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Sample preparation in matrix-assisted laser desorption/ionization mass spectrometry of whole bacterial cells and the detection of high mass (>20 kDa) proteins

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Three sample preparation strategies commonly employed in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOFMS) of whole bacterial cells were investigated for the detection of high mass signals; these included the dried droplet, the seed-layer/two-layer, and the bottom-layer methods. Different sample preparation approaches favoured the detection of high- or low-mass proteins. The low-mass peaks were best detected using the bottom-layer method. By contrast, the dried droplet method using a solvent with higher water content, and hence effecting a slower crystallization process, gave the best results for the detection of high-mass signals. Signals up to *m/z* 158 000 could be detected with this methodology for *Bacillus sphaericus*. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the same extracts used for MALDI-TOFMS showed bands in the molecular weight range in which high-mass peaks were observed in MALDI-MS, suggesting that the high-mass signals are not polymeric adducts of low-mass protein monomers. In addition, one of the high molecular weight proteins (~126 kDa) was putatively identified as an S-layer protein by an in-gel tryptic digest. The bacterial samples spotted on the target wells for MALDI-TOFMS, using the different sample preparation strategies, were examined under a scanning electron microscope and differences were observed between the different strategies, suggesting that the nature of the crystals and the distribution of the analytes amidst the crystals could influence the spectral pattern observed in MALDI-TOFMS of whole bacterial cells. Finally, evidence is presented to indicate that, although the determinands are intact cells, cell lysis occurs both before and during the MALDI process. Copyright © 2002 John Wiley & Sons, Ltd.

The application of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to whole microbial cells, for their rapid characterization and identification, has been the subject of many investigations in recent years.[1–8] The reason for this widespread interest can be traced to the importance of developing novel analytical techniques for the rapid detection of biological hazards. Soft ionization techniques such as electrospray ionization[9–11] and MALDI[1,6,7] have the potential to generate information-rich mass spectra from whole microbial cells. Mass spectrometry is particularly well suited because it largely aims at generating mass spectra that contain distinguishable spectral patterns or specific biomarkers unique to the microbial cell analyzed that can be used for rapid reproducible characterization within bacterial (chemo)taxonomy.[5] Several investigations have contributed to the development of MALDI-MS in terms of maximizing the signal content,[4] extracting information for fingerprinting,[12–14] improving spectral reproducibility,[15] and identifying potential biomarkers for specific bacteria.[8,16] The dependence of the spectral signal on both culture conditions[17] and analytical parameters[5,18] are also recognized.

The sample preparation strategies for whole-cell MALDI-MS analysis have been largely 'borrowed' from those developed for the analysis of peptides and proteins. Since the latter make up a significant fraction of dry cell matter in microbial cells, they would logically be ideal candidates for detection as biomarkers. The influence of matrix, solvent and spotting techniques on the quality of the MALDI mass spectra of peptides and proteins is well appreciated.[19–23] Although some attempts have been made to investigate and optimize sample preparation conditions for whole-cell analysis of certain bacteria,[4,19,24] optimization remains largely empirical. Little is understood about the physicochemical processes that contribute to the spectral information. Among the matrices attempted, the cinnamic acid derivatives, 4-hydroxy-α-cyanocinnamic acid (alpha), sina-

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pinic acid, and ferulic acid, have all been popular, although other matrices have also been shown to be useful. Generally, alpha has been employed for peptide analysis, while sinapinic and ferulic acids have been employed for the analysis of proteins.

A majority of the investigations thus far have concentrated on obtaining signals in the *m/z* range 2000–20000.[5] The signals in this range are generally attributed to cellular or cell-wall associated proteins.[7,14,16,17] While there are many proteins that fall within this mass range that could potentially be observed by MALDI-MS of whole bacterial cells, only a small fraction of these proteins are observed by current MALDI-MS techniques. In addition, the molecular mass distribution of proteins in this mass range[14] is such that, due to the comparatively low mass resolution, many of them are not uniquely discriminated by mass alone (and these can of course be compared with the protein databases of sequenced organisms), thereby decreasing the utility of the mass spectra for discrimination purposes. By contrast, the number of proteins in the higher mass range is lower and so (notwithstanding the lower mass resolution in absolute terms) potentially less likely to have similar molecular masses. One would therefore maximize the probability of finding *unique* protein biomarkers when analyzing at higher mass ranges. The ability to predict high-mass signals, as well as those in the lower mass ranges, would thus widen the scope and application of MALDI-MS for microbial characterization. Few investigations report the detection of high-mass signals in bacterial MALDI-MS. Madonna *et al.*[4] reported the use of ethanol applied to the sample spot to improve the signal content and detect some high-mass signals (up to *m/z* 75000) that were not observed otherwise. Winkler *et al.*[25] reported the detection of signals up to *m/z* 60000 in *Campylobacter* and *Helicobacter* strains, although the high-mass signals were less stable and were lost beyond 1 h of sample preparation. In an earlier investigation, Chong *et al.*[26] reported the detection of signals up to 500 kDa, albeit on cell lysates rather than on intact cells.

The objective of this investigation was to assess the commonly used sample preparation methods for observing signals from *m/z* 3000 to 200000. In particular we concentrated on detecting signals above *m/z* 20000, since these may be considered as unique biomarkers. Among the spotting techniques employed in whole-cell MALDI-MS, three basic methodologies can be identified; (a) mixing the matrix with the sample and spotting a known volume as a single layer (the dried droplet method as employed previously[3,16,17,24,27]), (b) spotting the sample matrix mixture as a second layer on top of a thin layer of matrix,[22,28] and (c) spotting the sample first and overlaying the dried sample layer with a layer of matrix on top.[8,25,29] These methods or their variations have been widely employed in whole-cell MALDI-MS investigations, and were hence investigated here. A factor that is known to influence the MALDI mass spectra of peptides and proteins is the rate of matrix crystal growth.[19] Botting[30] reported that the use of a slow crystallization method improves the MALDI-MS signals for large proteins. Moreover, it was noted by Figueroa *et al.*[31] that the rate of matrix crystallization and hence the MALDI mass spectra are influenced by the water content in the matrix solution from which the matrix crystals grow. We therefore used the commonly employed solvent consisting of a mixture of acetonitrile and 0.1% trifluoroacetic acid (TFA) and varied the water content by mixing the two in three different ratios of 1:3, 1:1, and 3:1. The MALDI mass spectra obtained under these sample preparation conditions were then examined. Finally, we studied the distribution of the matrix crystals and the microbial cells in the target well, under the different sample preparation conditions, using a scanning electron microscope.

EXPERIMENTAL

Sample preparation

Bacterial strains from eight species were employed in this investigation. These were the Gram-positive *Bacillus sphaericus* (DSM 28), *Brevibacillus laterosporus* (NCTC 7579), *Micrococcus luteus, Staphylococcus aureus*, and the Gram-negative *Escherichia coli* (UB5201), *Stenotrophomonas maltophilia* (2454 — a hospital isolate), *Pseudomonas aeruginosa* (PA01), and *Agrobacterium tumefaciens* (5A). These bacteria were grown on LabM blood agar base plates (LabM, Media and Consumables Suppliers, Bury, Manchester, UK), and incubated at 37 °C, for 16 h, except for *S. maltophilia* (incubated at 25 °C for 16 h), and *A. tumefaciens* (incubated at 25 °C for 40 h). Vegetative cells were carefully harvested using sterile plastic loops and resuspended in water (HPLC-grade; Fisher Scientific, Leicestershire, UK). After washing them twice these bacterial suspensions were spotted with the matrix solution on a stainless steel target plate supplied by Micromass Ltd. (Wythenshawe, Manchester, UK). All spots were prepared to give a final concentration of approximately 1 μg of dry bacteria/well. Sinapinic acid was used as the matrix and, unless stated otherwise, it was made up (50 mM) in acetonitrile/trifluoroacetic acid mixture (solvent). Three spotting methods were tested using three solvent combinations (*viz.*, acetonitrile/0.1% trifluoroacetic acid, in volumetric ratios of 3:1 (A), 1:1 (B), and 1:3 (C)), as detailed below:

Method I. Dried droplet method

An appropriate dilution of the bacterial suspension was mixed with the matrix solution in a volumetric ratio of 1:9, and 1 μL of this mixture was then immediately spotted onto a well of the stainless steel target plate, and air-dried at ambient temperatures.

Method II. Seed-layer/two-layer method

This method involved the application of two layers. The first layer consisted of 1 μL of the matrix in 50:50 methanol/acetonitrile (3 mg/mL). The spot was then air-dried, and a second layer prepared as in method I was spotted on top of the dried first layer and subsequently air-dried.

Method III. Bottom-layer method

An appropriate dilution of the bacterial suspension was made up in each of the three solvent combinations, spotted first as the first layer (1 μL), and air-dried. A second layer consisting of the matrix alone made up in the same solvent as

was used to dilute the bacterial suspension was then spotted (1 μL) on top of the dried first layer and air-dried.

MALDI-TOF analysis

The mass spectra were acquired using a ToFSpec2E™ MALDI-TOF mass spectrometer supplied by Micromass Ltd. (Wythenshawe, Manchester, UK), and operated in the positive ion linear mode. A nitrogen laser (337 nm) was employed for desorption/ionization. The mass spectra were acquired over two mass ranges: one up to *m/z* 20 000 (low-mass) and the other up to *m/z* 200 000 (high-mass). The acceleration voltage was kept at 20 kV and the pulse voltage was maintained at 1600 V for both the mass range acquisitions. Ion suppression was set to 1000 u for low-mass acquisitions and 5000 u for high-mass acquisitions. A high-mass detector whose voltage was set to 15 kV was used for the high-mass acquisitions.

The spectra were acquired in automated mode using random coordinates on the target well for firing the laser shots. Typically, 15 scans were combined to generate a raw spectrum, each scan being an average of 30 laser shots. The low-mass spectra were externally calibrated using a mixture of cytochrome C and myoglobin, and then lock mass corrected using cytochrome C, spotted in a central well of every five wells in a clover leaf arrangement in the target plate. For high-mass spectra, trypsinogen was used as the external calibrant and bovine serum albumin for lock mass correction. The calibrants were acquired from Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK). The combined raw spectra were then smoothed and baseline subtracted using the Masslynx software (Micromass Ltd., Wythenshawe, Manchester, UK).

SDS-PAGE

The cells of *B. sphaericus* and *Br. laterosporus* were grown and harvested as detailed above in the dried droplet method and suspended in solvent C to an approximate concentration of 1 μg/μL (dry cell weight), vortex mixed briefly (30 s) and the cell-free supernatants obtained by centrifugation were analyzed by SDS-PAGE. Prior to SDS-PAGE the cell-free supernatants were added to an equal volume of cracking buffer (Tris-HCl (3.75 mL, 0.5 M pH 6.8), 2-mercaptoethanol (1.5 mL), SDS (0.6 g), glycerol (3 g), bromophenol blue stock (1 mL, 0.1% w/v in distilled water), pH 6.8 made up to 10 ml with distilled water). Samples were heated in a boiling water bath for 5 min immediately prior to loading onto the stacking gels. The denatured protein samples were then run on a Protean II (Bio-rad) SDS-PAGE system with a 0.75 mm, 10% separating gel. A current of 600 V was applied to the gels from a Powerpack 300 (Bio-Rad) for approximately 3 h or until the bromophenol blue front reached the bottom of the gel. The gel was removed from its cast and silver stained.[32]

MALDI-MS of in-gel tryptic digest

One of the bands (~126 kDa in *B. sphaericus*), which corresponded to a protein in the SWISS-PROT database, was further characterized by performing an in-gel tryptic digest followed by MALDI-MS, as detailed elsewhere.[33] Sequencing-grade modified trypsin (Promega, Madison, WI, USA) was used for the digestion. The digests were further modified by guanidination of lysine[34,35] to increase the sensitivity of the lysine-containing peptides. The modified digests were Zip-tipped™ before analyzing by MALDI-MS. MALDI-MS of the digests was performed using the reflectron mode of the ToFSpec2E™ MALDI-TOF mass spectrometer (resolution 8000 FWHM).

Scanning electron microscopy

The incorporation of *E. coli* and *B. sphaericus* cells into the sinapinic acid matrix for the different sample preparation methods and solvent compositions, and the appearance of the spots after MALDI-MS analysis, were inspected using a scanning electron microscope (SEM) (JSM-840 SEMicroprobe, JEOL UK Ltd., Herts, UK) operating in the secondary electron mode. The samples were prepared as detailed for the MALDI-MS analysis on the wells of a piece of stainless steel target plate that was cut to size to fit into the SEM. The plates were secured on a mount using adhesive and coated with a thin layer of gold palladium mixture (~10 nm) using a Polaron E5000 coating unit, before analysis on the SEM.

RESULTS AND DISCUSSION

Microbial colonies can be directly mixed with a matrix and analyzed by MALDI-MS. Although this rapid method has been employed by several investigators,[1,2,29] because it is necessary for the laser to hit 'sweet spots' to attain reproducible results,[5] we employed a more repeatable microbial sample preparation protocol which allowed the different sample-matrix preparations to be compared directly. Thus the microbial cells were harvested, washed to remove contaminating media components and finally resuspended to a known microbial concentration. Sinapinic and ferulic acid are common matrices employed in whole-cell MALDI-MS for mass measurements above *m/z* 3000. Although ferulic acid has been shown to yield better signals than sinapinic acid,[4,23] it has a higher degree of crystal inhomogeneity. Sinapinic acid forms fine crystals (with commonly employed solvents, such as acetonitrile) that tend to be uniformly distributed when co-crystallized with the sample on the well of a target plate, thus enabling more truly random selection of spots for analysis. Moreover, greater shot-to-shot variability has been reported for ferulic acid and alpha matrices, compared to sinapinic acid, for the analysis of acetonitrile/water extracts.[28] Therefore, for convenience and better mass accuracy, sinapinic acid was used in this study. All mass spectra were acquired in the positive ion mode with two different settings for analysis in the low-mass end (*m/z* 3000–20 000) and the high-mass end (*m/z* 20 000–200 000). The method and solvent optimization were performed on strains from three of the eight bacterial species (*B. sphaericus, Br. laterosporus*, and *E. coli*), and the optimized methods were tested for the other bacteria.

Low-mass signals (*m/z* 3000–20 000)

Figure 1 shows the spectra at the low-mass end for the three bacterial strains (a–c), obtained using the three spotting methods (I–III) and solvent A. It can be seen from the figure that, of the three methods, method III gives relatively better spectra, for the three bacterial strains, in terms of the number

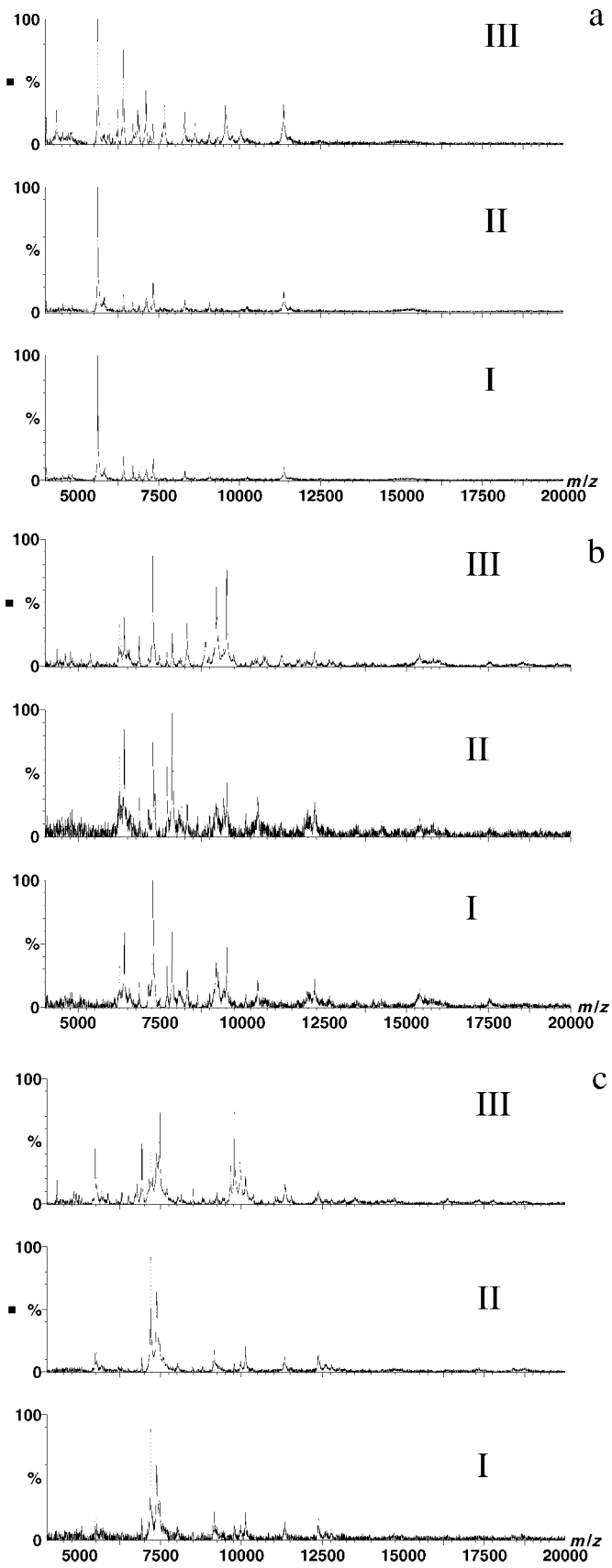

Figure 1. Positive ion MALDI-TOF mass spectra of (a) *B. sphaericus*, (b) *E. coli*, and (c) *Br. laterosporus* in the low-mass range (*m/z* 3000–20000), obtained using methods I, II and III, and solvent A (acetonitrile/0.1% TFA 3:1).

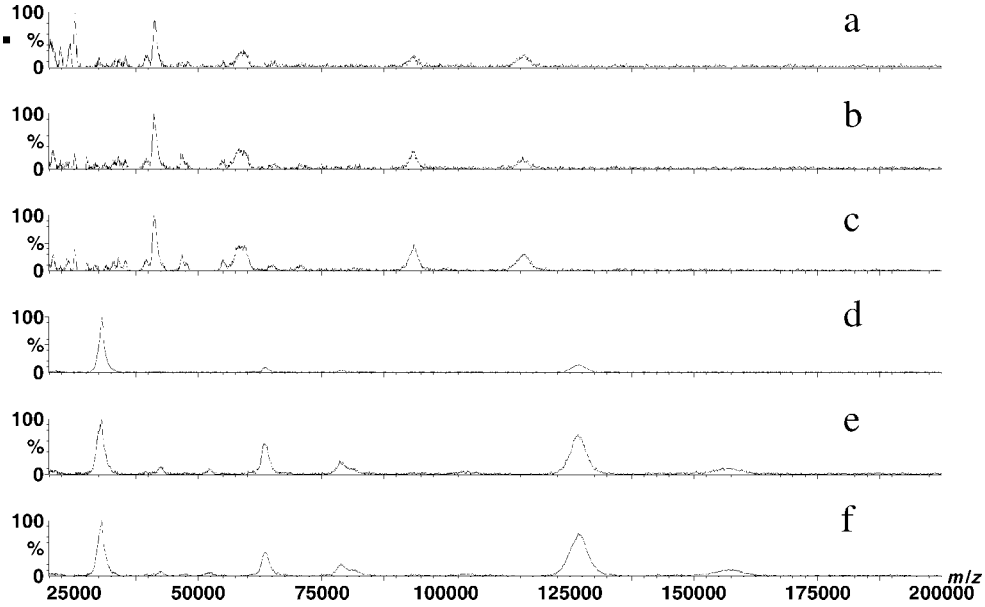

Figure 2. Positive ion MALDI-TOF mass spectra of *Br. laterosporus* (a–c) and *B. sphaericus* (d–f) in the high mass range (*m/z* 20 000–200 000) showing a *method*-wise comparison. Spectra were obtained using methods I (c, f), II (b, e) and III (a, d), and solvent C (acetonitrile/0.1% TFA 1:3).

of peaks and a higher signal-to-noise ratio. Similar results were obtained with solvents B and C (data not shown). Moreover, of the three methods, method III appeared to be less influenced by changes in the solvent composition and the differences between the spectra obtained using the three different solvent ratios appeared to be minimal. Dai *et al.*[22] noted that the analysis of proteins by the dried droplet method (method I in this study) using sinapinic acid showed poor shot-to-shot reproducibility although good sensitivity was observed, and reported that the two layer method (method II in this study) was very effective for analyzing complex mixtures. Nilsson[28] has employed a similar method for the analysis of *Helicobacter pylori*, and reported several peaks with sinapinic acid. However, his work was performed on bacterial lysates extracted with acetonitrile/water. In the present study, no significant difference was observed between methods I and II for the three solvents in the low-mass end. It was also observed that method III gave better results in terms of peak numbers and a higher signal-to-noise ratio for the other bacterial strains in comparison to method I (data not shown).

High-mass signals (*m/z* 20 000–200 000)

The results of the high-mass investigations are shown in Figs 2 and 3 for *B. sphaericus* and *Br. laterosporus*. A *method*-wise comparison, using solvent C (1:3 acetonitrile/0.1% TFA), is shown in Fig. 2. Of the three methods, method I (dried droplet method) can be seen to produce better signals for both the bacterial strains. A *solvent*-wise comparison, using method I (Fig. 3), shows that solvent C gives the best signal for both the bacterial strains. The number of repeatable peaks observed in both the low- and high-mass spectra, using the three methods and the three solvent combinations, is summarized in Fig. 4 in the form of a histogram, for each of the three bacterial strains. It is clear from this figure that method III clearly gives the maximum number of peaks in the low-mass end, for all the three bacterial strains, irrespective of the solvent used. Method I using solvent C gives the maximum number of repeatable peaks, at least for *B. sphaericus* and *Br. Laterosporus*, which show significant numbers of high-mass peaks (particularly $>m/z$ 50 000), compared with *E. coli* (Table 1). The influence of the solvent on the high -mass signals appears to be greater than that of the spotting method employed. It is therefore clear from this investigation that the sample preparation strategy used may have to be different for detecting proteins in different mass ranges.

SDS-PAGE of the crude cell extract of *B. sphaericus* and *Br. laterosporus* run in 10% acrylamide (Fig. 5) shows bands between the 98 and 148 kDa marker proteins, which are likely to be due to the peaks at about 115 and 126 kDa, observed in the two bacterial high-mass spectra (Fig. 2). The above result combined with the observation that the mass spectral intensity of the two peaks are identical to, if not greater than, those of peaks occurring at lower *m/z* (half or one-quarter the high-mass values), suggests that the two peaks observed are monomeric, singly charged species, rather than polymeric adducts. A SWISS-PROT/spTrEMBL protein database search[36] revealed that *B. sphaericus* possesses a surface layer protein whose molecular weight is 125 225 Da. In order to gather further evidence, the high mass band (at ~126 kDa) in the SDS gel (Fig. 5) was excised, digested with trypsin and the digest analyzed by MALDI-MS (in-gel digest), as detailed in the Experimental section. Simultaneously, the 125-kDa protein from the SWISS-PROT database was digested *in silico* using ProteinProspector,[37] and the results from the in-gel and *in silico* digests compared. It was observed that 18% (10 fragments) of the total peaks

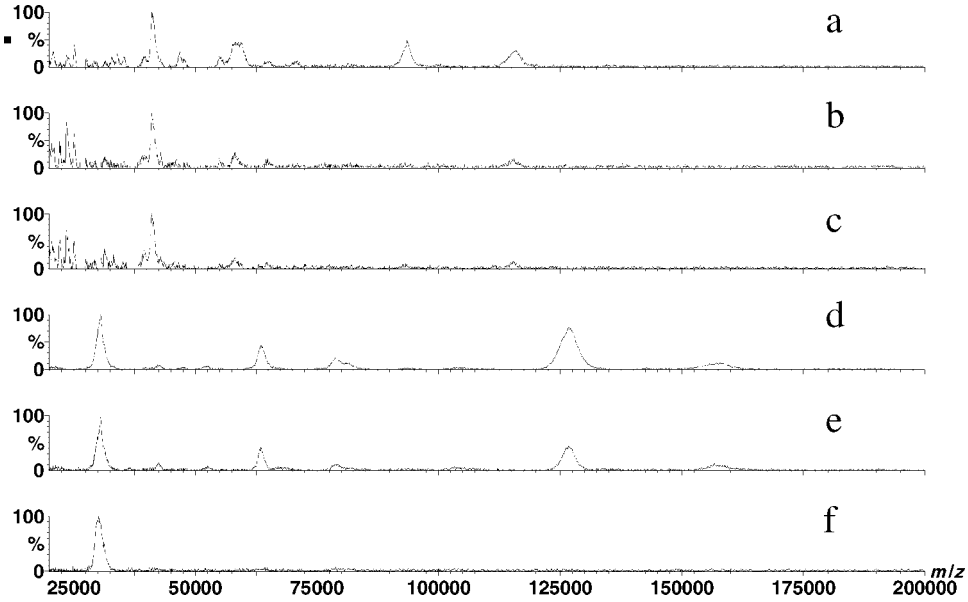

Figure 3. Positive ion MALDI-TOF mass spectra of *Br. laterosporus* (a–c) and *B. sphaericus* (d–f) in the high-mass range (*m/z* 20 000–200 000) showing a *solvent*-wise comparison. Spectra were obtained using method I, and the solvent combination of acetonitrile/0.1% TFA in ratios of 3:1 (c, f), 1:1 (b, e), and 1:3 (a, d).

(monoisotopic) in the in-gel digest correlated to those in the *in silico* digest to within 100 ppm, and 42% (24 fragments) to within 1000 ppm. The corresponding percentages of protein covered by the correlated digests were found to be 10 and 20%, respectively. Given this coverage,[38] it is likely that the ~126-kDa peak observed in the MALDI mass spectrum of *B. sphaericus* is due to the surface-layer protein.

Table 1 lists the repeatable peaks for three of the bacterial strains in both the low- and high-mass end ranges. Most of the peaks observed in the low-mass end for *E. coli* UB5201 have a match in the Swiss-prot/spTrEMBL database and are in good agreement (within ±5 *m/z*) with those reported in the literature for *E. coli*.[8,16,17,39] Although the resolution and mass accuracy of the high-mass peaks were poor, it is noteworthy that bacterial proteins at such high masses are rare in number and so likely to be highly useful for discriminatory purposes even with poor resolution and mass accuracy. The other bacterial strains used in the investigation showed fewer high-mass signals. It is possible that the high-mass signals observed with *B. sphaericus* and *Br. laterosporus* are due to proteins on the surface layer of these bacterial cells that are easily dislodged using the methods employed in this investigation, while the method is not entirely successful in extracting high-mass proteins from the other bacterial strains investigated. However, it is noteworthy that once high-mass proteins are extracted into the solvent, employment of method I, using solvent C, gave a better signal.

Earlier investigations[8,16,17] suggested that a large proportion of the peaks observed in the MALDI mass spectra of whole bacterial cells could be attributed to ribosomal proteins. This would imply that the bacterial cells are lysed somewhere in the analytical process. The mixing of the cells with the solvent prior to spotting could potentially cause cell lysis, in which case the proteins that are leached out of the cells are co-crystallized with the matrix when the sample is prepared for MALDI-MS, and are eventually desorbed during MALDI-MS. Alternatively, the cells associated with matrix crystals are lysed during the MALDI process when the laser is applied on the target spot for desorption. The distribution of the analytes in the matrix crystals and the nature of the crystals are known to influence MALDI-MS signals;[30,31,40] thus, investigating the influence of the different sample preparation strategies on the co-crystallization may help explain the differences observed in the mass spectral signals.

SEM investigations

Crystal morphology is known to influence spectral information in MALDI-MS of proteins using sinapinic acid matrix.[41–44] It has been shown that small crystals are completely volatilized by the laser shot, whole larger crystals undergo a layer-by-layer evaporation, pointing to the influence of sample preparation on the crystal size distribution and its consequence on volatilization.[43] It has also been demonstrated that proteins can be incorporated into a growing crystal by selective interaction with one of the crystal faces.[41] Crystal morphology and analyte distribution could thus be factors that can be useful in explaining the differences in the signal patterns observed with the different sample preparation strategies in whole-cell MALDI-MS.

In order to understand the observed differences in the nature of the signals under the different sample preparation conditions, the sample spots were examined under a scanning electron microscope. Figure 6 shows representative target wells for both *B. sphaericus* (Figs 6(a)–6(c)) and *E. coli* (Figs 6(d)–6(f)) samples, the former prepared using method I and the latter using method III, using the three solvents A (Figs 6(a) and 6(d)), B (Figs 6(b) and 6(e)) and C (Figs 6(c) and 6(f)). It can be seen that the matrix crystals are uniformly

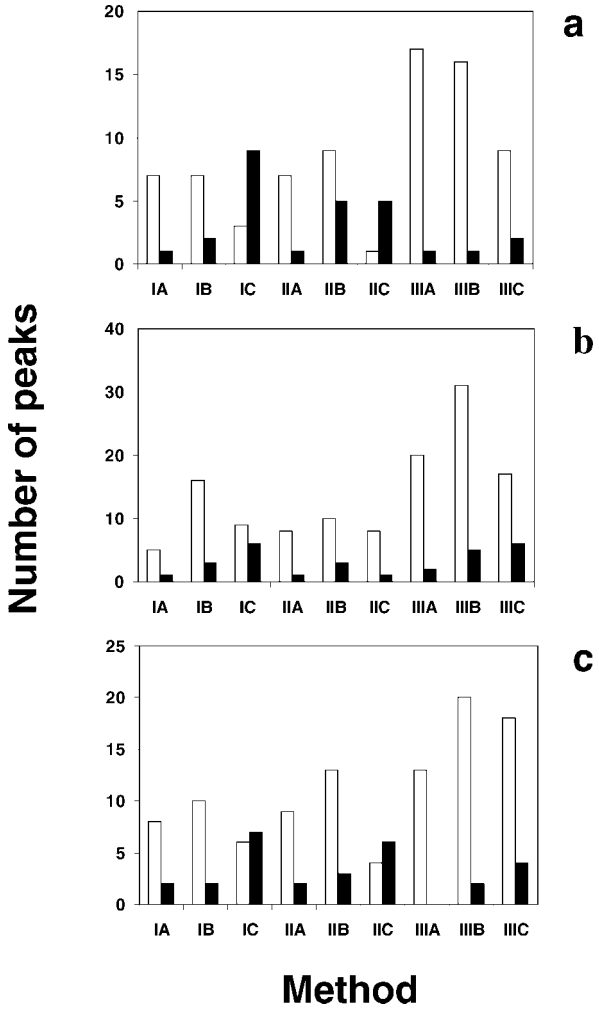

Figure 4. Comparative histogram showing the number of reproducible peaks obtained using the three methods (I, II, and III) and the three solvent combinations of acetonitrile/0.1% TFA in the ratios 3:1 (A), 1:1 (B) and 1:3 (C), for (a) *B. sphaericus*, (b) *E. coli* UB5201, and (c) *Br. laterosporus*. White (open) bars represent the low-mass (*m/z* 3000–20000) peaks and black (closed) bars the high mass (*m/z* 20000–200000) peaks.

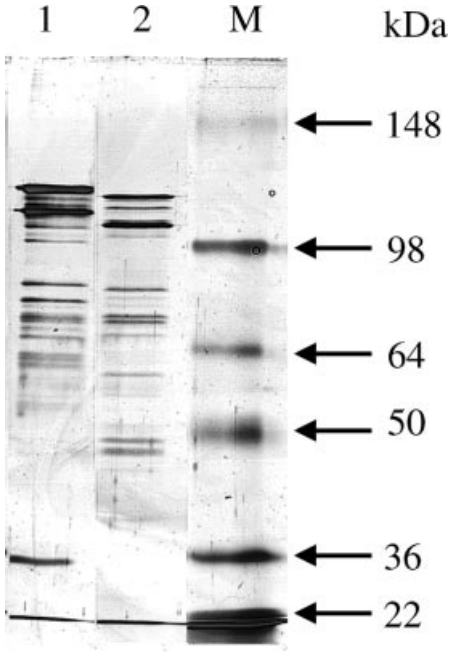

Figure 5. SDS polyacrylamide gel of the cell-free supernatants of crude bacterial extracts obtained by vortex mixing the washed bacterial cells in solvent C (acetonitrile:/0.1% TFA 1:3). Lanes are (1) *B. sphaericus*, (2) *Br. laterosporus*, and (M) standard protein markers whose molecular weights are listed alongside for comparison. Note the prominent bands with both the bacterial extracts, between 98 and 148 kDa, corresponding to the high-mass signals at 127 and 115 kDa for *B. sphaericus* and *Br. laterosporus*, respectively, in Fig. 3.

distributed across the plate in all the target wells, suggesting that irrespective of the water content of the sample, and the resultant variations in the surface tension of the solvent mixture, crystal distribution is uniform (similar results were also observed with method II). However, the nature of the crystals formed can be seen to differ with the type of solvent used. While solvent A appears to induce the formation of well-defined crystal structures, the crystals formed using solvent C are larger, flatter and ill-defined, and those formed using solvent B seem to contain both crystal types, although the former predominate. This is perhaps unsurprising since

Table 1. Repeatable markers (*m/z*) for the three bacterial strains in both the low (*m/z* 3000–20000) and the high (*m/z* 20000–200000) mass range

	m/z markers	
Bacterial strain	Low mass[a]	High mass[b]
B. sphaericus DSM 28	4026, 4336, 4793, 4818, 5615, 6076, 6417, 6852, 7109, 7240, 7689, 8308, 8632, 9597, 10053, 11365	30500, 42260, 52200, 63500, 78700, 93400, 104000, 127000, 158000
E. coli UB5201	3630, 4363, 4608, 4766, 6256, 6316, 6411, 6507, 6548, 6857, 7273, 7332, 7480, 7707, 7867, 8325, 8877, 8896, 8991, 9190, 9271, 9535, 9741, 10297, 10387, 10648, 10693, 11190, 11732, 12206, 15403	25100, 26250, 28500, 33450, 46650, 56900
Br. laterosporus NTCT 7579	3590, 4292, 4820, 6773, 6919, 7183, 7254, 7368, 7468, 8490, 8853, 9240, 9664, 9788, 9956, 10127, 11048, 11355, 11533, 12372	20750, 25100, 39500, 41150, 58300, 93300, 115500

[a] Using spotting method III; values are typically accurate to ±5 *m/z* units or less.
[b] Using spotting method I; values are rounded to the nearest approximation.

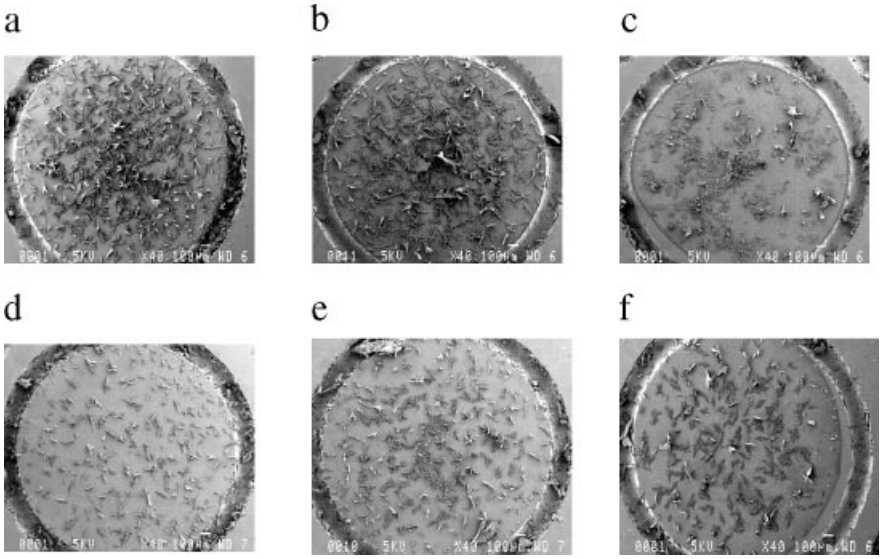

Figure 6. Distribution of sinapinic acid matrix crystals on the MALDI-MS target wells as observed by scanning electron microscopy (magnification ×40) for whole-cell bacterial samples from *B. sphaericus* using spotting method I (a–c) and *E. coli* employing spotting method III (d–f). All three solvent combinations are shown: A (a, d), B (b, e), and C (c, f).

it is known that varying the solvent composition influences the size of the crystals formed.[40]

A closer inspection of representative spots (Fig. 7) reveals the nature of the crystals and the distribution of bacterial cells amidst the crystals. The cells can be seen to be homogeneously distributed amongst the matrix crystals when method I (Figs 7(a) and 7(c)) is employed, compared to when method III (Figs 7(b) and 7(d)) is employed. The distribution for method II was similar to that of method I (data not shown). This is expected given that the cells and the matrix are mixed and spotted in methods I and II, while in method III the matrix is spotted as a separated layer on top of the bacterial sample layer. An inspection of the bacterial cell morphology (Fig. 8) shows that the cells appear to be intact, although *B. sphaericus* cells appear to have imploded, and there appears to be some evidence of pre-spores. The drying conditions employed in preparing the samples for MALDI-MS (and SEM in turn) seem to have caused the cells to lose water, but there is little evidence to suggest that the cells are extensively damaged by lysis. In fact, *E. coli* cells appeared

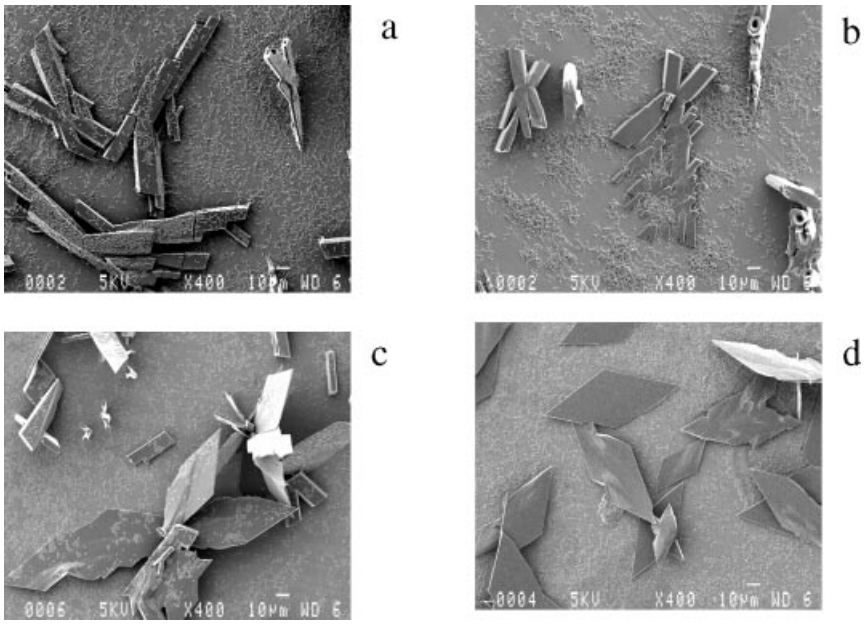

Figure 7. Nature of sinapinic acid crystals formed using spotting method I (a, c) and method III (b, d), using solvent A (a, b) and solvent C (c, d) and the distribution of bacterial cells (*B. sphaericus* (a, b) and *E. coli* (c, d)) amidst the matrix crystals, as observed by scanning electron microscopy (magnification ×400).

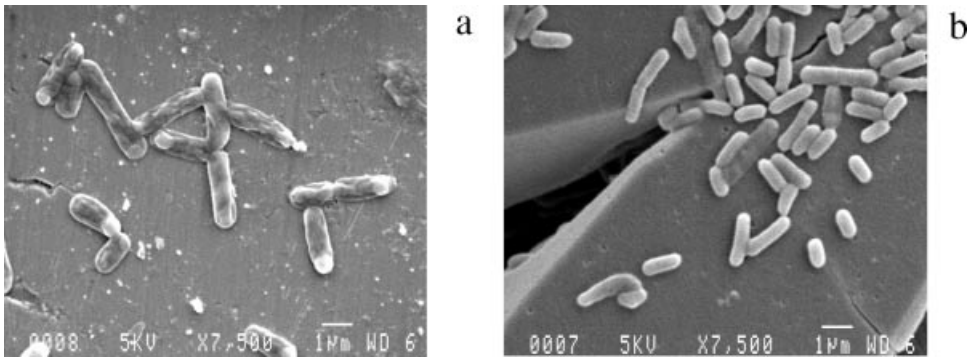

Figure 8. Morphology of the bacterial cells co-crystallized with the sinapinic acid matrix on the target well before MALDI-MS analysis, as seen by scanning electron microscopy (magnification ×7500). (a) *B. sphaericus* in matrix prepared by method II, using solvent B, and (b) *E. coli* prepared by method I, using solvent A.

intact with little damage to cell structure, even on examination at these higher magnifications.

Figure 9 reveals the appearance of selected sample spots after MALDI-MS analysis (30 laser shots). The remnants of matrix and bacterial cell debris after desorption seen in the figure suggest that the matrix crystals melt and associated cells are possibly disrupted when the laser shots are fired. The particles seen in Fig. 9(a) appear to be cell debris that is strewed across the area under the laser's influence. Some cells close to the area of impact can be seen to be intact in the inset. Lysis of the cells might well be occurring at this stage of the analysis. However, the results from the SDS-PAGE analysis, where the supernatants from the sample preparations were analyzed, show the presence of several proteins, suggesting cell breakage, even before analysis by MALDI-MS. Moreover, it was observed (data not shown) that the high-mass spectra of cell-free supernatants of *B. sphaericus* and *Br. laterosporus* were identical to those of whole bacterial cells, suggesting that at least these signals arise from proteins leached out into the solvent. It is therefore likely that the mass spectral signals arise from both cell lysis during sample preparation and during the MALDI-MS analysis itself.

Botting[30] reported that, by using a slow crystallization method, high-mass signals are less suppressed by low-mass proteins, and attributed the effect to differential partitioning of the analyte between the crystal and the bulk solution, a

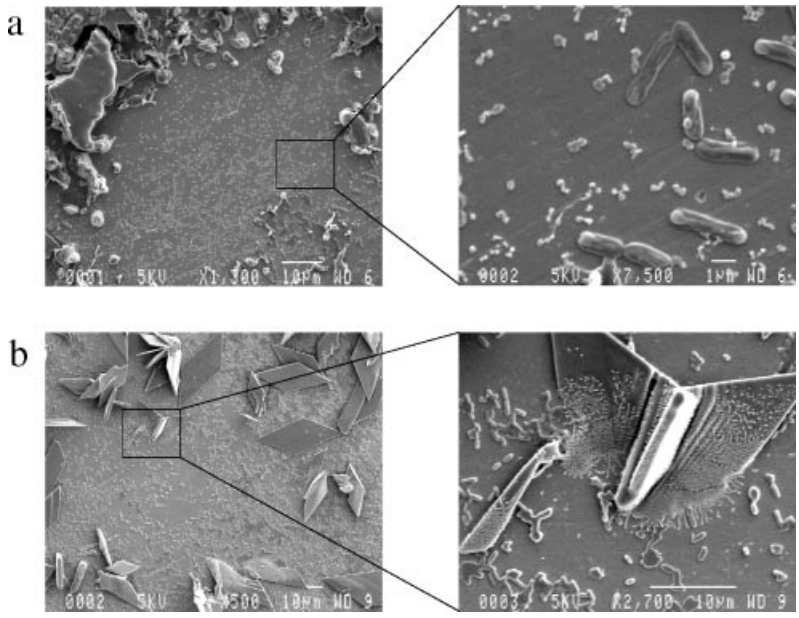

Figure 9. Apparent target spots after analysis by MALDI-MS seen by scanning electron microscope (magnification (a) × 1300, (b) × 500). A portion of each spot is further magnified in the inset ((a) × 7500, (b) × 2700). (a) *B. sphaericus* prepared by method II using solvent C, and (b) *E. coli* prepared by method III using solvent C. Note the debris strewed across (inset a), possibly from cell lysis and the remnants of melted matrix crystals (insets a and b).

statement hypothesised earlier by Cohen and Chait.[19] It was also noted that the slow crystallization process appears to concentrate the protein from the solution into the matrix crystal. In the present study, assuming that the cells are lysed prior to MALDI-MS, solvent composition would be the factor that would influence protein solubility and partitioning between crystal and bulk solution, while the spotting method would influence analyte (whole cells or leached proteins) distribution and incorporation within matrix crystals. Acidic and large proteins would be less soluble in the bulk solvent, which would become increasingly acidic on evaporation. These proteins would be more readily incorporated into the matrix crystals. Increasing the water content of the matrix solvent, as is the case with solvent C, would approximate the slow crystallization process adopted by earlier investigators,[19,30] at least in relative terms, and would explain the improved high-mass signals when using solvent C. In methods I and II, the bacterial cells are mixed with the matrix solution before spotting. There would therefore be a more homogeneous distribution of analytes amidst matrix crystals and a better chance for the less-soluble high-mass proteins to partition and be incorporated into the matrix crystals, given the inverse relation between the mass of the protein and its propensity to adhere to the matrix crystal.[19] Therefore, these two methods result in better signals for high-mass proteins. On the other hand, when method III is employed, small proteins are readily solubilized when spotting matrix solution on top of the bacterial cells, enabling co-crystallization of these in preference to large proteins or whole cells. Therefore, method III gives better results for small proteins compared with large ones. Although these explanations seem plausible, the presence of intact cells, which seem to form a significant portion of the cells in the target well, suggests that further investigations are required in order to understand and optimize the process involved in MALDI-MS of whole bacterial cells.

CONCLUSIONS

The sample preparation strategy adopted in MALDI-MS of whole bacterial cells influences the detection of signals with different masses. High-mass signals (>20 kDa) were detected well with the dried droplet method when a solvent with a higher water content and hence a slower crystallization process was employed, whilst lower mass signals were suitably detected with the bottom layer method, irrespective of the solvent used. SDS-PAGE analysis of the extracts used for MALDI-MS showed bands corresponding to the high-mass signals suggesting that these peaks do not arise from polymeric adducts of lower mass proteins. SEM was employed to investigate the sample spots that were prepared using the different strategies, in order to obtain an explanation for the observed MALDI-MS results. Under SEM the majority of the bacterial cells appeared to be intact suggesting the cells are lysed during the MALDI process itself. However, the detection of proteins by SDS-PAGE in the extracts *prior* to MALDI-MS suggests that some of the cells were lysed prior to MALDI analysis. This strongly indicates that both processes are involved in the production of the mass spectral signals from MALDI-MS analysis of bacteria.

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REFERENCES

1. Claydon MA, Davey SN, Edwardsjones V, Gordon DB. *Nat. Biotechnol.* 1996; **14**: 1584.
2. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO. *Rapid Commun. Mass Spectrom.* 1996; **10**: 1227.
3. Lynn EC, Chung M-C, Tsai W-C, Han C-C. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2022.
4. Madonna AJ, Basile F, Ferrer I, Meetani MA, Rees JC, Voorhees KJ. *Rapid Commun. Mass Spectrom.* 2000; **14**: 2220.
5. Lay JOJ. *Trends Anal. Chem.* 2000; **19**: 507.
6. vanBaar BLM. *FEMS Microbiol. Rev.* 2000; **24**: 193.
7. Krishnamurthy T, Rajamani U, Ross PL, Jabhour R, Nair H, Eng J, Yates J, Davis MT, Stahl DC, Lee TD. *J. Toxicol. Toxin Rev.* 2000; **19**: 95.
8. Ryzhov V, Fenselau C. *Anal. Chem.* 2001; **73**: 746.
9. *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications*, Cole RB (ed). John Wiley: New York, 1997.
10. Goodacre R, Heald JK, Kell DB. *FEMS Microbiol. Lett.* 1999; **176**: 17.
11. Vaidyanathan S, Rowland JJ, Kell DB, Goodacre R. *Anal. Chem.* 2001; **73**: 4134.
12. Arnold RJ, Reilly P. *Rapid Commun. Mass Spectrom.* 1998; **12**: 630.
13. Jarman KH, Daly DS, Petersen CE, Saenz AJ, Valentine NB, Wahl KL. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1586.
14. Demirev PA, Ho YP, Ryzhov V, Fenselau C. *Anal. Chem.* 1999; **71**: 2732.
15. Saenz AJ, Petersen CE, Valentine NB, Gantt SL, Jarman KH, Kingsley MT, Wahl KL. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1580.
16. Holland RD, Duffy CR, Rafii F, Sutherland JB, Heinze TM, Holder CL, Voorhees KJ, Lay JO. *Anal. Chem.* 1999; **71**: 3226.
17. Arnold RJ, Karty JA, Ellington AD, Reilly JP. *Anal. Chem.* 1999; **71**: 1990.
18. Wang ZP, Russon L, Li L, Roser DC, Long SR. *Rapid Commun. Mass Spectrom.* 1998; **12**: 456.
19. Cohen SL, Chait BT. *Anal. Chem.* 1996; **68**: 31.
20. Kussmann M, Nordhoff E, Rahbek Nielsen H, Haebel S, Rossel Larsen M, Jakobsen L, Gobom J, Mirgorodskaya E, Kroll Kristensen A, Palm L, Roepstorff P. *J. Mass Spectrom.* 1997; **32**: 593.
21. Roepstorff P, Larsen MR, Nielsen HR, Nordhoff E. In *Cell Biology: a Laboratory Handbook*. Academic Press, 1998; 556.
22. Dai YQ, Whittal RM, Li L. *Anal. Chem.* 1999; **71**: 1087.
23. Bornsen KO, Gass MAS, Bruin GJM, von Adrichem JHM, Biro MC, Kresbach GM, Ehrat M. *Rapid Commun. Mass Spectrom.* 1997; **11**: 603.
24. Domin MA, Welham KJ, Ashton DS. *Rapid Commun. Mass Spectrom.* 1999; **13**: 222.
25. Winkler MA, Uher J, Cepa S. *Anal. Chem.* 1999; **71**: 3416.
26. Chong BE, Wall DB, Lubman DM, Flynn SJ. *Rapid Commun. Mass Spectrom.* 1997; **11**: 1900.
27. Welham KJ, Domin MA, Scannell DE, Cohen E, Ashton DS. *Rapid Commun. Mass Spectrom.* 1998; **12**: 176.
28. Nilsson CL. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1067.
29. Evason DJ, Claydon MA, Gordon DB. *J. Am. Soc. Mass Spectrom.* 2001; **12**: 49.
30. Botting CH. *Rapid Commun. Mass Spectrom.* 2000; **14**: 2030.

31. Figueroa ID, Torres O, Russell DH. *Anal. Chem.* 1998; **70**: 4527.
32. Gooderham K. In *Methods in Molecular Biology: Proteins*, Walker JM (ed). Humana Press Inc.: Clifton, NJ, 1984; 113.
33. Shevchenko A, Wilm M, Vorm O, Mann M. *Anal. Chem.* 1996; **68**: 850.
34. Hale JE, Butler JP, Knierman MD, Becker GW. *Anal. Biochem.* 2000; **287**: 110.
35. Brancia FL, Oliver SG, Gaskell SJ. *Rapid Commun. Mass Spectrom.* 2000; **14**: 2070.
36. Available: http://us.expasy.org/sprot/.
37. Available: http://128.40.158.151/ucsfhtml3.4/msdigest.htm.
38. Mann M, Hendrickson RC, Pandey A. *Annu. Rev. Biochem.* 2001; **70**: 437.
39. Dai Y, Li L, Roser DC, Long SR. *Rapid Commun. Mass Spectrom.* 1999; **13**: 73.
40. Dai YQ, Whittal RM, Li L. *Anal. Chem.* 1996; **68**: 2494.
41. Beavis RC, Bridson JN. *J. Phys. D Appl. Phys.* 1993; **26**: 442.
42. Bolbach G, Riahi K, Spiro M, Brunot A, Breton F, Blais JC. *Analusis* 1993; **21**: 383.
43. Sadeghi M, Vertes A. *Appl. Surf. Sci.* 1998; **129**: 226.
44. Westman A, Huthfehre T, Demirev P, Sundqvist BUR. *J. Mass Spectrom.* 1995; **30**: 206.