein concentration. Results showed that the use of TFA for extraction in combination with either ethanol or methanol yields the highest protein concentration, 821 µg/mL and 1,131 µg/mL, respectively. The lowest protein concentrations were observed when adipic, malonic, and oxalic acid were used. These results suggest that TFA is capable of solubilizing larger amounts of proteins compared to all the other acids, while adipic, malonic, and oxalic acid have limited protein solubilization capabilities.

MALDI-MS analysis was then performed to evaluate the number of reproducible peaks obtained for every method. Data analysis results showed that the highest number of peaks, 28, was observed twice; i.e. when formic acid was used in combination with methanol treatment, and when HPLC-grade water in combination with ethanol was used for extraction. Both methods yielded intermediate protein concentrations 522 µg/mL and 687 µg/mL, respectively. This indicates that there is no direct relation between protein concentration and the number of reproducible peaks observed when adequate (detectable) concentration is present. At low concentrations, such as those obtained when either citric or oxalic acid in combination with methanol treatment was used, the lowest number of peaks was observed; 5 and 3, respectively.

When HPLC-grade water was used for extraction, no peak with m/z over 7,000 was observed. This suggests that proteins or peptides having a m/z larger than 7,000 are not soluble in HPLC-grade water. On the other hand, when either acetic acid, formic acid, or TFA was used, peaks with m/z greater than 9,000 were observed. The highest

observed peak had a m/z of 16,074, and was observed when TFA/ethanol method was used for extraction. Despite the higher protein concentration, TFA/methanol method yield a significantly lower number of reproducible peaks, 6, when compared to the TFA/ethanol method, 27. Moreover, no peak having m/z larger than 10,000 was observed.

The use of formic acid ethanol/methanol methods led to higher protein concentrations than HPLC-grade water methods. The number of peaks observed was 21 and 28 when ethanol or methanol treatment was used, respectively. Thus, peak reproducibility remains high despite the changes in the extraction protocol.

The only correlation between the pH of the 70% acid solutions and protein concentration was observed when TFA was used for extraction. The TFA solution was the most acidic among the group, pH = -0.67, and yielded the highest protein concentrations when used for extraction with either alcohol.

The use of formic acid/ethanol method yielded the highest number of common peaks, Table 3.4, sharing at least one peak with each extraction method when ethanol was used for treatment. Two peaks having a m/z of 1,835 and 5,039, were found to be consistently present in 8 out of 10 methods when ethanol was used for treatment. The peak at m/z 1,835, not present in TFA and formic acid extracts, and the peak at 5,039, not present when either acetic acid or HPLC-grade water was used for extraction. When methanol was used for treatment, only the peak with m/z 3,444 was shared by 7 of the extraction methods, not present when acetic, formic, or TFA was used for extraction.

Although we have shown that the formic acid/ethanol sample preparation method is efficient; nevertheless, our results suggest that two other methods should be assessed as prospects for MALDI-identification of micro-organisms. Both formic acid/methanol and TFA/ethanol methods presented a higher number of peaks, and thus larger datasets for analysis. Having more peaks, increases the probability of detecting variations between different strains of the same species.

4.2 Peptide Mass Fingerprinting

Two proteomic techniques that allow protein separation and visualization are 1-DE and 2-DE gel electrophoresis. In an attempt to identify proteins extracted using formic acid/ethanol method, PMF was performed on proteins separated by 1-DE and 2-DE gel electrophoresis.

No statistically significant protein identification was achieved after performing PMF on protein bands obtained from a 1-DE gel of SA-16 formic acid/ethanol extracts. This can be explained by presence of protein mixtures within a single band leading to failure of PMF (Gonnet *et al.*, 2003). Only 20 protein bands were observe on the 1-DE gel, while 50 protein spots were observed on the 2-DE gel, Figures 3.9 and 3.10. This indicates the higher resolving power of the 2D gel electrophoresis.

Nine proteins were identified using PMF on protein spots excised from 2-DE gels. The functions of the identified proteins were related to the processes of cell division, DNA repair, energy production, virulence and translation. None of the peaks acquired from the analysis of formic acid/ethanol extracts in linear mode by MALDI-MS matched the weights of the identified proteins.

Endonuclease IV (MW 33,131 Da/spot 1), is an enzyme that cleaves phosphodiester bonds at apurinic or apyrimidinic sites (AP sites), thus producing base-free deoxyribose 5-phosphate residues at 5'-ends. Endonuclease IV plays a main role in DNA repair and antibiotic resistance by selectively attacking altered AP sites created by bleomycin and neocarzinostatin. http://www.uniprot.org/uniprot/Q4L6Q7

DivIVA protein is a putative cytoplasmic protein (MW 17,300 Da/spot 2), predicted to play a role in the cell cycle and cell division. http://www.uniprot.org/uniprot/G8V1B1

Nucleoside diphosphate kinase (NDPk) (MW 16,564 Da/spot 3) is a cytoplasmic component responsible for nucleoside triphosphate synthesis (UTP and GTP). The cell deploys a ping-pong mechanism and a phosphorylated active-site intermediate, for the transfer of an ATP gamma phosphate to a NDP beta phosphate. http://www.uniprot.org/uniprot/A7X2H3

Two identified proteins with unknown functions are the UPF0342 protein SA1663 (MW 13,301 Da/spot 4) and UPF0457 protein SA1975.1 (MW 10,000 Da/spot 7). SA1975.1 is closely related to the uridylyltransferase family. http://www.uniprot.org/uniprot/Q7A4V3 , http://www.uniprot.org/uniprot/E5R9Q8

Cell cycle protein GpsB (13,143 Da/spot 5), is a divisome, plays a role in cell division. Together with EzrA (a membrane protein), GpsB is a key component of the system that regulates PBP1 (a cell cycle control protein) localization during cell cycle progression. GpsB is also thought to regulate cell shape. http://www.uniprot.org/uniprot/A7X2D9

Virulence factor EsxA (11,029 Da/spot 6), is a secretory protein that is crucial for the initiation of infection in the host. EsxA belongs to the ESAT-6 (esx) family. http://www.uniprot.org/uniprot/Q99WU4

50S ribosomal protein L31 type B (9,717 Da/spot 8), is a ribonucleoprotein. It is a structural constituent of the ribosome, specifically the 50S ribosomal subunit, and it plays a major role in translation. According to Champney and Tober (2000), macrolides are capable of stopping translation during bacterial cell growth by inhibiting the formation of the 50S ribosomal subunit. Nonetheless, this has not been confirmed due to the fact that macrolides have translation inhibitory effects that may not interfere with the 50S ribosomal formation. http://www.uniprot.org/uniprot/A7X4W8

SpoVG, is a stage V sporulation protein G homologue (MW 9,386 Da/spot 9). SpoVG is a negative regulator of asymmetric septation, and necessary for spore formation in Bacillus. In *S. aureus*, it is involved in biofilm formation, capsular polysaccharide synthesis, and methicillin-resistance. http://www.ncbi.nlm.nih.gov/gene/1002557

The function of the SpoVG protein has been experimentally proven by Voyich *et al.* (2009). All other protein functions are either inferred by coding gene or protein sequence similarity.

Recently, 105 *S. aureus* proteins have been identified using MALDI tandem mass spectrometry. The identified proteins cover a wide range of functions varying from

basic cell processes to antibiotic resistance and virulence (Monteiro at al., 2012). The aim of the study was to understand the diverse virulence and resistance mechanisms expressed by MRSA strains. The authors adapted a whole protein extraction method based on sonication and protein solubilization in SDS. However, other than the 50S ribosomal protein, none of the proteins identified in our study matched the proteins identified by Monteiro *et al.* (2012).

None of the genes coding for the detected proteins has been previously used for molecular typing of *S. aureus*. However, genotypic typing techniques are mostly based on the characterization of virulence genes. The protein products of these genes typically have molecular weights outside the MALDI-typing range (Kornienko *et al.*, 2013). Two identified proteins correlated to virulence are SpoVG and endonuclease IV. Differences detected in the expression of these two proteins may be suitable for typing of *S. aureus.* Endonuclease 4 has a molecular weight of around 30 kDa, which is outside the MALDI-typing range. Nevertheless, fragments of such a protein may be detected by MALDI-MS. One way to check for the presence of endonuclease 4 is by performing tandem mass spectrometry and identifying the peptides and proteins in the extract. On the other hand, the molecular weight of SpoVG, 9,386 Da, is within the MALDI-typing range. A peak with a m/z of 9,386 does not appear in MALDI-MS spectra acquired in linear mode for formic acid/ethanol extracts of SA16. The peak at 9,386 might have low intensity, and thus was masked by background noise (Vragha *et al.*, 2006). Another explanation could be the presence of a more easily ionizable protein or peptide within a close mass range (Lebrun *et al.*, 1997).

Chapter Five
Conclusion

- The evaluation of sample preparation methods showed that formic acid/methanol and TFA/ethanol methods yield the highest information content, thus increased probability of protein fingerprint variation among different strains of one microorganism.
- Two dimensional electrophoresis gels allowed separation and visualization of the formic acid/ethanol *S. aureus* extracted proteins. More than fifty protein spots were observed and nine proteins were successfully identified using PMF.
- The functions of the identified proteins involved cell division control, translation, and virulence.
- Two virulence related proteins were considered as prospects for MALDI-typing of *S.* aureus, SpoVG and endonuclease IV. The relation between virulence and typing has been previously demonstrated in genotypic techniques.

Future work may include the use of MALDI tandem mass spectrometry for the identification of the remaining 42 protein spots. This will allow the assessment of the types of proteins extracted using the formic acid/ethanol method. The suitability of the sample preparation method for MALDI-typing will then be reevaluated based on the identified proteins with respect to genus, species, and/or strain specificity.

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Appendix

Table A-1. Table of reproducible peaks for every acid/ethanol extraction method, the number of peaks is also displayed. The peaks presented were consistently present in ≥2/3 of extractions and ≥60% of MALDI replicates.

Solvent									
AcA	**AdA**	**BA**	**CA**	**FA**	**MA**	**OA**	**SA**	**TFA**	**Water**
801.2	825.3	1835.5	867.3	843.8	1835.9	829.5	1835.5	843.6	824.3
927.5	867.2	1863.6	1835.3	2090.1	2754.8	830.5	1863.7	2307.0	828.4
1792.3	1835.3	2516.9	1863.3	2306.4	2870.5	1835.0	1907.9	2685.9	829.4
1834.4	1862.9	2754.6	2870.1	2325.4	3412.7	1863.4	2222.1	3308.0	830.3
2089.8	2869.2	2765.6	2965.2	2344.3	3447.0	2869.8	2517.2	3450.3	842.4
2306.7	3446.5	2870.1	3447.3	3212.9	3464.4	3079.9	2652.1	3587.7	843.4
2344.5	5038.0	3080.5	5038.8	3307.6	5039.1	3410.3	2755.2	3878.0	844.2
3212.5	5071.2	3152.3	5072.1	3446.3	5429.3	3445.6	2765.9	4308.5	858.4
3308.2	5430.4	3446.6	5530.1	3876.6	5516.2	4177.9	2870.0	4514.4	859.2
3877.0	5527.3	4974.6	6913.6	4307.3	5552.2	5036.9	3009.1	4824.7	1278.2
4306.8	6587.0	5038.2		4452.2	6834.3	5427.1	3080.6	5040.4	1834.6
4514.1	6910.8	5427.9		4514.3	6907.0	5515.3	3152.2	5308.9	1862.6
4824.9		5515.5		4823.5		6582.0	3446.8	5522.4	2753.1
5515.6		6582.8		5039.4		6833.3	4974.9	5945.3	2762.8
6435.4		6834.1		5515.3		6903.6	5038.7	6370.7	2867.9
6626.5		6906.0		5945.0			5428.7	6436.2	3052.7
8121.0				6434.7			5515.7	6618.1	3077.9
8168.3				6907.7			6581.5	6912.5	3149.0
9669.0				8924.7			6835.4	7185.4	3375.6
				9669.6			6906.3	7584.2	3443.2
				10153.8				8132.7	3979.5
								8928.3	4300.7
								9207.1	5027.5
								9673.4	5417.1
								10527.8	5501.8
								13878.3	6005.0
								16074.1	6812.7
									6881.8
19	12	16	10	21	12	15	20	27	28
Number of peaks									

*Acids: AcA = acetic acid, AdA = adipic acid, BA = boric acid, CA = citric acid, FA = fromic acid, MA = malonic acid, OA = oxalic acid, SA = succinic acid, TFA = Trifluoroacetic acid.

Table A-2. Table of reproducible peaks for every acid/methanol extraction method, the number of peaks is also displayed. The peaks presented were consistently present in ≥2/3 of extractions and ≥60% of MALDI replicates.

Solvent									
AcA	**AdA**	**BA**	**CA**	**FA**	**MA**	**OA**	**SA**	**TFA**	**Water**
1790.3	841.7	842.5	3445.6	851.9	2221.8	841.7	2478.4	4303.5	842.7
2087.3	2466.8	2421.2	5032.7	1342.0	2468.0	3442.9	3443.4	4504.0	2467.7
2175.4	2477.7	2468.0	5060.9	2198.5	2478.1	5422.5	4174.6	5523.9	2516.5
2286.7	3109.7	3053.2	5422.4	2236.1	2647.9		4300.1	6420.5	2753.5
2304.9	3442.9	3444.3	6898.5	2260.1	3110.8		4817.6	6594.2	2868.6
2322.9	5423.7	4176.3		2286.3	3444.8		5031.8	9631.3	3443.5
2341.6	5507.1	4439.8		2304.7	5032.4		5424.0		4173.5
3006.7	6558.3	4819.0		2324.4	5425.4		5508.4		4301.4
3208.7	6888.9	5033.3		2342.9	5508.4		6565.7		4436.4
4812.5		5425.0		2363.9			6889.2		4816.5
5503.5		5508.9		2382.1					5028.3
6413.2		6566.5		2402.5					5418.1
6600.1		6890.2		2544.4					5502.9
9616.3				2578.7					6005.0
10094.1				2683.0					6557.8
				2721.6					6882.0
				2743.3					
				2759.9					
				2978.0					
				3005.7					
				3043.3					
				3775.1					
				3873.3					
				4526.6					
				4562.1					
				4816.5					
				6605.2					
				9656.6					
15	9	13	5	28	9	3	10	6	16
Number of peaks									

*Acids: AcA = acetic acid, AdA = adipic acid, BA = boric acid, CA = citric acid, FA = fromic acid, MA = malonic acid, OA = oxalic acid, SA = succinic acid, TFA = Trifluoroacetic acid.

Table A-3. Exclusion list used in the Peak erazor software version 2.0.1 for the processing of mass lists prior to peptide mass fingerprinting.

Peak (m/z)				
506.082	649.995	833.000	1232.473	2162.898
523.064	656.011	876.987	1234.586	2184.908
524.087	659.329	927.500	1307.609	2192.836
537.282	662.200	1020.428	1308.585	2272.994
550.071	669.365	1107.468	1337.511	2289.970
568.081	671.983	1111.514	1405.538	2294.995
578.604	677.303	1137.550	1433.614	2305.901
579.330	713.391	1153.493	1475.667	2551.041
581.309	757.419	1168.549	1490.636	3153.300
597.390	801.441	1175.506	1493.616	3154.221
625.339	832.000	1179.525	1880.800	3212.229

Table A-4. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot one on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The proteins identified were, endonuclease 4 of *S. haemolyticus* species and putative endonuclease 4 of *S. warneri*.

Peaks (m/z)			
572.312	572.320	632.351	572.320
615.352	632.347	637.356	585.338
632.337	637.353	665.998	632.352
777.319	681.337	681.346	637.355
802.404	745.397	802.432	681.339
805.386	777.338	805.397	777.344
807.373	802.410	807.382	802.427
824.398	805.391	824.407	805.396
829.415	807.379	829.424	807.383
897.385	824.408	897.395	824.407
949.471	829.423	949.491	829.428
951.445	897.387	951.461	861.051
973.498	949.478	973.515	897.397
1003.512	951.449	1033.493	949.489
1033.480	973.502	1036.538	951.462
1035.495	1003.519	1060.533	973.508
1036.522	1033.488	1064.591	1003.532
1037.504	1035.507	1068.516	1033.493
1060.526	1036.528	1117.511	1036.537
1064.574	1037.516	1131.578	1037.521
1065.522	1060.533	1194.591	1060.532
1068.492	1064.580	1201.644	1064.590
1090.500	1068.499	1300.535	1068.510
1117.494	1090.508	1302.658	1082.569
1131.561	1117.499	1320.576	1090.506
1165.549	1131.568	1329.651	1117.510
1184.547	1165.559	1358.684	1131.574
1194.572	1194.582	1434.737	1165.567
1201.620	1201.628	1512.704	1194.588
1252.574	1252.576	1638.838	1201.631
1278.658	1300.518	1698.749	1278.665
1300.511	1302.637	1716.813	1300.534
1302.639	1320.559	1775.810	1302.644
1320.549	1329.632	1882.850	1320.567
1329.625	1357.675	1914.861	1329.640
1357.673	1427.699	1969.894	1357.687
1362.641	1434.721	2172.987	1362.655
1427.714	1512.692	2200.976	1427.696
1434.712	1638.822	2206.986	1434.730
1638.814	1652.778	2225.116	1512.697
1652.771	1698.736	2312.074	1638.832
1699.756	1716.805	2318.077	1653.794
1708.739	1774.797		1698.746
1716.800	1882.828		1716.816
1774.793	1914.842		1774.808
1882.824	1969.879		1882.843
1914.834	2201.971		1914.848
1969.876	2225.104		1969.888
2225.101	2384.907		2201.973
2257.112	2873.314		2207.987
2331.103	3137.362		2225.106
2384.903			2312.060
3137.361			

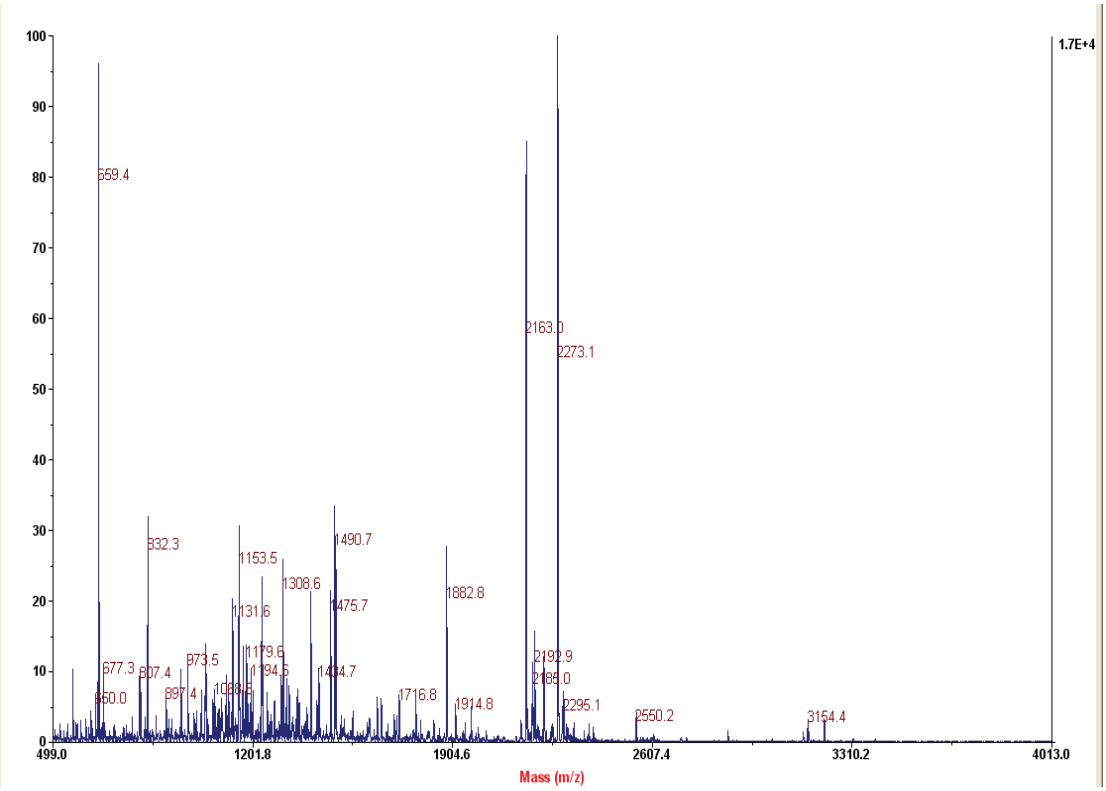

Figure A-1. MALDI-MS spectrum acquired from in-gel digestion of spot number 1 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-5. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot two on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, DivIVA domain protein of *S. aureus.*

Peak m/z			
645.312	617.015	645.319	645.315
780.350	645.316	802.445	861.025
861.016	780.355	861.033	945.453
945.455	802.419	945.457	1002.472
1002.562	855.014	967.450	1238.566
1054.543	861.027	1002.475	1254.541
1238.551	945.450	1190.575	1272.651
1254.531	1002.469	1217.593	1394.655
1272.638	1109.448	1238.571	1560.654
1394.641	1217.577	1254.547	2016.895
1560.635	1238.563	1272.662	2038.893
2016.871	1254.542	1364.646	2389.089
2039.815	1272.651	1394.664	2390.086
2389.054	1378.689	1410.646	2616.234
2390.048	1394.655	1560.654	2617.234
2616.185	1560.648	2016.902	3138.311
2617.196	1576.637	2038.896	
	2016.894	2389.084	
	2038.884	2390.085	
	2389.078	2616.236	
	2390.074	2617.225	
	2616.231		
	2617.217		

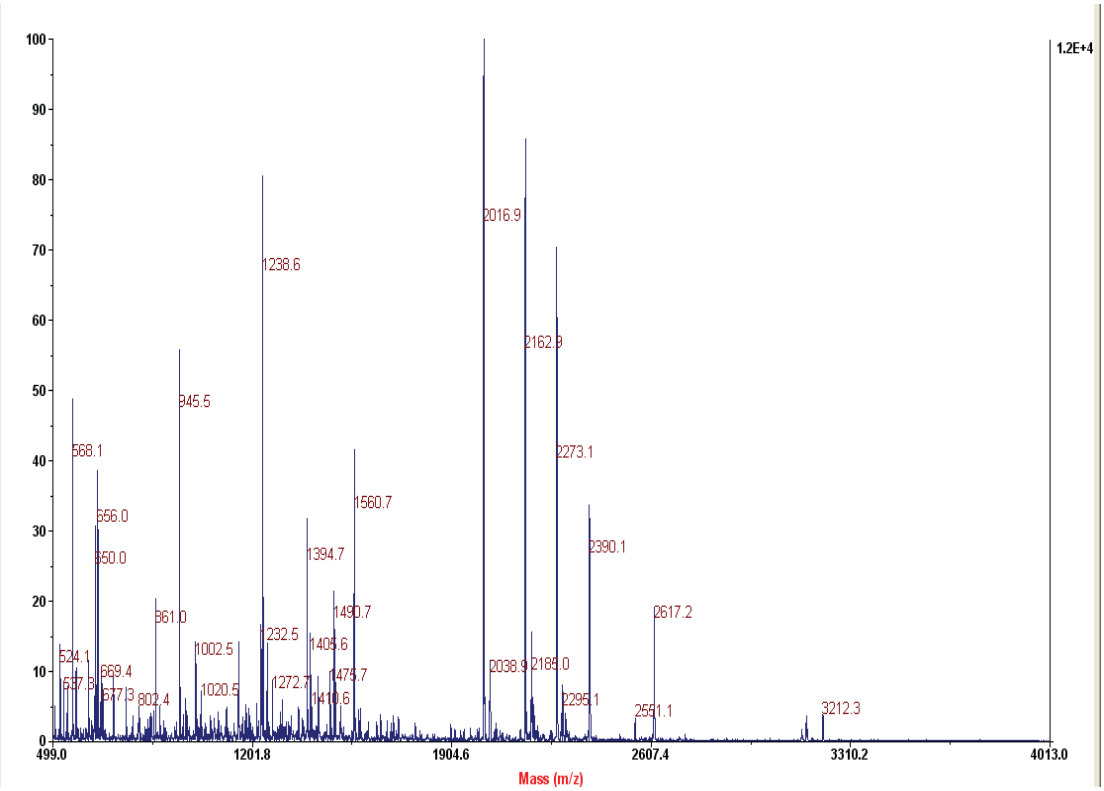

Figure A-2. MALDI-MS spectrum acquired from in-gel digestion of spot number 2 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-6. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot three on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, Nucleoside diphosphate kinase of *S. aureus.*

Peak m/z			
615.334	615.354	615.326	615.333
637.331	861.078	637.322	861.004
861.013	1000.515	802.374	1000.505
887.426	1418.730	861.009	1348.497
1000.509	1527.642	887.426	1418.674
1348.516	1722.745	1000.506	1527.630
1399.639	2193.978	1348.506	1722.742
1418.688		1399.624	
1527.636		1418.681	
1722.765		1434.630	
3137.260		1527.628	
		1722.753	
		2442.985	
		3137.233	

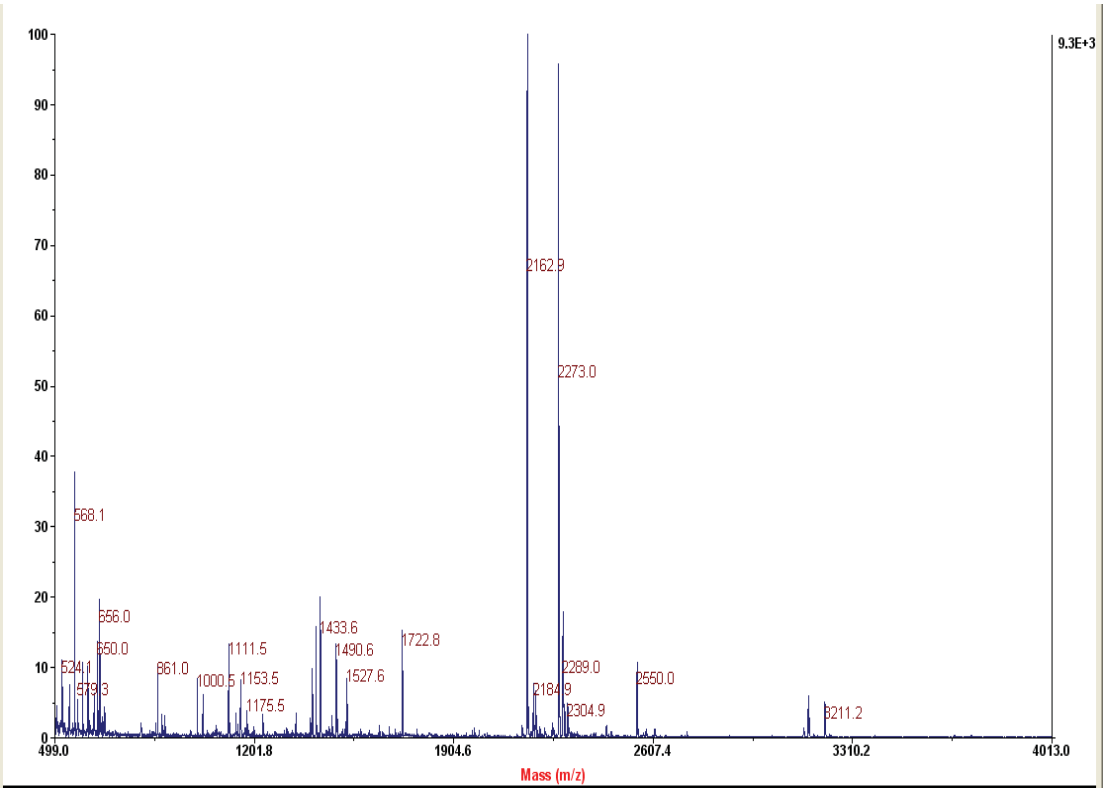

Figure A-3. MALDI-MS spectrum acquired from in-gel digestion of spot number 3 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-7. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot four on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, UPF0342 protein SA1663 of *S. aureus.*

Peak m/z			
526.161	665.987	628.021	628.027
778.373	778.371	637.335	644.002
798.371	798.365	644.000	665.984
805.384	805.387	665.978	778.370
807.370	825.350	778.366	798.362
811.373	826.377	798.359	805.385
825.351	845.487	800.356	825.351
826.389	848.353	805.374	826.375
848.355	855.028	825.349	839.045
883.395	861.038	826.371	848.351
935.489	954.470	845.487	855.019
937.520	976.444	848.352	861.034
954.485	998.419	855.018	954.469
976.446	1011.489	861.032	976.443
998.429	1135.525	870.332	1011.487
1011.489	1157.520	954.464	1066.032
1117.547	1205.586	976.439	1135.523
1135.533	1320.608	998.417	1157.518
1157.524	1630.680	1011.484	1205.586
1205.591	1647.677	1066.028	1320.607
1300.505	1663.668	1135.518	1630.676
1320.614	1669.668	1157.516	1647.671
1582.755	1857.841	1205.580	1663.659
1599.705	1878.861	1320.601	1669.669
1630.692	1879.854	1630.671	1857.834
1639.812	1902.851	1647.667	1878.858
1647.679	1919.828	1663.659	1879.847
1663.678	1924.830	1669.669	1902.848
1669.674	1937.893	1857.828	1918.824
1857.858	1942.876	1879.843	1924.830
1878.867	2001.951	1902.844	1937.893
1902.847	2559.123	1924.831	1942.873
1919.812	2576.119	1937.882	2559.112
1924.832	2581.120	1942.862	2576.115
1937.893	2593.190	2559.106	2581.100
1942.889	2609.182	2581.109	2593.178
2000.961	2615.198	2593.172	2610.162
2226.077	2647.141	2615.180	2615.185
2511.146	3487.487	2647.129	2647.134
2559.133			3487.462
2576.134			
2581.123			
2593.209			
2615.204			
2646.158			
3486.490			

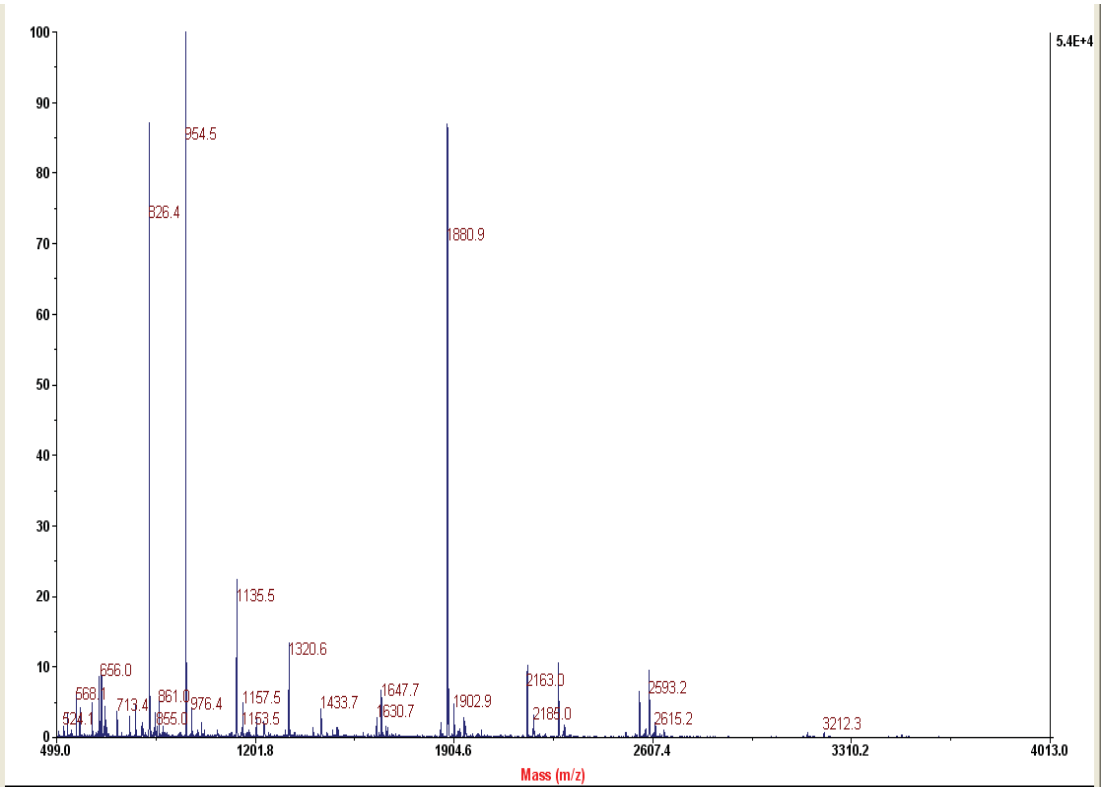

Figure A-4. MALDI-MS spectrum acquired from in-gel digestion of spot number 4 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-8. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot five on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, Cell cycle protein GpsB of *S. aureus*.

Peak m/z			
788.328	788.346	615.324	615.310
860.984	861.001	788.344	627.977
1186.436	1150.410	861.015	637.302
1439.669	1186.456	1150.416	665.938
1479.642	1439.646	1186.455	788.323
1567.586	1567.567	1351.593	838.985
1883.702	1883.694	1439.679	854.961
1907.726	2039.790	1567.587	860.975
2039.794	2299.881	1639.762	1065.956
2299.887		1661.630	1150.400
2416.940		1883.692	1186.435
		2039.785	1439.662
		2299.887	1661.607
		2415.919	1883.698
			2039.770
			2061.758
			2299.867
			2416.920

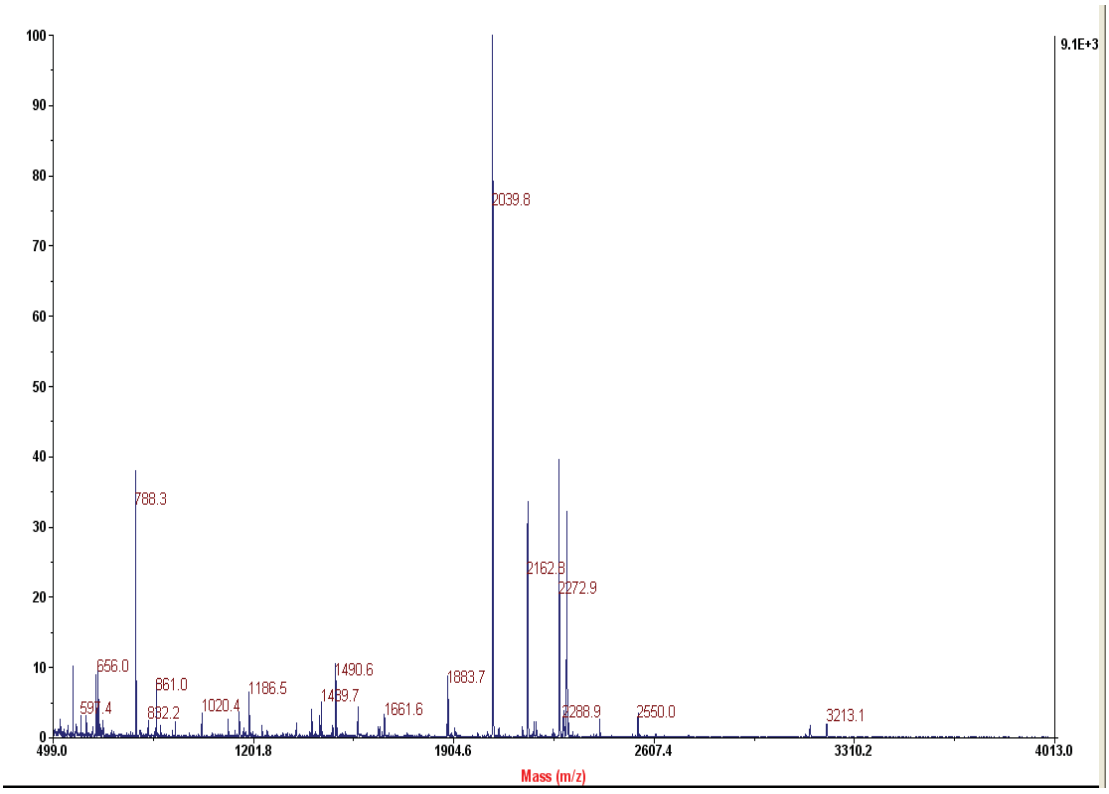

Figure A-5. MALDI-MS spectrum acquired from in-gel digestion of spot number 5 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-9. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot six on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, Virulence factor EsxA of *S. aureus.*

Peak m/z			
805.403	805.404	805.408	861.378
861.397	861.404	861.385	945.482
945.523	928.503	945.504	1090.553
1090.585	945.523	1090.564	1325.558
1325.593	1090.590	1325.575	1380.619
1380.651	1325.599	1380.633	1652.756
1623.790	1380.660	1653.771	
	1623.802		

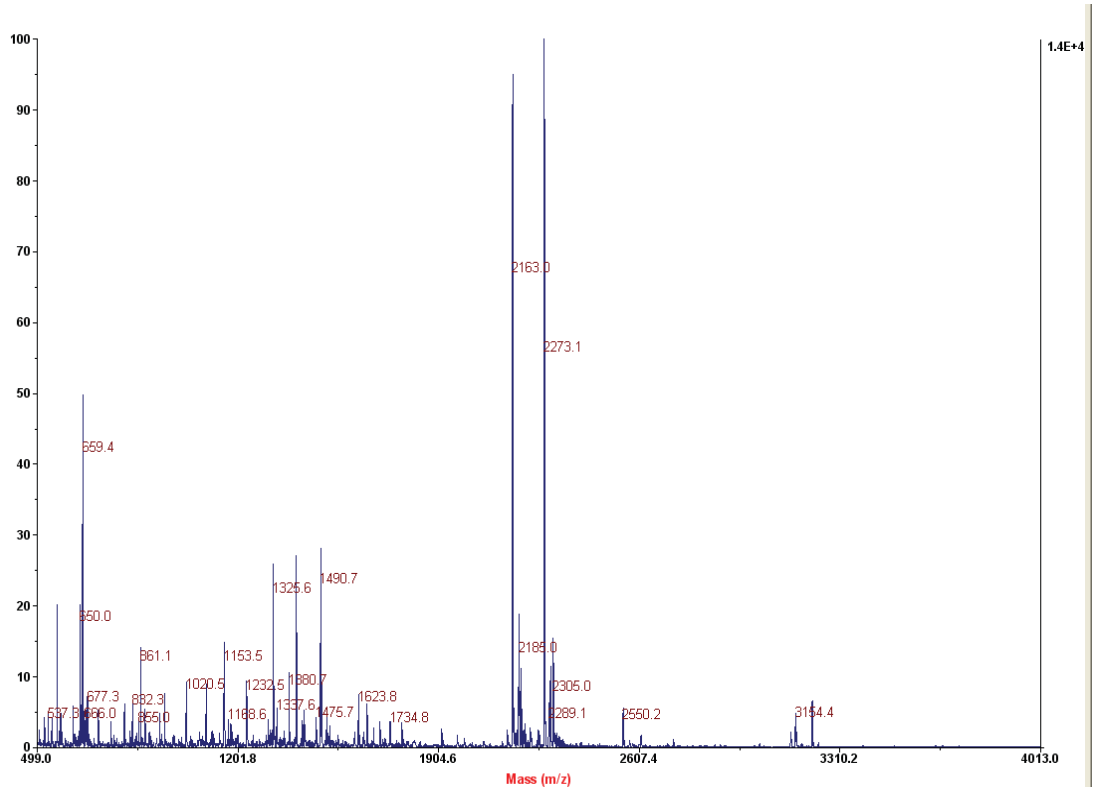

Figure A-6. MALDI-MS spectrum acquired from in-gel digestion of spot number 6 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-10. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot seven on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, UPF0457 protein SA1975.1 of *S. aureus.*

Peak (m/z)			
602.276	602.278	602.280	602.277
615.315	615.312	634.252	615.327
618.271	618.269	866.388	618.278
634.259	634.252	1169.436	634.260
643.964	860.991	1313.655	861.042
665.946	866.390	1439.672	866.396
854.977	1169.451	1478.631	1313.667
860.988	1313.662	1622.622	1439.709
866.398	1348.482	1644.620	1478.647
1169.460	1439.678	2336.911	1500.634
1313.652	1478.648	2358.871	1567.636
1335.639	1500.594		1622.657
1348.489	1522.579		1644.634
1439.690	1567.598		1851.750
1478.633	1622.690		2320.021
1500.618	1644.666		2336.921
1512.589	1851.712		2358.897
1516.586	2251.804		
1522.596	2319.996		
1567.606	2336.919		
1622.648	2358.871		
1639.786			
1644.638			
1851.730			
2320.004			
2336.920			
2358.904			

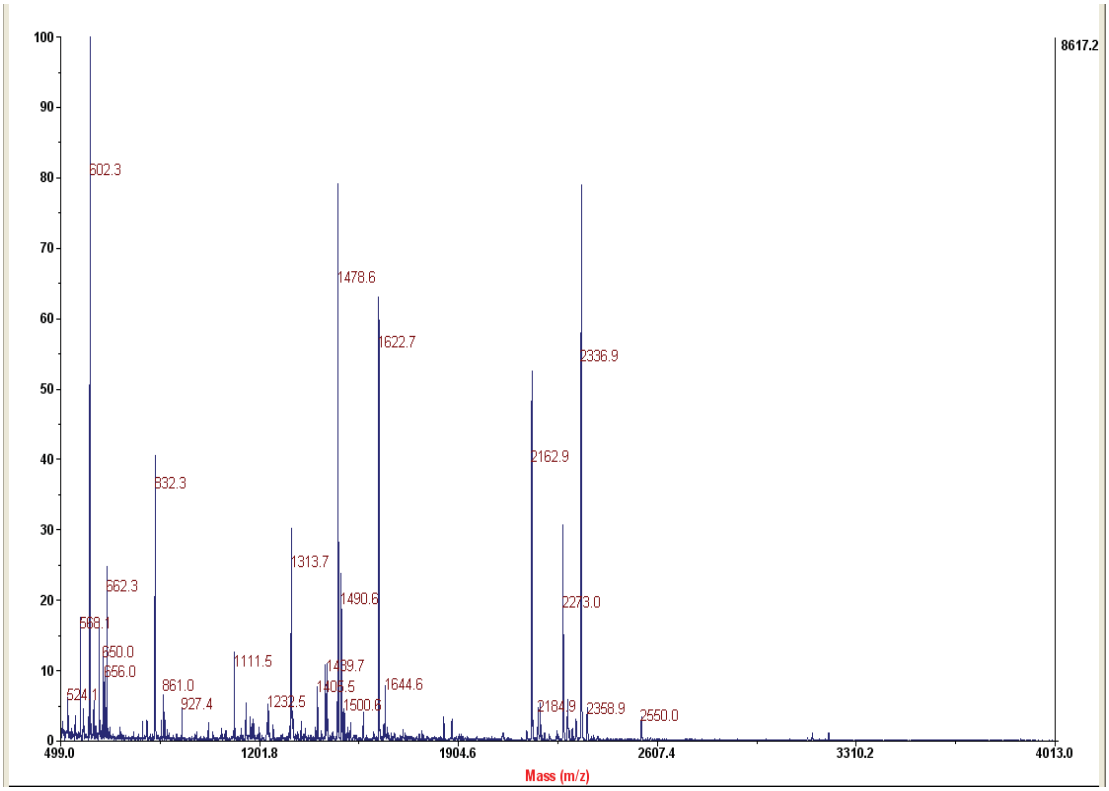

Figure A-7. MALDI-MS spectrum acquired from in-gel digestion of spot number 7 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-11. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot eight on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The protein identified was, 50S ribosomal protein L31 type B of *S. aureus.*

Peak (m/z)			
821.325	821.323	821.324	821.308
861.023	861.009	861.018	861.004
935.413	935.408	935.410	935.403
977.427	1061.452	977.425	977.405
1044.438	1268.532	1044.441	1044.424
1061.461	1284.513	1061.458	1061.440
1268.535	1327.625	1268.531	1268.515
1284.524	1642.633	1284.518	1284.499
1327.625	1911.940	1327.626	1327.604
1911.943	2453.917	1458.547	1458.530
2453.906	2613.021	1911.946	1911.919
2475.913	3137.293	2323.936	1933.874
3137.263		2453.917	2251.843
		2553.079	2322.892
		3137.287	2453.885
			2554.051
			3137.231
			3173.236

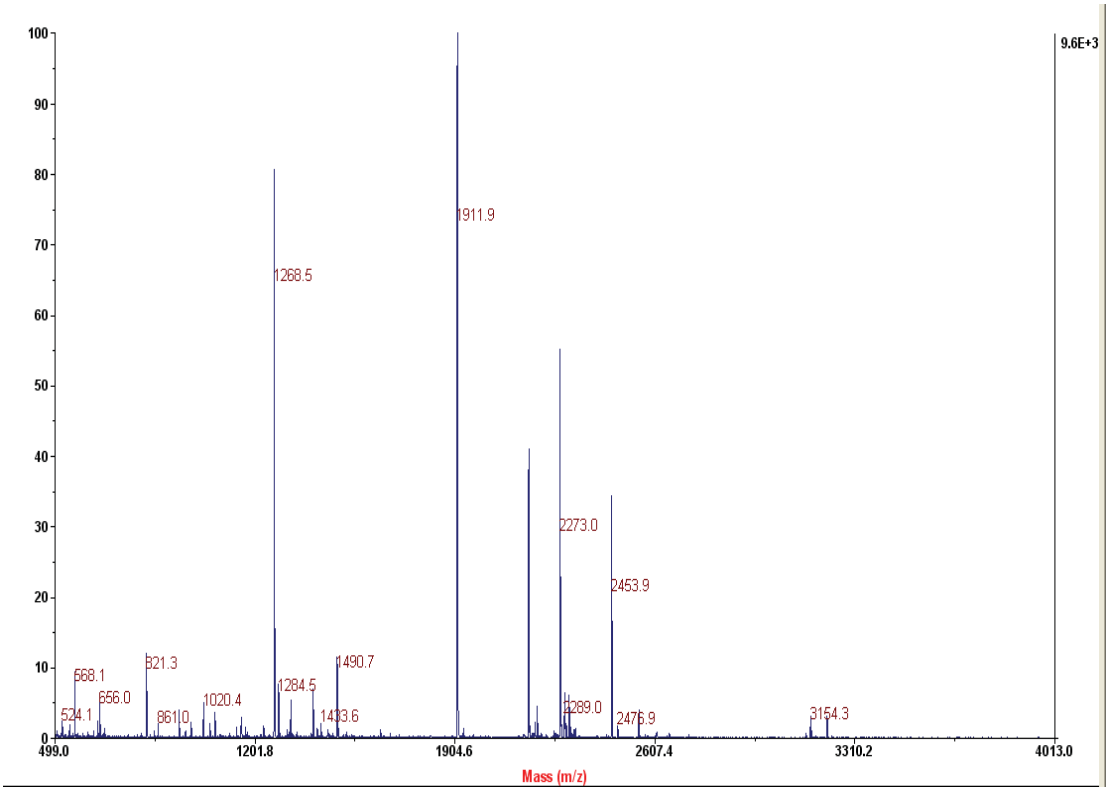

Figure A-8. MALDI-MS spectrum acquired from in-gel digestion of spot number 8 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-12. The four peak lists uploaded on Mascot 2.2 server for the identification of protein spot nine on the 2-DE gel. Peak lists were collected using the 4000 Series Explorer software version 3.5.1 (Applied Biosystems, USA), and processed using Peak erazor 2.0.1. The proteins identified were, putative septation protein spoVG and regulatory protein SpoVG of *S. aureus*

Peak (m/z)			
627.989	627.990	627.989	776.346
643.958	643.964	665.942	860.962
665.943	665.943	776.345	1091.446
776.348	776.344	838.992	1117.380
842.572	838.998	842.563	1200.407
854.968	845.443	845.442	1594.595
860.984	854.977	854.971	2186.773
886.592	860.984	860.980	2202.753
930.617	1065.978	886.584	2218.741
974.639	1091.443	889.461	2250.736
1065.975	1117.405	1065.968	2286.877
1091.447	1594.594	1091.442	2370.936
1117.400	2186.779	1117.396	2387.913
1594.600	2202.757	1594.583	3137.080
2186.779	2218.740	2186.758	
2202.757	2370.980	2202.740	
2218.748	2387.956	2218.728	
2370.993	2410.963	2370.964	
2387.964		2387.945	

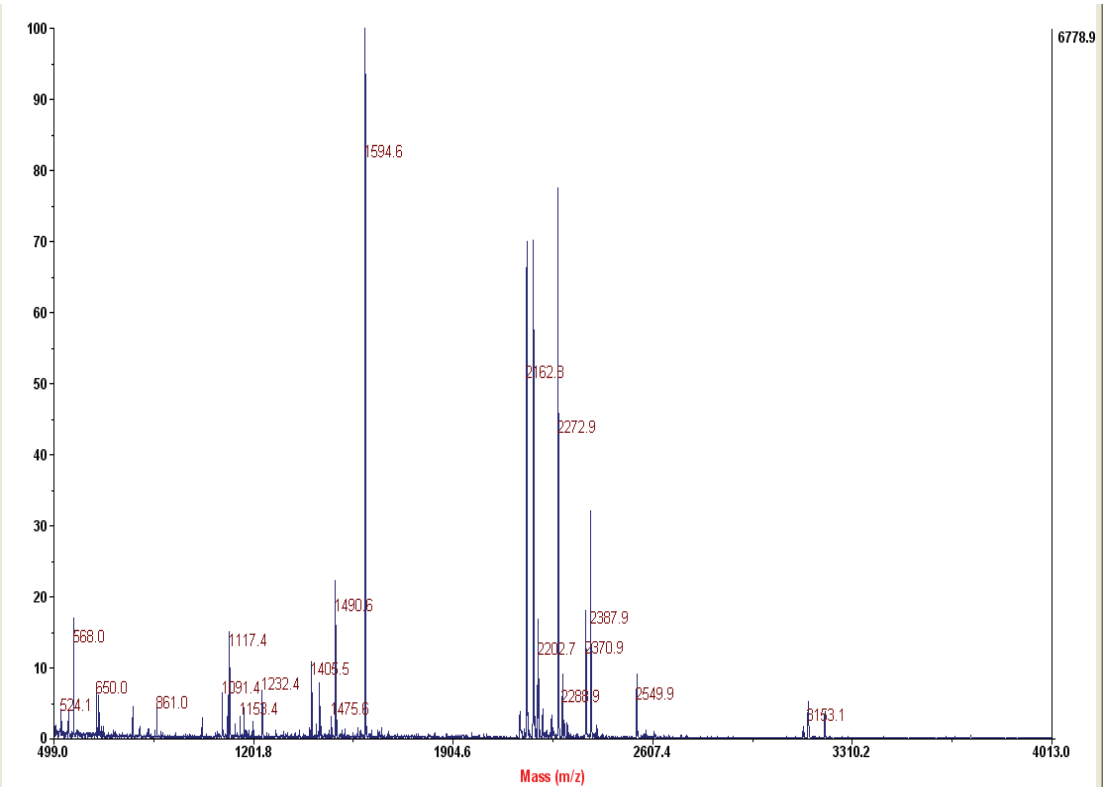

Figure A-9. MALDI-MS spectrum acquired from in-gel digestion of spot number 9 on the 2-DE gel. Spectrum was acquired in reflector mode with a mass range of 500-4,000 Da.

Table A-13. Peak list comparison between acetic acid-ethanol (E) and acetic acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Acetic-E	Acetic-M
801.1575	*	
927.5469	*	
1790.2930		*
1792.3117	*	
1834.4478	*	
2087.2955		*
2089.8070	*	
2175.3961		*
2286.7458		*
2304.8608		*
2306.7422	*	
2322.9158		*
2341.5727		*
2344.4741	*	
3006.7499		*
3208.6890		*
3212.4942	*	
3308.1515	*	
3876.9789	*	
4306.8297	*	
4514.0507	*	
4812.5420		*
4824.9473	*	
5503.5362		*
5515.6286	*	
6413.2414		*
6435.4218	*	
6600.0877		*
6626.5177	*	
8121.0244	*	
8168.2747	*	
9616.2925		*
9669.0305	*	
10094.1436		*

Table A-14. Peak list comparison between adipic acid-ethanol (E) and adepic acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Adipic-E	Adipic-M
825.314	*	
841.691		*
867.1839	*	
1835.252	*	
1862.901	*	
2466.84		*
2477.696		*
2869.198	*	
3109.681		*
3442.917		*
3446.479	*	
5037.953	*	
5071.216	*	
5423.677		*
5430.434	*	
5507.132		*
5527.26	*	
6558.329		*
6586.952	*	
6888.934		*
6910.771	*	

Table A-15. Peak list comparison between boric acid-ethanol (E) and boric acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Boric-E	Boric-M
842.5166		*
1835.515	*	
1863.623	*	
2421.225		*
2468.027		*
2516.913	*	
2754.582	*	
2765.568	*	
2870.051	*	
3053.233		*
3080.524	*	
3152.313	*	
3444.319		*
3446.575	*	
4176.307		*
4439.764		*
4819.041		*
4974.581	*	
5033.299		*
5038.221	*	
5425.02		*
5427.887	*	
5508.948		*
5515.507	*	
6566.483		*
6582.828	*	
6834.061	*	
6890.245		*
6905.989	*	

Table A-16. Peak list comparison between citric acid-ethanol (E) and citric acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Citric-E	Citric-M
867.346	*	
1835.326	*	
1863.311	*	
2870.066	*	
2965.172	*	
3445.559		*
3447.344	*	
5032.706		*
5038.833	*	
5060.934		*
5072.098	*	
5422.356		*
5530.123	*	
6898.459		*
6913.641	*	

Table A-17. Peak list comparison between formic acid-ethanol (E) and formic acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Formic-E	Formic-M
843.7559	*	
851.9117		*
1342.02		*
2090.094	*	
2198.531		*
2236.131		*
2260.121		*
2286.273		*
2304.696		*
2306.443	*	
2324.413		*
2325.442	*	
2342.935		*
2344.334	*	
2363.949		*
2382.122		*
2402.458		*
2544.375		*
2578.713		*
2682.955		*
2721.595		*
2743.273		*
2759.894		*
2977.989		*
3005.693		*
3043.329		*
3212.865	*	
3307.628	*	
3446.325	*	
3775.108		*
3873.334		*
3876.621	*	
4307.276	*	
4452.23	*	
4514.288	*	
4526.564		*
4562.062		*
4816.502		*
4823.549	*	
5039.357	*	
5515.334	*	
5945.037	*	
6434.655	*	
6605.212		*
6907.676	*	
8924.697	*	
9656.615		*
9669.585	*	
10153.76	*	

Table A-18. Peak list comparison between oxalic acid-ethanol (E) and oxalic acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Oxalix-E	Oxlic-M
829.5449	*	
830.5018	*	
841.659		*
1835.017	*	
1863.379	*	
2869.776	*	
3079.927	*	
3410.307	*	
3442.902		*
3445.579	*	
4177.878	*	
5036.872	*	
5422.517		*
5427.071	*	
5515.288	*	
6582.029	*	
6833.27	*	
6903.64	*	

Table A-19. Peak list comparison between succinic acid-ethanol (E) and succinic acid-methanol (M) treatment showing highly unique profiles.

Peak m/z	Succinic-E	Succinic-M
1835.468	*	
1863.734	*	
1907.914	*	
2222.075	*	
2478.358		*
2517.218	*	
2652.14	*	
2755.213	*	
2765.911	*	
2869.988	*	
3009.084	*	
3080.621	*	
3152.184	*	
3443.361		*
3446.788	*	
4174.59		*
4300.093		*
4817.573		*
4974.931	*	
5031.788		*
5038.658	*	
5423.959		*
5428.701	*	
5508.355		*
5515.748	*	
6565.68		*
6581.546	*	
6835.425	*	
6889.177		*
6906.333	*	

Table A-20. Peak list comparison between TFA-ethanol (E) and TFA-methanol (M) treatment showing highly unique profiles. One peak was common between the two extraction methods, 5523.176.

Peak m/z	TFA-E	TFA-M
843.6404	*	
2306.96	*	
2685.913	*	
3307.955	*	
3450.343	*	
3587.724	*	
3877.994	*	
4303.474		*
4308.491	*	
4503.957		*
4514.39	*	
4824.747	*	
5040.392	*	
5308.911	*	
5523.176	x	x
5945.284	*	
6370.719	*	
6420.457		*
6436.235	*	
6594.25		*
6618.091	*	
6912.507	*	
7185.421	*	
7584.209	*	
8132.748	*	
8928.35	*	
9207.138	*	
9631.257		*
9673.404	*	
10527.79	*	
13878.34	*	
16074.08	*	

Table A-21. Peak list comparison between water-ethanol (E) and water-methanol (M) treatment showing highly unique profiles. Ten peaks were common between the two extraction methods, 842.5554, 2753.319, 2868.246, 3443.314, 4301.048, 5027.903, 5417.575, 5502.373, 6005.004, and 6881.891.

Peak m/z	Water-E	Water-M
824.3382	*	
828.3617	*	
829.4235	*	
830.3257	*	
842.5554	x	x
843.373	*	
844.2199	*	
857.3493	*	
858.4112	*	
859.2349	*	
1278.208	*	
1834.631	*	
1862.614	*	
2467.713		*
2516.465		*
2753.319	x	x
2762.775	*	
2868.246	x	x
3052.75	*	
3077.938	*	
3148.999	*	
3375.615	*	
3443.314	x	x
3979.544	*	
4173.457		*
4301.048	x	x
4436.416		*
4816.511		*
5027.903	x	x
5417.575	x	x
5502.373	x	x
6005.004	x	x
6557.774		*
6812.712	*	
6881.891	x	x